Vascular Endothelial Growth Factor Is a Predictor of Relapse and Stage Progression in Superficial Bladder Cancer

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Abstract

Tumor development is angiogenesis dependent, and vascular endothelial growth factor (VEGF) is a key growth factor in this process. We demonstrate that high expression of VEGF mRNA in superficial bladder cancers was associated with earlier recurrence (P = 0.001; hazard ratio, 3.09) and progression to a more invasive phenotype (P = 0.02; hazard ratio, 5.33). VEGF mRNA expression correlated with protein levels in superficial tumors (r = 0.59, P = 0.003) and normal bladder (r = 0.65, P < 0.05), although the ratio of VEGF protein to mRNA was elevated in tumors compared to normal bladder (P = 0.004), suggesting posttranscriptional regulation. In this study, VEGF is implicated as a major downstream mediator of the effects of the p53 tumor suppressor gene by the association between high p53 protein (determined immunohistochemically) and high VEGF protein and mRNA expression (P < 0.02), although in cases without high p53 protein expression, high VEGF mRNA also predicts a poor prognosis. The relationship between VEGF and early tumor recurrence suggests that seeding via angiogenesis may be a major mechanism in the pathogenesis of recurrence. These studies indicate that VEGF can predict the behavior of superficial bladder tumors and is a therapeutic target for intravesical therapy.

Introduction

Angiogenesis, as quantitated by vascular density, is an independent prognostic tumor marker in several tumor types (1), and VEGF is a major factor in this process (2). High vascular density is associated with a poor prognosis in bladder cancer (3, 4), suggesting that VEGF may give prognostic information in these tumors. Clinical and molecular evidence point to different developmental pathways for superficial (stage T<sub>1</sub> and T<sub>2</sub>) and muscle invasive (stage T<sub>3</sub>-<sub>4</sub>) bladder cancer, and we have previously demonstrated differential up-regulation of VEGF mRNA in superficial disease (5). Over 50% of stage T<sub>1</sub> bladder cancers recur within 12 months, and 30% progress to muscle invasion with a poor prognosis, despite treatment (6). The mechanisms leading to relapse and stage progression are poorly understood but are particularly relevant to well and moderately differentiated (G<sub>2</sub> and G<sub>3</sub>, respectively) T<sub>1</sub> tumors, which account for a large proportion of bladder cancer. This highly heterogeneous group of tumors exhibits variable clinical behavior (7), making determination of the risk of relapse and stage progression difficult. Mutations of the p53 tumor suppressor gene indicate a poor prognosis in these tumors (8, 9) and have been implicated in the regulation of VEGF expression in cells. Here we show that high VEGF mRNA expression strongly indicates a poor prognosis in stage T<sub>1</sub> bladder cancer and that overexpression of p53 protein, detected by immunohistochemistry, is related to VEGF up-regulation, which may, therefore, be one of the key downstream pathways in vivo for the adverse effects of mutations of the gene. The study demonstrates that the endothelial-specific angiogenic factor VEGF is a major determinant of biological behavior of early bladder cancer, indicating the possibility for treatment using anti-VEGF strategies.

Materials and Methods

Patients and Sample Collection. Primary superficial transitional cell carcinomas of the bladder were obtained from 94 patients undergoing transurethral bladder cancer resection at The Churchill Hospital. Twenty-nine specimens of macroscopically normal bladder were obtained from patients undergoing surgery for bladder cancer (n = 22) or from cadaveric organ donors at the time of donor nephroureterectomy (n = 7).

RNA and Protein Preparation. RNA was prepared according to the method of Chomczynski and Sacchi (10). All RNA samples were run on a 1% agarose gel (under RNase-free conditions), and the concentrations were measured spectrophotometrically prior to RNase protection analysis. Tumor protein cytosols were prepared in HEPES buffer as described previously (11).

RNase Protection Assay. Plasmid pBluescript KS+ containing the full length of VEGF 121 was linearized with EcoRV, and a 520-nucleotide antisense fragment was generated with T7 polymerase. Antisense riboprobes, labeled with [32P]dCTP, were hybridized to 10 μg of total cellular RNA, and the free unhybridized probe was digested with RNase A and T1. Following electrophoresis in a 6% polyacrylamide/urea-sequencing gel, the protected fragments (Fig. 1) were analyzed using the ImageQuant Phosphorimager (version 3.3; Molecular Dynamics, Inc., Sunnyvale, CA). Analysis was performed in a blinded fashion prior to determining patient outcome.

Antisense and sense GAPDH riboprobes were generated (after linearizing the pBluescript KS+1/GAPDH construct with HindIII and BamHI, respectively) using T3 and T7 polymerase, respectively. Hybridization of these riboprobes, including the 30-bp Bluescript plasmid sequence shared by both probes, gives rise to a 150-bp protected fragment, whereas a 120-bp protected fragment corresponded to the antisense GAPDH riboprobe hybridized to endogenous GAPDH. All signals were normalized to the external control formed by hybridization of the sense and antisense GAPDH riboprobes (12) and were expressed relative to a positive control run on all electrophoresis gels.

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2 To whom requests for reprints should be addressed, at Institute of Molecular Medicine, University of Oxford, Oxford OX3 9DU, UK.

3 The abbreviations used are: VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EGFr, epidermal growth factor receptor; CI, confidence interval.
ELISA. The Quantikine<sup>®</sup> ELISA (R&D Systems, Abingdon, UK) was used to measure VEGF protein. Prior to the study, the ELISA was validated for use with tumor protein preparations.

**Immunohistochemistry.** Five-μm paraffin sections were available for all 55 T<sub>1</sub>G<sub>1</sub> and T<sub>1</sub>G<sub>2</sub> bladder tumors that had VEGF mRNA quantified. Sections were analyzed for p53 and EGFr protein expression using an avidin-biotin-complex immunoperoxidase technique (13). All steps were performed at room temperature. In brief, following deparaffinization endogenous peroxidase was blocked by immersing sections in methanol with 0.3% hydrogen peroxide, followed by incubation in 5% normal human serum for 30 min. Following high pressure antigen retrieval, tissue sections were incubated for 2 h with the primary antibody. PAb1801, at a concentration of 200 ng/ml (Oncogene Science, Inc., Manhasset, NY), was used as the primary antibody for p53 analysis, whereas NCL-EGFr, at 5 μg/ml (Novacastra Laboratories, Ltd., Peterborough, UK), was used for the analysis of EGFr. Breast cancer specimens with high expression of either p53 or EGFr were used as positive controls, and a negative control antibody of the same isotype was run for all sections. Sections were incubated with secondary biotinylated goat anti-mouse antibody for 1 h at a 1:100 dilution (Dako Ltd., High Wycombe, UK) and with avidin-biotin-peroxidase complexes for 1 h (Dako Ltd., High Wycombe, UK). Diaminobenzidine was the chromogen, and sections were counterstained in hematoxylin. All sections were washed twice for 3 min in PBS between incubations. Sections were reviewed independently by two investigators. Tumors with p53 nuclear staining in over 20% of cells or strong EGFr membrane staining were considered positive for p53 and EGFr, respectively. The regions of strongest staining were analyzed.

**Statistics.** All statistics was performed using Statview 4.5 (Abacus Concepts, Berkeley, CA) or Stata 5.0 (Stata Corp., TX). The effect of VEGF and p53 on time to recurrence and stage progression was assessed using Cox proportional hazards regression (Table 2). Regression analysis was also performed on characteristics of the tumor (multifocality, morphology, grade, and EGFr status) and patient (age, sex, and smoking history). In multivariate analysis, VEGF, p53, tumor multifocality, and morphology were analyzed. All other analyses and correlations were performed using a Mann-Whitney U test or Spearman’s rank correlation, respectively.

**Results**

The Prognostic Significance of VEGF mRNA on Recurrence and Stage Progression in Stage T<sub>1</sub> Bladder Cancer. Using RNase protection analysis, VEGF mRNA was quantified in 55 primary T<sub>1</sub>G<sub>1</sub> and T<sub>1</sub>G<sub>2</sub> transitional cell carcinomas of the bladder and 12 normal bladder specimens (7 cadaveric organ donors and 5 patients undergoing surgery for bladder cancer; Fig. 1). There was a 300-fold variation in expression of VEGF mRNA in the tumors, with expression greater in tumors than normal bladder (P < 0.001; Table 1). VEGF mRNA was higher in tumors that recurred within 6 months (n = 28; P < 0.001) or that underwent stage progression to a muscle-invasive phenotype (n = 12; P = 0.02; Fig. 2).

The effect of VEGF mRNA on time to relapse and stage progression was assessed in univariate and multivariate analysis. Bladder tumors with VEGF mRNA levels above the median underwent earlier recurrence (P = 0.001; hazard ratio, 3.09; 95% CI, 1.54—6.20) and had a greater risk of stage progression to an invasive phenotype (P = 0.02; hazard ratio, 5.33; 95% CI, 1.16—24.4) than those expressing levels below the median (Fig. 2). Similarly, VEGF mRNA as a continuous variable was significantly associated with time to tumor recurrence (hazard ratio, 1.11; 95% CI, 1.02—1.21; P = 0.01), and there was evidence that progression-free survival was shortened in patients with higher VEGF mRNA (hazard ratio, 1.14; 95% CI, 0.99—1.31; P = 0.06).

**Table 1 VEGF protein, mRNA, and protein:mRNA ratios in normal bladder, superficial bladder cancer, and p53-positive and -negative T<sub>1</sub> bladder cancer**

<table>
<thead>
<tr>
<th>VEGF protein level (pg VEGF/mg protein)</th>
<th>VEGF mRNA expression (arbitrary units)</th>
<th>VEGF protein:mRNA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>n</strong></td>
<td><strong>Median</strong></td>
<td><strong>Range</strong></td>
</tr>
<tr>
<td>Normal bladder</td>
<td>29</td>
<td>60</td>
</tr>
<tr>
<td>Superficial bladder cancer</td>
<td>68</td>
<td>910</td>
</tr>
<tr>
<td>p53-positive T&lt;sub&gt;1&lt;/sub&gt; bladder cancer</td>
<td>12</td>
<td>2586</td>
</tr>
<tr>
<td>p53-negative T&lt;sub&gt;1&lt;/sub&gt; bladder cancer</td>
<td>17</td>
<td>1322</td>
</tr>
</tbody>
</table>
VEGF IN SUPERFICIAL BLADDER CANCER

Fig. 2. Kaplan-Meier survival curves demonstrating relapse free (A) and stage progression free (B) survival in 55 T1G1 and T1G2 bladder cancers expressing low VEGF mRNA (i.e., below the median, \( n = 27 \)), high VEGF mRNA (i.e., above the median) and p53 negative (\( n = 15 \)), and high VEGF mRNA and p53 positive (\( n = 13 \)). All but one p53-positive tumor expressed high levels of VEGF mRNA.

The Relationship between VEGF mRNA and Protein. VEGF protein was higher in superficial bladder tumors compared to normal bladder (Table 1; \( P < 0.0001 \)), although there was no difference between stages Ta and T1 (\( P = 0.32 \); Fig. 3). In both normal bladder (\( n = 12 \); \( r = 0.65; P < 0.05 \)) and T1 tumors (\( n = 29 \); \( r = 0.59; P = 0.003 \)), VEGF protein correlated closely with mRNA expression. However, the ratio of VEGF protein to mRNA was 4-fold greater in superficial tumors compared to normal bladder (Table 1; \( P = 0.004 \)), suggesting that there is posttranscriptional regulation of VEGF in some tumors.

The Relationship between Abnormal p53 Expression and VEGF. Expression of VEGF mRNA (\( n = 55 \)) was 3-fold greater (\( P < 0.001 \); Table 1), and protein (\( n = 29 \)) was 2-fold greater in tumors that were p53 positive compared with those that were p53 negative (\( n = 29 \); \( P = 0.02 \); Table 1; Fig. 3), although protein:mRNA ratios were the same (\( P = 0.78 \)).

Interaction of High VEGF and High p53 with Recurrence and Stage Progression. VEGF mRNA was strongly related to earlier recurrence and stage progression. Similarly, overexpression of p53 protein (\( n = 14 \)), as shown in other studies, was associated with a shorter time to recurrence (hazard ratio, 3.57; \( P = 0.001 \)) and stage progression (hazard ratio, 3.19; \( P = 0.05 \)). Tumors that expressed high VEGF mRNA and were p53 positive had the poorest prognosis with respect to early recurrence (\( P < 0.001 \); hazard ratio, 4.19; 95% CI, 2.00–8.84) and progression (\( P = 0.04 \); hazard ratio, 3.39; 95% CI, 1.09–10.6; Fig. 2); however, in p53-negative tumors, high expression of VEGF mRNA also resulted in increased risk of early recurrence compared to low VEGF expression (\( P = 0.06 \); hazard ratio, 2.10; 95% CI, 0.92–4.80; Fig. 2). This suggests an interaction of VEGF and p53 in determining bladder tumor behavior and indicates that VEGF may give additional prognostic information in p53-negative tumors.

In multivariate analysis, VEGF mRNA was an independent prognostic factor for early recurrence (\( P = 0.02 \); hazard ratio, 1.12; 95% CI, 1.02–1.22) when allowing for tumor multifocality and morphology (the most significant parameters in univariate analysis; Table 2), although the association between p53 and VEGF reduced the effect of VEGF when allowing for p53.
Table 2  Cox Proportional Hazard Regression analysis for VEGF mRNA and characteristics of the tumor or patient in superficial bladder tumor recurrence and stage progression-free survival

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Recurrence-free survival</th>
<th>Stage progression-free survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio</td>
<td>P</td>
</tr>
<tr>
<td>p53 (positive or negative)</td>
<td>3.57</td>
<td>0.001</td>
</tr>
<tr>
<td>VEGF (continuous variable)</td>
<td>1.11</td>
<td>0.01</td>
</tr>
<tr>
<td>Multifocality (single or multiple)</td>
<td>2.21</td>
<td>0.03</td>
</tr>
<tr>
<td>Morphology (solid or papillary)</td>
<td>1.89</td>
<td>0.09</td>
</tr>
<tr>
<td>EGFR (positive or negative)</td>
<td>1.73</td>
<td>0.15</td>
</tr>
<tr>
<td>Size (&gt;=2 cm)</td>
<td>1.49</td>
<td>0.26</td>
</tr>
<tr>
<td>Sex</td>
<td>1.49</td>
<td>0.29</td>
</tr>
<tr>
<td>Grade (G1 or G2)</td>
<td>0.72</td>
<td>0.37</td>
</tr>
<tr>
<td>Age (continuous variable)</td>
<td>1.01</td>
<td>0.50</td>
</tr>
<tr>
<td>Smoking history (smoker or nonsmoker)</td>
<td>1.04</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Discussion

Although VEGF expression has been related to systemic metastasis via angiogenesis, a role in local tumor growth, invasion, and recurrence has not been demonstrated in patients. In this study, VEGF mRNA and protein expression are shown to correlate in T1G1 and T1G2 bladder cancer. The association between high VEGF levels in the tumors and a poor prognosis presents a means of determining the biological nature and behavior of this heterogeneous group, which could allow treatment to be tailored to individual patients.

The strong association between high VEGF mRNA expression and bladder tumor relapse (hazard ratio, 3.09; P = 0.001 for tumors with mRNA levels above the median) suggests a role for VEGF in the pathogenesis of recurrence. One mechanism for metachronous bladder tumor recurrence is the implantation of clones of cells that originate from the primary tumor (14). Tumors with high VEGF expression may recur frequently by virtue of the survival advantage conferred upon malignant cells through an ability to implant and grow rapidly due to enhanced angiogenesis. Alternatively, the significant association between tumor multifocality, earlier recurrence, and stage progression also demonstrated in the study (Table 2) supports a field change within the bladder, although no association between primary tumor VEGF mRNA expression and multifocality (n = 55, P = 0.56) was evident. The two theories of recurrence are not mutually exclusive, and it is likely that both are probably involved in the pathogenesis.

The increased risk of stage progression associated with high VEGF (hazard ratio, 5.33; P = 0.02 for tumors with mRNA levels above the median) could result from the direct angiogenic effect of the growth factor as well as induction of proteolytic enzymes such as urokinase-type plasminogen activator in endothelial cells. Furthermore, although evidence is conflicting, VEGF may interfere with the immune response to the tumor, facilitating invasion and stage progression (15, 16).

The difference in VEGF protein:mRNA ratios between superficial bladder tumors and normal bladder may result from posttranscriptional regulation through factors including the eukaryotic initiation factor 4e. This polypeptide facilitates translation by increasing binding efficiency of mRNA to the ribosome and has been demonstrated to induce malignant transformation and increased VEGF expression in vitro and in vivo (17, 18). High protein levels could equally represent high mRNA and protein expression are shown to correlate in T1G1 and stage progression also demonstrated in the study (Table 2) supports a field change within the bladder, although no association between primary tumor VEGF mRNA expression and multifocality (n = 55, P = 0.56) was evident. The two theories of recurrence are not mutually exclusive, and it is likely that both are probably involved in the pathogenesis.

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