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Original contribution

Biologic correlates and significance of axonogenesis in prostate cancer☆,☆☆,★

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Summary Cancer-related axonogenesis and neurogenesis are recently described biologic phenomena. Our previously published data showed that nerve density and the number of neurons in the parasympathetic ganglia are increased in prostate cancer (PCa) and associated with aggressive disease. Tissue microarrays were constructed from 640 radical prostatectomy specimens with PCa. Anti–protein gene product 9.5 (PGP 9.5) antibodies were used to identify and quantify nerve density. Protein expression was objectively analyzed using deconvolution imaging, image segmentation, and image analysis. Data were correlated with clinicopathological variables and tissue biomarkers available in our database. Nerve density, as measured by PGP 9.5 expression, had a weak but significant positive correlation with the lymph node status ($\rho = 0.106$; $P = .0275$). By Cox univariate analysis, PGP 9.5 was a predictor of time to biochemical recurrence, but not on multivariate analysis. Increased nerve density correlated with increased proliferation of PCa cells. It also correlated with expression of proteins involved in survival pathways (Phosphorylated alpha serine/threonine-protein kinase, NFκB, GSK-2, PIM-2, c-Myc, SKP-2, SRF, P27n, PTEN), with increased levels of hormonal regulation elements (androgen receptor, estrogen receptor α), and coregulators and repressors (SRC-1, SRC-2, AIB-1, DAX). Axonogenesis is a recently described phenomenon of paramount importance in the biology of PCa. Although the degree of axonogenesis is predictive of aggressive behavior in PCa, it does not add to the information present in current models on multivariate analysis. We present data that corroborate that axonogenesis is involved in biologic processes such as proliferation of PCa, through activation of survival pathways and interaction with hormonal regulation.
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1. Introduction

Although genetic damage is the molecular signature of cancer, it is now recognized that the tumor microenvironment has an influence of critical importance on tumor development, progression, and survival. The tumor microenvironment encompasses stromal elements (fibroblasts, extracellular matrix), immune and inflammatory cells, blood and lymphatic vessels, and nerves. These elements provide an essential communication network, via secretions of various molecules, providing the necessary signals that turn on different transcription factors. Therefore, the tumor microenvironment is an integral part of tumor physiology, structure, and function [1].

Numerous animal studies emphasized the role of the autonomic nervous system in regulating the structure and function of the prostate gland, demonstrating that its control is not regulated exclusively by hormones [2-8]. These studies also highlighted the importance of the interactions between the nerve fibers and the secretory prostatic epithelial cells, concluding that the nervous input is of paramount importance for their function.

Recently, we described a novel biologic phenomenon: cancer-related axonogenesis and neurogenesis [9]. Using 3-dimensional reconstructions of whole prostate mounts, we showed increased nerve density in preneoplastic and neoplastic lesions of the human prostate. Furthermore, patients with prostate cancer (PCa) had increased numbers of neurons in their prostatic ganglia compared with controls. Neurogenesis was correlated with features of aggressive behavior and recurrence in PCa. Our laboratory has also shown that semaphorin 4F, a member of a family of proteins with roles in embryological axon guidance, was involved in the regulation of cancer-induced neurogenesis. In supporting the evidence of increased neuroepithelial interactions in PCa, other studies reported a lower incidence of PCa in patients with early sustained spinal cord injury (age matched) [10,11].

Protein gene product 9.5 (PGP 9.5) is a member of the ubiquitin hydrolase family of proteins, confined to the cytoplasm of nerves and neurons. PGP 9.5 is a cytoplasmic neuron-specific protein structurally and immunologically distinct from neuron-specific enolase. Abnormal cytoplasmic PGP 9.5 protein expression has been described in abnormal tissues, most commonly in neuroendocrine carcinomas [12], squamous cell carcinomas, and keratoacanthomas [13]. A study described abnormal protein expression in 2 PCa cell lines [14].

The objectives of this study are to examine the axon density in PCa by using PGP 9.5 as an axonal marker and to analyze the biologic significance of axonogenesis in PCa. By using a large cohort of patients and an associated database of biologic markers previously performed on the same cohort of patients, we show the influence that nerve fibers increased by axonogenesis have on cancer cells.

2. Materials and methods

2.1. Clinical and pathologic characteristics

The initial cohort consisted of 1210 patients who underwent radical prostatectomy at Baylor College of Medicine (Houston, TX)–affiliated hospitals between 1983 and 1998. We qualified 640 cases for building tissue microarrays (TMAs) based on the following criteria: (1) patients did not receive preoperative treatment, (2) patients had surgery between 1983 and 1998, and (3) sufficient PCa tissue was available for building TMAs. The full cohort patient characteristics had been previously published [15]. A total of 435 patients had analyzable PGP 9.5 data for this study. Twenty-nine patients had lymph node metastasis, 188 had extracapsular extension (ECE), 53 had seminal vesicle invasion (SVI), 63 had positive surgical margins, 28 had biochemical recurrence, and 12 patients died of PCa. Tissue recruitment was in accordance with institutional review board approval.

2.2. TMA construction

For this TMA, whole-mount prostate slides were reviewed and mapped. The *tumor index*, defined as the largest and/or highest Gleason score (GS), was identified on the slide, and areas representative of the highest GS were circled. Two-millimeter cores were obtained from these areas and transferred to a recipient paraffin block. The TMAs were built using a manual tissue microarrayer (Beecher Instruments, Silver Spring, MD). The characteristics of this array had been previously published [15].

2.3. Immunohistochemistry

Immunohistochemical staining with antibodies against PGP 9.5 (Novacastra Laboratories, Newcastle upon Tyne, UK) on TMA slides was conducted by using an automated immunostainer (Dako, Carpinteria, CA). Briefly, sections were deparaffinized in xylene, rehydrated through decreasing concentrations of alcohol ending in phosphate-buffered saline, subjected to steam heat in 10 mmol/L citrate buffer (pH 6.0) for 40 minutes in a vegetable steamer, then allowed to cool off at room temperature for an additional 10 minutes. After endogenous peroxidase activity was quenched in 3% hydrogen peroxide solution in distilled water, sections were incubated with rabbit polyclonal antibody against PGP 9.5 (1:40, overnight at 48°C; cat no. 9462). Sections were washed, and the bound antibody was detected by using a Dako Envision Plus kit (Dako) with diaminobenzidine as chromogen. Finally, sections were counterstained with hematoxylin and eosin, dehydrated, and mounted. Negative controls were sections immunostained as above, but normal rabbit serum was used instead of primary antibody.

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