Progressive Increase of Apoptosis in Prostatic Intraepithelial Neoplasia and Carcinoma

Comparison Between In Situ End-labeling of Fragmented DNA and Detection by Routine Hematoxylin-eosin Staining

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- **Objective.**—Apoptosis has attracted significant attention in the study of tumors during recent years. The first goal of this study was to evaluate the number of apoptotic cells and bodies in benign glands, in high-grade prostatic intraepithelial neoplasia, and in malignant prostatic glands. The second objective was to compare the effectiveness of in situ end-labeling of fragmented DNA (ISEL) with the use of routine hematoxylin-eosin (H&E) stains in the assessment of apoptosis rates.

**Methods.**—The percentage of apoptosis was measured with ISEL and H&E stains in sections from 16 prostatectomies performed for previously untreated peripheral prostatic adenocarcinomas.

**Results.**—Both methods showed progressive increase of the rates of apoptosis from benign glands (0.34% to 0.38%), to high-grade prostatic intraepithelial neoplasia (1.44% to 1.39%), to carcinoma (2.69% to 2.75%). The increase in apoptosis rate in prostatic intraepithelial neoplasia and carcinomas is one more indication of the continuum in the pathogenetic process leading to invasive prostatic carcinoma. Student’s t test revealed no statistically significant difference in the percentage of apoptosis rendered by ISEL and H&E staining.

**Conclusions.**—From a practical point of view, evaluation of apoptosis with H&E stains can be readily performed using routine clinical material. The procedure is inexpensive, and it gives good tissue morphology. However, quantitative measurements may be time-consuming and observer-dependent. The apoptotic bodies are clearly identifiable with ISEL, making quantitation easy and even amenable to automated counting methods. Disadvantages of ISEL are significantly higher costs and poor tissue morphology. We conclude that accurate evaluation of apoptosis may be performed reliably with both routine H&E staining and the ISEL method. The decision to choose one method over the other depends on the economic resources available and the amount of material to be evaluated.

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Only a small proportion of the large number of prostatic adenocarcinomas diagnosed every year will behave in an aggressive manner. The small or quiescent carcinomas, which are amenable to earlier diagnosis, represent a significant therapeutic dilemma. Studying the elements related to tumor biology would help the effort to separate aggressive tumors from indolent ones. Gleason’s grading system, which is based on patterns of histologic tumor differentiation, is the most accurate tool available for predicting the rate of prostatic cancer progression and determining the prognosis. In addition to the degree of differentiation, the biologic behavior of a tumor and its metastatic potential are directly related to its size, which generally reflects the tumor growth rate. The latter is determined by the ratio between tumor cell proliferation and tumor cell loss.

The patterns of cell death within the microenvironment of growing tumors are important because they express fundamental properties of tumor biology and therefore may relate to prognosis. Extensive studies in recent years have shown the two main mechanisms of cell loss in growing tumors (and tissues in general) to be apoptosis and necrosis. Necrosis occurs in confluent zones within tumors and results from nonphysiologic perturbations of the cellular environment (eg, ischemia). Apoptosis, on the other hand, is a programmed process of active cellular self-destruction, which requires the expression of a number of genes and usually affects single cells surrounded by viable neighbors.

See also p 83.

The light microscopic evaluation of apoptosis is impaired by the fact that the potentially visible process may last a relatively short time (minutes to hours). Morphologic evaluation is impaired by the resemblance of the apoptotic nuclear fragments to pyknotic lymphocytes or fragments of polymorphonuclear leukocytes.

Apoptosis can be biochemically confirmed by the demonstration of chromatin cleavage in agarose gel electrophoresis. The genomic DNA extraction and analysis method, which shows the well-known "ladder" of nucleosomal DNA fragments, does not, however, allow for the identification of individual apoptotic cells. In contrast, by

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using in situ end-labeling of the DNA fragments on tissue sections, early stages of apoptosis, which presumably are not detectable by routine hematoxylin-eosin (H&E) stains, can be identified against the viable background.11,12

Previous studies of apoptosis in human prostatic carcinomas using routine H&E sections showed a positive correlation between apoptotic bodies and Gleason grade. Other investigators have also demonstrated a progressive increase in the amounts of apoptosis in prostatic intraepithelial neoplasia (PIN) on routine H&E sections.12,14

Based on these factors, the purpose of our study was twofold: (1) to determine the frequency of apoptotic cells in benign glands, high-grade prostatic intraepithelial neoplasia, and malignant prostatic glands and (2) to compare the use of routine H&E sections with in situ end-labeling of fragmented DNA (ISEL) in the evaluation of apoptosis in prostatic pathology.

Concordance between ISEL and H&E staining has been demonstrated previously in the evaluation of experimentally induced apoptosis in the liver; in some cases, H&E staining appeared to be more sensitive in detecting apoptotic nuclei (1% vs 0.4%).19

**MATERIALS AND METHODS**

Histologic material from 16 prostatectomies for stage B (10 cases) and C (6 cases) peripheral prostatic adenocarcinoma was studied. Carcinomas with patterns of growth of 2 and/or 3 by the Gleason's scheme were chosen. The patients had not received previous chemotherapy, hormone therapy, or radiation therapy. The ages of the patients ranged from 62 to 73 years. The tissue was fixed in 10% neutral-buffered formalin and routinely embedded in paraffin. Four-micrometer sections from two blocks from each case were stained with H&E and with the ApopTag in situ apoptosis detection peroxidase kit (Oncor, Gaithersburg, Md). This kit labels the 3'-OH DNA ends created by DNA fragmentation.

After deparaffinization, the tissue sections were digested with proteinase K (Oncor, 20 µg/mL) for 15 minutes at room temperature. Subsequently, the endogenous peroxidase was quenched by treating the sections with 2% hydrogen peroxidase in phosphate-buffered saline for 5 minutes.

Application of terminal deoxynucleotidyl transferase followed, so as to bind residues of digoxigenin-nucleotide (UTP) to the 3'-OH ends of single or double-stranded DNA.

Antidigoxigenin conjugated peroxidase was then applied to bind to the heteropolymer of digoxigenin-11-DUTP and dATP. Diaminobenzidine was used as a substrate chromogen to visualize the apoptotic nuclei and bodies.

The tissue sections were counterstained with methyl green-pyronin. Between each step, the sections were rinsed with phosphate-buffered saline at a pH of 7.46 for 5 minutes.

Morphologic criteria for apoptosis were based on previously published studies. Briefly, in H&E-stained sections apoptotic bodies and cells consist of condensed cells with contracted cytoplasm and hyperchromatic pyknotic nuclei, dense chromatin fragments, and/or cellular fragments (Fig 1). The apoptotic cells and bodies are usually surrounded by a clear halo. Only structures with unequivocal features of apoptosis were counted.

On ISEL stains, only well-defined and darkly stained apoptotic cells and bodies were counted. Apoptotic fragments identified in the glandular lumina or in the stroma were not counted.

From each case, four areas of carcinoma and four areas of benign glands without any preservation or fixation artifacts were selected. The benign areas included hyperplastic glands (20% to 70% of the total surface). In addition, 12 areas of high-grade PIN from 10 cases were chosen. The studied areas were all separate from each other. In each area, 1000 cells were evaluated by one pathologist using a ×40 objective, and the percentage of apoptosis was recorded.

**RESULTS**

Marked variability was observed from area to area and from case to case. Figure 1 depicts typical examples of the morphology of apoptosis on H&E stain. Apoptotic cells and apoptotic bodies (fragments) appeared dark brown with the ISEL stains (Figs 2, a and 3, a). Apoptotic fragments were occasionally seen in the lumen of glands (Fig 2, a). A clear halo was often observed around the darkly stained apoptotic cells or fragments, similar to that seen on H&E stains (Fig 3, a and b).

Benign prostate glands displayed minimal amounts of apoptosis overall, with focal areas of increased values in glands with hyperplastic changes. The mean values in benign glands were 0.34% (SD 0.26) on H&E stains and 0.38% (SD 0.25) with the ISEL stains (range 0.025% to 1% in both).

In glands with high-grade PIN, the mean number of apoptotic cells was 1.44% (SD 0.31) on H&E stains and 1.39% (SD 0.32) with the ISEL stains (range 0.8% to 2% for H&E and 0.7% to 2.1% for ISEL) (Fig 2). In benign and dysplastic glands, apoptosis was predominantly localized in the basal layers; however, occasional dysplastic glands showed luminal clusters of apoptotic cells on ISEL stains.

The amount of apoptosis in carcinomas showed the greatest degree of variability from area to area and from tumor to tumor. The mean value was 2.75% (SD 0.86) on H&E stains and 2.69% (SD 1.07) with the ISEL stains (range 0.5% to 10% for H&E and 0.5% to 9% for ISEL stains) (Fig 3, a and b).

Carcinomas with patterns 2 and 3A (infiltrating discrete acini) generally showed lesser amounts of apoptosis (mean 2.6% in H&E and 2.9% in ISEL stains) than the cribriform pattern 3B (mean 3.2% in H&E and 3.0% in ISEL stains).

Occasional apoptotic cells in the interglandular stroma were also seen; this was more marked in areas of chronic prostatitis.

Student's t test demonstrated no statistically significant difference between the two staining methods (P = 0.3). The rate of increase in apoptosis from benign, to PIN, to and malignant glands with both methods was statistically highly significant by analysis of variance (P < .0001). To test reproducibility, evaluations were repeated in four cases by a second pathologist, and no statistically signifi-
cant differences were found with either method ($p = .05$, t test).

**COMMENT**

Researchers have found the rate of cell loss in growing tumors to be considerable. Particularly, tumors with high cell-proliferation rates are characterized by increased cell death. This is also noted in proliferating benign tissues, such as lymph node germinal centers. Accordingly, high degrees of apoptosis have been observed in rapidly growing tumors known to respond to chemotherapy (eg, small cell carcinoma, Ewing's sarcoma, and Burkitt's lymphoma), whereas low apoptosis rates were seen in more indolent neoplasms, such as follicular carcinoma of the thyroid and follicular lymphomas.

This finding appears at first to be paradoxical, since an increase in the apoptosis rate would conceivably result in a decrease in the rate of tumor growth. It is possible that the positive correlation with the traditional measurement of tumor aggressiveness relates to the multiple regulatory mechanisms that can affect apoptosis. The latter include oncogenes (eg, bcl-2, myc, ras, and human papillomavirus), ischemia, radiation, and chemotherapy.

Studies performed on multiple tumor types indicate that these differ in their inherent susceptibility to apoptosis, and that often an inverse relationship exists between apoptosis and necrosis. The latter observation was explained by the hypothesis that the intrinsic cellular susceptibility to apoptosis may also determine to some degree the probability of necrosis. According to this theory, cells primed for apoptosis are liable to die following this pattern in several types of adverse conditions (eg, mild ischemia). Tumors with less propensity for apoptosis have a rapid growth and eventually necrosis will be the mode of death when the tumor growth exceeds the blood supply. In prostate cancer the relationship of apoptosis and necrosis does not follow this general model. Presence of necrosis is a feature of the highest Gleason's pattern of growth.

Fig 3.—Prostatic adenocarcinoma stained with routine hematoxylin-eosin stain (A) and with the DNA fragment end-labeling method (B). Comparable numbers of apoptotic bodies are observed (original magnification ×400).

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The second goal of our study was to compare the use of routine H&E stains with the ISEL method. The Table provides a summary of the advantages and disadvantages of each method. Our findings indicate that although ISEL is supposed to detect all phases of apoptosis, including those that are not morphologically evident, there was no statistically significant difference in the number of apoptotic bodies and cells identified by the two methods.

Advantages and disadvantages are associated with each of the various methods used for the quantification of apoptosis. Although it is possible to demonstrate very low levels of DNA fragmentation in tissue extracts using special techniques, the "single cell" sensitivity of the method ISEL in tissue sections represents a significant advantage. Poor morphology and high costs could argue against using the ISEL method, particularly for routine diagnostic purposes. Also, it is necessary to keep in mind that necrotic-appearing cells can contain a sufficient amount of DNA fragments to cause false-negative staining with the ISEL method.

With the ISEL method the evaluation of apoptotic cells and bodies can be performed faster; this rapid examination is not possible with the H&E stains, which require high-power examination to identify each individual apoptotic body with confidence. Furthermore, on routine stains apoptotic cells should be distinguished from lymphocytes, fragments of inflammatory cells, and mitoses. In favor of evaluating apoptosis on H&E stains are the low cost and the fact that the morphologic features can be evaluated better in this context.

In summary, this study further establishes the clear association between progression to malignancy in the prostate and an increase in apoptotic rates. Although multiple studies have determined the significance of apoptosis in the evaluation of tumors, clinical correlation and longer follow-up of patients is necessary to determine if the rates of apoptosis have value as independent prognostic factors in prostatic carcinoma.

We conclude that in the evaluation of apoptosis rates the routine H&E stain and the ISEL method are comparable. The main disadvantages of DNA fragment labeling are related to tissue morphology and cost; conversely, the interpretation of these stains is easy and large tissue sections can be evaluated semiquantitatively faster. Apoptotic bodies and cells can be identified on H&E stains with considerable certainty; this is the method of choice in diagnostic settings. For research purposes, however, the evaluation of apoptosis in H&E stains may be extremely time-consuming since only high-power evaluation is acceptable, and the quantitation depends on the observer's subjectivity and experience. The decision to select one method over the other depends on the economic resources available and the amount of material to be evaluated.

References

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