Influence of chronic inflammation on Bcl-2 and PCNA expression in prostate needle biopsy specimens

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Abstract. The association between inflammation and cancer has been established in certain forms of human malignancies; however, its role in prostate cancer remains unclear. The present study investigates a possible association between chronic inflammation and the development of epithelial neoplasia in the prostate. Needle biopsy specimens were obtained from patients with serum prostate-specific antigen levels >4 ng/ml, evaluated for morphological findings, and immunostained for Bcl-2 and proliferating cell nuclear antigen (PCNA). Bcl-2 is a survival protein that appears to lie at a nodal point in pathways involved in cell survival, carcinogenesis, and development of therapeutic resistance in certain cancer types. Similarly, PCNA is a critical protein for DNA replication, repair of DNA damage, chromatin structure maintenance, chromosome segregation and cell-cycle progression. The association between these two proteins was examined in prostate tissues with and without chronic inflammation, as well as tissues with and without evidence of neoplastic changes. Of the 106 needle biopsies examined, 18% exhibited atrophy with inflammation. Proliferative inflammatory atrophy/post-atrophic hyperplasia were observed in 42%, high-grade prostatic intraepithelial neoplasia (HGPIN) in 8%, prostatic adenocarcinoma in 11%, and 2% had atypical acinar proliferation suspicious for malignancy. A total of 36 specimens were stained for Bcl-2 and PCNA. Bcl-2 was expressed widely in inflammatory and epithelial tissue; however, more intense expression was observed in the areas of chronic inflammation, predominantly in infiltrating immune cells. The highest proliferation index was observed in the epithelia of HGPIN and cancer. An inverse correlation between the expression of Bcl-2 and the expression of PCNA was observed in the epithelium. The areas of chronic inflammation were associated with increased Bcl-2 expression, whereas the highly proliferative epithelium minimally expressed Bcl-2. These results suggest that Bcl-2 alters the phenotype of particular epithelial cells with a gain in neoplastic characteristics, leading to a likely precursor that may later progress into HGPIN and cancer.

Introduction

Persistent chronic inflammation has emerged as a potential risk factor in the development of carcinoma of several organ types, including liver, large bowel, urinary bladder, gastric mucosa, lung, colon and pancreas (1-3). Experimental evidence suggests that longstanding chronic inflammation promotes prostate carcinogenesis (4,5). In fact, a positive link between clinical history of prostatitis and increased relative risk of prostate cancer has been documented among 70,000 North American men (6). Chronic inflammation is frequently identified in prostate biopsies, radical prostatectomy specimens and tissue resected from benign prostatic hyperplasia (7,8). A number of possible etiological factors have been proposed in the development of chronic inflammation in the prostate, including dietary imbalances, exposure to environmental pollutants, alterations in testosteron:estrogen ratio, pathogen infection, race or genetic alterations (9-12). The resulting microenvironment, characterized by the accumulation of various types of immune cells, generates reactive oxygen and nitrogen species, inducing oxidative stress in proliferating epithelium that may interact with DNA to generate permanent genomic alterations (13). Extensive research efforts have been undertaken to investigate a possible link between inflammation and the development of prostate cancer (14,15). We previously reported the effect of
chronic inflammation on prostate carcinogenesis in a 5-year follow-up study of needle biopsy specimens from 177 patients with clinical parameters suspicious for malignancy. In follow-up biopsies over a 5-year period, 20% of patients with only chronic inflammation on initial biopsy exhibited a new diagnosis of adenocarcinoma, and an additional 6% exhibited high-grade prostatic intraepithelial neoplasia (HGPIN) lesions; by contrast, among patients initially showing no inflammation, only 6% subsequently developed cancer (16).

In recent years, an injury-and-regeneration model has been proposed suggesting that repeated bouts of injury to the prostatic epithelium, presumably as a result of inflammation in response to pathogens or autoimmune disease and/or dietary factors, results in proliferation of epithelial cells that possess a phenotype intermediate between basal cells and mature luminal cells (17). These alterations may result in the modulation of a delicate balance between cell proliferation and cell death. A defect in the normal regulation of programmed cell death by a subset of cells that are able to survive and continue to proliferate may favor the acquisition of further genetic alterations that would promote the development of malignancy. A current hypothesis is that morphological changes in acinar or ductal epithelia result from the continuous influence of inflammation (18). These findings are characterized as HGPIN, a lesion that is a putative precursor of prostatic adenocarcinoma. A pathological entity of chronic inflammation accompanied by focal glandular atrophy and epithelial cell proliferation, termed proliferative inflammatory atrophy (PIA) and post-atrophic hyperplasia (PAH), has been recognized (19,20). PIAs are atrrophic lesions frequently observed in the peripheral zone of the prostate, occasionally adjacent to the foci of HGPIN and cancer (19). It has been hypothesized that the atrophic epithelial cells in PIA lesions are the targets of neoplastic transformation and potentially give rise to carcinoma. PAH has been shown to be closely associated with persistent chronic inflammation (21). PAH represents ‘post-inflammatory’ atrrophic lesions. It is hypothesized that proliferative regeneration of secretory cells following disruption of the epithelium may generate small, hyperplastic glands arranged in a lobular configuration, which is a typical feature of PAH. PAH is occasionally confused with carcinoma due to its overlapping architectural and nuclear features. In particular, these lesions are predominately composed of small crowded glands, usually surrounded by centrally situated and dilated large ducts, irregular and atrophic-appearing contours lined by flattened-to-cuboidal epithelial cells, with nuclear enlargement and prominent nucleoli (21,22). PIA/PAH lesions have been reported to show increased proliferative activity compared with benign prostatic epithelium and simple atrophy (SA) (23). The signs as well as other morphological evidence may suggest the transition from a PIA/PAH lesion to the development of HGPIN; however, the molecular evidence for cell survival and proliferation under the influence of chronic inflammation is lacking. To gain knowledge in this area, the present study examined the morphology of 106 needle-biopsy specimens. Immunohistochemistry was used to investigate the association between the expression of Bcl-2, a cell survival protein that plays a role in apoptosis by blocking programmed cell death (24), and the expression of proliferating cell nuclear antigen (PCNA), a proliferation marker (25). The results suggest an inverse correlation between the expression of Bcl-2 and that of PCNA in epithelial cells under the influence of chronic inflammation. Since, in our opinion, PAH represents the end result of longstanding PIA, these morphological lesions were grouped together and designated as PIA/PAH lesions for the sake of the present analysis.

Materials and methods

Prostate biopsy material. The study was performed in formalin-fixed, paraffin-embedded prostate biopsy specimens obtained between August 2001 to June 2003 from male patients aged >40 years who had serum prostate-specific antigen levels of >4 ng/ml. Needle biopsies were performed at the University Hospitals Cleveland Medical Center (Cleveland, OH, USA). Specimens from all ethnic groups were included. The study was approved by the Institutional Review Board (08-03-38) at the University Hospitals Cleveland Medical Center.

Characterization of extent and intensity of inflammatory findings. All specimens were examined by two expert urological pathologists (QR and GTM) for the presence and distribution of chronic inflammatory infiltrate, which was assigned a score of 1 (mild), 2 (moderate), 3 (strong) or 4 (very strong), as previously reported (16). The degree of inflammation was assigned according to the approximate percentage of stroma and glands in the biopsy specimen involved by aggregates of ≥50 lymphocytes, or by granulomatous inflammation on a scale of mild inflammation (1-10% involvement), moderate inflammation (11-50% involvement) and severe/strong inflammation (>50% involvement). A total of 106 hematoxylin and eosin-stained needle-biopsy specimens were examined.

Classification of prostatic lesions. Prostatic needle-biopsy specimens were further evaluated and categorized for foci of SA, PIA and/or PAH, HGPIN, and cancer. The morphology of these lesions and the criteria for their diagnosis are well-established, and readily found in standard journals of surgical pathology (26,27).

Immunohistochemistry. Immunohistochemical staining for Bcl-2 and PCNA was performed in 36 needle-biopsy specimens previously categorized in groups as SA, PIA/PAH, HGPIN and cancer, using standard techniques. Paraffin-embedded prostate needle-biopsy specimens were cut at a 4-µm thickness and the sections were incubated with target retrieval solution (cat. no. S2367; Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) in a steamer for 45 min. The sections were permeabilized in a solution containing 100 mM Tris (pH 7.5), 150 mM NaCl, 0.5% blocking agent, 0.3% Triton X and 0.2% saponin for 20 min at room temperature. Sections were incubated overnight in a humid chamber at 4°C with antibodies against Bcl-2 (cat. no. M0887; Dako; Agilent Technologies, Inc.) at 1:50 dilution. Control sections were incubated with antiserum in the presence of 10-fold excess of these antibodies or with isotype-matched IgG (cat. no. sc-516102; Santa Cruz Biotechnology Inc., Dallas, TX) in normal goat serum (cat. no. 927502; BioLegend, Inc., San Diego, CA) for 30 min at room temperature. Subsequent to washing three times in TBS, detection was achieved using standard HRP-labeled LSAB2 system (cat. no. K0609; Dako; Agilent Technologies,
Inc.), with 3-3-diaminobenzidine as the chromogen. For PCNA (cat. no. sc-71858; Santa Cruz Biotechnology, Inc.), the immunoreactive complexes were detected using AEC+ substrate-chromogen from the Dako EnVision+ System (cat. no. K4002; Dako; Agilent Technologies, Inc.), consisting of 3-amino-9-ethylcarbazole as the chromogen. Slides were then counterstained in Mayer's hematoxylin, mounted in crystal mount medium, and dried overnight on a level surface, as previously described (28).

**Scoring of stained cells.** The immunostained sections were examined independently by the pathologist (QR) and co-investigators (MG and SS) using light microscopy. Sections were viewed under an Olympus BH2 inverted microscope (Olympus Corporation, Tokyo, Japan) and images were acquired with Image-Pro Plus v.7 software (Media Cybernetics Rockville, MD, USA) and digitally stored on a computer. The intensity of immunohistochemical staining for Bcl-2 was scored as 0 (negative), 1 (weak), 2 (moderate) or 3 (intense). Bcl-2 staining was heterogeneous, exhibiting areas with intense staining and other areas with moderate-to-weak staining. Because the majority of the observed lesions were heterogeneous, the overall score assigned represented the average for the entire lesion. The PCNA staining was scored by counting the positively stained cells and total number of cells, quantified in random microscopic field with a minimum 500-cell count (magnification, ×40) with the assistance of a software program (Olympus Microsuite v.5; Olympus America Inc. Center Valley, PA). A minimum of four screenshots per specimen, representing individual microscopic fields, were counted for determining the tissue staining distribution.

**Statistical analysis.** Statistical analysis was performed using Kruskal-Wallis test, a nonparametric test based on Wilcoxon scores, to examine the median differences in Bcl-2 and PCNA among the four subcategories of tissues (SA, PIA/PAH, HGPIN and cancer), followed by pair-wise comparison of any two tissue types where the P-value was not adjusted for multiple comparisons. The association between various grades of inflammation (mild, moderate, strong and very strong) with Bcl-2 and PCNA was also estimated using Spearman correlation coefficient. All tests were two-sided and P<0.05 was considered to indicate a statistically significant difference.

**Results**

As shown in Table I, of the 106 specimens examined, 20% (20/106) showed unremarkable prostatic tissue, 8% (8/106) of specimens exhibited SA, whereas 42% (45/106) of cases showed PIA/PAH lesions. HGPIN was observed in 8% (8/106) of specimens, and adenocarcinoma was diagnosed in 11% (12/106) of specimens. Additionally, 2% (2/106) of the specimens exhibited atypical small acinar proliferations, suspicious for, but not diagnostic of, malignancy. Representative images of these lesions observed from needle biopsies are shown in Fig. 1A-D.

**Bcl-2 protein expression.** A total of 36 needle biopsy specimens that contained areas of inflammation were randomly selected for equivalent portions of strong/very strong, moderate or mild/absent inflammatory infiltrates detected in the biopsy core for various lesions and stained for Bcl-2 by immunohistochemistry (Fig. 2A-D). The samples were analyzed for the percentage of cells expressing Bcl-2 as well as intensity of expression. The average intensity of the stain was graded from 0 (no expression) to 3 (strong expression). In these samples, basal epithelial cells exhibited prominent Bcl-2 staining, which was noted to be in close proximity to the inflammatory lesions, with 77.1% of basal epithelial cells showing strong expression. However, negative-to-weaker Bcl-2 expression was noted in the majority of luminal epithelial cells (>90%), with heterogeneous staining pattern in the normal/benign areas. Average epithelial cell stain intensity was in the low-medium range (mean, 1.67). The immune cells in the specimens also expressed Bcl-2, but only 38.2% exhibited immunoreactivity to this antigen. The immune cells expressing Bcl-2 exhibited a higher staining intensity (mean, 2.04) than the epithelial cells. Cytoplasmic Bcl-2 expression was almost exclusively observed amongst epithelial cells, whereas immune cells were observed to have extensive Bcl-2 expression within the nucleus. Only 3.7% of inflammatory cells and epithelial cells in areas adjacent to but not involved by inflammation showed Bcl-2 expression, with an average staining intensity of 0.95. Compared to SA (7.0%), the highest percentage (17.4%) of Bcl-2-positive cells was observed in PIA/PAH lesions (P<0.0001), followed by HGPIN (10.1%) and cancer lesions (9.2%), respectively (Fig. 3A).

**PCNA protein expression.** The staining for the proliferative marker PCNA was also examined in the prostate needle-biopsy specimens (Fig. 2A-D). In normal/benign glands, PCNA expression was more often noted in the basal epithelial cells, than in the luminal epithelial cells. The proliferation index analyzed from numerous microscopic fields revealed a progressive increase in proliferation index in epithelial cells from SA to cancer. Compared to SA (2.8%), a significant increase in PCNA was observed in PIA/PAH (4.4%), HGPIN (8.1%) and cancer (17.6%) (P<0.0001), respectively (Fig. 3B).

**Relationship between Bcl-2 and PCNA expression.** A negative correlation was observed within the serial specimens stained for PCNA and Bcl-2. Cells expressing Bcl-2 were observed to
have low levels or an absence of PCNA expression. A similar association was observed in samples that exhibited high PCNA expression, wherein little-to-no Bcl-2 expression was detected. PCNA stainings was observed within the nucleus among epithelial cells, as compared with the predominant cytoplasmic staining observed in epithelial cells stained for Bcl-2. Immune infiltrate cells did not express high levels of PCNA as compared with the Bcl-2 expression (Fig. 4A and B).

Relationship between inflammation, cell survival and neoplastic progression. Subsequently, the correlation between inflammation, cell survival and neoplastic progression was assessed with regard to the Bcl-2 and PCNA scoring by a pathologist. (Fig. 5). As shown in Fig. 5A, a strong positive association between inflammation and Bcl-2 expression was observed (r=0.88; P<0.0001) suggesting that cell survival increases with the increasing inflammation. A negative association (r=-0.197; P=0.249) between inflammation and proliferation was identified (Fig. 5B), suggesting that prolonged inflammation facilitates the initiation of normal prostate epithelial cells through increased survival, which then gain independence to proliferate and progress to malignancy.
Discussion

The purpose of the present study was to determine the association between epithelial cell survival and proliferation under the influence of chronic inflammation, and whether molecular changes may be an indicator of neoplastic progression. Prostate carcinogenesis is a continuous process with distinct stages of atrophy, hyperplasia, prostatic intraepithelial neoplasia and carcinoma, which can be identified on the basis of cellular morphology and topographical features (18-23). During the initiation of prostate cancer, most of the proliferation occurs in the luminal compartment rather than in the basal compartment (18-20). This shift is present in most of the carcinoma precursor lesions, including HGPIN. It has long been hypothesized that PIA may be a precursor of prostate cancer, as several studies have reported a morphological transition from PIA to HGPIN (18,21). This suggestion is based on the following findings: i) Morphological overlap between PIA and HGPIN; ii) the phenotypes of many of the cells in PIA are most consistent with that of immature secretory-type cells, similar to that of the cells of HGPIN; and iii) PIA, HGPIN and carcinoma all occur with high prevalence in the peripheral zone and low prevalence in the central zone of the prostate. In the present study on needle-biopsy specimens, a close topographic...
association between PIA/PAH and HGPIN was observed, in agreement with the findings in previous studies (18,21).

As PIA is predominantly associated with inflammation, we speculated whether chronic inflammation may lead to neoplastic progression. It has been postulated that, under the influence of chronic inflammation, the proliferating epithelial cells of the prostate tend to adapt to the altered microenvironment by altering the delicate balance between cell death and proliferation (29). Chronic inflammation with the accumulation of lymphocyte infiltrate in the prostate may lead to repeated tissue damage and regeneration, with release or increased expression of cytokines, growth factors, and oncogenes, leading to a highly neoplastic state (30,31). This has been observed by a marked increase in the Bcl-2 protein, a marker of cell survival, in PIA/PAH lesions by our group and several other investigators (32,33). Based on immunohistochemical staining, the present study revealed that Bcl-2 is upregulated in epithelial cells in areas of chronic inflammation. The Bcl-2 protein is a product of the BCL2 gene; it is a powerful inhibitor of apoptotic cell death and may lead to malignant transformation and tumor formation (34,35). Bcl-2 expression in prostate cancer is associated with the androgen-independent phenotype (36). High expression of Bcl-2 was also noted in the infiltrating immune cells in the present study. Further studies by De Marzo et al (37) have shown that protection from repeated oxidative or electrophilic DNA damage is achieved in PIA/PAH lesions by increased expression of glutathione S-transferase pi (GSTP1), which catalyzes the detoxification of reactive electrophiles and oxidants and therefore can have a protective effect against neoplastic transformation; by contrast, this does not occur in HGPIN and carcinoma (37). Further recent studies have shown that GSTP1 CpG island hypermethylation changes occur in a proportion of PIA/PAH lesions, providing evidence that at least some of these lesions harbor cells already initiated to progress to HGPIN and/or prostate adenocarcinoma (38,39).

The rate of proliferation observed in the PIA lesion in the present study is comparatively less than that of HGPIN and cancer, suggesting that the majority of the cells have not achieved a neoplastic state. Therefore, it was investigated whether chronic inflammation may influence the development of cancer through the precursor HGPIN or PIA by rendering the cells to achieve a neoplastic state. Previous studies have shown that persistent chronic inflammation adjacent to PIA lesions may drive the epithelial cells to achieve increased survival (18-21). These may be regarded as initiated cells, and acquisition of further genetic alterations under the influence of continuous inflammation may drive the cells towards malignancy. It has been postulated that these initiated cells, over a period of time, may or may not be further influenced by inflammation. An independent ability to proliferate in an altered microenvironment may lead to cancer progression (18-21). This is evident from the increased proliferation rate, as demonstrated by intense PCNA staining, where the majority of the neoplastic/malignant cells exhibited a high proliferation index along with Bcl-2 staining. This suggestion is consistent with the previous findings, wherein some PIA/PAH lesions have been shown to acquire somatic chromosome 8 abnormalities.

Figure 5. Scatter plot of inflammation vs. (A) Bcl-2 and (B) PCNA. A strong positive association between inflammation and expression level of Bcl-2 (r=0.882) was observed: The expression of Bcl-2 identified (r=-0.197); however, this was not statistically significant (P=0.249). r-values represent Spearman correlation coefficient. PCNA, proliferating cell nuclear antigen.

Figure 6. A hypothetical model showing how chronic inflammation leads to cancer development. Under the influence of chronic inflammation epithelial cells acquire increased survival with an increase in Bcl-2 expression. The survival signal alters the phenotype of these particular epithelial cells, which gain neoplastic characteristics, leading to precursors that may later acquire increased proliferation rate leading to cancer development and subsequent progression.
and mutations in the tumor suppressor gene TP53 (genetic alterations that are associated with prostate cancer and HGPIN lesions) (40). Aberrant expression of these two molecules may lead to extended survival of neoplastic cells and increased likelihood of mutational aberrations, which are responsible for proliferation and aggressiveness.

Genetic alterations in the number of tumor suppressor genes, oncogenes, proliferation, apoptosis, and cellular stress response genes have been reported to be associated with HGPIN and prostate cancer (41,42). In the present study, a strong association of PIA/PAH lesions with chronic inflammation was demonstrated, which seems to predispose cells to neoplastic development in the prostate. Furthermore, an inverse correlation between Bcl-2 and PCNA in the glandular epithelial cells seems to indicate that extended survival and apoptosis inhibition by Bcl-2, and low proliferation rate under the influence of low-grade persistent inflammation, may promote malignant transformation. Fig. 6 illustrates this hypothetical model. However, broader studies of the apoptosis-regulatory proteins and proliferative proteins should be performed in an effort to elucidate the relationship between inflammation and neoplastic progression. In the present study, we attempted to minimize the pitfalls by categorizing specimens into four different subcategories (SA, PIA/PAH, HGPIN and cancer) associated with uniformly high chronic inflammation. Taken together, the findings of the present study suggest that persistent chronic inflammation is a common distinct entity resulting in increased survival of cells, and facilitates neoplastic development leading to a state of increased proliferation.

In conclusion, the present study identifies chronic inflammation as a contributor to neoplastic progression of prostate epithelial cells. Additional studies are required to illustrate the molecular/genetic alterations that occur during the events of inflammation that could lead to disease progression.

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