Highly Metastatic Human Prostate Cancer Growing within the Prostate of Athymic Mice Overexpresses Vascular Endothelial Growth Factor

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ABSTRACT

Angiogenesis is essential for tumor progression and metastasis. It is mediated by the release of angiogenic factors by the tumor or host. We analyzed the expression of angiogenic factors by the prostate cancer cell line LNCaP and two derived variants, in vitro and in vivo, to determine whether metastatic cell lines express higher levels of these factors. The production of three angiogenic factors, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and interleukin 8 (IL-8), by LNCaP and its variants, LNCaP-LN3 (highly metastatic) and LNCaP-Pro5 (slightly metastatic), was measured by ELISA. VEGF, bFGF, and IL-8 mRNA expression was determined in vitro by Northern blot analysis. VEGF mRNA expression was determined in vivo by in situ hybridization. VEGF and flk-1 protein expression and microvessel density of LNCaP cell tumors were quantified by immunohistochemistry. In vitro, VEGF production by LNCaP-LN3 (3.15 ± 0.04 pg/ml/10^3 cells) was significantly higher than that of both LNCaP (2.38 ± 0.34 pg/ml/10^3 cells) and LNCaP-Pro5 (1.67 ± 0.37 pg/ml/10^3 cells; P = 0.049 and 0.001, respectively). None of the three cell lines produced detectable levels of bFGF or IL-8 in vitro. In vivo, LNCaP-LN3 tumors exhibited higher levels of VEGF mRNA and protein (152.2 ± 28.5 and 200.5 ± 28.3) and of flk-1 protein (156.5 ± 20.6) and had higher microvessel density (16.4 ± 4.2) than either LNCaP tumors (89 ± 17.5, 173.3 ± 23.0, 124.6 ± 21.6, and 12.4 ± 3.5, respectively) or LNCaP-Pro5 tumors (63 ± 14.7, 141.2 ± 38.1, 126.1 ± 20, and 5.8 ± 2.2, respectively). In conclusion, metastatic human prostate cancer cells exhibited enhanced VEGF production and tumor vascularity compared with prostate cancer cells of lower metastatic potential. Thus, VEGF may play an important role in prostate cancer metastasis.

INTRODUCTION

Tumor growth and metastasis depend upon the induction of a blood supply (1, 2). This process, angiogenesis, is regulated by a diverse group of molecules, including VEGF (2, 3), bFGF (4, 5), and IL-8 (6). The prevascular phase of a tumor is usually associated with local, nonmetastatic tumors; a vascular phase precedes invasion and metastasis (7). The vascular density of prostate cancer, a histological indicator of angiogenesis, correlates with invasion and metastasis (8, 9).

The acquisition of an angiogenic phenotype is mediated by angiogenic factors released by the tumor or host cells and depends upon the balance between stimulatory and inhibitory factors released by the tumor and its microenvironment (10). Following the induction of vascularization, the rate of tumor growth increases exponentially (11, 12).

The specific angiogenic factors regulating prostate cancer growth and metastasis have not been elucidated. It is highly unlikely that any one factor will be solely responsible for angiogenesis in all prostate cancers, and furthermore, multiple factors may be necessary for angiogenesis to occur in a single tumor.

VEGF is expressed by both benign and malignant prostate cells as well as by neuroendocrine cells (13). The level of expression by malignant cells is greater than that by benign prostate cells (14, 15). VEGF expression enhances the tumorigenicity of human prostate cancer. Administration of VEGF to mice receiving whole-body irradiation increased the growth of human prostate cancer xenografts and led to rapid tumor progression (16). In addition, administration of an anti-VEGF antibody to nude mice that were growing the human prostate cancer cell line DU145 completely inhibited neovascularization within the tumor (17). In androgen-responsive cells, VEGF expression seems to be androgen dependent. For instance, VEGF expression by the androgen-responsive PC-82 and A-2 human prostate lines growing s.c. in severe combined immunodeficient mice was inhibited by castration (18). Androgen withdrawal also down-regulates VEGF production by human prostate cancer (19, 20).

The abbreviations used are: VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; IL-8, interleukin 8; GAPDH, glyceraldehyde phosphate dehydrogenase; IHC, immunohistochemistry; ISH, in situ hybridization.
Here, we evaluated the expression of angiogenic factors in an orthotopic model of human prostate cancer. We previously established this model by directly implanting the human prostate cancer cell line LNCaP into the prostates of athymic nude mice. We selected from the parental LNCaP cell line distinct subpopulations that either were more tumorigenic within the prostate (LNCaP-Pro5) or had a greater propensity to metastasize (LNCaP-LN3; Ref. 21). The purpose of the study described herein was to evaluate whether the expression of the angiogenic factors VEGF, bFGF, and IL-8 by LNCaP and its variants, LNCaP-Pro5 and LNCaP-LN3, correlated with enhanced angiogenesis and metastasis.

**MATERIALS AND METHODS**

**Tumor Cell Lines.** The three cancer cell lines were maintained as monolayers in RPMI 1640 supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, 1-glutamine, nonessential amino acids, and penicillin-streptomycin. The low metastatic LNCaP-Pro5 and high metastatic LNCaP-LN3 variants were isolated by intraprostatic injection of LNCaP and sequential selection for nonmetastatic and metastatic variants as described previously. The LNCaP cell line is intermediate in its metastatic potential compared with these variant lines (Table 1; Ref. 21).

**Animals.** Male athymic BALB/c nude mice were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility (Frederick, MD). The mice were maintained in a laminar air flow cabinet under specific pathogen-free conditions and used at 8–12 weeks of age. Animal facilities were approved by the American Association for Accreditation of Laboratory Animal Care in accordance with the standards of the United States Department of Agriculture, Department of Health and Human Services, and IACUC.

**ELISA.** Fifty thousand viable cells from the LNCaP, LNCaP-Pro5, and LNCaP-LN3 cell lines were seeded onto 35-mm Petri dishes. Conditioned medium was removed after 9 h of incubation and centrifuged at 5000 rpm for 5 min before washing with 1 ml of HBSS. Both conditioned medium and cell suspensions were stored at −20°C prior to assay. The levels of cell-associated and supernatant VEGF, bFGF, and IL-8 were measured using commercially available ELISA kits (Quantikine; R&D Systems, Minneapolis, MN). The protein level for each angiogenic factor was quantified by comparing its optical density to the standard curve for each factor and normalizing for cell number.

**Northern Blot Analysis.** Polyadenylated mRNA was extracted from the parental LNCaP cell line Tumorigenicity and production of metastasis by LNCaP cells and two variants subsequent to orthotopic implantation in athymic nude mice.

**Table 1** Tumorigenicity and production of metastasis by LNCaP cells and two variants subsequent to orthotopic implantation in athymic nude mice

<table>
<thead>
<tr>
<th>LNCaP cell line</th>
<th>Tumorigenicity</th>
<th>Prostate weight, g (mean ± SD)</th>
<th>Para-aortic lymph node metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>24/43</td>
<td>1.2 ± 1.0</td>
<td>12/43</td>
</tr>
<tr>
<td>LNCaP-Pro5</td>
<td>10/17</td>
<td>3.3 ± 1.7^a</td>
<td>2/17</td>
</tr>
<tr>
<td>LNCaP-LN3</td>
<td>19/19^b</td>
<td>1.2 ± 0.9</td>
<td>13/19</td>
</tr>
</tbody>
</table>

^a P < 0.05, compared with LNCaP and LNCaP-LN3.
^b P < 0.001, compared with LNCaP; P = 0.002, compared with LNCaP-Pro5.
^c P < 0.003, compared with LNCaP; P < 0.001, compared with LNCaP-Pro5. Adapted from Pettaway et al. (21).

**Orthotopic Implantation of the Tumor Cells.** For the *in vivo* portion of the study, cultured LNCaP, LNCaP-Pro5, and LNCaP-LN3 cells (70% confluent) were prepared for injection as described previously (21). Mice were anesthetized with methoxyflurane, a lower midline incision was made, and the prostate was exposed. Viable tumor cells (2 × 10⁶ cells in 40 μl of HBSS) were injected into one of the dorsal lobes of the prostate. The formation of a “bleb” was the sign of a satisfactory injection. Organs were returned to their proper locations, and the abdominal wall was closed in a single layer with metal clips.

**Necropsy.** The mice were killed 5 weeks after injection. Prostate tumors were harvested, weighed, and either embedded in OCT solution (Sokera Inc., Torrence, CA) for frozen sections or fixed in formalin for paraffin sections.

**Immunohistochemical Determination of VEGF, bFGF, IL-8, and flk-1.** The expression of VEGF, bFGF, IL-8, and VEGF receptor flk-1 was detected in paraffin sections of tumors using rabbit polyclonal IgG antibodies for VEGF, bFGF, and IL-8; GAPDH was used as a control for loading. The cDNA probes used were (a) a 1.3-kb PstI cDNA for GAPDH (22); (b) a 1.4-kb cDNA fragment of bovine bFGF (23); (c) a 204-bp BamHI-EcoRI fragment of the human VEGF cDNA (a gift of Dr. Brygida Berse, Harvard Medical School, Boston, MA; Ref. 24); and (d) a 0.5-kb EcoRI cDNA fragment corresponding to human IL-8 (a gift of Dr. K. Matsushima, Kanazawa, Japan; Ref. 25). The probes were radiolabeled by a random primer technique and [α-32P]dCTP (Amersham Corp.). Autoradiography of the membrane was performed after washing. Densitometry scanning permitted quantification of the bands.
Table 2  In vitro production of bFGF, IL-8, and VEGF in LNCaP cells and selected variants

<table>
<thead>
<tr>
<th>Cell line</th>
<th>bFGF</th>
<th>IL-8</th>
<th>VEGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>0</td>
<td>0</td>
<td>2.38</td>
</tr>
<tr>
<td>LNCaP-Pro5</td>
<td>0</td>
<td>0</td>
<td>1.67</td>
</tr>
<tr>
<td>LNCaP-LN3</td>
<td>0</td>
<td>0</td>
<td>3.17</td>
</tr>
<tr>
<td>253JB-V</td>
<td>0.50</td>
<td>5.00</td>
<td>2.98</td>
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<thead>
<tr>
<th>Cell-associated supernatants</th>
</tr>
</thead>
<tbody>
<tr>
<td>bFGF</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

*Tukey’s honestly significant difference test Ps: LNCaP vs. LNCaP-Pro5, P = 0.064; LNCaP-LN3 vs. LNCaP, P = 0.049; LNCaP-LN3 vs. LNCaP-Pro5, P = 0.001.

**Oligonucleotide Probes.** Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts of the three angiogenesis-related genes based on the published reports of the DNA sequence: VEGF/vascular permeability factor, TGG`TGA`TG`TGG`ACT`CCT`CAG`TGG`GCU, 57.7% guanosine-cytosine content (24); bFGF, CGG`GAA`GGC`GCC`GCT`GCC`GCC`, 85.7% guanosine-cytosine content (23); IL-8, CTC`CAC`CCA`CCT`CTG`CAC`CC, 65% guanosine-cytosine content (25). The specificity of the oligonucleotide sequence was initially determined by a Gene Bank European Molecular Biology Library database search with the use of the Genetics Computer Group sequence analysis program (GCC; Madison, WI) based on the FastA algorithm (27). These sequences showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each of the sequences was also confirmed by Northern blot analysis. A poly(dT)29 oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3’ end via direct coupling using standard phosphoramidite chemistry (Research Genetics, Huntsville, AL; Ref. 28). The lyophilized probes were reconstituted to a stock solution at 1 µg/µl in 10 mm Tris (pH 7.6) and 1 mm EDTA. The stock solution was diluted with Probe Diluent (Research Genetics) immediately before use.

**In Situ mRNA Hybridization.** Paraffin-embedded sections of fixed tissue (3–5 µm) were mounted on ProbOn slides (Fisher Scientific, Pittsburgh, PA). The slides were de waxed and prepared, and ISH was performed using the MicroProbe system (Fisher Scientific) as described previously (29, 30). Slides were rinsed three times in Tris buffer for 30 s; the probes were then hybridized at 45°C for 45 min. The slides were washed three times for 2 min each time with 2× SSC at 45°C. The samples were then incubated with alkaline phosphatase-labeled avidin for 30 min at 45°C, rinsed in 50 mm Tris buffer (pH 7.6), and then briefly (1 min) rinsed in alkaline phosphatase enhancer.
The samples were then incubated with chromogen substrate for 20 min at 45°C. If necessary, an additional incubation was performed with fresh chromogen to enhance a weak reaction. The samples were then covered with Universal Mount mounting medium (Research Genetics), heat-dried, and examined. Red staining indicated a positive reaction in this assay. Appropriate controls for endogenous alkaline phosphatase were included by treating the samples in the absence of biotinylated probe, using chromogen alone. No immunoreactivity was observed in the controls.

**Fig. 2** ISH and immunohistochemical staining of LNCaP, LNCaP-Pro5, and LNCaP-LN3 growing in the prostates of athymic nude mice. Note the increased expression of both VEGF mRNA and protein in the highly metastatic LNCaP-LN3 tumors. Poly(dT) staining served as the control for mRNA integrity. Microvessel density by anti-CD31 staining of endothelial cells and flk-1 expression on tumoral vessels were also greater in LNCaP-LN3 cell tumors.
Table 3 In situ mRNA and immunohistochemical analysis of VEGF, VEGF receptor flk-1, and microvessel density in LNCaP and selected variants

Mice were injected with 2 × 10⁶ cells and necropsied 5 weeks after injection. Five different areas of three separate tumors were analyzed. Values are means ± SD of 15 determinations of each cell line. Statistical comparisons were performed using Tukey’s honestly significant difference test.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>VEGF (ISH)</th>
<th>VEGF (IHC)</th>
<th>flk-1</th>
<th>CD31</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP</td>
<td>89 ± 17.5</td>
<td>173.3 ± 23.0</td>
<td>124.6 ± 21.6</td>
<td>12.4 ± 3.5</td>
</tr>
<tr>
<td>LNCaP-Pro5</td>
<td>63 ± 14.7</td>
<td>141.2 ± 38.1</td>
<td>126.1 ± 20.0</td>
<td>5.8 ± 2.2</td>
</tr>
<tr>
<td>LNCaP-LN3</td>
<td>152.2 ± 28.5</td>
<td>200.5 ± 28.3</td>
<td>156.5 ± 20.6</td>
<td>16.4 ± 4.2</td>
</tr>
</tbody>
</table>

Tukey’s honestly significant difference test Ps

LNCaP vs. LNCaP-Pro5 0.117
LNCaP vs. LNCaP-LN3 0.001
LNCaP-LN3 vs. LNCaP-Pro5 0.001

Table 3 In situ mRNA and immunohistochemical analysis of VEGF, VEGF receptor flk-1, and microvessel density in LNCaP and selected variants

To check the specificity of the hybridization signal, the following controls were used: (a) RNase pretreatment of tissue sections, (b) substitution of the antisense probe with a biotinylated sense probe, and (c) competition assay with unlabeled antisense probes. Markedly decreased or no signal was obtained after all of these treatments (31).

Densitometry Quantification of IHC and ISH. The intensity of IHC staining and ISH was evaluated in five fields at the periphery of the tumors representing areas of most intense staining. Each field was evaluated using the ImageQuant analyser and Optimas software program (Bioscan, Edmonds, WA). IHC staining intensity of each sample was compared with the staining intensity of the normal prostate glands in the sample and expressed as a ratio (tumor cell normal glandular cell). ISH was quantified in a similar manner using serial sections of the same tumor block. Normal prostate glands served as the internal control for mRNA expression, and poly(dT) staining controlled for mRNA preservation. Results were expressed as the ratio of the intensity of tumor ISH staining to that of normal glandular cell staining and normalized for poly(dT) expression.

Statistical Analysis. One-way ANOVA was used to examine group differences in VEGF production by the cell lines in vitro and in VEGF mRNA and protein expression as well as flk-1 protein expression on tumor sections in vivo. When ANOVA indicated a significant (P < 0.05) difference, a post hoc test was performed using Tukey’s honestly significant difference multiple comparison test.

RESULTS

In Vitro Expression of VEGF, bFGF, and IL-8. The in vitro production of cell-associated and secreted VEGF, IL-8, and bFGF protein by the parental LNCaP cell line and the variant LNCaP-Pro5 and LNCaP-LN3 cell lines is shown in Table 2. The 253J B-V cell line served as a positive control for bFGF and IL-8 production (32). By ELISA, VEGF was the only angiogenic peptide detected among the LNCaP cell lines. The highly metastatic LNCaP-LN3 cell line secreted significantly more VEGF (3.15 ± 0.04 pg/ml/10⁶ cells) than the LNCaP cell line (2.38 ± 0.34 pg/ml/10⁶ cells) or the LNCaP-Pro5 line (1.67 ± 0.37 pg/ml/10³ cells; P = 0.049 and 0.001, respectively).

Northern blot analysis confirmed the results of ELISA. Densitometric analysis indicated that the relative steady-state gene expression of VEGF by LNCaP-LN3 was 4-fold greater than that by LNCaP-Pro5 and 1.5-fold greater than that by LNCaP (Fig. 1). Four VEGF transcripts were identified by Northern blot analysis. We did not identify bFGF or IL-8 mRNA transcripts in the three LNCaP cell lines (data not shown).

In Vivo Expression of VEGF, bFGF, IL-8, flk-1, and Microvessel Density. We used IHC and ISH to compare the expressions of VEGF, bFGF, IL-8, and flk-1 and the microvessel densities of the LNCaP, LNCaP-Pro5, and LNCaP-LN3 cell lines growing in the prostates of nude mice. Representative tumor sections analyzed for immunoreactivity of VEGF are shown in Fig. 2. The immunoreactivity of VEGF was highest in the LNCaP-LN3 tumors, intermediate in LNCaP tumors, and lowest in LNCaP-Pro5 tumors, which is in accordance with our in vivo findings. Computer-assisted analysis of representative sections confirmed these results (Table 3). Using ISH, we observed that steady-state mRNA expression of VEGF was also greatest in the LNCaP-LN3 tumors, intermediate in the LNCaP tumors, and least in the LNCaP-Pro5 tumors (Fig. 2). Relative expressions of VEGF mRNA and protein were 2.4-fold higher in the LNCaP-LN3 tumors than in the LNCaP-Pro5 tumors (Fig. 2 and Table 3). In vivo bFGF and IL-8 protein (by IHC) and mRNA (by ISH) were expressed at similar low levels in all three tumors (data not shown).

By anti-CD31 immunostaining, microvessel density was significantly greater in the LNCaP-LN3 tumors than in either LNCaP or LNCaP-Pro5 tumor tissue (P = 0.001; Fig. 2 and Table 3). By image analysis, the VEGF receptor flk-1 was also significantly overexpressed on endothelial cells within the
LNCAp-LN3 tumors compared with either LNCAp or LNCAp-Pro5 tumors (Fig. 2 and Table 3).

**DISCUSSION**

Tumor growth and metastasis depend upon the induction of a blood supply. This process of angiogenesis is mediated, in part, by the secretion of angiogenic factors such as VEGF by tumors growing in their relevant microenvironment. VEGF is secreted by a wide variety of tumor cells, and its secretion correlates with the metastatic potential of these tumor cells (33–35). Our data indicate that the malignant potential of the human prostate carcinoma cell line LNCAp and its variant lines, LNCAp-LN3 and LNCAp-Pro5, correlated with their VEGF expression. In *vitro* evaluation revealed that the highly metastatic cell line LNCAp-LN3, which we selected for its ability to metastasize to regional lymph nodes, overexpressed VEGF protein compared with the poorly metastatic cell line LNCAp-Pro5, which was selected for its growth within the prostate. Increased steady-state gene expression of VEGF mRNA was also observed in the LNCAp-LN3 cell line. By Northern blot analysis, we observed four mRNA transcripts. The relative expressions of these four VEGF mRNA transcripts by the three cell lines were compared following normalization to GADPH. These transcripts represent both secreted and freely soluble VEGF (36). The increased expression of VEGF by LNCAp-LN3 was confirmed *in vivo* using IHC and ISH. Assessment of VEGF mRNA and protein was performed at the invasive edge of each tumor because the centers of the tumors were often necrotic (and potentially hypoxic and acidotic), making VEGF expression within these regions difficult to interpret because of artifacts (37, 38). Using antibodies directed against murine CD31, we also identified increased microvessel density in the periphery of the LNCAp-LN3 prostate tumor, which corresponded to the area of greatest VEGF expression. This suggests that the local production of VEGF by metastatic prostate cancer cells induced the increase in neovascularization, which presumably resulted in enhanced metastasis. VEGF is likely an important angiogenic factor secreted by LNCAp cells because we did not detect significant expression of either bFGF or IL-8 by these cells *in vitro* or *in vivo*. However, other angiogenic molecules, such as platelet-derived endothelial cell growth factor, transforming growth factor-β, and angiogenin, were not measured in this study.

VEGF promotes the formation of new capillaries by stimulating endothelial cell division and migration and by increasing capillary permeability following ligand binding to the endothelial cell surface membrane (39–41). We observed that the VEGF receptor protein flk was overexpressed within all LNCAp tumors compared with normal prostate epithelium (data not shown). In addition, the intensity of immunoreactivity to anti-flk-1 was increased on the cell membrane of the endothelial cells within LNCAp-LN3 tumors compared with either LNCAp or LNCAp-Pro5, suggesting that VEGF up-regulates its own receptors on the endothelial cells within the tumor. These observations are consistent with previous reports in which the VEGF receptor protein flk was up-regulated in gastrointestinal tract adenocarcinomas (33), renal cell carcinoma (34, 42), and transitional cell carcinoma of the bladder (42). In conclusion, these results indicate that the metastatic potential of a human prostate cancer cell line correlates with its VEGF expression. We further speculate that VEGF plays a role in spontaneous metastasis subsequent to orthotopic implantation of LNCAp cells. Studies are currently in progress to directly test this hypothesis.

**REFERENCES**


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