Stage-Specific Characterization of the Vascular Endothelial Growth Factor Axis in Prostate Cancer: Expression of Lymphangiogenic Markers Is Associated with Advanced-Stage Disease

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ABSTRACT

Purpose: The vascular endothelial growth factor (VEGF) family plays a critical role in tumor angiogenesis and lymphangiogenesis. We characterized, at the mRNA and protein levels, the expression of VEGF-A and VEGF-D and their cognate receptors, VEGFR-1, VEGFR-2, and VEGFR-3 in early- and advanced-stage prostate cancer specimens. Experimental Design: The levels of VEGF-A and VEGF-D mRNA in early- and advanced-stage specimens were compared using an angiogenic gene array and were confirmed by quantitative real-time PCR. Receptor protein levels and activation status were determined by immunoblotting. Spatial expression of the proteins was evaluated using immunohistochemistry with fresh and archival tissues from benign prostatic hypertrophy specimens, early-stage prostate specimens, and advanced-stage metastatic specimens. Circulating plasma levels of these growth factors were measured using ELISAs.

Results: We observed that expression patterns of VEGF isotypes corresponded to the prostate cancer stage: high expression of angiogenic growth factor VEGF-A was observed in early-stage prostate specimens, whereas high expression of lymphangiogenic growth factor VEGF-D was associated with advanced-stage metastatic disease. All VEGF receptors were present at variable levels in all specimens, but their activation states varied in a stage-specific manner.

INTRODUCTION

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer-related mortality among men in the United States. For 2004, the American Cancer Society projected 230,110 prostate cancer diagnoses and estimated 29,900 deaths (http://www.cdc.gov/cancer/prostate, Cancer Facts and Figures 2004, ACS, 2004). The majority of prostate cancer–related deaths are associated with incurable, advanced-stage disease. Localized early-stage prostate cancer progresses to invasive, hormone-sensitive disease and then to metastatic hormone-resistant disease. The sites of metastasis are predominantly localized in the bone and lymph nodes, suggesting a combination of hematogenous and lymphatic routes for dissemination of the disease. Whereas several clinical features of disease progression are known, molecular signatures that contribute to the progression of prostate cancer disease from localized to the metastatic state are not fully understood.

Angiogenesis, the development of new blood vessels from the preexisting vasculature, is essential for tumors to sustain growth and metastasis to distant sites (1). Numerous proangiogenic and antiangiogenic growth factors and their cognate receptors have been identified (2). For vascular network–mediated tumor growth and metastasis, the tight balance between proangiogenic and antiangiogenic factors regulates the angiogenic switch (3). In addition to angiogenesis, lymphangiogenesis, the growth of new lymphatic vessels, is becoming recognized as a key component of metastatic spread in several types of cancer (4–7). However, the status of lymphangiogenesis in prostate cancer disease progression is not yet known.

Among various proangiogenic growth factors, the vascular endothelial growth factor (VEGF) family plays an important role in the development of angiogenesis (2) and lymphangiogenesis (7) in a wide variety of tumors. VEGF-A and VEGF-B promote vascular angiogenesis predominantly by activating their receptors VEGFR-1 (Flt1) and VEGFR-2 (Flk1 and KDR). VEGF-C (8) and VEGF-D (also known as c-fos–induced growth factor; refs. 9, 10) have been identified as important mediators of lymphangiogenesis and tumor metastasis by activating receptors VEGFR-2 and VEGFR-3 (Flt4; ref. 11). VEGF-C and VEGF-D each contain the central VEGF-homology domain characteristic of the VEGF family, along with distinct N- and C-terminal pro-domains. VEGF-C and -D are each converted into their bioactive forms through proteolytic processing of the terminal pro-domains.

VEGFR-1 and, to a limited extent, VEGFR-2 were activated in early-stage specimens, whereas VEGFR-2 and VEGFR-3 were activated in advanced-stage specimens.

Conclusions: Our results suggest that lymphangiogenic markers, such as VEGF-D and VEGFR-2 and VEGFR-3, may be better than angiogenic markers as targets of therapeutic intervention in advanced-stage prostate disease.
and forming disulfide-linked dimers of the VEGF-homology domains (12, 13). Mature, bioactive VEGF-C and VEGF-D bind to their cognate receptors VEGFR-2, primarily localized on vascular endothelial cells, and VEGFR-3, localized on lymphatic endothelial cells. Binding growth factor activates VEGFR-2 and VEGF-3 via tyrosine phosphorylation and results in induction of angiogenic and lymphangiogenic signals (14).

Expression of angiogenic growth factor VEGF-A and its receptors has been previously reported in prostate cancer, and it seems that VEGF-A is a critical element in promoting angiogenesis in prostate cancer (15–22). The molecular events leading to the development of tumor vascularization have also been previously identified using xenograft nude mice (23) and a transgenic model of prostate cancer (24, 25). Although metastasis to the lymph nodes is recognized as correlating with poor prognosis in prostate cancer, there is no report on the involvement of lymphangiogenic growth factors and their receptors in advanced-stage prostate cancer.

In the current study, we characterized global changes in the VEGF axis in early- and advanced-stage prostate cancer specimens. Our results show that, whereas cancer cells from early-stage prostate cancer patients predominantly express the angiogenic growth factor VEGF-A and its activated receptor VEGFR-1, the majority of the advanced-stage prostate tumors express higher levels of the lymphangiogenic growth factor VEGF-D and its activated cognate receptors VEGFR-2 and VEGFR-3. This suggests that lymphangiogenesis plays a role in progression of prostate cancer and that VEGF-D signaling may provide useful markers of advanced-stage disease.

**MATERIALS AND METHODS**

**Sample Collection.** Prostate tissues from patients with early-stage prostate cancer undergoing radical prostatectomy (n = 10) and from advanced-stage prostate cancer patients undergoing trans-urethral resection of prostate (n = 8) for bladder outlet obstruction were collected after approval of the protocol by the Institutional Human Research Advisory Committee and signing of the informed consent by the patients. The early-stage cancer group showed no evidence of bone or lymph node metastasis on clinical or pathologic examination. All patients in the advanced-stage group had clinical evidence of either bone or lymph node metastasis and were undergoing androgen-deprivation therapy. Four of the eight advanced-stage patients in this group were also undergoing systemic chemotherapy for hormone refractory disease. These samples were not differentiated as treated and untreated for data analysis because of a very small sample size. Only one patient was included in the study before and after chemotherapy, but his postchemotherapy tissue sample was unavailable.

Freshly resected tissues were snap frozen in liquid nitrogen and stored at −80°C until analysis. Blocks of formalin-fixed and paraffin-embedded tissues from the same set of patients were obtained from the Surgical Pathology Laboratory of Central Arkansas Veterans Healthcare System. Patient blood samples were collected in sodium citrate tubes (4.5:0.5, blood-to-saline volume ratio). The samples were centrifuged at 2,500 × g to collect platelet-poor plasma and then batched for analysis of angiogenic growth factors.

**Gene Expression Profiling.** Gene array analysis for angiogenic markers was done using the GEArray Q Series Human Angiogenesis Gene Array (SuperArray Bioscience Co., Frederick, MD) and the supplier-recommended protocol. Total RNA was extracted from a portion of the frozen tumor specimen using TRIzol method (Invitrogen Co., Carlsbad, CA). Total RNA was further purified with RNeasy (Qiagen, Valencia, CA). The purity and integrity of the RNA samples was determined by spectrophotometry and by denaturing agarose gel electrophoresis. Intact RNAs were successfully isolated from 16 (radical prostatectomy, n = 9; trans-urethral resection of prostate, n = 7) out of 18 specimens and were used for gene array analysis and quantitative real-time PCR (QRTPCR).

Approximately 2 μg of individual RNA samples were used to prepare 32P-labeled cDNA probes using array-specific primer mix provided in the GEArray and Ampolabeling kits (SuperArray Bioscience). The labeled probes were mixed with hybridization solution provided, and were used for hybridizing all membranes at 60°C overnight. Membranes were washed twice with wash buffer A (2 × saline-sodium citrate, 1% SDS) at 60°C for 15 minutes each, followed by two washes with wash buffer B (0.1 × saline-sodium citrate, 0.5% SDS) at 60°C for 15 minutes each. The images of all membranes were obtained using a Storm phosphorimager and were digitized using ImageQuant 5.1 (Amersham Biosciences, Piscataway, NJ). We superimposed a grid of eight columns and 14 rows onto the raw image. Each cell of the grid contained a tetra-spot on the array, allowing us to convert the image data into numerical data. All data were analyzed using GEArrayAnalyzer (SuperArray Bioscience). Each membrane was normalized to signal from β-actin cDNA on the array, and the data table (not shown) was generated depicting the relative intensity of each cDNA as compared with β-actin.

**QRTPCR.** Expression levels of selected angiogenic and lymphangiogenic genes were confirmed by QRTPCR, using SYBR green chemistries, and the Prism 7700 Sequence Detection System (Applied Biosystems). Primer pairs for QRTPCR (Table 1) were designed with Primer Express v1.5 software (Applied Biosystems), in conjunction with the National Center for Biotechnology Information database, and were synthesized by Integrated DNA Technologies (Coralville, IA). Two different sets of primers from different areas of the gene were used for VEGFR-2 to confirm its expression levels. For each prostate cancer specimen, cDNA was synthesized using 2 μg of total RNA, random hexamers, and the Taqman Reverse Transcription Reagents (Applied Biosystems). To optimize assay efficiency, PCR standard curves were produced using a pool containing each sample cDNA. Reaction variables for each gene of interest are listed in Table 1. Standard curve data points were generated using 4-fold serial dilutions of cDNA pool along with 40 cycles of amplification. Gene expression in individual samples was compared using 16, 4, or 1 ng RNA equivalent of cDNA, based on the range of the standard curve. All expression levels were normalized to 18s rRNA.

**Western Blot Analysis.** Total protein extracts were prepared by tissue homogenization in lysis buffer [10 mmol/L Tris-HCl (pH 7.5) 150 mmol/L NaCl, 1% Triton X-100, 30 mmol/L sodium pyrophosphate, 2 mmol/L Na3VO4, 5 mmol/L of NaF, 1 mmol/L phenylmethyl sulfonyl fluoride, and 1× protease inhibitor cocktail (Sigma, Saint Louis, MO)]. Only 9 of
10 early-stage specimens and six of eight advanced-stage specimens were analyzed for protein expression due to insufficient material. About 20 µg of protein from each tissue sample were denatured by boiling for 10 minutes in loading buffer [125 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 1% SDS, and 0.01% β-mercaptoethanol], and electrophoretically separated on 4% to 15% gradient SDS-PAGE (Bio-Rad, Hercules, CA). After gel-separation, proteins were transferred onto nitrocellulose membrane (Bio-Rad). Membranes were blocked with nonspecific protein (5% dry nonfat milk in PBS) then incubated with rabbit polyclonal primary antibody specific for VEGF-D (sc-13085, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:2,000, rabbit polyclonal antibody specific for VEGF-A (sc-507, Santa Cruz Biotechnology) diluted 1:1,000, and mouse monoclonal antibody specific for β-actin (Clone AC-15, Sigma) diluted 1:5,000 in PBS for 4 hours at ambient temperature with continuous rocking. Membranes were washed extensively with TBST [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, and 0.05% Tween 20] and incubated for 2 hours with 1:2,000 dilution of horseradish peroxidase–conjugated secondary antibody (Amersham Biosciences). Membranes were washed with TBST and developed with enhanced chemiluminescence detection system (Enhanced Chemiluminescence Plus, Amersham Biosciences) and exposed to Hyperfilm-enhanced chemiluminescence system (Amersham Biosciences). The Western blot analysis was scored positive if the band of interest was present at the expected molecular weight appropriate for each marker protein. All analyses were done in duplicate. Intensities of all bands on immunoblots were quantified with UnScanIt-gel software (Silk Scientific Co., Orem, UT).

**Immunoprecipitation.** The three VEGF receptors were immunoprecipitated using the Seize X immunoprecipitation kit (Pierce Biotechnology, Rockford, IL). Briefly, receptor-specific antibodies (sc-9029 for VEGFR-1, sc-6251 for VEGFR-2, and sc-321 for VEGFR-3; Santa Cruz Biotechnology) were immobilized to Protein A gel using the cross-linker disuccinimidyl suberate. Equal amounts (200 µg) of tissue lysates from patient samples (described above) were incubated with immobilized antibody to form immune complexes which were washed extensively to remove non-specifically bound proteins. Bound antigen was dissociated using low pH glycine solution. Eluted proteins were immediately neutralized with Tris base and processed for electrophoresis and immunoblotting as described above. The levels and activation status of all immunoprecipitated receptors was assessed by probing with receptor-specific and phospho-tyrosine antibodies (sc-508, Santa Cruz Biotechnology) respectively. Intensities of bands of interest on immunoblots were quantified with UnScanIt-gel.

**Immunohistochemistry.** Formalin-fixed and paraffin-embedded blocks of specimens were obtained from the Surgical Pathology Laboratory of CAVHS. Five-micrometer sections were cut out of the paraffin blocks and mounted on Silane-Prep slides (Sigma). Slides were hydrated with graded alcohol and equilibrated in PBS. Antigen retrieval was done by steaming the slides in 10 mmol/L citrate buffer (pH 6.0) for 20 minutes. Endogenous peroxidase activity was quenched by short incubation with 3% hydrogen peroxide in methanol. Nonspecific binding was blocked with a 5% serum solution in PBS. Slides were incubated with specific antibodies diluted 1:100 in PBS, followed by extensive washes with PBS. Subsequently, slides were incubated with horseradish peroxidase–conjugated secondary antibody, and washed. Immunoreactivity was detected with the mouse avidin-biotin complex method staining system (Vector Laboratories, Burlingame, CA) according to the manufacturer’s protocol. Sections were counterstained with Gill’s hematoxylin, dehydrated with graded alcohol and xylene, and mounted under glass coverslips. Appropriate isotype antibodies were used as controls to evaluate specificity of staining.

**ELISA.** Patient plasmas from the current trial (n = 18), and from patients with advanced-stage disease from a previous trial (n = 12) were tested for circulating levels of VEGF-A and VEGF-D.

### Table 1

<table>
<thead>
<tr>
<th>Gene and accession no.</th>
<th>5'-3' Forward/ reverse primer</th>
<th>Standard range</th>
<th>Sample input</th>
<th>Primer concentration</th>
<th>Product Tm</th>
<th>Primer-dimer Tm</th>
<th>% E1</th>
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<tr>
<td>VEGF-D NM_004469</td>
<td>TGGACACAGAACCCACTCTCTATC/ GCAACGATCTTTGCTCAACATC</td>
<td>80-1.25</td>
<td>16</td>
<td>160</td>
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<td>97</td>
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<td>VEGF-A M32977</td>
<td>AGACTCCTGCGGAGAACAT/ AATGGGCAATTTCAATCC/A</td>
<td>20-0.31</td>
<td>4</td>
<td>200</td>
<td>83.2</td>
<td>n.d.</td>
<td>99</td>
</tr>
<tr>
<td>VEGFR-1 AF035121</td>
<td>CACCACTCAAACGGCTGACATG/ CAACTCAGGCTAACCATGGA</td>
<td>20-0.31</td>
<td>4</td>
<td>120</td>
<td>80.1</td>
<td>75.3</td>
<td>99</td>
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<tr>
<td>VEGFR-2 15 NM_002019</td>
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<td>20-0.31</td>
<td>4</td>
<td>100</td>
<td>72.4</td>
<td>74.6</td>
<td>96</td>
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<tr>
<td>VEGFR-2 21 NM_002019</td>
<td>CCCCAGGCGCAGACTA/ AAGGGCTGTCTGCACTCTTAGT</td>
<td>20-0.31</td>
<td>4</td>
<td>155</td>
<td>81.6</td>
<td>n.d.</td>
<td>92</td>
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<td>VEGFR-3 NM_002020</td>
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<td>100</td>
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<td>75.8</td>
<td>81</td>
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<td>TCCGGACGCTGCTGCCCTATCA/ ATGGTAGACCCAGGGACTA</td>
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<td>1</td>
<td>300</td>
<td>78.5</td>
<td>n.d.</td>
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*Standard curve range and sample input units are ng RNA equivalent of cDNA.

1Primer concentrations are in nmol/L.
2PCR product and primer-dimer Tm units are °C.
3%E is the amplification efficiency of the PCR assay.
2Two different sets of primers from different areas of the gene were used for VEGFR-2 to confirm the expression levels.
4Primers were purchased from Applied Biosystems.
VEGF-D using Human VEGF and VEGF-D Quantikine ELISA kits (R&D Systems, Minneapolis, MN), as previously reported (22).

**Statistical Analysis.** All statistical analyses were conducted in Excel v 5.0 (Microsoft). The two-sided Wilcoxon rank-sum test with t-approximation (i.e., Student’s t test conducted on the ranks) was used at $\alpha = 0.05$ to assess the two groups for differences in gene expression using QRTPCR and plasma protein levels.

**RESULTS**

**VEGF-D Is a Highly Expressed Gene in Prostate Cancer.** Angiogenic gene arrays were used to identify angiogenic genes that are prominently expressed in early-stage (radical prostatectomy) and advanced-stage (trans-urethral resection of prostate) patient samples (Fig. 1). All specimens showed the highest hybridization intensity for the tetra-spot corresponding to VEGF-D (Fig. 1, circled spot, array B1). Expression levels are presented as fold intensity relative to the internal control gene, $\beta$-actin. VEGF-D expression levels in early-stage specimens ranged from 1.4- to 13.9-fold, with a mean level of 9.58-fold; levels in trans-urethral resection of prostate samples ranged from 2.76- to 19.3-fold, with a mean value of 10.9-fold. Hybridization intensities of VEGF-A, VEGF-B, and VEGF-C were low in both specimen types (Fig. 1, boxed spots, array B2) with mean values of 0.48-fold versus 0.24-fold, 0.31-fold versus 0.21-fold, and 0.21-fold versus 0.24-fold in early-stage and late-stage specimens, respectively. The levels of VEGF-A, although low, varied significantly between the two specimen types. Levels of VEGF-B and VEGF-C were not significantly different between the two groups of patients.

Relative expression levels between the early- and late-stage specimens were confirmed for the prototype angiogenic growth factor, VEGF-A, and lymphangiogenic growth factor, VEGF-D, using QRTPCR. We have normalized expression levels to those of 18s rRNA by presenting them as units (i.e., multiples) of this invariant internal control. As shown in Fig. 2A, the median expression level (and interquartile range) of VEGF-A was 4.13 (1.73-5.51) units in early-stage patients, compared with 0.89 (0.54-1.26) units in advanced-stage patients ($P = 0.0058$). In contrast, the median expression level (and interquartile range) of VEGF-D was 14.20 (11.71-16.06) units in early-stage patients, compared with 22.99 (12.58-38.41) units in advanced-stage patients ($P = 0.3238$). Thus, these two growth factor mRNAs may not be coordinately regulated during cancer progression. VEGF-A expression is greatest in early-stage prostate cancer patients whereas VEGF-D expression was higher in advanced-stage patients.

**Expression of VEGFRs in Prostate Cancer Specimens.** The array data indicated that the differences in expression levels

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**Fig. 1** Expression profiles of angiogenic genes in prostate cancer specimens. Angiogenic gene arrays were hybridized with radioactive-ly labeled cDNA from early-stage (A1-A7) and advanced-stage (B1-B3) specimens from prostate cancer patients. The tetra-spot for VEGF-D is circled on one representative array (B1). Boxed tetraspots in array B2: VEGF-A, VEGF-B, and VEGF-C, respectively. Boxed tetraspots in array B3, adjacent to VEGF-D: Flk-1 and Flt-1, respectively.
of VEGFR-1 (Fig. 1, left spot in the box, array B3) were not significant between the two groups of subjects (0.20-fold versus 0.38-fold). However, the relative expression levels of VEGFR-2 were higher (0.63- and 0.72-fold respectively) than those of VEGFR-1 in all specimens from both stages of the disease (Fig. 1, right spot in the box, array B3). cDNA corresponding to VEGFR-3, the second receptor for VEGF-D was not represented on this array.

We also used QRTPCR to quantitate and compare the expression levels of VEGF receptors in the two sets of patients (Fig. 2B). The relative expression level of VEGFR-1 had a median (interquartile range) of 3.37 (3.14-3.56) in early-stage patients, versus 3.15 (1.79-5.70) in advanced-stage patients ($P = 0.8182$). The relative level of VEGFR-2 had a median (interquartile range) of 3.08 (2.82-3.69) in early-stage patients, versus 2.67 (1.46-5.40) in advanced-stage patients ($P = 0.7438$). The relative expression level of VEGFR-3 had a median (interquartile range) of 5.03 (3.67-5.80) in early-stage patients and 4.58 (4.04-5.82) in advanced-stage patients ($P = 0.8793$). Thus, the expression levels of these receptors were not significantly different in early- and advanced-stage specimens. However, the expression levels of VEGFR-3 were the highest among these three receptors.

Protein Levels of VEGF-A and VEGF-D Vary According to Disease Stage. Protein levels of VEGF-A and -D in tissue lysates of prostate cancer specimens were analyzed using Western blot analysis (Fig. 3A). As shown in a, all nine specimens from early-stage disease showed high levels of the 21-kDa VEGF-A protein, whereas only three of six advanced-stage specimens showed VEGF-A protein, and it was present in low levels. The average level of expression as normalized to $\beta$-actin after densitometric quantitation was 1.18- and 0.309-fold in early- and advanced-stage specimens, respectively. On the contrary, levels of the 21-kDa processed VEGF-D protein were higher in advanced-stage (5.15-fold) as compared with early-stage samples (0.572-fold; b). Protein levels of $\beta$-actin (c) were assessed for loading control. This data suggests that the VEGF-A and -D protein levels correspond to their gene expression levels: in early-stage patients VEGF-A levels are higher, whereas in advanced-stage patients VEGF-D levels are elevated.

Protein Levels and Activation Status of VEGFR in Prostate Cancer. The effects of growth factors are mediated by their binding to cognate receptors, which subsequently induce downstream intracellular signaling pathways. The activation status of VEGF receptors is reflected by their tyrosine phosphorylation status ([11]). We evaluated the protein levels of the receptors VEGFR-1, VEGFR-2, and VEGFR-3 by immunoprecipitation followed by Western blot analysis using receptor-specific antibodies. We also evaluated the activation status of the immunoprecipitated receptor proteins using phospho-tyrosine antibodies (Fig. 3B).

VEGFR-1 protein was present in all specimens, and the intensity of the 180-kDa band was on the average 1.23-fold of $\beta$-actin in early and advanced-stage specimens in four of nine early-stage samples and in three of six advanced-stage samples (a). When the activation status of VEGFR-1 was examined, on the average only 0.07-fold of this receptor was activated in early-stage specimens and only 0.05-fold was activated in advanced-stage specimens (b).

All nine early-stage samples show 0.52-fold normalized expression of the 190- to 235-kDa doublet (c), indicating the presence of VEGFR-2 and the normalized levels of activated receptor were 0.36-fold in early-stage specimens (d). On the other hand, VEGFR-2 was present at higher levels (1.19-fold) in all advanced-stage samples (c) and was highly (0.68-fold) activated in five of these six samples (d).

Five of nine early-stage patient samples showed high levels (mean, 2.5-fold) of VEGF-3 protein (150-170 kDa), whereas all six advanced-stage patient samples showed very high levels (mean, 3.82-fold; e). However, VEGF-3 was activated in five of six advanced-stage samples (0.36-fold) and 0.14-fold in five of nine early-stage samples (f). Our data suggests that, in advanced-stage prostate cancer, VEGFR-2 and VEGFR-3 are predominantly in their activated states and are capable of promoting lymphangiogenic responses.

Circulating Levels of VEGF-A and VEGF-D. We compared circulating levels of diffusible forms of VEGF-A and VEGF-D in platelet-poor plasma from early-stage ($n = 9$) and advanced-stage ($n = 21$) patients. The results are presented in Fig. 4. Plasma levels of VEGF-A had a median (interquartile range) of 30 pg/mL (0 to +47) in early-stage samples, versus 115 pg/mL (14.5-338.2) in advanced-stage samples ($P = 0.0249$). VEGF-A is reported to be 61 pg/mL in historical control EDTA plasma (R & D systems Quantikine manual). Plasma levels of VEGF-D had a median (interquartile range) of 332 pg/mL (309-342) in early-stage samples, compared with 436 pg/mL (366-867) in advanced-stage samples ($P = 0.0043$). VEGF-D is reported to be 208 pg/mL in historical control EDTA-plasmas (R & D Systems
Quantikine manual). Although the small sample size prevents any correlation between the stage-specific differences in the levels of these growth factors and clinicopathologic variables, it is noted that the specimen with the highest VEGF-D protein levels was acquired from a patient with high levels of prostate-specific antigen.

Spatial Expression of VEGF-A, VEGF-D, and their Receptors in Prostate Cancer Specimens. We extended our studies, using immunohistochemistry to compare the expression levels of VEGF-A and VEGF-D in fresh histology specimens from the current trial [early-stage (n = 9) and advanced-stage (n = 6) disease] and archival specimens [benign prostatic hyperplasia (n = 27), early-stage (n = 23), and advanced-stage (n = 16) disease]. Using standard immunohistochemistry procedures, we evaluated the localization of VEGF-A and VEGF-D and VEGFR-1, VEGFR-2, and VEGFR-3 in formalin-fixed prostate tissue sections. The staining results of the representative cases are presented in Fig. 5. VEGF-A and VEGF-D proteins were seen in glandular epithelial cells (Fig. 5A and B). We observed VEGF-A staining predominantly in epithelial cells of two of nine early-stage fresh specimens (Fig. 5A), whereas VEGF-D stained tumor cells of the poorly differentiated tissues from two of six advanced-stage specimens (Fig. 5B). Neither VEGF-A nor VEGF-D was detected in any of the archival tissues from any stage of the disease.

We observed VEGFR-1 staining only in glandular epithelial cells for 2 of 9 early-stage fresh specimens (Fig. 5C). VEGFR-2 was present at moderate levels in tumor cells for 5 of 9 early-stage fresh specimens and at high levels in 1 of 9 early-stage specimens and 4 of 5 poorly differentiated tissues from
advanced-stage specimens (Fig. 5D and E). This histologic data agrees with the expression patterns we observed for the growth factors VEGF-A and VEGF-D. As was the case for VEGF-A and VEGF-D, neither VEGFR-1 nor VEGFR-2 stained any of the archival tissues. In contrast, VEGFR-3 staining was observed in epithelial cells in 17 of 32 early-stage specimens (Fig. 5F) and in 10 of 27 benign prostatic hyperplasia samples. Tumor cells in 3 of 22 advanced-stage specimens were positive for VEGFR-3 (Fig. 5G-H).

In endothelial cells of blood vessels and lymphatic vessels, no staining was observed for VEGF-A or VEGF-D or for any of their cognate receptors.


**DISCUSSION**

Tumor cell metastasis to regional lymph nodes is clinically recognized as an early event in the dissemination of prostate cancer. Although lymphatic involvement has been associated with poor prognosis in prostate cancer, there has not yet been study of lymphangiogenic molecular markers that may signal a transition from dormant early-stage disease to active metastatic disease. In this study, we investigated the expression profiles of some angiogenic and lymphangiogenic genes in local, early-stage prostate cancer and in metastatic, advanced-stage prostate cancer. Our results show that, whereas early-stage disease is associated with increased expression of the angiogenic isoform VEGF-A, the lymphangiogenic isoform VEGF-D is up-regulated in the advanced stages of prostate cancer disease. This conclusion is based on five lines of evidence: microarray analysis of angiogenic cDNAs, QRT-PCR analysis of mRNA levels, Western blot analysis of protein levels, circulating levels of growth factors by ELISA, and immunohistochemical analysis of protein localization.

VEGF-D is an important lymphangiogenic factor in vivo and a stimulator of endothelial cell proliferation and migration in vitro (26). VEGF-D transcripts have been detected in lung, heart, skeletal muscle, skin, adrenal gland, and GI tract, and its protein is sequestered in dispersed neuroendocrine cells in these tissues (27). VEGF-D is up-regulated in glioblastoma (28), melanoma (29), colorectal carcinoma (30), breast carcinoma (31), and cervical intraepithelial neoplasia (32). Its expression is an independent prognostic marker for survival in colorectal carcinoma (30) and correlates with lymph node metastasis in lung (33), colorectal carcinoma (34), and ovarian carcinoma (35). In cervical intraepithelial neoplasia, expression of VEGF-D and its receptors correlates with progression of the disease to higher grade (32). Nevertheless, there are contradictory conclusions on the prognostic value of VEGF-D in various tumor types, and the clinical significance of its expression is, therefore, under intense scrutiny (36–38). The expression levels of VEGF-D and its receptors in advanced-stage prostate cancer have not been previously reported.

Previous reports of prostate cancer angiogenesis have focused exclusively on the expression of angiogenic growth factor VEGF-A and its cognate receptors, VEGFR-1 and VEGFR-2. Results of these studies implicated a strong role for VEGF-A—mediated angiogenesis in prostate cancer. Ferrer et al. (15) and Latil et al. (16) reported that VEGF-A overexpression is associated with early-stage prostate cancer; however, there are conflicting reports regarding differences in expression of VEGF-A in control and prostate cancer samples (24, 39).

Our initial examination of angiogenic and lymphangiogenic genes in early- and advanced-stage prostate cancer specimens revealed VEGF-D as the most highly expressed of the genes examined in all specimens. On further examination of these results using QRT-PCR, we confirmed that VEGF-D expression levels were 1.83-fold higher in advanced-stage samples than in early-stage samples ($P = 0.054$). Examination of the 21-kDa isoforms of VEGF-A and VEGF-D in protein extracts from early- and advanced-stage specimens using immunoblotting also revealed that VEGF-A protein was elevated 3.81-fold in early-stage specimens and VEGF-D was elevated 9-fold in advanced-stage specimens. Protein localization was also examined using immunohistochemistry in early- and advanced-stage prostate cancer specimens, along with benign prostatic hyperplasia specimens as controls. Results from the freshly resected samples from the current trial confirmed the presence of VEGF-A and VEGF-D proteins in tumor cells of early- and late-stage samples, respectively; however, the archival specimen set showed no staining in any of the specimens. This could be due to heterogeneity in the tumor samples, diffusion of protein in circulation, or detection sensitivity of the antibody in the formalin-fixed archival tissue.

If mRNA levels of these growth factors are reflected in their levels of circulating protein, then VEGF-A and VEGF-D protein levels could be used as markers for monitoring disease progression. Circulating levels of VEGF-A and VEGF-D in platelet-poor plasma collected preoperatively from patients in both early- and advanced-stage prostate cancer indicate that the differences in VEGF-A levels are significant ($P = 0.02$) when used to investigate progression from early- to advanced-stage prostate cancer. On the other hand, circulating levels of VEGF-D protein were highly significantly different in advanced-stage than in early-stage prostate cancer specimens ($P = 0.004$) and seemed to correlate with clinical observations of widespread metastasis to bone and lymph nodes. Therefore, our data suggest that a study with a large sample size and longitudinal clinical follow-up may prove that measuring plasma levels of VEGF-D is a useful prognostic or predictive tool.

To elicit a cellular response, growth factors must bind their cognate receptors; therefore, the activity of VEGF signaling pathways can be assessed based on the activation status of the appropriate receptors. We evaluated VEGF-A and VEGF-D signaling by the expression profiles and phosphorylation states of all three known VEGF receptors. Expression analysis showed that VEGFR-1, VEGFR-2, and VEGFR-3 were present in varying degrees in all cancer specimens analyzed. However, when we measured tyrosine phosphorylation levels of immuno-precipitated receptors, we found that their functional states differed considerably in specimens from different stages of prostate cancer. Whereas activation of VEGFR-1 was observed in early-stage specimens, activation of VEGFR-2 and VEGFR-3 was more evident in advanced-stage specimens. The VEGFR-2 was detected as a doublet on all these immunoblots. This could represent the proteolytically cleaved receptor (lower band) and the native receptor (upper band). We speculate that during disease progression, the receptor is cleaved and released in the circulation. This may contribute to decreased intensity of the upper band and failure of its detection using IHC. If true, measuring circulating levels of the receptor may serve as potential biomarker of the disease progression and of antiangiogenic therapy.

Because both the lymphangiogenic growth factor VEGF-D and its receptors VEGFR-2 and -3 are expressed and active in advanced-stage specimens, it is likely that these specific molecular pathways play a significant role during advanced-stage prostate cancer. Prostate cancer cells expressing VEGFR-2 reportedly activate the integrins αVβ3 and αVβ5 on their surfaces, and these cells preferentially metastasize to bone (40). Therefore, the growth factor-mediated VEGFR-2 signaling pathway may be important for angiogenesis and lymphangiogenesis in prostate cancer progression. In fact,
Becker et al. (41) showed significant reduction in tumor size in xenograft and transgenic model of prostate cancer after intervention with soluble VEGFR-2.

VEGF-C— and -D—associated activation of VEGF-R-3 induce lymphatic vessel growth in several advanced stage tumor types (32, 36, 42). Also, inhibiting VEGF-R-3 suppresses tumor growth and lymph node metastasis in various cancers (43–47), indicating the emerging significance of the VEGF-3 signaling pathway during lymph node metastasis in advanced tumor stages. In our study, the localization of this receptor on tumor cells in advanced-stage disease signifies that it may have paracrine function in lymphangiogenesis. Our findings also support very recent reports of the expression of VEGF-R-3 (48) and its splice variant (49) in prostate cancer patients with recurring or advancing disease.

This report lays the foundation for further study and provides the framework for a working model that highlights the importance of lymphangiogenesis and VEGF-D signaling in progression of prostate cancer from early- to advanced-stage. In the present study, all patients in the advanced-stage group had clinically detectable lymph node and bone metastasis. Therefore, our observation of consistent expression of VEGF-D and activation of VEGF-R-2 and VEGF-R-3 in the advanced-stage group suggests a role for these factors in lymph node and bone metastasis during advanced stages of prostate cancer. Conversely, in the early-stage group, increased VEGF-A expression and VEGF-R-1 and VEGF-R-2 activation indicate a central role for these molecules during early-stage prostate cancer angiogenesis. The low level of VEGF-D expression and VEGF-R-3 activation in specimens from patients with radical prostatectomy and no lymph node metastasis is consistent with the involvement of these molecular pathways in lymphangiogenesis and metastasis. The functional significance of VEGF-R-1, VEGF-R-2, and VEGF-R-3 in the advancement of prostate cancer might be further elucidated by determining their activation levels in situ using antibodies specific for their phosphorylated forms. This warrants for generation of antibodies specific against activated receptors.

In conclusion, our data provide evidence that prostate cancer disease progression correlates with expression of lymphangiogenic growth factor VEGF-D and with activation of its cognate receptors. Measuring the relative serum levels of VEGF-A and VEGF-D isoforms may indicate early- or advanced-stage disease, respectively. Our results suggest that using quantitative RNA analysis and immunoblotting to examine angiogenic and lymphangiogenic markers in tissue biopsies may provide prognostic data beyond basic histologic morphology. Furthermore, our results imply that lymphangiogenic markers may be useful targets for new therapeutic interventions for advanced-stage prostate cancer. Experiments in xenografted mouse models and transgenic animals are needed to fully explore this promising possibility.

ACKNOWLEDGMENTS

We thank Jennifer James in the Department of Pathology Core Histopathology Laboratory under the direction of Dr. Laura Lamps for immunostaining the specimen slides and Dr. Margaret Brenner in the Office of Grants and Scientific Publications at UAMS for editorial assistance during the preparation of this article.

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