Vascular Endothelial Growth Factor-B and Vascular Endothelial Growth Factor-C Expression in Renal Cell Carcinomas: Regulation by the von Hippel-Lindau Gene and Hypoxia

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ABSTRACT

Angiogenesis is essential for tumor growth and metastasis. It is regulated by numerous angiogenic factors, one of the most important being vascular endothelial growth factor (VEGF). Recently VEGF-B and VEGF-C, two new VEGF family members, have been identified that bind to the tyrosine kinase receptors flt-1 (VEGFR1), KDR (VEGFR2), and flt-4 (VEGFR3). Although the importance of VEGF-A has been shown in renal carcinomas, the contribution of these new ligands in kidney tumors is not clear. We have, therefore, measured the mRNA level of VEGF-B and VEGF-C together with their receptors by RNase protection assay (RPA) in 26 normal kidney samples and 45 renal cell cancers. We observed a significant up-regulation of VEGF-B (P = 0.002) but not VEGF-C (P = 0.3) in neoplastic kidney compared with normal tissues. In addition, although VEGF receptors were higher in tumors than normal kidney, there was a significant up-regulation of only flt-1 (P = 0.003) but not KDR (P = 0.12) or flt-4 (P = 0.09). There was also a significant correlation between VEGF-B and both of its receptors flt-4 (P = 0.006) and KDR (P = 0.03) but no association between VEGF-B and its receptor flt-1 (P = 0.23). A significant increase was observed in flt-1 (P < 0.001), KDR (P = 0.02), and flt-4 (P = 0.01) but not VEGF-B (P = 0.82) or VEGF-C (P = 0.52) expression in clear cell compared with chromophob (papillary) carcinomas. No significant association was demonstrated between VEGF-B, VEGF-C, flt-1, KDR, and flt-4 with patient sex, patient age, or tumor size (P > 0.05). The effect of von Hippel-Lindau (VHL) gene and hypoxia on VEGF-B and VEGF-C expression in the renal carcinoma cell line 786–0 transfected with wild-type and mutant VHL was determined by growing cells under 21% O2 and 0.1% O2. In wild-type VHL cells, whereas VEGF-A was significantly up-regulated under hypoxic compared with normoxic conditions (P < 0.001), expression of VEGF-C was reduced (P < 0.002). Nevertheless, the repression of VEGF-C was lost in mutant VHL cell lines under hypoxia. In contrast VEGF-B was not regulated by VHL despite clear up-regulation in vivo. These findings strongly support an enhanced role for this pathway in clear cell carcinomas by regulating angiogenesis and/or lymphangiogenesis. The study shows that clear cell tumors are able to up-regulate angiogenic growth factor receptors more efficiently than chromophob (papillary), that clear cell tumors can use pathways independent of VHL to regulate angiogenesis, and that this combined regulation may account for their more aggressive phenotype, which suggests that targeting VEGFRI (flt-1) may be particularly effective in these tumor types.

INTRODUCTION

Although RCCs2 account for only 1–3% of visceral malignancies, these tumors pose a significant health problem with over 23,000 new cases diagnosed annually in the United States alone. Its clinical course is notoriously unpredictable with numerous instances of spontaneous regression of metastasis with frequent recurrences in many who survive 10 or more years. A major problem in the management of patients with RCC is predicting this tumor behavior. Stage is the most useful determinant, with other features, including tumor grade and histology, being of lesser value. Because these tumors are highly angiogenic, several studies have measured the molecules that regulate neovascularization such as VEGF.

Studies have shown that VEGF mRNA and protein are significantly elevated in tumors compared with normal kidney tissues with some evidence to suggest a relationship to microvessel density (1–4). Furthermore, serum VEGF-A protein was related to grade and stage and may be useful in predicting prognosis in nonoperable patients only (5–7). Nevertheless, there is some conflicting evidence as to the presence of a significant relationship between VEGF-A and the above clinicopathological parameters (1, 8), suggesting that other angiogenic factors participate in kidney tumor neovascularization. This is likely based on our studies in breast cancers in which up to seven angiogenic factors are expressed (9). It also suggests that rather than a single angiogenic factor giving useful clinical information for patient management, it is likely that several factors, combined as an index, will be required for use in the clinic. Recently several new members of the VEGF family have been identified including VEGF-B (10, 11) and VEGF-C (12, 13), which may help to develop this approach.

VEGF-B binds to flt-1 (VEGFR1), receptor activation resulting in up-regulation of urokinase Plasminogen Activator and its inhibitor plasminogen activator inhibitor-1 (PAI-1), which suggests a role in endothelial cell migration and matrix remodeling (14). VEGF-C stimulates migration and proliferation of endothelial cells in addition to increasing vascular permeability (15). These processes mediated through KDR (VEGFR2) and another related tyrosine kinase receptor flt-4 (VEGFR3; 16). The latter appears to be largely restricted to lymphatic endothelium but is also present on tumor neovascularature, which suggests a role in both angiogenesis and lymphatic spread (17–20).

However, unlike the numerous studies demonstrating the importance of VEGF-A in human RCCs, there are no data available for VEGF-B or VEGF-C. Therefore, we have examined the level of expression of VEGF-B and VEGF-C together with their receptors flt-1, KDR, and flt-4 by RPA in a series of clear cell and papillary carcinomas together with matched normal kidney tissue. Furthermore, because dysregulation of the VHL gene is closely associated with clear cell carcinoma, we have also investigated the effects of VHL on VEGF-B and VEGF-C expression in the VHL-negative renal carcinoma cell line 786–0 transfected with wild-type and mutant VHL under normoxic and hypoxic conditions. Our aims were to investigate VEGF-B and VEGF-C in renal cell clear cell and papillary renal carcinomas, correlate their level of expression in clear cell carcinomas with standard clinicopathological characteristics, and determine the role of VHL in the regulation of these VEGF family members.

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2 The abbreviations used are: RCC, renal cell carcinoma; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; RPA, RNase protection assay; VHL, von Hippel-Lindau; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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MATERIALS AND METHODS

Tumors and Patients

Renal carcinomas (n = 45) and histologically normal kidney tissues (n = 26; taken from uninvolved kidney tissue distant from tumor) were derived from patients undergoing surgery at the Churchill Hospital, Oxford, United Kingdom, and Christchurch Hospital, Christchurch, New Zealand. Histological subtypes included 35 clear cell carcinomas and 10 chromophil (papillary) carcinomas. Grading of RCCs was performed according to the Fuhrman grading system (21). The clinicopathological characteristics of the patient series are contained in Table 1.

Renal Cell Transfection and Culture

786–0 cells (which have a mutant pVHL) stably expressing wild-type pVHL, a truncated mutant (ARZ), or empty vector (PRC) were a gift from W. G. Kaelin (22). Cells were grown in αMEM (Imperial Cancer Research Fund, Clare Hall, United Kingdom) supplemented with 10% FCS and l-glutamine (2 μM). Studies were performed on cells approaching confluence, and exposure to hypoxia was for 16 h. Parallel incubations were performed on cells in normoxia or hypoxia. Hypoxic conditions were generated in a Napco 7001 incubator (Precision Scientific) with 0.1% O_2, 5% CO_2, and balance N_2.

Preparation of RNA.

Tissue was snap-frozen after surgical resection and total RNA was prepared by either the method of Chomczynski and Sacchi or the guanidinium thiocyanate lysis and cesium chloride gradient method (24).

RPA Protocol.

Two hundred ng of each cDNA template was linearized and antisense 32P-RNA probes generated using a Riboprobe Combination System T3/T7 kit (Promega, Madison, WI). The probes were designed to generate different sized protected fragments to allow several factors to be assayed within the same total RNA sample, thereby avoiding the problem of interassay variability, and enabling measurement and direct comparison of the levels of each factor in the same sample. These comprised a 471-bp VEGF-A (positive control for cell line experiments), 340-bp VEGF-B probe (Dr. Dawn Clark, Ag Research, Mosgiel, New Zealand), and a 415-bp VEGF-C probe (Dr. Kari Alitalo, Helsinki, Finland), together with a 218-bp flt-1, a 350-bp KDR, and a 273-bp flt-4 probe (25). The DNA template was removed with DNase1, and the reaction was purified using a mini Quick Spin RNA Column (Boehringer Mannheim Corp.).

Twenty μg of RNA samples were resuspended in hybridization buffer containing the respective labeled RNA probes and the samples were denatured and incubated overnight at 45°C. The RNA remaining after hybridization was digested with RNase A and RNase T1 (Boehringer Mannheim GmbH, Mannheim, Germany). RNases were inactivated using proteinase K and proteins removed by phenol/chloroform/isooamyl alcohol extraction. Hybridized RNA was ethanol precipitated and size separated on an 8% polyacrylamide gel. Gels were vacuum dried (Bio-Rad model 583) and exposed to X-ray film (Eastman Kodak Co., Rochester, NY) between intensifying films at −80°C overnight. Aliquots of native labeled probes and labeled probes subjected to RNase digestion were included in all of the assays to determine probe integrity and efficacy of RNase digestion.

As we have previously described, internal controls can be variable (26). To avoid this, 20 μg of total RNA was loaded for each sample, and to control for any intraexperimental losses, known amounts of transcribed GAPDH sense probe were also added to each sample and assayed. Scanning laser densitometry was used to quantitate mRNA levels, and mRNA signal was standardized against the sense GAPDH control spike signal. Twenty μg of tRNA was used as a negative control for each experiment.

Statistical Analysis. Spearman rank correlation coefficients were used for studying the association between continuous variables. Tests of hypotheses on the location parameter (median) were done using rank statistics (Mann-Whitney, Kruskall-Wallis, and adjusted Kruskall-Wallis for ordered groups). The χ^2 test was used to test for independence of categorical variables including categorized continuous variables and logistic regression/multivariate analysis to confirm any significant statistical associations. All of the tests were performed using the Stata package release 5.0 (Stata Corporation, College Station, TX).

Table 1. Clinicopathological data* for RCCs studied

<table>
<thead>
<tr>
<th>Histology</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Tumor size (mm)</th>
<th>Tumor grade (high vs. low)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>Mean (range) n</td>
<td>Mean (range) n / 1 + 2 / 3 + 4 / n</td>
</tr>
<tr>
<td>Clear cell</td>
<td>18</td>
<td>17</td>
<td>65 (40–79) 35</td>
<td>6.1 (2.5–14) 9 15 5 20</td>
</tr>
<tr>
<td>Chromophil</td>
<td>8</td>
<td>2</td>
<td>66 (45–84) 10</td>
<td>3 3 6</td>
</tr>
</tbody>
</table>

* When n < 45, data were unavailable.
RESULTS

VEGF-B, VEGF-C, FLT-1 KDR, and FLT-4 Expression in Normal and Neoplastic Renal Tissues. VEGF-B and its receptor flt-1 mRNA expression as well as VEGF-C and its receptors KDR and flt-4 mRNA expression were detected in normal and neoplastic kidney samples by RPA (representative cases are shown in Figs. 1 and 2). The differences observed for VEGF-B, VEGF-C, flt-1, KDR, and flt-4 expression in normal samples and tumor samples by RPA and densitometry are shown in Figs. 3 and 4. VEGF-B and flt-1 mRNA were significantly increased in tumor compared with normal kidney tissue \((P = 0.002)\) and \((P = 0.003)\), respectively (Fig. 3), whereas, although an increase was observed, no significant up-regulation in VEGF-C \((P = 0.30)\) or its receptors KDR \((P = 0.12)\) and flt-4 \((P = 0.09)\) mRNA were observed (Fig. 4). There was a significant relationship between VEGF-C and its receptors KDR \((P = 0.03)\) and flt-4 \((P = 0.006)\), but no significant association was observed between VEGF-B and its receptor flt-1 \((P = 0.23)\).

Comparison of VEGF-B, VEGF-C, and Receptor Expression in Clear Cell and Chromophil RCCs. To determine whether the VEGF pathways are used differently by the major histological types, we compared VEGF-B, VEGF-C, and the VEGF receptor expression between clear cell and chromophil (papillary) carcinomas. We observed a significantly greater mRNA expression of flt-1 \((P < 0.001)\), KDR \((P = 0.02)\), and flt-4 \((P = 0.01)\) but not VEGF-B \((P = 0.82)\) or VEGF-C \((P = 0.52)\) in clear cell compared with chromophil (papillary) RCCs (Table 2; Figs. 5 and 6). The mean \pm SD are given for VEGFRs because these demonstrated a normal distribution and a parametric test (\(t\) test) was used, whereas the median and range are given for VEGF-B and VEGF-C because these demonstrated a

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Mean densitometry values standardized to a GAPDH spike control of VEGF-B, VEGF-C, flt-1, KDR, and flt-4 mRNA with Ps in clear cell and chromophil (papillary) RCCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clear cell RCC</td>
</tr>
<tr>
<td>flt-1</td>
<td>Mean (SD) n</td>
</tr>
<tr>
<td></td>
<td>25.3 (10.1) 22</td>
</tr>
<tr>
<td>KDR</td>
<td>1.7 (1.0) 21</td>
</tr>
<tr>
<td>flt-4</td>
<td>1.1 (0.6) 10</td>
</tr>
<tr>
<td></td>
<td>Median (range)</td>
</tr>
<tr>
<td>VEGF-B</td>
<td>13 (6.3–39.4) 31</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>0.4 (0–1.2) 30</td>
</tr>
</tbody>
</table>

Fig. 3. VEGF-B \((n = 20; \text{clear cell}, n = 15; \text{papillary}, n = 5)\) and flt-1 \((n = 15; \text{clear cell}, n = 11; \text{papillary}, n = 4)\) gene expression in matched normal (gray bars) and neoplastic (black bars) kidney samples as measured by RPA and densitometry and standardized to GAPDH spike control. Data are expressed as mean \pm SEM.

Fig. 4. VEGF-C \((n = 19; \text{clear cell}, n = 14; \text{papillary}, n = 5)\), KDR \((n = 14; \text{clear cell}, n = 10; \text{papillary}, n = 4)\), and flt-4 \((n = 14; \text{clear cell}, n = 10; \text{papillary}, n = 4)\) gene expression in matched normal (gray bars) and neoplastic (black bars) kidney samples as measured by RPA and densitometry, standardized to GAPDH spike control. Data are expressed as mean \pm SEM.

Fig. 5. Flt-1, KDR, and flt-4 gene expression in clear cell \((n = 22; \text{gray bars})\) and chromophil (papillary; \(n = 7; \text{black bars}\)) RCCs. mRNA abundance was measured by RPA and densitometry, standardized to a GAPDH spike control.
skewed distribution, and a nonparametric test was used (Mann-Whitney; Table 2).

Relationship between VEGF-B, VEGF-C, FLT-1, KDR, and FLT-4 and Clinicopathological Variables in Clear Cell Carcinomas. There was no significant correlation in clear cell carcinomas between the VEGFRs, VEGF-B, or VEGF-C and tumor grade (stratified Fuhrman 1/2 versus 3/4), patient sex, patient age, or tumor size ($P > 0.05$).

The Effects of VHL and Hypoxia on VEGF Gene Expression. In VHL wild-type transfected 786–0 cells, VEGF-A was significantly up-regulated under hypoxic compared with normoxic conditions ($P < 0.001$; a significant increase was observed in the ARZ because of increased mRNA stabilization (27, 28)). This effect was opposite to that observed for VEGF-C with which a significant ($P < 0.002$) down-regulation of expression was observed under hypoxia compared with normoxia. The suppression effect for VEGF-B was 45% but of borderline significance ($P = 0.07$), whereas for VEGF-C, it was 20% but significant. However, the repression of VEGF-B and VEGF-C was lost in cells transfected with ARZ- and PRC-mutant VHL. Indeed there was an increase in VEGF-C in ARZ ($P = 0.08$) and PRC mutants ($P < 0.002$) and, although small and not significant, a decrease in VEGF-B in ARZ ($P = 0.36$) and PRC ($P = 0.12$) VHL mutants. Overall, therefore, VEGF-B was not regulated by pVHL mutation, whereas VEGF-C was, but in the opposite direction from VEGF-A (Figs. 7–9).

DISCUSSION

VEGF-A has previously been demonstrated to be an important growth factor regulating tumor neovascularization in highly angiogenic RCC tumors. However, the role of other VEGF family members is not known. We have demonstrated that both VEGF-B and VEGF-C mRNA are expressed by normal and tumor kidney tissues and, thus, are also likely to play a role in renal neovascularization. Indeed their presence in normal kidney suggests that like VEGF-A, these related family members may also play a role in renal microvascular assembly and repair (29, 30).

In tumors, we observed a significant increase in VEGF-B and its receptor flt-1 but no difference in VEGF-C and its receptors between normal and tumor tissues. Although this suggests a more prominent role for VEGF-B in neoplastic situations, we cannot exclude a similar role for VEGF-C because it and its receptors were still expressed in this series of tumors. It also supports the concept that different tumors use different angiogenic pathways to establish a neovasculature with those tumors unable to use primary potent cytokines such as VEGF-A, expressing other related proteins including VEGF-B and/or VEGF-C (31).

No significant correlation was observed between expression of either VEGF-B or VEGF-C and any of the clinicopathological param-
growth factor (43), and emphasizes that genes can be up-regulated or
reduction in mRNA has been reported for other angiogenic factors,
VEGF-C demonstrated reduction in mRNA in wild-type VHL under
hypoxic responsiveness of tumors. The role of levels (37) and plasmin-mediated proteolysis (38) but also affects the
renal cell line. The
gate the genetic basis in clear cell subtypes, we examined the effect of
also by increasing cognate receptors, an observation that may account
for their more aggressive phenotype. Nevertheless, to further investi-
gate the presence of metastasis which, like its ligand VEGF-C, has been
proposed as a lymphatic marker. This is likely attributable to loss of
lymphatic restricted expression of flt-4 to involve vascular endothe-
lium (20), because of the tumor environment that may cause vascular
endothelial cells to revert to an embryological phenotype.
Because clear cell and chromophil subtypes have a different genetic
basis, we also looked for differences between these histological types.
The results suggest that clear cell tumors might increase their neo-
vascularization not only via the expression of angiogenic ligands but
also by increasing cognate receptors, an observation that may account
for their more aggressive phenotype. Nevertheless, to further investi-
gate the genetic basis in clear cell subtypes, we examined the effect of
the VHL gene and hypoxia on VEGF-B and VEGF-C expression in a
renal cell line. The VHL tumor suppressor gene that is mutated in
sporadic clear cell renal cancers not only modulates growth factor
levels (37) and plasmin-mediated proteolysis (38) but also affects the
hypoxic responsiveness of tumors. The role of VHL in hypoxic regu-
lation has recently been demonstrated (39, 40). pVHL is a compo-
ent of an active E3 ubiquitin-ligase complex and binds HIF-1α and
HIF-2α through its α domain and elongin C via its β domain, thus
enabling HIF to be routed for proteosomal degradation. pVHL muta-
tions are unable to degrade HIF, which allows its accumulation under
noxic conditions; this, in turn, will allow binding to a specific DNA
hypoxic response element, which will increase mRNA transcrip-
tion of angiogenic genes such as VEGF-A (41). In this study,
VEGF-C demonstrated reduction in mRNA in wild-type VHL under
hypoxia, an effect opposite to that observed for VEGF-A, although the
decline in VEGF-C does not appear to be biologically large. A
reduction in mRNA has been reported for other angiogenic factors,
including angioptiotin-1 (42) and the VEGF family member placenta
growth factor (43), and emphasizes that genes can be up-regulated or
down-regulated by hypoxia (44). Although the mechanism is unclear,
because VEGF-C does not have hypoxic response elements in the
promoters examined to date (45, 46), sequences outside of the
promoter elements not studied or other non-HIF related mecha-
nisms, such as modulation of RNA stability at the 3’ untranslated
region (27, 28), may still be responsible. Nevertheless, although the
cytokine induction of VEGF-C (42) may account for its high levels in
other types of cancer, the small loss of VEGF-C repression observed
in mutant VHL cell lines in hypoxic conditions suggests that it would
make little difference in vivo. Although VEGF-B was not regulated by
VHL, it was markedly up-regulated in renal cancer, which suggests an
additional mechanism by which clear cell tumors subvert the normal
regulatory controls. This is further supported by the lack of difference
between papillary and clear cell tumors in VEGF-B expression, i.e.,
expression does not depend on VHL mutations. This is in contrast to
flt-1, known to be induced by hypoxia, and KDR, which is up-
regulated by VEGF, which is itself hypoxically inducible, both of
which were up-regulated in clear cell tumors compared with papillary
tumors. The mutation of VHL up-regulating HIF may explain these
different patterns. In further support of the independent regulation, no
immunohistochemical up-regulation of either VEGF-B or VEGF-C
has been reported in necrotic areas of tumors (the presumed area of
hypoxia; Refs. 25, 47). These results show that an additional pathway
to VHL is involved in regulation of angiogenic factors in renal cancer
and suggest that flt-1 may be a particularly relevant target for VEGF
kinase inhibitors.

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Fig. 9. Upper panel, graph demonstrating VEGF-A and GAPDH spike mRNA abun-
dance in RCC 786–0 transfected with wild-type VHL, truncated VHL mutant (ARZ), and
empty vector (PRC) under normoxia (gray bars) and hypoxia (black bars) measured by
RPA and densitometry (n = 4 experiments). Lower panel, representative examples of
VEGF-A and GAPDH spike mRNA from a RPA.
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