Association between Immunohistochemical Expression of Vascular Endothelial Growth Factor (VEGF), VEGF-expressing Neuroendocrine-differentiated Tumor Cells, and Outcome in Prostate Cancer Patients Subjected to Watchful Waiting

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ABSTRACT
Tumor growth is dependent on angiogenesis, which is thought to be controlled by angiogenic factors. Therefore, the immunoreactivity of the angiogenic cytokine vascular endothelial growth factor (VEGF) was semiquantitatively scored in archival prostate tumors obtained at diagnosis in 221 patients followed expectantly. At diagnosis, 125 patients suffered from clinically localized disease. Median length of follow-up was 15 years, and 57% of the patients eventually died of prostate cancer. All of the tumors exhibited cytoplasmic staining for VEGF. The staining intensity was weak in 47 tumors and moderate and strong in 107 and 67, respectively. VEGF expression was significantly correlated with microvessel density (MVD; median, 43; range, 16–151; \( P = 0.014 \)), increasing T-classification (\( P = 0.001 \)), dedifferentiation (\( P < 0.001 \)), and disease-specific survival (\( P = 0.013 \)). Strongly VEGF-immunoreactive, neuroendocrine-differentiated (NE) tumor cells were observed in 125 tumors. NE expression was significantly correlated with increasing MVD, increasing T-classification, dedifferentiation, and survival (all, \( P < 0.001 \)). MVD and NE tumor cell expressions were significant variables in a multivariate analysis that included patients with clinically localized prostate cancer only. VEGF and NE expression were significantly correlated with MVD, clinical characteristics, and disease-specific survival. NE expression was a significant prognostic marker in localized prostate cancer patients, whereas the applied semiquantitatively scoring of VEGF expression was inadequate to make this growth factor provide any additional prognostic information. Moreover, the significant VEGF expression of NE tumor cells suggests an additional important character of these cells in the involvement in disease progression.

INTRODUCTION
It is known that malignant tumors depend on angiogenesis, the formation of new blood vessels from an extant microvascular bed (1). In prostate carcinoma, it has also been suggested that the degree of tumor angiogenesis is related to clinical stage (2, 3), progression after radical prostatectomy (4), and survival (5–7). The secretion and activation of various endothelial growth factors, called angiogenic factors, by tumor cells have been shown to play crucial roles in the formation of neovascu-lature (8–11). VEGF, also known as vascular permeability factor, is one of the most potent and specific angiogenic factors isolated (12). It is a multifunctional cytokine that stimulates endothelial cell growth and angiogenesis (13–16) and increases microvascular permeability (17). VEGF expression is induced by, for example, exposure to hypoxia (18), transforming growth factor-\( \beta \) (19), and the mutated form of the murine p53 tumor suppressor gene (20); and it acts through two tyrosine kinase receptor proteins found on endothelial cells, flt-1, and KDR (21, 22). Immunohistochemical studies have shown that prostate cancer cells can produce VEGF (23–25), and the granular cytoplasmic staining has previously been demonstrated to be especially intense in NE prostate tumor cells (23). NE tumor cells probably play a significant role during prostatic growth and differentiating via endocrine, lumencrine, or neurocrine mechanisms (26).

The aim of this study was to investigate whether immunohistochemical expression of VEGF and VEGF-immunoreactive NE tumor cells were correlated with MVD, clinical characteristics, and disease-specific survival in prostate cancer patients subjected to watchful waiting.

PATIENTS AND METHODS
A complete population of patients with prostate cancer consisting of 719 inhabitants of Aarhus County was diagnosed

Received 5/10/99; revised 12/13/99; accepted 2/17/00.

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1 Supported by grants from The Danish Cancer Society, Clinical Research Unit at Aarhus Oncological Center.

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3 The abbreviations used are: VEGF, vascular endothelial growth factor; NE, neuroendocrine differentiated; MVD, microvessel density; TURP, transurethral resection of the prostate; vWF, von Willebrand factor.
in a 5-year period (January 1, 1979–December 31, 1983). They were retrospectively followed from the time of diagnosis until death (27). The tumors were retrospectively reclassified (27) according to the Union International Contre le Cancer 1992 classification system (28), whereas the original histopathological malignancy grade according to WHO (29) was used. The patients were followed expectantly and palliated at symptoms only. Archival tumor tissue was theoretically available in 280 patients after the exclusion of patients diagnosed postmortem and patients with incomplete data registration. Unfortunately, because the tissue from one specific county hospital had been lost, tumor tissue was not accessible in an additional 59 cases. The current study population thus included 221 patients (31%), representing a premortem-diagnosed cohort in which both archival tumor specimens obtained at diagnosis as well as complete data records were available.

TURP specimens for the immunostaining procedures were retrieved from the formalin-fixed, paraffin-embedded tissue

Fig. 1  

a, immunohistochemical distribution of VEGF in prostate cancer; b, intensively stained polymorphonuclear neutrophils acting as internal immunohistochemical positive control; c and e, immunohistochemical distribution of VEGF; d and f, chromogranin A in adjacent sections of a prostate carcinoma.
used for the original histopathological grading at the time of diagnosis. One representative section (4-μm thick) per patient was chosen, without knowledge of the clinical outcome.

**VEGF Immunohistochemical Assay.** The tissue was deparaffinized and rehydrated before it was microwaved three times in a buffer of 10 mM Tris and 0.5 mM EGTA, for 5 min at 650 W. After a 20-min cooling at room temperature, the slides were rinsed with Tris/PBS 1:9 for 5 min. Next, the tissue was incubated in 1:20 35% H$_2$O$_2$ in distilled water for 20 min at room temperature, followed by incubation with the primary antibody (rabbit polyclonal IgG, 100 μg/ml; Santa Cruz Biotechnology) diluted 1:200 in antibody (diluent code S0809; Dako) for 60 min. The slides were rinsed two times for 5 min in Tris/PBS with 0.02% detergent (BRIJ35, protein grade; Calbiochem). It was blocked by Dako protein block 0.02%, followed by an incubation in 1:20 35% H$_2$O$_2$ in distilled water for 20 min at room temperature, followed by further washing in distilled water and rinsing two times for 5 min with a Tris/PBS with 0.02% detergent (BRIJ35, protein grade; Calbiochem) and incubated for 10 min in 3,3’-diaminobenzidine (liquid DAB, substrate-chromogen system; Dako). Finally, the slides were counterstained with Mayer’s hematoxylin. The chromogranin A-positive controls consisted of paraffin-embedded sections of a known chromogranin A-positive, lymph-node metastatic, neuroendocrine tumor of the pancreas.

**vWF-VIII Immunohistochemical Assay.** Microvessels were highlighted by staining endothelial cells for vWF (polyclonal P226; Dako). The method has been described previously in detail (7).

**Quantitation.** The distributions of both VEGF and vWF-VIII immunoreactivity were homogeneous, and there existed a broad variation in staining intensity and number of endothelial cells stained. In case obvious differences in the intratumoral VEGF staining intensity and number of stained endothelial cells existed, the area with the most intense VEGF staining and most stained endothelial cells (“hot spots”) was chosen. Because the quantitation of VEGF and MVD immunohistochemical staining was not performed on the same tissue slide, there is no documentation that areas scored as VEGF +3 actually represent MVD hot spots. The procedures were done by a single observer (M. B.) unaware of the clinical outcome.

**VEGF staining was considered positive if unequivocal red staining was seen in the tumor cell cytoplasm, and the immunoreactivity was scored semiquantitatively as the intensity of the immunoreactive reaction: 0 to 3 (0, no immunoreactivity; 1, weak intensity; 2+, moderate intensity; 3+, strong intensity; intensity was assessed comparable with the intensity of the polymorphonuclear neutrophils and NE prostate cancer cells; Fig. 1, a and b).

Because not all of the cases were stained for chromogranin A, the NE cells were defined as focally occurring, intense (3+) VEGF-immunoreactive tumor cells. In an adjacent slide the reactivity with chromogranin A was demonstrated (Fig. 1, c–f).
As described previously (7), MVD was quantified in the most vascularized areas (hot spots) of the tumor at ×200 high-power field using a grid in the eyepiece (grid area, 0.25 mm²). The method had been validated in a previous methodical study (6).

**Statistical Analysis.** Statistical analysis was performed using the SPSS 8.0 for Windows (SPSS Inc., Chicago, IL) program package. The two-sided χ² test was used to test for an association between categorical data, and the Spearman rank correlation coefficient was used to characterize the correlation between ordinal and continuous variables. The survival functions were calculated according to the method of Kaplan and Meier, and the differences between the survival curves were tested by the log rank test. The Cox proportional hazards regression model was used to analyze the prognostic value of the clinical and biological characteristics determined at the time of diagnosis. Disease-specific deaths were defined as all of the deaths presumably caused directly by prostate cancer, excluding deaths from coexisting disease, accidents, and unknown causes. All of the P values were based on two-sided testing.

**RESULTS**

The distribution of clinical characteristics between the original complete population (n = 719) and the current 221 patients was compared in a previous study (7). The median age at diagnosis was 75 years (range, 49–95 years). At the time of diagnosis 125 patients (57%) suffered from clinically localized (T1–2, N0, M0) disease, whereas 96 patients (43%) either had

[Fig. 2 VEGF expression (1+, 2+, 3+) correlated with disease-specific survival. A, in all of the 221 patients with prostate cancer irrespective of tumor stage; B, in 125 patients with clinically localized prostate cancer (T1–2, N0, M0).]
locally advanced or disseminated (T$_{\text{loc}}$, N$_{\text{c}}$, and/or M$_{\text{c}}$) disease. The patients have been followed expectantly and palliated at symptoms only, and 108 (49%) of the patients had received endocrine treatment during disease, and at the end of registration, 215 patients (98%) had died. According to the hospital charts and death certificates, 125 deaths (57%) were caused by prostate cancer, whereas 90 patients (41%) had died of other causes. The median time to death for those who died was 3.5 years (range, 0.01–15.6 years).

All of the 221 tumors exhibited cytoplasmic staining for VEGF. However, the immunoreactivity was heterogeneously distributed, and there existed a broad variation in staining intensity. Diffuse immunoreactivity of variable intensity was also observed in periglandular stroma in areas of tumor. In 47 (21%) tumors, the intensity of the immunoreactive reaction was considered weak (1+), whereas in 107 (49%) and 67 (30%) tumors, the immunoreactive intensity was moderate (2+) and strong (3+), respectively. Thereby, VEGF expression was increasing significantly with T-classification ($P = 0.001$), M-classification ($P = 0.007$), and histopathological malignancy grade ($P < 0.001$; Table 1). Moreover, VEGF was significantly associated with disease-specific survival in the entire population ($P = 0.01$; Fig. 2A) as well as in the subpopulation consisting of patients with clinically localized prostate cancer ($P = 0.03$; Fig. 2B). However, using overall survival as end point, the correlation was insignificant. Including all of the 221 patients, the correlation between MVD (median 43; range, 16–151) and VEGF expression was significant (Spearman correlation coefficient = 0.17; $P = 0.01$), whereas the correlation was borderline significant only (Spearman correlation coefficient = 0.18; $P = 0.05$) when focusing on the clinically localized subpopulation (Table 2). The highly statistically significant association ($P < 0.001$) between MVD and the clinicopathological characteristics as well as survival has been described previously ($P < 0.001$).

NE tumor cells, often identified focally as 3+ VEGF-immunoreactive tumor cells (Fig. 1, c and e), were observed in 125 (57%) of the tumors. To verify the identification of the intensively (3+) VEGF-immunoreactive cells as NE tumor cells, chromogranin-A immunostaining was performed successfully in adjacent slides in 10 tumors (Fig. 1, d and f). Not all of the 221 cases were stained for chromogranin A; therefore, focally 3+ VEGF was taken to mean “chromogranin A positivity.” The correlation between the focally distributed 3+ VEGF-immunoreactive NE tumor cells and the VEGF expression of the tumors was statistically significant in the entire population ($P < 0.001$) as well as in the clinically localized tumor subpopulation ($P = 0.001$; Table 3). The presence of NE tumor cells was significantly correlated with MVD ($P < 0.001$), T-classification ($P < 0.001$), histopathological grade ($P < 0.001$), and disease-specific survival ($P < 0.001$; Fig. 3).

Tables 4 and 5 demonstrate the results of univariate and multivariate analyses using disease-specific death as end points. Analysis A includes all of the 221 prostate cancer patients, whereas analysis B focuses solely on patients with clinically localized disease. Initially, VEGF and NE tumor cell expression were analyzed together with the classic clinicopathological characteristics (Table 4). Contrary to VEGF, NE tumor cell expression turned out to be a significant prognostic marker in both analysis A ($P = 0.02$) and B ($P = 0.002$). Furthermore, in analysis B, NE expression was the only significant marker. Then, MVD was included in the multivariate analysis, and, as shown in Table 5, it demonstrated significant prognostic value ($P = 0.0004$) in both of the populations. In the clinically localized prostate cancer subpopulation (B) MVD was accompanied by NE expression as a significant ($P = 0.04$) predictor of disease-specific survival, whereas the independent prognostic value of VEGF remained insignificant.

**DISCUSSION**

The current study was based on a previously described complete prostate cancer population subjected to watchful waiting (27), and the present subpopulation represents the patients with both available archival histological tumor samples obtained at the time of diagnosis as well as complete data records. The follow-up was nearly complete, and the ratios of patients dying from prostate cancer in the two populations were nearly identical. However, retrospectively obtained information will never be optimal, and understaging as well as inaccuracies of determination of the cause of death are well-known problems. Unfortunately, the consequence of the long-term follow-up was the lack of available prostate-specific antigen at the time of diagnosis. Although, the nature of prostate cancer has changed with prostate-specific antigen screening, the spontaneous prognosis of patients with favorable marker status provides important information that cannot be achieved in patients treated with
intent to cure. Because the patients were subjected to watchful waiting, endocrine therapy was considered a surrogate marker for a poor prognosis, which led to the exclusion of endocrine therapy in the survival models. Survival analyses could to some extent be distorted by the fact that the TURP giving rise to the diagnosis may have rendered some of the patients free of disease. Moreover, the “true” phenotype classification of prostate tumors based on TURP chips material is impeded by the pronounced heterogeneous and multifocal nature of these tumors.

Despite, the risk of inaccuracy in data because of retrospectively obtained patient characteristics as well as immunohistochemically quantification of angiogenesis, the results of a previous study (7) concerning the association between angiogenesis as measured by MVD and survival suggested that the pattern of neovascularization is important in the natural history of prostate cancer. The capability of producing VEGF has been demonstrated in prostate cancer cells (23–25), but to our knowledge, VEGF expression has not previously been correlated with survival in prostate cancer patients. As in the present study, all of the 30 prostate tumors stained positively for VEGF in the study by Jackson et al. (24), whereas the majority of prostate cancer specimens stained positively for VEGF in two other studies (23, 25). In the latter study (25), technical error in slide preparation was suggested as being responsible for the lack of staining in 5 of 25 tumors. In contrast to Ferrer et al. (25), who suggested a

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**Fig. 3** NE tumor cell expression (positive versus negative) correlated with disease-specific survival. A, in all of the 221 patients with prostate cancer irrespective of tumor stage; B, in 125 patients with clinically localized prostate cancer (T1–2, N0, M0).

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A

- NE negative (n=96)
- NE positive (n=125)

B

- NE negative (n=70)
- NE positive (n=55)

$p<0.001$
loss of VEGF expression in the poorly differentiated tumor cells, we and Harper et al. (23) found an increasing VEGF expression with the dedifferentiation, whereas Jackson et al. (24) noted no difference in the intensity or distribution of VEGF immunoreactivity between well-, moderately, and poorly differentiated tumors. Although, a "switch" to the angiogenic phenotype mediated by angiogenic factors has been demonstrated as an early event in tumor development (30), nothing indicates that the switch is inactivated during tumor progression. On the contrary, MVD has been demonstrated to increase significantly with dedifferentiation, tumor growth, and metastasis (2, 7).

Table 4  Univariate and Cox multivariate analyses including clinical characteristics, VEGF expression, and VEGF-expressing NE tumor cells for disease-specific survival

<table>
<thead>
<tr>
<th>Factor</th>
<th>Univariate P</th>
<th>Multivariate RR</th>
<th>CI (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A: all PC patients (n = 221)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-classification(^b)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>1.94</td>
</tr>
<tr>
<td>M-classification(^c)</td>
<td>&lt;0.0001</td>
<td>0.3</td>
<td></td>
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<tr>
<td>Grade(^d)</td>
<td>&lt;0.0001</td>
<td>0.049</td>
<td>1.29</td>
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<tr>
<td>VEGF(^e)</td>
<td>0.01</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>NE(^f)</td>
<td>&lt;0.0001</td>
<td>0.02</td>
<td>1.60</td>
</tr>
<tr>
<td>Group B: clinically localized PC patients (n = 125)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>T-classification(^g)</td>
<td>0.03</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Grade(^d)</td>
<td>0.004</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>VEGF(^e)</td>
<td>0.04</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>NE(^f)</td>
<td>0.002</td>
<td>2.40</td>
<td>1.37–4.18</td>
</tr>
</tbody>
</table>

\(^a\) RR, relative risk; CI, confidence interval; PC, prostate cancer.
\(^b\) T1\(^a\) versus T1\(^b\) versus T2 versus T2
\(^c\) M0 versus M1.
\(^d\) Well versus moderate versus poor differentiation.
\(^e\) VEGF expression 1+ versus 2+ versus 3+.
\(^f\) NE positive versus negative.
\(^g\) T1\(^a\) versus T1\(^b\) versus T2.

Table 5  Univariate and Cox multivariate analyses including clinical characteristics, VEGF expression, VEGF-expressing, NE tumor cells and MVD for disease-specific survival

<table>
<thead>
<tr>
<th>Factor</th>
<th>Univariate P</th>
<th>Multivariate RR</th>
<th>CI (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A: all PC patients (n = 221)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-classification(^b)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>1.89</td>
</tr>
<tr>
<td>M-classification(^c)</td>
<td>&lt;0.0001</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Grade(^d)</td>
<td>&lt;0.0001</td>
<td>0.03</td>
<td>1.33</td>
</tr>
<tr>
<td>VEGF(^e)</td>
<td>0.01</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>NE(^f)</td>
<td>&lt;0.0001</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>MVD(^g)</td>
<td>&lt;0.0001</td>
<td>0.0004</td>
<td>1.01</td>
</tr>
<tr>
<td>Group B: clinically localized PC patients (n = 125)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-classification(^g)</td>
<td>0.03</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Grade(^d)</td>
<td>0.004</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>VEGF(^e)</td>
<td>0.04</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>NE(^f)</td>
<td>0.002</td>
<td>1.84</td>
<td>1.04–3.26</td>
</tr>
<tr>
<td>MVD(^g)</td>
<td>&lt;0.0001</td>
<td>0.0004</td>
<td>1.03</td>
</tr>
</tbody>
</table>

\(^a\) RR, relative risk; CI, confidence interval; PC, prostate cancer.
\(^b\) T1\(^a\) versus T1\(^b\) versus T2 versus T2
\(^c\) M0 versus M1.
\(^d\) Well versus moderate versus poor differentiation.
\(^e\) VEGF expression 1+ versus 2+ versus 3+.
\(^f\) NE positive versus negative.
\(^g\) median versus median.
\(^h\) T1\(^a\) versus T1\(^b\) versus T2.

loss of VEGF expression in the poorly differentiated tumor cells, we and Harper et al. (23) found an increasing VEGF expression with the dedifferentiation, whereas Jackson et al. (24) noted no difference in the intensity or distribution of VEGF immunoreactivity between well-, moderately, and poorly differentiated tumors. Although, a "switch" to the angiogenic phenotype mediated by angiogenic factors has been demonstrated as an early event in tumor development (30), nothing indicates that the switch is inactivated during tumor progression. On the contrary, MVD has been demonstrated to increase significantly with dedifferentiation, tumor growth, and metastasis (2, 7).

There exist no established or adequate methods quantitating VEGF immunohistochemical expression. However, using the semiquantitative (0–3+) analysis of cytoplasmic VEGF staining previously carried out successfully by Harper et al. (23), we were able to demonstrate that VEGF correlates with clinicopathological characteristics, and, more importantly, it is significantly associated with the spontaneous outcome of patients with clinically localized prostate cancer. Still, the inadequateness of the applied method could be responsible for the VEGF not providing additional prognostic information when excluding MVD from the multivariable analysis.
The interesting observation by Harper et al. (23) that NE tumor cells exhibited intense (3+) cytoplasmic granular immunolocalization of VEGF was reproduced in the current study. These strongly stained VEGF cells were observed in 55 (23) and 57% (current study) of the patients, respectively. In the current study, verification of the intensively stained VEGF cells defined as NE tumor cells was successfully done by chromogranin A immunostaining performed in adjacent sections in 10 tumors (Fig. 1. c-f). Harper et al. (23) have previously found a significant correlation between the total number of chromogranin A-positive cells and the total number of VEGF-containing NE cells in individual tumors. As previously suggested (31–33), by investigating the chromogranin A-immunoreactive NE cells, the current study demonstrated that the presence of VEGF-expressing NE tumor cells was correlated with poor prognoses. In a multivariate analysis using disease-specific death as an end point, NE tumor cells provided additional prognostic information in the clinically localized prostate cancer subpopulation (Table 5). NE prostate tumor cells have been demonstrated to constitute an androgen-insensitive, nonproliferating cell population (34), producing a number of bioactive hormone-related substances (35), and the significant VEGF expression of these cells suggests an additional important characteristic of these cells in the involvement of prostate cancer progression.

It should be emphasized that the current data are based on material primarily removed by TURP, whereas the future clinical utility of several prognostic markers will depend on biopsy techniques, which might turn out to be a critical issue caused by the less available material from this distinctly heterogeneous cancer (36, 37).

In conclusion, VEGF and NE expression were significantly correlated with increasing MVD, tumor extension, dedifferentiation, and poor survival. Although angiogenesis and NE tumor cell expression were significant prognostic markers in localized prostate cancer patients, the applied semiquantitative scoring of VEGF expression was inadequate to provide any additional prognostic information. However, the significant VEGF expression of NE tumor cells suggests an additional important characteristic of these cells in the involvement in disease progression.

REFERENCES


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