Expression of Vascular Endothelial Growth Factor Receptor-3 by Lymphatic Endothelial Cells Is Associated with Lymph Node Metastasis in Prostate Cancer

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ABSTRACT
Purpose: The molecular mechanisms underlying lymph node metastasis are poorly understood, despite the well-established clinical importance of lymph node status in many human cancers. Recently, vascular endothelial growth factor (VEGF)-C and VEGF-D have been implicated in the regulation of tumor lymphangiogenesis and enhancement of lymphatic invasion via activation of VEGF receptor-3. The purpose of this study was to determine the expression pattern of the VEGF-C/VEGF-D/VEGF receptor-3 axis in prostate cancer and its relationship with lymph node metastasis.

Experimental Design: The expression pattern of VEGF-C, VEGF-D, and VEGF receptor-3 in localized prostate cancer specimens (n = 37) was determined using immunohistochemistry.

Results: Widespread, heterogeneous staining for VEGF-C and VEGF-D was observed in all cancer specimens. Intensity of VEGF-C staining was lower in benign prostate epithelium than in adjacent carcinoma, whereas no difference between benign epithelium and carcinoma was observed for VEGF-D staining. VEGF receptor-3 immunostaining was detected in endothelial cells of lymphatic vessels in 18 of 37 tissue samples. The presence of VEGF receptor-3-positive vessels was associated with lymph node metastasis (P = 0.0002), Gleason grade (P < 0.0001), extracapsular extension (P = 0.0382), and surgical margin status (P = 0.0069). In addition, VEGF receptor-3 staining highlighted lymphatic invasion by VEGF-C-positive/VEGF-D-positive carcinoma cells.

Conclusions: Together, these results suggest that paracrine activation of lymphatic endothelial cell VEGF receptor-3 by VEGF-C and/or VEGF-D may be involved in lymphatic metastasis. Thus the VEGF-C/VEGF-D/VEGF receptor-3 signaling pathway may provide a target for antilymphangiogenic therapy in prostate cancer.

INTRODUCTION
Lymph node status provides important information in both diagnosis and treatment of prostate cancer. The presence of lymph node metastasis is a poor prognostic sign for patients with prostate cancer (1). In addition, lymph node status influences clinical management because a curative treatment approach (such as perineal prostatectomy or brachytherapy) has a low probability of success in prostate cancer patients with lymph node metastasis (2). Although combined use of clinical data such as tumor stage, serum prostate-specific antigen level, and Gleason grade can help predict the risk of having lymph node metastasis, there are no noninvasive techniques to accurately predict the presence of lymph node metastasis, in particular, microscopic metastasis (1). The emphasis on earlier detection and the need for more effective treatment of prostate cancer patients with lymph node metastasis require a better understanding of the molecular mechanisms involved.

The molecular mechanisms involved in lymph node metastasis are poorly understood, partly because of the lack of specific lymphatic endothelial markers and specific lymphatic growth factors. This situation has improved somewhat since vascular endothelial growth factor (VEGF) receptor-3 was described (3). In embryos, VEGF receptor-3 is initially expressed in venous vasculature (4), but in adults, it is absent in endothelia of all large blood vessels and is generally restricted to lymphatic endothelial cells and a subset of capillary endothelia (5, 6). VEGF receptor-3 is reactivated in the blood vessel endothelium in some tumors, and the up-regulation of its two ligands, VEGF-C and VEGF-D (7), may accompany this (8–10).

VEGF-C and VEGF-D belong to the VEGF family (11, 12), and like all VEGF family members, they contain a central region called the VEGF homology domain (11, 12). They have NH2-terminal and COOH-terminal propeptides and can bind VEGF receptor-2 and VEGF receptor-3 (10, 13, 14). Recent studies in tumor models have provided direct evidence that
VEGF-C and VEGF-D can induce lymphangiogenesis through VEGF receptor-3 and/or promote angiogenesis through VEGF receptor-2 (15–19) and that induction of lymphangiogenesis is often associated with lymph node metastasis in these model systems. In breast carcinoma models, VEGF-C induced only lymphangiogenesis (15, 16, 19), and this was associated with lymph node metastasis in two studies (15, 19). In contrast, in melanoma and epithelioid-like (293EBNA cell line) tumor models transfected with VEGF-C and VEGF-D, respectively, both lymphangiogenesis and angiogenesis were observed (17, 18), and lymph node metastasis was observed only in the 293EBNA model. The differences in these results most likely reflect variations in the relative expression of the VEGF-C/VEGF-D receptors VEGF receptor-2 and VEGF receptor-3 in the model systems.

Studies in clinical specimens have shown that VEGF-C expression is positively associated with lymph node metastasis in several tumor types. In prostate cancer, Tsursusaki et al. (20) found that VEGF-C mRNA levels were significantly higher in lymph node-positive tumors and that VEGF receptor-3-positive vessels were increased in the stroma of VEGF-C-positive tumors. Moreover, VEGF receptor-3 expression was correlated with Gleason score, preoperative prostate-specific antigen levels, and lymph node metastasis in prostate cancer (21). However, there are no reports documenting VEGF-C protein localization in human prostate cancer. Recent reports in breast and ovarian cancer suggest that VEGF-D positivity is a prognostic factor (22–24); however, VEGF-D localization in prostate cancer has not been described previously. In the present study, the protein expression patterns of VEGF-C, VEGF-D, and VEGF receptor-3 were examined by immunohistochemistry to explore whether this signaling axis is associated with lymph node status in primary prostate cancer.

MATERIALS AND METHODS

Prostate Tissue Samples and Patient Characteristics. Immunohistochemical examination was performed on 37 prostate cancer radical prostatectomy specimens obtained from men treated at either St. Vincent’s Hospital, Melbourne between 2001 and 2003 or St Vincent’s Hospital, Sydney between 1990 and 1998. The pathological characteristics of the resected primary tumors and regional lymph nodes are summarized in Table 1. Sections that contained areas of adenocarcinoma with adjacent benign tissue were selected to allow comparative evaluation. Serial 4-μm sections were cut from formalin-fixed, paraffin-embedded tissue and mounted on slides for immunohistochemical detection of VEGF-C, VEGF-D, and VEGF receptor-3 as described below. The studies were conducted with ethical approval of the St. Vincent’s Hospital Human Ethics Committee and were in accordance with Australian National Health and Medical Research Council Guidelines.

**Antibodies.** Goat polyclonal anti-VEGF-C (AF752), goat polyclonal anti-VEGF receptor-3 (AF349), and mouse monoclonal anti-VEGF-D (MAB 286) were purchased from R&D Systems (Minneapolis, MN) and used for immunohistochemistry at the following concentrations: anti-VEGF-C, 2.5 μg ml⁻¹; anti-VEGF receptor-3, 1.67 μg ml⁻¹; and anti-VEGF-D, 8.33 μg ml⁻¹. Mouse monoclonal antibody D2-40, which reacts with an O-linked sialoglycoprotein found on lymphatic endothelium, was purchased from Signet Laboratories (Dedham, MA) and used at a dilution of 1:100.

**Immunoprecipitation, Western Blotting, and Analysis of Antibody Specificity.** A plasmid encoding FLAG-tagged, full-length VEGF-D and transfection into 293EBNA cell have been described previously (14). For VEGF-C, an expression plasmid encoding full-length VEGF-C tagged at the COOH terminus with three Myc tags (single Myc epitope, EQKLISEEDL) was generated. 293EBNA cells were transiently transfected with this plasmid using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer’s instructions. VEGF-C and VEGF-D were purified from conditioned medium collected over 24–48 h. A c-Myc antibody (9E10; Zymed Laboratories Inc., San Francisco, CA) coupled to cyanogen bromide-activated Sepharose (Amer sham Biosciences, Castle Hill, New South Wales, Australia) and M2 beads (anti-FLAG M2-Agarose mouse; Sigma-Aldrich, St. Louis, MO) were used to pull down VEGF-C and VEGF-D, respectively.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No. of specimens</th>
<th>% of specimens with VEGFR-3⁺ vessels</th>
<th>Relative risk (95% confidence limit)</th>
<th>P</th>
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<tr>
<td>Combined Gleason grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>6</td>
<td>17</td>
<td>12</td>
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<tr>
<td>7</td>
<td>14</td>
<td>71</td>
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<tr>
<td>≥ 8</td>
<td>6</td>
<td>100</td>
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<tr>
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<td>63</td>
<td>2.71 (0.96–7.66)</td>
<td>0.0382†</td>
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<tr>
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<td>23</td>
<td></td>
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<tr>
<td>Surgical margin status</td>
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<tr>
<td>Positive</td>
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<td>79</td>
<td>2.58 (1.31–5.08)</td>
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<tr>
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<td>Lymph node metastasis</td>
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Abbreviations: VEGFR, vascular endothelial growth factor receptor; n/a, not applicable.
* χ² test.
† Fisher’s exact test.
For Western blotting, the proteins were transferred to a nitrocellulose membrane (Hybond-C Super; Amersham Biosciences, Buckinghamshire, United Kingdom) and probed with primary antibody against VEGF-C or VEGF-D, followed by the appropriate secondary antibody. Signal was developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology, Rockford, IL).

Immunohistochemistry. Tissue sections were deparaffinized in shelllex, followed by rehydration in graded ethanol. After quenching of endogenous peroxidase activity, antigen retrieval (citrate buffer, pH 6.0), and protein blocking (DAKO protein block serum free; DAKO Corp., Carpinteria, CA), tissue sections were incubated overnight at 4°C with the specified primary antibody. The tissue sections were then incubated with the appropriate biotinylated secondary rabbit antigoat or antimouse antibodies (DAKO, Glostrup, Denmark) and streptavidin-biotin-peroxidase complex (DAKO). Peroxidase reactivity was visualized using 3,3′-diaminobenzidine (DAKO). The sections were counterstained with hematoxylin and coverslipped. Negative controls included immunostaining of normal human cerebrum (25) and substitution of normal goat IgG (R&D Systems) or mouse IgG1 (DAKO) for the primary antibodies. Normal human heart was used as a positive control tissue for VEGF-C (11) and VEGF-D (24) staining, human melanoma was used as a positive control tissue for VEGF receptor-3 (26) staining, and normal lymph node was used as a positive control for D2-40 staining. Two trained observers (K. O. and E. D. W.) evaluated and interpreted the results of immunohistochemical staining without knowledge of the clinical data of each patient. Staining was scored by both observers simultaneously, using a multihead microscope. Staining of adenocarcinoma, benign epithelia, stroma, lymphatic vessels, vascular endothelial cells, and smooth muscle was recorded as strong, weak, or negative and recorded on standardized data sheets. Primary and secondary Gleason grades were assigned by a pathologist (K. O.).

Statistics. The relationships between the presence of VEGF receptor-3-positive vessels and clinicopathological parameters were evaluated by Fisher’s exact test or χ² test as indicated. P < 0.05 was considered statistically significant. All calculations were performed using the statistical computer program Prism+Instat bundle (GraphPad Software, San Diego, CA).

RESULTS

Antibody Specificity. Western blotting demonstrated specific detection of VEGF-C and VEGF-D using the anti-VEGF-C and anti-VEGF-D antibodies, respectively (Fig. 1A), and proteins of the expected size were detected (13, 14). There was no cross-reactivity between either the anti-VEGF-C antibody and VEGF-D or the anti-VEGF-D antibody and VEGF-C (Fig. 1A). The VEGF-C/VEGF-D-positive control tissue, normal human heart (11), exhibited strong granular staining in all cardiac muscle cells for both VEGF-C and VEGF-D (Fig. 1B). The VEGF receptor-3-positive control tissue, human melanoma (26), showed strong staining for VEGF receptor-3 in the endothelial cells of all lymphatic and some blood vessels in the tumor and adjacent dermis (Fig. 1B). Lymphatic vessels in normal lymph node showed strong positive staining using the lymphatic endothelial marker D2-40, with no staining in other tissue components (Fig. 1B). Normal human brain, the negative control tissue for the four antibodies (25), did not show any staining (Fig. 1B). Substitution of isotype-matched controls for the primary antibody also showed no immunostaining in each specimen (data not shown).
Localization of Vascular Endothelial Growth Factor C in Human Prostate Tissue. Positive staining for VEGF-C was observed in prostate cancer cells in all samples (37 of 37 samples). Tumor nests showed heterogeneous granular cytoplasmic staining (Fig. 2A) and membrane-associated staining (Fig. 2B). By comparison, benign prostate glands adjacent to tumor nests demonstrated weak or no cytoplasmic staining for VEGF-C in epithelial cells (Fig. 2C). VEGF-C was also de-
ected at low levels in some stromal cells, in smooth muscle cells of arteries, and occasionally in blood vessel endothelial cells. This is consistent with the previous demonstration of VEGF-C protein expression in smooth muscle and endothelial cells in normal fetal and adult tissues (6).

Localization of Vascular Endothelial Growth Factor D in Human Prostate Tissue. Carcinoma cells in all specimens stained positively for VEGF-D (37 of 37 specimens). Heterogeneous cytoplasmic staining was observed in cancer cells (Fig. 2, D–F), with membrane-associated staining detected in some cancer cells (Fig. 2F). Furthermore, staining was much stronger at the edges of tumor nests than in the central parts in most samples (data not shown). Heterogeneous expression of VEGF-D was also noted in the benign glandular epithelial cells adjacent to tumor areas in all 37 cases (Fig. 2E). The staining pattern of the hypertrophic glands and atrophic glands was similar to that of prostate cancer glands, showing heterogeneity within each sample.

A strong and consistent pattern of VEGF-D expression was observed within the fibromuscular stroma of all prostate cancer specimens (37 of 37 specimens; Fig. 2F). The smooth muscle cells of blood vessels in and adjacent to areas of tumor expressed VEGF-D strongly (37 of 37 samples; Fig. 2G). In contrast, microvessel endothelial cells showed inconsistent staining. Vascular endothelia adjacent to carcinoma showed positive staining in some specimens (Fig. 2H).

Localization of Vascular Endothelial Growth Factor Receptor-3 in Prostate Tissue. VEGF receptor-3 immunostaining decorated the endothelial cells lining a subpopulation of vessels in 18 of 37 specimens. The median number of VEGF receptor-3-positive cross-sections of vessels was 45 per tissue section (range, 5–519 per tissue section). The vessels were thin-walled and devoid of erythrocytes and neutrophils, suggesting a lymphatic nature (Fig. 3, A–D and F). Furthermore, the vessels stained positively using the lymphatic endothelium marker D2-40 (Fig. 3E). The adjacent blood vessels were always negative for VEGF receptor-3 (Fig. 3A). VEGF receptor-3-positive vessels were located within the tumor nests, at the tumor periphery, and in benign tissue. Usually, intratumoral VEGF receptor-3-positive vessels were very small or collapsed (Fig. 3, B and C), although large intratumoral VEGF receptor-3-positive vessels were observed occasionally (Fig. 3D). In five cases, four of which had known clinical lymph node metastasis, cancer cells were visible within the lumen of D2-40-positive (Fig. 3E), VEGF receptor-3-positive (Fig. 3F) vessels adjacent to tumor areas. The cancer cells were positive for VEGF-C (Fig. 3G) and VEGF-D (Fig. 3H). The vessels containing the cancer cells were thin-walled, devoid of erythrocytes, and D2-40 positive, indicating lymphatic invasion.

VEGF receptor-3 expression was not detected in either epithelial components or stromal cells in the majority of the prostate cancer specimens. Only 3 of 37 samples (8%) showed focal positive staining for VEGF receptor-3 (data not shown) in cytoplasm of cancer cells.

Correlation between Vascular Endothelial Growth Factor Receptor-3 Expression and Clinicopathological Factors. As shown in Table 1, the presence of VEGF receptor-3-positive vessels (one or more) was significantly associated with Gleason grade ($P < 0.0001$), surgical margin status ($P = 0.0069$), and lymph node status ($P = 0.0002$). Consistent with our results, Li et al. (21) found a significant correlation between VEGF receptor-3 expression and lymph node metastasis.

DISCUSSION

Lymphatic invasion is one of the major routes for prostate cancer cell dissemination, and pelvic lymph node involvement is the first sign of metastasis in many prostate cancers (2, 27). However, studies on the role of tumor lymphangiogenesis in lymphatic spread have been overshadowed by the focus on tumor angiogenesis. Recent work on the VEGF-C/VEGF-D/VEGF receptor-3 axis has begun to elucidate the molecular mechanisms involved in lymphangiogenesis and lymphatic metastasis. Our study has revealed the presence of VEGF-C, VEGF-D, and VEGF receptor-3 protein in human prostate cancer and suggests that together, these molecules may play a role in the formation of prostate cancer lymph node metastasis.

In this study heterogeneous staining was demonstrated in prostate cancer cells for both VEGF-C and VEGF-D. The intensity of VEGF-D staining was much stronger on the edge than in the center of the tumor, indicating the heterogeneity of prostate cancer, which contains subpopulations of cells with different biological properties such as invasive potential (28, 29). Indeed, in human gastric carcinoma, VEGF-C immunoreactivity was more intense in the invasive tumor component compared with in situ tumor (30). In several human cancers, including prostate cancer, immunohistochemical analysis has shown that expression of genes and proteins associated with angiogenesis and invasion is higher in peripheral zones of cancers than in their centers (28).

Epithelial-stromal interactions are thought to play a critical role in the initiation and promotion of carcinogenesis in prostate cancer (31). Expression of several growth factors involved in tumor growth, angiogenesis, invasion, and metastasis has been demonstrated in both prostate epithelia and stroma (32–34). It has been suggested that growth factors may have a role in inducing epithelial proliferation and prostatic carcinogenesis via both autocrine and paracrine pathways (32, 33, 35, 36). In the present immunohistochemical study, VEGF-C and VEGF-D were localized to both cancer epithelial cells and stromal cells in all prostate carcinoma specimens. VEGF-C and VEGF-D have been shown to enhance tumor growth in tumor mouse models (16, 18). It is possible that VEGF-C, which was overexpressed in carcinoma cells compared with benign epithelial cells, may contribute to prostate cancer growth. In contrast, VEGF-D was readily detected in both epithelial and carcinoma cells. Because VEGF receptor-3 was detected in carcinoma cells in only 3 of 37 samples, it is likely that, if VEGF-C and VEGF-D elicit any direct biological actions on tumor cells, this is achieved via other receptor(s). This may be VEGF receptor-2, or alternatively, there might exist as yet unidentified specific receptor(s) for VEGF-C and VEGF-D.

VEGF receptor-3 is predominantly localized to lymphatic endothelial cells in adult tissue (4, 5, 37), although its up-regulation in angiogenic blood vessel endothelium has been detected in breast cancer (9) and some vascular tumors (38). In the present study, expression of VEGF receptor-3 was restricted to a small proportion of vessels that had morphology character-
Immunohistochemical staining of VEGF receptor-3 in human prostate cancer. Lymphatic vessels stained positively for VEGF receptor-3, whereas blood vessels stained negatively (A). Positive staining was observed in peritumoral lymphatics (A), small intratumoral vessels (B), collapsed intratumoral vessels (C), and large intratumoral vessels (D). In serial sections (E–H), endothelial cells of vessels were positive for lymphatic marker D2-40 (E) and VEGF receptor-3 (F), and tumor cells inside the vessels showed positive staining for VEGF-C (G) and VEGF-D (H). Arrows, lymphatic vessels; arrowheads, blood vessels; t, tumor. Magnification, ×400.
istic of lymphatic vessels and stained positively using the lymphatic vessel marker D2-40. There was a strong association between positive staining of prostatic lymphatic vessels for VEGF receptor-3 and the presence of lymph node metastasis (Table 1). Occasionally, tumor emboli were detected within peritumoral VEGF receptor-3-positive vessels. An essential prerequisite for the formation of lymphatic metastasis is the entry of cancer cells into lymphatic vessels (29). Our study suggests that peritumoral lymphatic vessels may serve as a route for nodal metastasis in human prostate cancer. Similar to our findings, tumor invasion into peritumoral lymphatic vessels has been demonstrated in human colorectal carcinoma (24) and head and neck cancer (39). Tumor emboli have also been detected in intratumoral lymphatics of VEGF-C- and VEGF-D-transfected tumor mouse models (15, 18).

VEGF-C has been shown to be capable of increasing vascular permeability and may enhance cancer cell dissemination via lymphatic vasculature in some human tumors (9, 40, 41). Because VEGF-D shares 61% sequence with VEGF-C (12), it is conceivable that VEGF-D may have similar functional roles to those of VEGF-C. Our study demonstrates the presence of VEGF-C- and VEGF-D-positive carcinoma cells within VEGF-receptor-3-positive vessels. Moreover, the presence of VEGF receptor-3-positive vessels was correlated with lymph node metastasis in prostate cancer. Taken together, these results suggest that VEGF-C and VEGF-D secreted by cancer cells may activate VEGF-receptor-3 expressed on the endothelial cells of adjacent lymphatic vessels via a paracrine mechanism to induce lymphatic invasion, possibly by modifying vessel permeability (9, 40, 41). This would provide a route for tumor metastasis via the lymphatic vessels to the lymph nodes.

In summary, our results demonstrate that both VEGF-C and VEGF-D are widely expressed in human prostate carcinoma. VEGF-D, but not VEGF-C, is also abundantly expressed in adjacent benign prostate epithelia. In contrast, VEGF receptor-3 is up-regulated in vessels in a subset of prostate cancers. The demonstration of tumor emboli in VEGF receptor-3-positive vessels and the significant correlation between the presence of prostatic VEGF receptor-3-positive vessels and lymph node metastasis provide tantalizing evidence for a role of VEGF receptor-3 signaling in the development of lymph node metastasis. A humanized monoclonal antibody against VEGF-A has recently been developed for therapeutic use in metastatic colorectal cancer (42), and it is possible that VEGF-C or VEGF receptor-3 may provide useful clinical targets for developing new therapeutic agents toward prostate lymph node metastasis. VEGF-D may also provide a potential therapeutic target for inhibiting prostate cancer progression, given the stronger staining for VEGF-D observed at the edge of tumor nests; however, the role of VEGF-D in prostate cancer requires further analysis.

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