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# Occurrence of cell death (apoptosis) in prostatic intra-epithelial neoplasia

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**Abstract.** The aim of our study was to assess the frequency and location of apoptotic bodies (ABs) in haematoxylin and eosin-stained sections of prostatic intra-epithelial neoplasia (PIN) and then to compare the patterns with those in benign prostatic hyperplasia (BPH) and prostatic invasive adenocarcinoma (PAC). ABs were identified in all epithelial cell layers of the ducts, acini and tumour islands, as well as in the lumina contained in such structures. In the epithelial cell layers, ABs were found in general in the intercellular space and occasionally in the cytoplasm of epithelial cells. The frequency of ABs increased from BPH through PIN up to PAC. The proportions of ABs in PIN lesions of low grade (PINlow) and high grade (PINhigh) were greater than in BPH, the values decreasing from the nuclei in the basal position towards those in the luminal layer. In PINlow, the mean category values were 0.85% (standard error, SE, 0.311%) in the basal, 0.623% (SE 0.065%) in the intermediate and 0.474% (SE 0.138%) in the luminal position. In PINhigh, the mean category values were 1.006% (SE 0.16%) in the basal position, 0.713% (SE 0.182%) in the intermediate and 0.618% (SE 0.172%) in the luminal position. The proportions of ABs in adenocarcinoma with cribriform pattern decreased from the basal towards the luminal layer, as for PIN: 1.806% (SE 0.346%) in the basal position, 1.15% (SE 0.172%) in the intermediate and 0.886% (SE 0.137%) in the luminal position. In the solid/trabecular adenocarcinomas, the mean category value in the cell layer adjacent to the stroma was 2.154% (SE 0.203%), whereas in the other cell layers it was 2.052% (SE 0.239%). In small and large acinar adenocarcinomas, the proportions of positive nuclei were 1.022% (SE 0.1%) and 0.922% (SE 0.163%), respectively. The evaluation of the frequency and location of ABs gives accurate information on cell death in PIN in comparison with BPH and PAC.

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**Key words:** Prostate gland – Apoptosis – Benign prostatic hyperplasia – Prostatic intraepithelial neoplasia – Invasive adenocarcinoma of the prostate

## Introduction

Prostatic intra-epithelial neoplasia (PIN) is a premalignant lesion affecting prostatic ducts and acini, (Bostwick and Brawer 1987; Kastendieck and Helpap 1989; McNeal and Bostwick 1986). It is defined as proliferation and anaplasia of luminal (or secretory) cells (Bostwick and Brawer 1987) and the diagnosis is based on the subjective evaluation of architectural features (epithelial crowding, stratification and spacing), cytological features (nuclear enlargement and size variability, chromatin pattern, and nucleolar frequency and prominence), together with the associated features of intactness or disruption of the basal cell layer and basement membrane (McNeal and Bostwick 1986). These groups of features have been quantified, thus allowing us to acquire accurate information on PIN and its progression towards the invasive phase of adenocarcinoma (Deschenes and Weidner 1990; Hansen and Ostergard 1990; Helpap 1988; Montironi et al. 1990a, b, 1991, 1992a, b; Patein et al. 1991).

In a previous study we investigated the state of PIN proliferation by evaluating the expression and location of the proliferating cell nuclear antigen (PCNA) (Montironi et al. 1993). Quantitatively, we found that the degree of proliferation increased from PINlow to PINhigh and to prostatic adenocarcinoma. Qualitatively, PCNA immunostaining was confined to the nucleus, with the exception of nuclei with pyknotic chromatin, which were not stained. We assumed that these nuclei belong to the group of apoptotic cells, which are not cycling and therefore not expressing the PCNA antigen. This prompted us to evaluate the occurrence of cell death (apoptosis) in histological sections in PIN as compared with benign prostatic hyperplasia and PAC.

## Materials and methods

The study was performed on 60 prostatectomy/prostatic adenectomy cases included in a previous paper dealing with the expression and location of PCNA (Montironi et al. 1993). The ages of the patients ranged from 60 to 70 years. No patient had received chemotherapy, hormone therapy or radiation therapy before surgery.

The archive material was fixed for 24–48 h in neutral buffered formalin (4%), dehydrated in alcohols (50%, 70%, 90%, 95% twice, 100% twice), cleared in xylene (twice) and embedded in paraffin. Using haematoxylin and eosin stained sections, one of our team (MS) reviewed all the histological slides, controlled the quality of the material and selected the slides for quantitative evaluations. Because prostate lesions were being sought, the slides selected mainly covered the peripheral zone of the prostate. The following morphological patterns of prostate diseases were investigated: BPH (20 cases), PINlow (10 cases) and PINhigh (10 cases), and PAC (5 cases with cribriform pattern or PACcri, 5 with solid/trabecular or PACsolid, 5 with small acinar or PACsmac, and 5 with large acinar or PAClgac) (Mostofi et al. 1980). All the invasive adenocarcinomas were stage B, according to Jewett (1975). Areas with acute and chronic inflammation were carefully avoided. PIN was selected in slides where PAC was not present, so as to avoid the risk of measuring the intraductal spread of adenocarcinomatous cells; in those cases where the PIN grade was not homogeneous, the highest was always selected. Since the prostatic epithelium adjacent to PIN and PAC usually shows a proliferative pattern (Bostwick and Brawer 1987), BPH was selected in cases where PIN and PAC were not present.

Conventional 3 μm-thick histological sections (microtome setting) were cut from formalin-fixed, paraffin-embedded material. Using haematoxylin and eosin-stained sections, the part of the lesion to be analysed was marked with a pen and the sections were evaluated by one of our team using a Leitz Orthoplan microscope equipped with a × 63 objective and an eyepiece graticule (Montironi et al. 1993). The criteria used to identify the apoptotic bodies and to distinguish them from mitotic figures were detailed in a previous publication (Montironi et al. 1988). ABs are round or oval in outline and variable in size, made up of masses of pyknotic chromatin surrounded by narrow cytoplasmic rims. In contrast, mitoses are characterised by the absence of nuclear membrane, absence of a clear zone at the centre, presence of hairy instead of triangular or spiky projections, and basophilia of the surrounding cytoplasm. When several apoptotic fragments were believed to represent the remains of a single cell on the basis of their size and clustering, they were recorded as a single apoptotic cell (Ijiri 1989). Care was taken to distinguish ABs from small intra-epithelial lymphocytes, which showed round nuclei with distinguishable chromatin granules. The percentage of ABs from a minimum of 1000 nuclei per case was calculated separately for each cell layer (see the Results section). The epithelial nuclei in the lumina of ducts, acini and tumour islands whose cell layers were evaluated for ABs were counted separately, subdivided into apoptotic or not, and recorded as the ratio of the number of apoptotic nuclei to the total number of nuclei present in the lumina, multiplied by 100, that is, as percentage of ABs in the lumina. The time needed to analyse each case was approximately 60 min.

The data were stored in an Apple Macintosh II computer. StatView II software was used for the calculation of the category mean and SE, as well as for statistical analyses (Kruskal-Wallis and Mann-Whitney tests; Spearman's rank correlation).

Reproducibility was tested for by duplicate evaluations of the AB-related feature in 6 cases (two of BPH, two of PIN and two of PAC), but no statistically significant differences were found.

## Results

From the histological point of view, ABs were identified in all epithelial cell layers of the ducts, acini and tumour islands as well as in the lumina contained in such structures (Fig. 1, 2). For the epithelial cell layers, ABs were found in general in the intercellular space and occasionally seemed in the cytoplasm of epithelial cells. Even though rare lymphocytes were observed among the epithelial cell layers, they did not appear in close association with ABs. No close AB association was found for typical and atypical mitoses of the epithelial cells (the latter only observed in PINhigh and PAC). ABs recorded in the lumina were occasionally seen in the cytoplasm of macro-

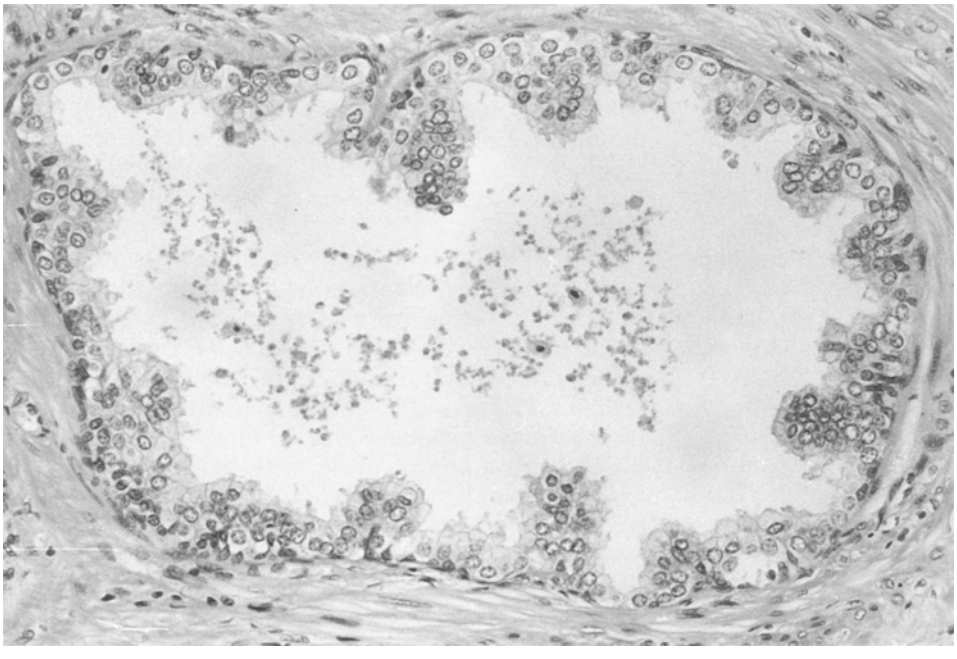

**Fig. 1.** Prostatic intraepithelial neoplasia (PIN) of low grade. Two apoptotic bodies (ABs) are clearly visible in the lumen. Other ABs are observable in the epithelial cell layers. H&E, × 100

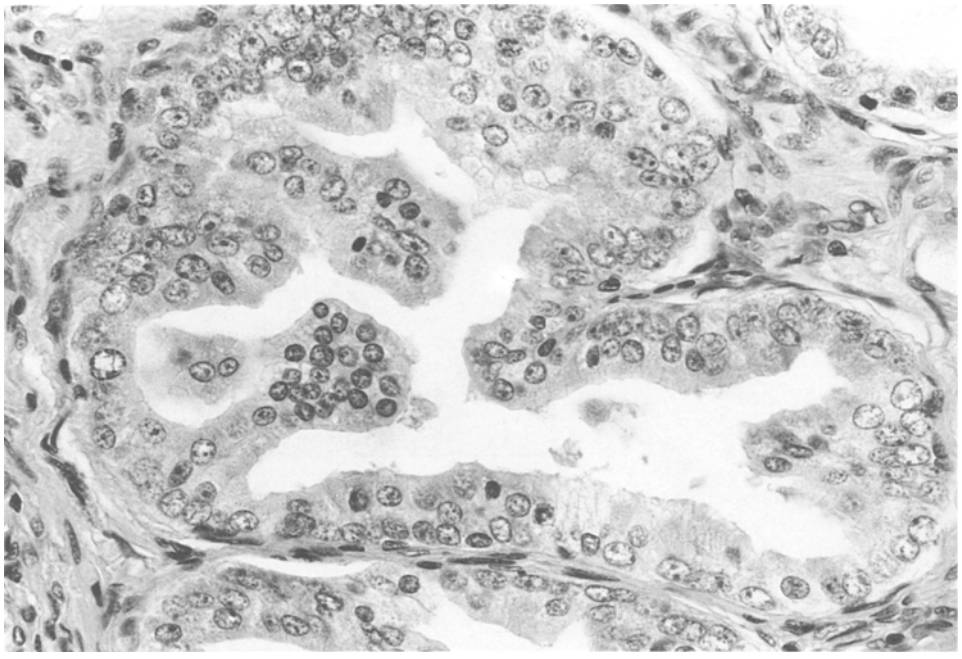

**Fig. 2.** PIN of high grade. ABs are present in all the epithelial cell layers. H&E. × 250

**Table 1.** Apoptotic bodies in piostatic intra-epithelial neoplasia (PIN) and adenocarcinoma

| Comparison | Epithelial component | | Lumen | |
|---|---|---|---|---|
| | No. of cases | *p* value | No. of cases | *p* value |
| Prostatic intraepithelial neopasia (PIN) | | | | |
| – PIN[a] vs BPH | 40 | 0.0001 | 40 | 0.002 |
| – PIN vs PAC[a] | 40 | 0.0004 | 35 | n.s. |
| – PIN low vs PIN high | 20 | n.s.[c] | 20 | n.s. |
| – PIN vs BPH vs PAC[b] | 60 | 0.0001 | 55 | 0.0001 |
| Adenocarcinoma (PAC) | | | | |
| – PAC vs BPH | 40 | 0.0001 | 35 | 0.0001 |
| – PACcri vs PACsolid | 10 | 0.02 | / | / |
| – PACcri vs PACsmac | 10 | n.s. | 10 | 0.05 |
| – PACcri vs PAClgac | 10 | n.s. | 10 | n.s. |
| – PACsolid vs PACsmac | 10 | 0.009 | / | / |
| – PACsolid vs PAClgac | 10 | 0.009 | / | / |
| – PACsmac vs PAClgac | 10 | n.s. | 10 | n.s. |
| – PACcri vs PACsolid vs PACsmac vs PAClgac[b] | 20 | 0.009 | / | / |
| – PACcri vs PACsmac vs PAClgac[b] | / | / | 15 | n.s. |

[a] PIN is considered as a single category, that is, without distinguishing between low and high grade; the same was applied to PAC.
[b] Kruskal-Wallis test; all others employ Mann-Whitney test, [c] n.s. not significant

phage-appearing cells. The frequency of ABs in the epithelial cell layers increased from BPH through PIN up to PAC, the differences being statistically significant for some of the comparisons (Table 1): BPH, mean category value, 0.264% (SE 0.033%); PINlow, 0.679% (SE 0.146%); PINhigh, 0.749% (SE 0.108%); PACcri, 1.282% (SE 0.137%); PACsolid, 2.098% (SE 0.186%); PACsmac, 1.022% (SE 0.1%) and PAClgac, 0.922% (SE 0.163%) (Fig. 3). The frequency of ABs in the lumina also increased from BPH through PIN up to PAC: BPH, 5.41% (SE 4.99%); PINlow, 17.30% (SE 9.95%); PINhigh, 37.70% (SE 12.50%); PACcri, 80.00% (SE 20.00%); PACsmac, 20.66% (SE 7.58%) and PAClgac, 22.66% (SE 6.39%) (Fig. 4). The differences for some of the comparisons were statistically significant (Table 1). There was a significant correlation between epithelial cell layer and lumen AB changes ($r_s = 0.55$, $p = 0.0001$) (Fig. 5).

In BPH, the ducts and acini appeared lined by epithelium made up of two cell types, the basal cell layer and the luminal (secretory) cell layer. ABs were almost exclusively seen in the basal cell layer, whose mean category value was 0.5577% (SE 0.069%). For the luminal cell

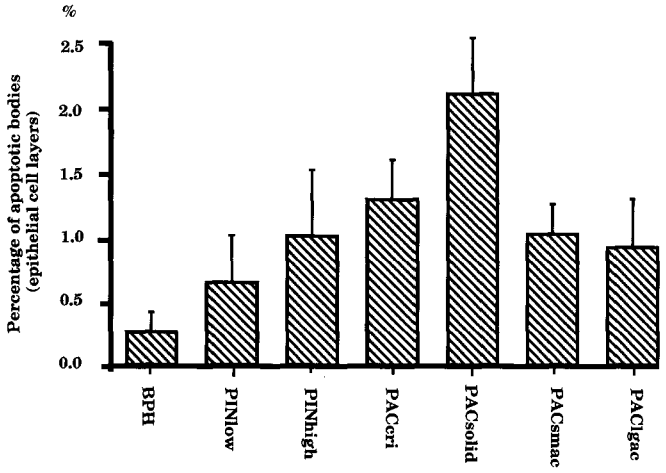


**Fig. 3.** Histogram of the distribution of the ABs of the epithelial cell layers among the diagnostic categories. *Columns* and *bars* represent mean ± SE. The frequency of ABs increases from benign prostatic hyperplasia (BPH) through PINlow and PINhigh up to adenocarcinoma with cribriform and solid/trabecular patterns (PACcri and PACsolid). The percentage of ABs in the small acinar pattern (PACsmac) was quite similar to that of PINhigh and close to that in the large acinar pattern (PACIgac)

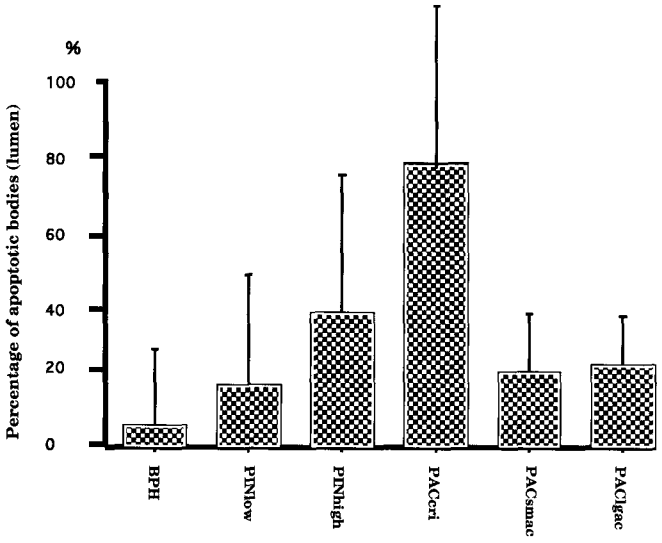


**Fig. 4.** Histogram of the distribution of the ABs of the lumen among the diagnostic categories. *Columns* and *bars* represent mean ± SE. The frequency of ABs increases from BPH through PINlow and PINhigh up to PACcri. The percentage of ABs in PACsmac was similar to that of PINlow and close to that in PACIgac

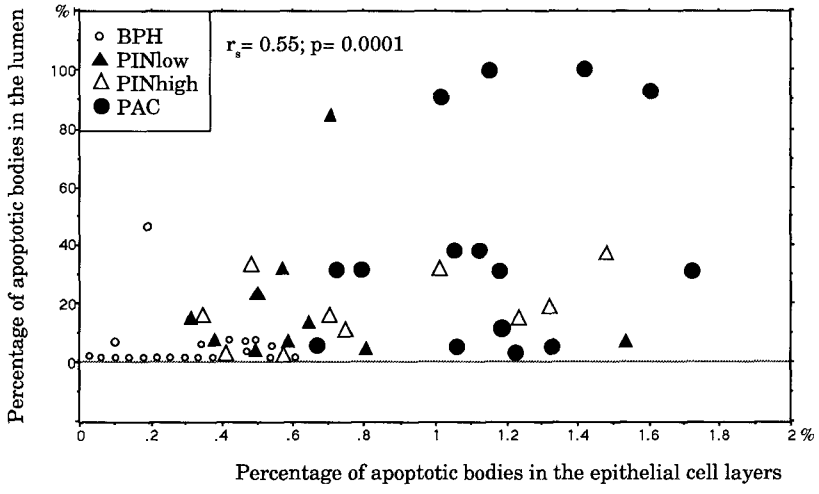


**Fig. 5.** Percentage of ABs in the epithelial cell layers plotted against percentage of ABs in the lumen. The scatterplot shows a good separation between the diagnostic categories (BPH; PINlow and PINhigh; PAC)

layer, ABs were rare, with mean category value of 0.087% (SE 0.039%).

PIN was characterised by the presence of cells appearing stratified and crowded. PINlow included cases that showed morphological features of mild and/or moderate dysplasia, PINhigh showing those of severe dysplasia and/or carcinoma in situ (Montironi et al. 1992b). In both grades the percentage of ABs was counted separately for the cells adjacent to the basement membrane, for the cells bordering the lumen and for the cells in the position intermediate between the basal and the luminal layers. In both, the proportions of ABs decreased from the basal position through the intermediate cell layers to the cell layer bordering the lumen. For PINlow, the mean category values were 0.85% (SE 0.311%) in the basal, 0.623% (SE 0.065%) in the intermediate and 0.474% (SE 0.138%) in the luminal position. For PINhigh, the mean category values were 1.006% (SE 0.16%) in the basal position, 0.713% (SE 0.182%) in the intermediate and 0.618% (SE 0.172%) in the luminal position. In PINlow the percentages were slightly lower than in PINhigh.

For adenocarcinomas with cribriform pattern (large acinar structures filled with epithelial cells forming multiple gland-like lumens), the proportions of ABs, slightly greater than in PINhigh, decreased from the basal position, or adjacent to the stroma, through the intermediate cell layers to the cell layer bordering the lumen. In fact, the mean category values were 1.806% (SE 0.346%) in the basal position, 1.15% (SE 0.172%) in the intermediate and 0.886% (SE 0.137%) in the luminal position. In the solid/trabecular pattern, the percentage of ABs was counted separately for the cells adjacent to the stroma

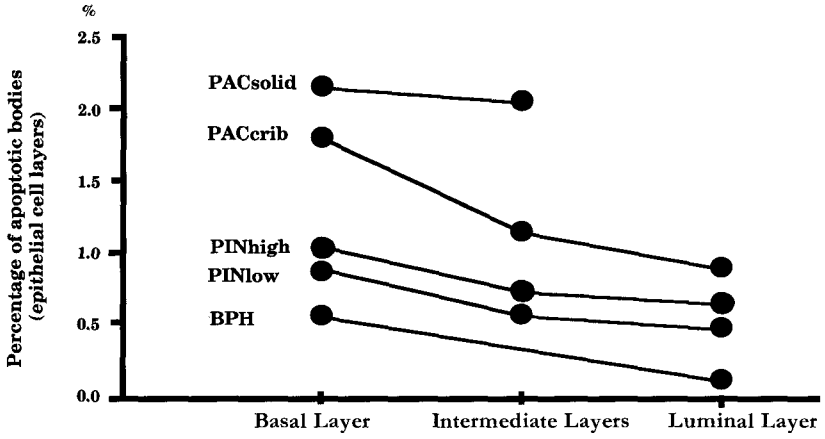


**Fig. 6.** Line chart of the frequency of the ABs in the basal, intermediate and luminal cell layers. The proportions in PINlow and PINhigh are greater than in BPH, the values decreasing from the basal position towards the luminal cell layer. For PINlow, the values are slightly lower than in PINhigh. The percentages in PACcri and PACsolid are higher than in the other categories; as with PIN, the values in the cribriform decrease from the basal towards the luminal cell layer

**Table 2.** Statistical analyses for apoptotic body (AB) frequency by epithelial cell layer

| | Kruskal-Wallis test | Mann-Whitney test *p* value | | |
|---|---|---|---|---|
| | *p* value | basal vs intermediate | basal vs luminal | intermediate vs luminal |
| BPH | / | / | 0.0001 | / |
| PINlow | n.s.[a] | n.s. | n.s. | n.s. |
| PINhigh | n.s. | n.s. | n.s. | n.s. |
| PACcri | 0.04 | 0.02 | n.s. | n.s. |
| PACsolid | / | n.s. | / | / |

[a] n.s. not significant

and for the cells in the other cell layers. The mean category value in the cell layer adjacent to the stroma was 2.154% (SE 0.203%), whereas in the other cell layers it was 2.052% (SE 0.239%). The proportions of ABs were higher than for other adenocarcinoma categories. The percentage of ABs in the small acinar pattern (simple glandular structures much smaller than normal ducts and acini and lined by a single layer of cuboidal cells) was quite similar to that of PINhigh and close to that in the large acinar pattern (glandular structures approximately the same size as, or somewhat smaller than, normal ducts and acini, but without convolutions and lined by a single layer of cuboidal or columnar cells), i.e., 1.022% (SE 0.1%) and 0.922% (SE 0.163%), respectively. Figure 6 shows the AB changes through the cell layers in the different diagnostic categories graphically; the results of the statistical analyses are reported in Table 2. Some comparisons showed statistically significant differences.

## Discussion

Apoptosis has been described in several experimental and spontaneous pathological conditions; it is involved in various types of atrophy. It occurs spontaneously in premalignant and malignant lesions, where it frequently retards growth significantly and is enhanced by radiation, cytotoxic drugs and hormone therapy in both malignant neoplasms and rapidly proliferating normal cell populations. It is seen in cell-mediated immune attack on cells (Clifton Stephens et al. 1991; Kerr et al. 1984; Searle et al. 1975; Wyllie and Morris 1982; Wyllie 1987). Recently, Armas et al. (1993) and Magi Galluzzi et al. (1993) observed that the apoptotic phenomenon in PAC is greatly enhanced by combination endocrine therapy (LHRH agonist and flutamide). To the best of our knowledge there are no published studies on the frequency and location of cell death (apoptosis) in PIN.

The present investigation, which deals with untreated BPH, PIN and PAC lesions, has shown that AB frequency increases from BPH through PIN up to PAC. In particular, the values in PINlow were close to those in BPH, whereas the percentages in PINhigh were close to and partly overlapped with those in PAC. This observation is in agreement with the findings of Columbano et al. (1984) obtained from a sequential study performed to investigate the occurrence of cell death in preneoplastic and neoplastic liver cells. They found that cell death, morphologically similar to that defined in our paper as apoptosis, was evident in foci of preneoplastic hepatocytes of rats 10 weeks after orotic acid feeding; an increased frequency was observed in nodules 20 weeks after starting the dietary regimen, followed by a further frequency increase in hepatocellular carcinomas that developed after 1 year. Moreover, for the preneoplastic and neoplastic lesions of the present research project, the increased frequency of ABs from BPH to PIN and to

PAC parallels that observed with PCNA previously investigated in the same cases (Montironi et al. 1993). However, the AB-related values were approximately an eighth to a tenth of those obtained in the PCNA study.

When considering quantitative analysis of AB location, in BPH ABs were almost exclusively seen in the basal cell layer; very few were counted in the secretory cell layer and in the lumen of the ducts and acini. The fact that ABs have a preferential location in normal tissues has already been observed by some authors in the crypts of the small and large intestine and in the endometrium, mainly in experimental studies (Hopwood and Levison 1976; Ijiri 1989; Kotler et al. 1986; Rotello et al. 1989; Searle et al. 1982) and in human pathological conditions. For instance, Kotler et al. (1986), investigating rectal biopsies from patients with AIDS, observed that focal crypt epithelial cell degeneration (apoptosis) was detectable at or near the crypt base. The same pattern was also found in the small intestine of mice by Searle et al. (1982) 4 h after intraperitoneal injection of cytosine arabinoside. Hopwood and Levison (1976) investigated apoptosis in human endometrial mucosa and reported that most of the ABs lie in the gland close to the base of the endometrium. In these papers the preferential location of ABs was linked to the site of the proliferative compartment, thus pointing to the close relationship between cell renewal and apoptosis.

In PIN, ABs were present in all cell layers, including the luminal, and in the lumina of ducts and acini. However, ABs were mainly located in the layer adjacent to the stroma, the frequency decreasing progressively towards the lumen. A similar pattern was seen in PACcri. The results of the analysis of AB location parallel those related to PCNA previously investigated in the same cases, which showed that PCNA-positive nuclei were present in all cell layers, that the main location was the cell layer adjacent to the stroma and that the frequency decreases towards the lumen, indicating that the width of the proliferative compartment expands from the basal cell layer (as seen in BPH) towards the luminal cell layer, as found in preneoplastic and neoplastic lesions of the prostate. This means that the location of ABs basically corresponds to the extension of the proliferative compartment and to the site of cell proliferation, and may point out the main site of AB production and/or accumulation. No mention of the tissue distribution of such bodies was made in the study by Columbano et al. (1984). Our observations in PAC disagree in part with the findings of Moore et al. (1985) from bronchial and cervical squamous cell carcinomas. They demonstrated that in tumour islands, mitoses are most numerous near the stroma and the blood vessels in the putative stem-cell region, while dead cells predominate in the remote, "older" part. In contrast, del Vecchio et al. (1991) reported that diffusely growing non-Hodgkin's lymphomas do not exhibit such a distinct histo-architectural pattern.

Histologically, some of the ABs were seen in the intercellular space, some in the cytoplasm of epithelial cells and some in the lumen where the bodies appeared in the cytoplasm of macrophages. This morphological relationship of ABs with viable cells observed in our study may indicate how ABs are eliminated, thus confirming the conclusions made by Kerr et al. (1984). This group of authors stated that ABs formed in tissues are dispersed from their site of origin along intercellular spaces; some of those arising in epithelia are extruded into the lumen, but most are phagocytosed and degraded by the adjoining cells; epithelial cells as well as cells of the mononuclear phagocytic system participate in this disposal. In malignant neoplasms many of the bodies are taken up and digested by surrounding neoplastic cells. Savill et al. (1989) clearly showed and demonstrated how macrophages present in the synovial cavity ingested apoptotic cells, mainly neutrophils, in acutely inflamed joints.

We do not know the precise causes of the increasing occurrence of apoptosis in PIN and PAC. However, the phenomenon may arise from several reasons, such as intrinsic mechanisms of growth control, abnormal cell division, abnormalities in DNA content, and immune attack. The apoptotic phenomenon may be linked in part to abnormally dividing epithelial cells in PIN and PAC, thus resulting in the formation of ABs (Colombel et al. 1992; El-Labban and Osorio-Herrera 1986). El-Labban and Osorio-Herrera (1986) pointed out that the mechanisms involved in the formation of ABs in squamous cell carcinoma were either that the microtubules forming the mitotic spindle were not correctly assembled or that the microtubules found represent only part of and not the whole spindle, as found in calcemid-treated human D98/AH2 cells containing calcemid-resistant chromosomal microtubules, or obtained with microtubule-disrupting drugs in cultured human lymphoma cells (Crenshaw et al. 1981; Takano et al. 1993). Colombel et al. (1992) identified a defective cell cycle leading to apoptosis in the prostate gland experimentally. In particular, they observed that high expression of mRNA encoding p53 during the cell cycle after active re-entry of androgen-deprived prostatic epithelial cells into the cell cycle initiated by testosterone replenishment can divert this cycle into apoptosis. Alternatively, the apoptotic phenomenon could also be one way to eliminate genetically aberrant cells or cells with serious abnormalities in DNA content, resulting from either abnormal mitotic division or genotoxicity due to an unknown agent (Falkvoll 1990; Ijiri 1989; Kerr et al. 1984; Potten 1977; Wargovich et al. 1983). Nuclear aberrations with consequent apoptosis were experimentally induced into mice by several authors (Ijiri 1989; Wargovich et al. 1983). We cannot excluded the possibility that cell death in PIN and PAC may also be related to an immunological attack (Savill et al. 1989).

In conclusion, the evaluation of the frequency and location of ABs gives accurate information on the apoptotic phenomenon in PIN in comparison with BPH and PAC. Its occurrence may indicate that the development of PIN and its progression to PAC could be associated with the presence of abnormally dividing cells and of cells with acquired DNA abnormalities.

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