Immunohistochemical Analysis of Inflammatory Cells in Benign and Precancerous Lesions and Carcinoma of the Prostate

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Key Words

Prostatitis • Prostate carcinoma • Lymphocytes • Macrophages • CD68 • CD204

Abstract

Objective: Inflammation is an important cause of tumorigenesis in various types of malignancy. Mediators derived from inflammatory cells are associated with cancer proliferation, angiogenesis, and DNA damage. In the present study, we immunohistochemically examined the infiltration patterns of inflammatory cells in benign glands including glandular hyperplasia, and in prostatic intraepithelial neoplasia and adenocarcinoma. **Methods:** Formalin-fixed, paraffin-embedded tissues were obtained from 100 patients with prostate cancer. All patients underwent radical prostatectomy. We assessed the number of infiltrating T cells (CD3⁺), B cells (CD20⁺, CD79α⁺), and macrophages (CD68⁺, CD204⁺) in benign and malignant prostate tumors. **Results:** CD68⁺ macrophages infiltrated benign glands to a higher extent than those of adenocarcinoma. In contrast, the number of CD204⁺ cells was higher in malignant glands than in benign glands. There was no significant difference in the number of infiltrating T cells between benign and malignant tumors; however, the number of infiltrating B cells was significantly

reduced in malignant glands. **Conclusions:** Inflammation of the prostate may act on prostate carcinomas; particularly that involving M2 macrophage infiltration may play a significant role in prostate carcinogenesis.

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Introduction

Chronic prostatitis is diagnosed using needle biopsy, transurethral resection, or radical prostatectomy specimens. Epidemiological studies of prostatic carcinoma show significant associations between infection and cancer. Inflammatory stress causes repeated genomic damage leading to transformation, vascularization, apoptosis, and DNA mutation. During bacterial infection, inflammatory cells such as lymphocytes, neutrophils, and macrophages frequently infiltrate into or around the tumor foci. This results in the production of reactive oxygen and nitrogen species, and various cytokines leading to the destruction of the environmental microcosm. In addition to bactericidal effects, these free radicals degrade proteins and damage tissues, resulting in irreversible DNA damage and malignant transformation. Inflammatory cells secrete a number of cytokines and chemokines that

Table 1. Characteristics of prostatic cancer for immunohistochemistry

Cases, n	100
Benign gland	81
Normal	55
Glandular hyperplasia	26
PIN	25
Adenocarcinoma	68
Age (range), years	67.3 (52–77)
PSA (range), ng/ml	11.8 (3.9–139)
Gleason score, n	
≤6	16
7	71
≥8	13

promote the outgrowth of neoplastic cells, in addition to autocrine growth factor production by the tumor cells [1]. Reactive free radical overload and some types of cytokines have been recognized as important risk factors for tumorigenesis [1, 2]. The release of such mediators induces the interaction of inflammatory cells with tumor cells resulting in the simultaneous advance of tumor growth and the extension of inflammation. On the other hand, other proteins or cytokines have been associated with tumor suppression or antitumorigenesis. For example, we isolated a highly expressed protein that we originally designated as prostate cancer antigen-1 (PCA-1), which repairs methylmethane sulfonate-induced alkylation damage in human cells [3].

In the prostate, normal glands, glandular hyperplasia, and adenocarcinoma are often accompanied by inflammation to some extent. Although the infiltration of inflammatory cells might be associated with the proliferation of glandular cells, eventually leading to cancer development, it remains unclear what types of inflammatory cells infiltrate and how these cells affect benign and malignant glandular cells.

Several reports have indicated an important role for infiltrative monocyte chemotactic protein-1 (MCPs) in cancer development and stated that tumor-associated macrophage (TAM) infiltration was found to be closely correlated with poor outcomes in several types of human malignancies [4–7]. In lung cancer, pancreatic cancer, and glioma, CD204⁺ macrophages infiltrate around the tumor and contribute to progression. Macrophages are divided into 2 types according to their function: a tumor-suppressive (M1) function and a tumor-supportive (M2) function [8]. M2 macrophages play important roles in tu-

morigenesis, angiogenesis, matrix remodeling, and metastasis [9, 10].

In the present study, we examined the composition of the inflammatory infiltrate in malignant and benign foci using radical prostatectomy specimens from patients with glandular hyperplasia, prostatic intraepithelial neoplasia (PIN), and adenocarcinoma.

Materials and Methods

Patients

A total of 100 patients with prostate cancer underwent radical prostatectomy at the Nara Medical University Hospital. All patients signed an institutional review board-approved informed consent form. The clinicopathologic data used in this study were reviewed by 2 urological pathologists (K.S. and N.K., Department of Pathology, Nara Medical University Hospital) and are summarized in table 1.

Histopathological Studies and Evaluation of Inflammation

All surgical specimens were fixed with 10% formalin and embedded in paraffin. Prostate sections (3–4 μ m) were stained with hematoxylin and eosin (HE) stain. Three observers (T.F., O.A., and N.K.) evaluated the histological type: benign glands, glandular hyperplasia, PIN, and adenocarcinoma, and samples containing leukocytes surrounding glands were selected. Inflammatory grade was determined according to a classification proposed as mild: individual inflammatory cells, most of which are separated by distinct intervening spaces; moderate: confluent sheets of inflammatory cells with no tissue destruction or lymphoid nodule/follicle formation, or severe: confluent sheets of inflammatory cells with tissue destruction or lymphoid nodule/follicle formation [11].

Immunohistochemistry

After reviewing the HE-stained slides of selected specimens, we followed the same tissue fixation and processing procedure as described in previous reports [12–14]. The slides were deparaffinized in xylene, dehydrated in a graded ethanol series, and subsequently rinsed with distilled water. Antigen retrieval methods and the final dilution of antibodies for immunohistochemistry are indicated in table 2. Three pathologists (T.F., O.A., and N.K.) counted CD20⁺, CD79 α ⁺, CD3⁺, CD4, CD8, CD68⁺, CD204⁺, and CD163 cells in a high-power microscopic field ($\times 400$; 0.00625 mm²).

Statistical Analysis

We diagnosed prostate glands according to histopathological findings as benign, glandular hyperplasia, PIN, or adenocarcinoma with inflammation, and classified the grading of inflammation as mild, moderate, or severe. The significance of the association of inflammation between benign, glandular hyperplasia or PIN, and adenocarcinoma was assessed using Student's *t* test (Prizm4; GraphPad Software, Inc.). *p* < 0.05 was considered statistically significant.

Table 2. Immunohistochemical analysis of the prostatic tissue

Antigen	Antibody clone	Dilution	Antigen retrieval method	Source
CD3	PS1	Prediluted	Autoclave	Nichirei, Japan
CD4	1F6	1:40	Autoclave	Leica, Germany
CD8	C8/144B	Prediluted	Autoclave	DAKO, Denmark
CD20	L-26	Prediluted	–	Nichirei, Japan
CD79 α	HM57	Prediluted	Autoclave	Nichirei, Japan
CD68	Kp-1	1:100	Protease	DAKO, Denmark
CD204	SRA-C6	1:100	Autoclave	Cosmo Bio, Japan
CD163	10D6	1:100	Autoclave	Leica, Germany

Results

Grade of Inflammatory Cell Infiltration in Benign and Precancerous Lesions and Adenocarcinoma of the Prostate

In order to examine whether the grade of inflammatory cell infiltration is associated with the histological pattern, the grade of the inflammatory cell was classified as mild, moderate, or severe, according to the grading system described previously in Materials and Methods (fig. 1a). The number of inflammatory cells within benign glands including glandular hyperplasia was much higher than that in PIN or adenocarcinoma (fig. 1b). The grade of inflammation was not significantly different among the Gleason patterns (fig. 1c). In a past report, no significant difference was found between mild and severe inflammation when considering patient age, preoperative serum prostate-specific antigen (PSA), Gleason score, or the percentage of organ-confined disease [15]. Thus, our result revealed that inflammatory cells contribute to cancer progression, but they are not involved in the differentiation of prostate cancer.

Identification of Leukocyte Types in Benign and Cancerous Glands of the Prostate

Immunohistochemical results of leukocyte distribution around the benign glands, PIN, and adenocarcinoma are shown in figure 2. The inflammatory cells present consisted of CD3⁺ T cells, CD20/CD79 α ⁺ B cells, and macrophages, in both the benign and the malignant glands. However, plasma cells, neutrophils, and eosinophils were rare. It is evident that CD3⁺ T and CD20/CD79 α ⁺ B lymphocytes predominantly infiltrated the benign glands including glandular hyperplasia, PIN, and adenocarcinoma.

Theyer et al. [16] previously indicated that the kinds of lymphocytes infiltrating the prostate were composed of approximately 70% T cells, 15% B cells, and 15% macrophages. In our results, the frequency of B cells in benign glands was 59%; interestingly, the ratio of infiltrating B cells was significantly reduced in both PIN and adenocarcinoma (PIN: 29%, adenocarcinoma: 31%). The rate of CD3⁺ T cells was not significantly different among benign glands, PIN, and adenocarcinoma (fig. 3a). Similarly, the rate of CD4⁺ and CD8⁺ T cells was not significantly different among these glands, but the rate of CD8⁺ T cells was lower than that of CD4⁺ T cells in each gland (fig. 3b).

Infiltration of CD68⁺/CD204⁺ Macrophages Was Higher in Cancerous Glands of the Prostate

Recent studies indicated that CD68⁺, CD163⁺, or CD204⁺ macrophages were observed with significantly high expression in borderline and malignant tumors, such as prostate cancer, glioma, and pancreatic cancer, and were associated with the grade of histological malignancy and a poor prognosis [4, 5, 17]. We examined the involvement of M2 macrophages in inflammation in benign glands, PIN, and adenocarcinoma of the prostate. The relationships between infiltrating CD68⁺ and CD204⁺ macrophages are shown in figure 4. The infiltration of CD68⁺ macrophages was higher in benign glands compared with PIN and adenocarcinoma. In contrast, CD204⁺ cells were more abundant in PIN and adenocarcinoma when compared with benign glands. Consequently, the CD204⁺:CD68⁺ ratio was increased in PIN and adenocarcinoma. Identification of CD68⁺ using the pan macrophage marker indicated that these cells infiltrated into benign glands, PIN, and adenocarcinomas to the same extent (fig. 4b). These data indicate that infiltration of CD204⁺ macrophages correlated with the early step of carcinogenesis of the prostate (fig. 4).

Fig. 1. Grade of inflammation in different types of prostate tissue (a). The grade of inflammation was classified as follows: mild, individual inflammatory cells, most of which are separated by distinct intervening spaces; moderate, confluent sheets of inflammatory cells with no tissue destruction or lymphoid nodule/fol-

licle formation, and severe, confluent sheets of inflammatory cells with tissue destruction or lymphoid nodule/follicle formation. (b, c) The percentage of mild, moderate, and severe inflammation in benign, PIN, and adenocarcinoma (b), or each Gleason patterns (c). mod. = Moderate; sev. = severe.

Prostatic Inflammation and Serum PSA

In our study, 98 patients exhibited high serum PSA values (>4 ng/ml) at the initial diagnosis, but there was no significant difference in PSA values among patients with severe, moderate, or mild inflammation (data not shown).

Discussion

In the present study, we indicate for the first time that different types of inflammatory cells infiltrate the tissue during prostatitis in malignant glands compared to those found in benign glands or precancerous lesions. Macrophages are immune-response cells that surround cancer-

ous tissue to affect the immune response of the host. Previous studies have indicated that TAM infiltration functions as an adverse prognostic factor in certain tumors, such as glioma, and lung and pancreatic cancer [4, 5, 7]. Several cytokines associated with TAM recruitment and differentiation have been reported. M2 macrophages are CD204⁺ cells and are associated with tumorigenesis, angiogenesis, and matrix remodeling [4, 10, 17, 18]. CD204⁺ cells contribute to several factors involved in local infiltration including lymphovascular invasion. Thus, CD204⁺ macrophages contribute to the local invasion by cancer cells.

The CD68⁺ macrophage is known as a pan-monocyte/macrophage marker; however, all of the TAMs are not positive for CD68. CD68⁺ macrophages reportedly in-

Fig. 2. Immunohistochemical staining of infiltrated inflammatory cells in prostate glands. The normal glands with severe inflammation, glandular hyperplasia with moderate inflammation, PIN with mild inflammation, and adenocarcinoma with mild inflammation were immunostained for CD3, CD20, CD79alpha, CD68, CD204, and CD163. The DAB staining represents a positive reaction.

filtrate esophageal squamous carcinoma [6]. Moreover, in bladder cancer, infiltration of CD68⁺ macrophages was higher in muscular invasive tumors than in noninvasive and superficial invasive phenotypes [19].

Our results indicated that the infiltration of CD68⁺ macrophages was high in benign glands but reduced in PIN and adenocarcinoma. In contrast, CD204⁺ cells

were more abundant in PIN and adenocarcinoma when compared to benign glands. Consequently, the CD204⁺/CD68⁺ ratio was increased in PIN and adenocarcinoma. We therefore suggest that the presence of CD204⁺ macrophages indicates a tumor-progressive phenotype; however, we cannot suggest that the increase in CD204⁺ cells results in tumor formation as the increase in number

Fig. 3. Immunohistochemical detection of T-cell and B-cell (a), or CD4 and CD8 (b) infiltration in benign and malignant prostate. Positive cells were counted in the high-power field. The bars represent the percentage of positive cells from the number of infiltrating cells in 200 cells.

Fig. 4. CD68 and CD204 infiltration in benign and malignant prostate. Immunohistochemical detection of CD68⁺ and CD204⁺ cells; positive cells were counted in the high-power field. Among the benign glands, PIN, and adenocarcinoma, the infiltrating macrophages correlated significantly with CD204/CD68.

may be a mechanism to prevent further tumor progression. Recent reports indicated that CD204 was capable of attenuating immunostimulatory adjuvant-induced anti-tumor immunity [20]. Thus, the CD204⁺ macrophage may function as a tumor suppressor; however, conflicting data regarding this have been accumulated. Therefore, the manner in which CD204⁺ macrophages influence prostate carcinogenesis and cancer progression should be further examined. Although CD68⁺ macrophages are pan-monocyte/macrophage markers, not all of the M2 macrophages are necessarily positive for CD68. The number of CD204⁺ cells is slightly higher than that of CD68⁺ cells. CD204⁺ cells that are not macrophages may also occur.

Previous studies indicated that asymptomatic chronic prostatitis is observed in 68–100% of patients, and inflammatory cells of the prostate are found to be entirely composed of T lymphocytes (70–80%) in needle biopsy, transurethral resection, or radical prostatectomy specimens [16, 21–25]. In the present study, no statistical difference in the number of infiltrating T cells was observed between the benign and malignant glands. Interestingly, B cells comprised 57% of infiltrating cells in benign glands; this number was significantly reduced in malignant glands. Inflammatory cytokines including TNF α , IL-6, and IL-8 play an important role in oncogenesis and tumor progression, whereas other kinds of cytokines including IL-17E have antitumour activity [26–29]. Thus, B cells contribute to both tumor progression and suppres-

sion. The prostate is frequently exposed to bacterial/viral infection, and the T cell/B cell-mediated immune system functions as a defense mechanism. Th1 and Th2 are induced by IL-12 and PGE2 secreted from antigen-presenting cells, respectively. Th1 cells contribute to cell-mediated immunity, whereas Th2 cells mediate humoral immunity. We speculate that the Th1/Th2 balance determines whether T cells or B cells will function as the predominant immune modulator in chronic prostatitis. Unlike in Western populations, the Th2-dependent B-cell elimination of extrinsic organisms may be involved in chronic inflammation of the prostate in Japanese patients. As the sustained Th2 predominant status leads to tumorigenesis of various types of organs including the prostate, we should consider that B-cell-rich inflammation may be associated with a rapid increase in the num-

ber of patients with prostate cancer in Japan. In contrast to chronic prostatitis, T cells predominantly infiltrate in or around prostate adenocarcinomas, as observed in our current study. This result indicates that cell-mediated immunity primarily acts in response to the invasion of prostate cancer cells.

In conclusion, the current study indicated that CD204⁺ macrophages surrounding cancerous glands could be a marker for tumor-related macrophages. Inflammation of the prostate, particularly that involving infiltrating M2 macrophages, is essential for the progression of the tumor. Therefore, a more accurate insight into the role of CD204⁺ macrophages and consideration of the microenvironment in the cancerous gland of the prostate has important implications for prostate carcinogenesis and cancer progression.

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