Abstract. Background: Immune cells (lymphocytes and macrophages) provide the microenvironment for immune surveillance of metastatic prostate cancer (PCa) cells in pelvic lymph nodes. We have hypothesized that degeneration and/or apoptosis of metastatic PCa cells in pelvic lymph nodes can distinguish between aggressive and non-aggressive metastatic disease in patients. Our objective was to define the relationship between metastatic cell lysis and the presence of immune cells. Materials and Methods: We studied archival primary PCa (n=38) and cancer-positive regional pelvic nodes (n=32) from the same patients undergoing radical retropubic prostatectomy at the Minneapolis Veterans Affairs Medical Center. Results: Using morphological and immunohistochemical features of immune and metastatic cancer cells, we have identified progression of metastasis in the nodal compartments. Nodal parenchyma contained small, intermediate and large metastatic nodules/tumors. Immune surveillance occurred primarily in small tumors and surveillance was either absent or greatly reduced in intermediate and large tumors in nodes. Metastatic nodules/cells were lysed or became apoptotic when under immune-surveillance, as indicated by pyknotic nuclei and cytoplasm, the latter still had remnants of prostate specific antigen (PSA) staining. Metastatic cells without surveillance did not exhibit morphological evidence of cell degeneration (lysis) or apoptosis. Metastatic cells under immune-surveillance had an inverse relationship with those without immune-surveillance. This relationship differed from node to node and patient to patient. Conclusion: We have shown that at least two populations of metastatic cells were present in the nodes; the first group of cells was under immune surveillance, as indicated by limited to wide-spread cell lysis/apoptosis, and the second group did not exhibit morphological evidence of cell lysis indicating emergence of surveillance-unresponsive (resistant) metastatic cells. These criteria can be used to distinguish metastatic cancer that is expected to be responsive to immunotherapy from that which would show little or no benefit from such treatment. Enhancement of immune surveillance and other treatments can be used to treat surveillance-unresponsive (resistant) disease to improve survival of patients.

Metastasis of solid-organ cancer is responsible for about 90% of deaths from human cancer, including from human prostate cancer (PCa) (1-3). Localization of prostate specific antigen (PSA) in nodes positively identifies metastatic cells of prostatic origin. Nodal metastasis occurs in approximately 9% of patients with PCa (4,5). Disease-positive regional pelvic lymph nodes are often used to predict prognosis in patients (3, 5-8). Primary PCa is a complex tumor because of morphological and genetic heterogeneity, epigenetic factors, proteases, and multifocal origin and pathological progression (9-12). In primary PCa, immune cells are relatively few when compared to nodal metastases (13-15). Metastatic cells reaching nodes, however, encounter an immunosuppressive microenvironment significantly greater than that in the primary tumor (16). The microenvironment of metastatic cancer cells and immune cells is complex and plays a critical role in primary PCa and nodal metastasis (12,17). For example, the microenvironment surrounding PCa is associated with reactive stroma when compared to benign (normal) prostate or benign
prostatic hyperplasia (BPH); both have well-organized stroma and glandular structures (18, 19). Microvessel density is higher in primary PCa and prostatic intraepithelial neoplasia (PIN) than in BPH or benign prostate (20). Proliferation of primary PCa cells occurs at a higher level of dihydrotestosterone (DHT) than in nodal metastatic cells (21). Nodes with metastatic PCa have lower levels of androgen receptors than those in the primary PCa (22). Cathepsin B, a lysosomal cysteine protease involved in degradation of the glanular basement membrane and stromal connective tissues, is expressed at a higher level in the primary PCa than in nodal metastases (14). The microenvironment of PCa metastases in nodes, pelvic bones, lungs and other organs is poorly understood and under-investigated.

Biological aggressiveness of PCa metastases in nodes is closely related to cancer volume and tumor grade in the primary organ (9, 12). Prout et al. and others considered nodal involvement as a prognostic indicator of PCa progression (3, 8, 12). Lymph node metastasis generally indicates the worst prognosis, however, some patients with nodal metastases have long-term survival (8, 12). The reasons for these differences are unknown, but we have provided some evidence (see later). Positive prostatic margins are associated with locally advanced cancer involving the regional lymph nodes in 85% of cases (23). Capsular penetration, cancer volume and nodal metastasis were strongly interrelated (8, 24). A recent study has shown that examination of pelvic adipose tissue provides a more accurate description of nodal metastasis (25). The initial sites of PCa cell dissemination, usually via hematogenic and lymphogenic routes, are the pelvic lymph nodes (5, 7, 26). Nearly 80% of solid organ metastasis occurs via the lymphatic system, while 20% of metastasis occurs via the blood vascular system and by direct seeding of periprostatic tissue (5, 27-29). Pelvic lymph nodes and pelvic bones are the most common sites of PCa metastasis (7). Lymph nodes have a cytokine-rich microenvironment, but limited ability to eradicate metastatic cancer cells (30). Reduced infiltration of tumor-associated macrophages was observed in PCa when compared to tumor-positive nodes (13). Our review of the extensive literature on PCa indicates that cancer cells are selected for their ability to migrate from glands to prostatic stroma, to prostatic capsule, and then to pelvic lymph nodes, as well as to other organs. We have hypothesized that degeneration and apoptosis in metastatic PCa cells in pelvic lymph nodes can distinguish between aggressive from non-aggressive metastatic disease in patients. Our objective was to define the relationship between the lysis of metastatic cells and the presence of immune cells.

Materials and Methods

We collected archival primary PCa (N=38) and cancer-positive regional pelvic nodes (N=32) from the same patients undergoing radical retropubic prostatectomy at the Minneapolis Veterans Affairs (VA) Medical Center. Areas of tumor-negative nodes and primary prostate without cancerous tissue were used as controls. We examined tissue sections from eight cases with benign prostate and BPH as controls. Patients were not treated with hormones or cytotoxic agents prior to surgery. Specimens were collected according to the informed consent guidelines of the Minneapolis VA Human Studies Committee and the Institutional Review Board: Human Subject Committee, University of Minnesota. We also collected data on age at prostatectomy, lymph node metastasis, clinical stage, clinical diagnosis, including pre-surgery serum total PSA levels and mortality/survival data. Prostate and node tissue samples were fixed in neutral, buffered formalin, embedded in paraffin or paraplast, and sectioned at 4 to 6 μm for hematoxylin and eosin (H&E) staining for pathological grading, and unstained sections were used for immunohistochemical (IHC) localization of selected antigens. Primary PCa and metastatic tumors in lymph nodes were graded on H&E sections by Dr. Donald F. Gleason or S. L. E according to the Gleason grading system (31). Later, Bazinet et al. used Gleason grading criteria of PCa and applied them to grade metastatic tumors in lymph nodes (32).

Antibody IgGs against PSA, Cathepsin B and macrophage marker (CD68). A rabbit antibody against human PSA was selected to identify PCa cells in nodes. The antibody was obtained from Dako (Dako Corporation, Carpinteria, CA, USA). A mouse monoclonal antibody to human macrophage, CD68 (clone PG-M1), obtained from Dako, was selected to identify macrophages/monocytes in nodes, but not myeloid cells (manufacturer’s data). A polyclonal, monospecific, rabbit antibody to-human liver cathepsin B (PC41) from Oncogene Research Products (Calbiochem, Cambridge, MA, USA) was selected to identify lysosomal cathepsin B in macrophages found in nodes and primary organs (33-35). Phosphate buffered saline (PBS) and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sources of secondary antibodies and other chemicals are identified below.

IHC localization of PSA, cathepsin B and CD68. Node and prostate tissue sections were immunostained for PSA, cathepsin B and CD68 antigens using appropriate antibodies and IHC techniques reported previously (15, 36). Immunostained sections were usually not counterstained with hematoxylin except for selected cases. Since we have not reported on localization of CD68 in macrophages, the technique is described briefly. De-paraffinized sections were rehydrated to distilled water via graded ethanol. After a peroxidase block using 0.6% H2O2 in methanol, antigen retrieval was performed using boiling citrate buffer in Decloaking Chamber Pro machine (Biocare Medical, Walnut Creek, CA, USA) and blocking was carried out using 2% BSA and 2% normal horse serum. Sections were incubated overnight at 4°C or one hour at room temperature with primary antibody (1:100 dilution) and processed using a secondary antibody followed avidin-biotin-peroxidase complex (ABC kit from Vector Labs, Burlingame, CA, USA) and blocking was carried out using 2% BSA and 2% normal horse serum. Sections were incubated overnight at 4°C or one hour at room temperature with primary antibody (1:100 dilution) and processed using a secondary antibody followed avidin-biotin-peroxidase complex (ABC kit from Vector Labs, Burlingame, CA, USA). The reaction products were developed with fresh-filtered 3,3′-diaminobenzidine (DAB) solution (0.25 mg/ml; Sigma) in PBS with 0.01% H2O2 as the substrate. Chromogenic development was viewed under a light microscope while reaction products developed, usually in less than 10 min. Sections were washed, dehydrated in graded ethanol and mounted in Permount (Fisher Scientific, Fair Lawn, NJ, USA). Along with experimental tissue sections, negative control sections were incubated with preimmune rabbit or mouse serum in lieu of the primary antibodies.
**Quantification of localization by the Metamorph image analysis system.** Distribution of cathepsin B and CD68 immunostained macrophages was evaluated in PCa-positive and -negative nodes and selected primary tumor sections with a Zeiss microscope (14, 36, 37). Photomicrographs of immunostained areas were acquired directly from slides via a Zeiss Axioplan microscope connected to a Nikon digital camera (Cool Snap HQ Monochrome camera: 14 Bit, 20 MHz Digital Monochrome camera, IEEE-1394 interface) (Nikon Instruments, Inc., Melville, NY, USA) which was attached to a computer. Recent photomicrographs were acquired using the Nikon digital camera system, this system was not used for image analysis. Another system was used for image analysis of immunostained sections, as reported (36, 37). We used a Photometric digital camera (Photometrics, Tucson, AZ, USA) which was attached to the Zeiss microscope and Metamorph software image analysis program (Universal Imaging Corp., West Chester, PA, USA). Images showing reaction products of cathepsin B, CD68 and PSA were acquired at magnifications of ×200 directly from microscope slides to a computer. Photomicrographs were acquired using the two types of software systems and thus, they required adjustment of image density using Photoshop software. Differences in immunostaining of PSA, CB and CD68 were determined using Student’s t-test. Statistical significance was determined at p≤0.05.

Montage reconstruction of PCa-positive pelvic lymph node. After obtaining low-power micrographs of tumor-positive nodes, we reconstructed montage images of nodal metastases. This allowed us to establish the distribution of cathepsin B and CD68 immunostained macrophages in nodes.

**Results**

**Profile of PCa patients.** The range of ages of patients with PCa was between 53 and 76 years at prostatectomy, with a mean age (±SD) 66.82±5.59. Distribution of node and prostate cancer patients is shown in Figure 1. The mean Gleason score for tumor-positive nodes was 7.47±0.92 and for prostate was 7.53±1.08. Student’s t-test did not show any statistical difference between scores of nodes and PCa. Tumor-positive nodes had a mean±SEM of 59.92±0.11 for PSA whereas negative nodes had a mean of 0.078±0.01. Localization of PSA in tumor-positive nodes was significantly different from that in negative nodes (p<0.001). According to the TNM classification system (12, 38), patients had clinical stages of pT2-3, pN1-3 and Mo. According to Jewett and Whitmore and Mayo classification, patients had B1 to D2 tumors (38).

**PSA-positive PCa cells in pelvic nodes.** The localization of PSA in nodes positively identified the prostatic origin of metastatic cells (Figures 2c and 3). Localization of PSA in positive nodes was significantly different from that in negative nodes (Figure 4). The lymph node was enclosed by fat cells and capsular dense collagenous fibers which in turn enclosed variably sized and shaped subcapsular sinuses (Figure 2a-d). The subcapsular fibers were contiguous in the nodal trabeculae before reaching the hilus, which contained blood vessels and lymphatics (Figures 2a, c, d and 5a). The subcapsular sinuses probably contained lymph prior to fixation which was, undoubtedly, destroyed during processing of the samples. In nodal connective tissue, metastatic PCa cells were usually not observed in the capsule (Figure 2a-d), but were observed in the subcapsular sinuses and the nodal parenchyma (Figure 2b-d). Isolated or groups of metastatic cells entered the nodal capsule, but were probably transitory. These cells formed metastatic nodules in the subcapsular sinuses (Figure 2b, d). The presence of nodules containing numerous metastatic cells indicated that they had proliferated in the subcapsular sinuses prior to invading the nodal parenchyma. In favorable sections, subcapsular sinuses containing metastatic nodules continued in the parenchyma (Figure 2c, d). Metastatic cells in the subcapsular sinuses exhibited cathepsin B immunostaining, but the reaction products were usually weak in the nodal parenchyma (Figure 5a, b, d). Metastatic cells invading the parenchyma encountered lymphatic nodules (outer cortex) which enclosed the inner medulla (germinal centers) (Figure 2a, c). CD68-immunostained macrophages were present in trabeculae and sometimes in nodal parenchyma (Figure 2e, f).

In the nodal parenchyma, metastatic nodules were surrounded either by sinus-like spaces (similar to those found in the subcapsular sinuses) (Figure 3a, b) or were completely surrounded (without any sinus-like spaces) by immune cells (Figure 3c-f). At the focal points of contact between metastatic cells and immune cells, numerous cancer cells exhibited degeneration (lysis) or were apoptotic, some of these with fragmented nuclei and remnants of PSA immunostaining in the cytoplasm (Figure 3a, b). The number of focal points varied from node to node, as did the sinus-like spaces. Figure 3 shows progressive degeneration/apoptosis in response to immune surveillance in metastatic cells. For example, the tumor cells exhibited lysis/apoptosis at the focal points of contacts between tumor and immune cells (Figure 3a, b), but not when the metastatic cells were separated by the sinus-like spaces (Figure 3a, b). Some nodes...
Figure 2. (a) Cathepsin B (CB)-immunostained section of a tumor-positive node shows absence of immunostained cells in the capsule, subcapsular fibers and fat cells, including immune cells in nodal parenchyma. Connective tissue fibers enclose subcapsular sinuses which are contiguous in nodal trabeculae in the parenchyma. The section was counterstained with hematoxylin: (b) The micrograph illustrates subcapsular sinuses containing groups of CB-immunostained metastatic prostate cancer cells in metastatic nodules. The section was counterstained with hematoxylin: (c) Metastatic cells immunostained with an antibody against prostate specific antigen (PSA) can be seen invading nodal parenchyma and trabeculae. Immune cells exhibit hematoxylin staining: (d) Metastatic cells immunostained for cathepsin B can be seen invading the nodal parenchyma via the nodal trabeculae. Subcapsular sinuses are surrounded by connective tissue fibers and fat cells: (e) Micrograph showing the distribution of CD68-immunostained macrophages in trabeculae of nodal parenchyma, but not in fat cells and subcapsular connective tissue and lymphatic nodules. The section was not counterstained with hematoxylin: (f) Macrophages immunostained for CD68 are associated with trabecular connective tissue, but some of them invade lymphatic nodules (boxed areas), as shown by the presence of CD68 immunostaining in macrophages and their secretory products. No hematoxylin counterstaining. An adjacent lymphatic nodule illustrates invasion by CD68-immunostained macrophages.
Figure 3. (a) Metastatic nodules/cells in the nodal parenchyma were separated by sinus-like spaces between cancer and lymphatic cells, except at the focal contacts with immune cells (boxed area). In the areas of contact with immune cells, many metastatic cells exhibit degeneration or apoptosis. The cytoplasm had remnants of prostate specific antigen (PSA) immunostaining, not illustrated here. In contrast, subjacent to the sinus-like spaces, cells were not lysed or apoptotic cells: (b) High magnification view of the area in the inset (a). Lysed/apoptotic cells, including immune cells, can be seen. Metastatic cells had PSA immunostaining (not illustrated). (c) Metastatic cells in close association with immune cells exhibited lysis or apoptosis, as indicated by pyknotic nuclei and cytoplasm. This small nodule was completely surrounded by immune cells and did not exhibit sinus-like spaces shown in (a). Metastatic cells, including lysed/apoptotic cells, exhibit PSA immunostaining. (d) PSA-immunostained metastatic cells can be seen to be closely associated with immune cells. Some of them are showing lysis/apoptosis while others do not. (e) This micrograph illustrates numerous lysed/apoptotic metastatic cells surrounded by immune cells. Metastatic cells had PSA immunostaining (not illustrated). (f) This micrograph illustrates numerous degenerating/apoptotic metastatic cells without discernible nuclear or cytoplasmic structures. Degeneration of metastatic cells is almost complete. Metastatic nodules had been replaced by fibrous structure. These metastatic nodules had remnants of PSA immunostaining (not illustrated).
had few lysed or apoptotic metastatic cells (Figure 3b-d), but others had numerous such cells (Figure 3e). In some nodes, metastatic cells were completely lysed, but still showed remnants of PSA immunostaining (Figure 3f). Figure 3f also indicates that completely lysed metastatic cells were replaced by fibrous connective tissue which was surrounded by immune cells. In addition to the lysed/apoptotic cells, nodes contained metastatic cells without any morphological evidence of cell degeneration (see Figures 2c, d, and 3a-c).

**Complexity of nodal metastases.** Since the distribution of metastatic cells varied from node to node, we have categorized metastatic nodules into three subgroups, namely, small- intermediate- and large-sized tumors. Small tumors were usually confined to the subcapsular sinuses and nodal parenchyma; the latter also had intermediate- or large-sized metastatic nodules. The large-sized tumors often occupied most of the node (Figure 5a). Interactions between metastatic and immune cells differed in small-intermediate-and large-volume tumors. Small metastatic nodules/cells separated from immune cells by sinus-like spaces did not show features of degenerating/apoptotic cells (Figure 2a, b). Intermediate- to large-volume metastatic tumors had limited interactions with immune cells, except at the edges of the tumor, where numerous cathepsin B and CD68-immunostained macrophages were located (Figures 5c, d). Nodal metastasis, as illustrated in the montage photomicrograph, did not exhibit sinus-like spaces but contained numerous metastatic cells which were sometimes infiltrated by immune cells, primarily macrophages (Figures 5a-d).

**Distribution of monocyte/macrophage in primary PCa and metastatic nodes.** Macrophages, immunostained by both cathepsin B and CD68, were primarily distributed along the nodal trabeculae (Figures 2e, f and 5c, d). Many lymphatic nodules contained numerous CD68-positive macrophages and ‘secretory molecules’ in the nodal parenchyma, whereas others had few macrophages (Figure 2e, f). Immunostained macrophages exhibiting localization of cathepsin B in tumor-positive nodes had a mean±SEM of 5.59±0.403 and in tumor-negative nodes of 5.98±0.85. Likewise, macrophages immunostained for CD68 in tumor-positive nodes had a mean±SEM of 5.24±1.74 and in tumor-negative nodes of 5.87±0.35. Cathepsin B and CD68 localizations in macrophages were not statistically different between positive and negative nodes (also see Figure 4). This indicates that the presence of metastatic PCa cells did not increase the numbers of macrophages in nodes. Lymphocytes did not localize cathepsin B, CD68 or PSA. Macrophages in the primary PCa were usually found in the stroma surrounding acini and occasionally in the glandular lumina (not illustrated). There were more CD68 immunostained macrophages in PCa than in BPH and benign prostate tissues. Lymphocyte B- and T-cells varied in size and usually exhibited deeply stained nuclei containing dense chromatin and scant and pale cytoplasm in hematoxylin-stained sections.

**Discussion**

Our study is consistent with the earlier observation that metastatic cells initially enter the nodal capsule and are sequestered in the subcapsular sinuses and nodal parenchyma (26). The exact duration of the dormancy (latency) period for metastatic cells in the capsule, subcapsular sinuses and nodal parenchyma is unknown, but it appears to be longer in the nodal parenchyma than in the subcapsular sinuses. In addition, progression of metastatic cells from one node to another is also unknown. Metastatic cells, undoubtedly, proliferate in subcapsular sinuses as shown by their increased number before invading the nodal parenchyma. Since macrophages were not observed in the subcapsular sinuses, the latter appear to be privileged sites for metastatic cell proliferation. An earlier study has shown that cell proliferation (as assessed by localization of Ki-67) in nodes is predictive of clinical outcome in patients with PCa (39). A relationship has been shown between grade and stage of PCa in pelvic lymph node, including lymph node metastasis (38, 40). In this study, we have shown that macrophages in the nodal parenchyma invaded some metastatic nodules, but not others. Earlier, Alitalo and Demar suggested that enhanced lymphangiogenesis may function as a permissive “lymphovascular niche” for the survival of metastatic cells (27). Our study indicates that immune surveillance usually occurs in small metastatic tumors in parenchyma.
Figure 5. (a): A low-power micrograph shows cathepsin B-immunostained metastatic cells occupying most of the pelvic lymph node, which is surrounded by capsule and fat cells (b): Detail of metastatic cells in the inset shown in (a) exhibits weak CB immunostaining (compare with figure 2b and d). There were few immune cells in this area; (c) Reconstruction of a node by montage illustrates cathepsin B-immunostained macrophages found primarily at the edges of the tumor and the nodal trabeculae; (d) Detail of the inset in (c) illustrates CB-immunostained macrophages.
Morphological and IHC evidence have shown that immune surveillance is usually limited to the areas of close association between metastatic and immune cells, including the focal areas of contact between them. A previously published photomicrograph had shown areas of focal contacts between metastatic and immune cells in nodal parenchyma (41). In contrast, metastatic nodules/cells have limited/decreased interactions with immune cells in intermediate to large tumor nodules except at the peripheral edges of the tumors. The presence of degenerating/lysed and apoptotic metastatic cells indicates that immune surveillance primarily occurred in the nodal parenchyma compartment. The lack of close association of immune and metastatic cells and little or no lysis of these cells indicate that immune surveillance was limited (partial) or undetectable by morphological methods. This indicates that at least two populations of metastatic cells are present in a node; the first group of cells is under immune surveillance and the second group has little to no immune surveillance, suggesting the emergence of surveillance-unresponsive (resistant) metastatic cells. The population of metastatic cells under immune surveillance has an inverse relationship to those without surveillance. This relationship differs from node to node and patient to patient. These criteria can be used to distinguish metastatic cancer that is expected to be responsive to immunotherapy from those who would show little or no benefit from such treatment. Responsive populations of metastatic cells may account for the differences observed in treatment response in patients; some patients survive longer even with metastatic disease. Patients with unresponsive populations of metastatic cells are expected to show survival ranging from a few years to many years, but this is the subject of another study.

We have shown that the microenvironment in which metastatic PCa cells proliferate and survive in nodes is distinctly different from that which is found in the primary PCa (11, 12, 42). The microenvironment for metastatic PCa cells in nodes also differs from that found in pelvic bone, lung and other organs (unpublished observation). We postulate that the microenvironment for metastatic cells, including the types of cells, in nodes also differs in other types of solid cancer (such as colon, breast, lung, ovarian, and pancreatic cancer). We suggest that an organ-specific microenvironment ought to be considered for successful treatment of metastatic disease. Additional studies in progress are expected to show genetic and epigenetic bases of differences in metastatic and primary tumors.

To the best of our knowledge, this is the first report to show the presence of two populations of metastatic PCa cells in pelvic nodes.

**Conflicts of Interest**

The Authors have no conflicts of interest in publication of this manuscript.