VEGF_{165}b, an Inhibitory Splice Variant of Vascular Endothelial Growth Factor, Is Down-Regulated in Renal Cell Carcinoma

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ABSTRACT

Angiogenesis is essential for tumor growth. Vascular endothelial growth factor (VEGF) is the most potent growth factor of tumor neovascularization, has been shown to be up-regulated in every tumor studied thus far, and is correlated with tumor stage and progression. To determine whether specific VEGF splice variants were differentially expressed in renal cell carcinomas, 18 polar tumor samples were analyzed by reverse transcription-PCR using primers designed to differentiate between VEGF splice variants. Control tissue was derived from the opposite normal pole. An amplicon of length consistent with the previously described variant VEGF_{148} was found in normal kidney tissue. Subsequent sequencing revealed a new VEGF isoform formed by differential splicing from the end of exon 7 into the 3’ untranslated region of the mRNA. Cloning of this transcript showed that translation would result in a 165-amino acid peptide with an alternative terminal 6 amino acids, followed by a stop codon. We have termed this new isoform VEGF_{165}b. This isoform was present in 17 of 18 normal kidney samples but only 4 of 18 cases from matched malignant tissue. VEGF_{148,b} was therefore expressed in a significantly higher proportion of normal tissue than malignant tissue from the same patients (P < 0.001). To determine the functional significance of this new isoform, we expressed the full-length protein in a heterologous expression system. Conditioned medium containing this isoform significantly and dose dependently inhibited VEGF_{165}-mediated proliferation, migration of endothelial cells, and vasodilatation of mesenteric arteries. This novel isoform VEGF_{165}b is therefore an endogenous inhibitory form of VEGF that is down-regulated in renal tumors and, therefore, may be anti-angiogenesis.

INTRODUCTION

Tumor survival, growth, and subsequent metastasis are underpinned by the creation of new blood vessels. The physiological or pathological formation of new vessels is a complex process controlled by at least 10 endothelium-specific growth factors from the VEGF, angiopoietin, and ephrin families. Other non-endothelial cell-specific growth factors, molecules, and enzymes also play a role, including cytokines, proteinases, adhesion, and junctional molecules. The terms vasculogenesis, angiogenic remodeling, and angiogenic sprouting refer to specific stages of new vessel formation, and each appears to be stimulated by a specific pattern of molecular interactions (reviewed in Refs. 1, 2).

Although the exact orchestration of tumor vascularization is still debated, VEGF-A seems to be the predominant vascular growth factor in most tumors. VEGF-A inhibition has therefore received the most attention in relation to potential anti-angiogenesis/anti-tumor therapy. Exon splicing of the VEGF pre-RNA results in three main mRNA species that code for three secreted isoforms, VEGF_{189}, VEGF_{165}, and VEGF_{121} (3). A number of minor splice variants, VEGF_{206}, VEGF_{183}, VEGF_{145} and VEGF_{148} have been reported, but their significance remains uncertain (4–7).

An increase in VEGF mRNA expression has been identified in almost all known tumors (3). A positive correlation between tumor VEGF-A expression and tumor vascularity has been described, and in many studies a correlation with prognosis has been shown (8). This is also true in RCC. Increased RCC VEGF-A expression has been shown by semiquantitative RT-PCR (9), competitive quantitative RT-PCR (10), in situ hybridization (11), and Western blotting (9) increase compared with normal tissue (9)]. These reports were recently underpinned by a quantitative “real-time” RT-PCR study based on the use of fluorogenic probes that suggested that VEGF mRNA in RCC was 200-2000 times higher than in adjacent normal tissue (12). This increase appears to be true for all three common VEGF isoforms (121, 165, and 189). However, one study suggested that not all RCCs express all three common isoforms, and that the presence of VEGF_{189} was a bad prognostic sign being associated with the higher pathological grades (13). This contrasts with our experience in which we find all three common isoforms in United Kingdom nephrectomy specimens (7). Similar to other tumors, VEGF expression in conventional RCCs has been shown to correlate with tumor vascularity and in addition be a significant predictor of outcome (14). Specific VEGF inhibitors have been shown to inhibit renal tumor growth and metastasis in animal models (15).

We have reported previously the identification of VEGF_{148} mRNA in a study of VEGF-A isoform expression in single glomeruli in which the terminal half of exon 7 is spliced out (7). If translated, this mRNA species would code for a truncated form of VEGF_{165}, because a frameshift prevents translation of exon 8 by insertion of a stop codon. Because the COOH terminal of VEGF-A appears to be essential for mitogenesis and VEGF-A exerts its effects as a dimer (16), we hypothesized that VEGF_{148} may act as a native inhibitor of angiogenesis through the formation of heterodimers, as has been described for other dimerized molecules, e.g., p73 (17).

We were therefore prompted to study the expression of VEGF_{148} in renal cell carcinomas and normal renal tissue from the same kidney, the polar nature of renal cell carcinomas lending itself well to the comparison of normal and malignant tissue. During this study, we identified a previously undescribed isoform of VEGF, VEGF_{165}b, and investigated its expression characteristics and functional properties.

MATERIALS AND METHODS

Human Tissue. Non-Von Hippel Lindau nephrectomy tissue was derived from patients undergoing nephrectomy for unilateral renal tumor (age range, 47–75 years). All patients were non-diabetic and normotensive with normal excretory renal function and no urininary sediment. The non-affected renal tissue was normal on H&E staining. Immediately after nephrectomy 1 × 1 × 1-cm
Metastasis classification of malignant tumors (UICC, 1997; Ref. 18) with nuclear grading using the Fuhrman grades 1–4 (19).

RT-PCR. One hundred to 200 mg of tissue were homogenized in Trizol reagent, and mRNA was extracted using the method of Chomczynski and Sacchi (20). Eight % of the mRNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase and poly(dT) as a primer. The cDNA was then amplified using primers designed to detect VEGF_165b (Fig. 2). One μM of a primer complementary to the 3' UTR primer (3' UTR, 5' -ATG GAT CCG TAT CAG TCT TTC CT-3') and 1 μM of primers complementary to exon 7a (Exon7a, 5' -GTA AGC TTG TAC TAC AGT AAC ATC CGC AGA CG-3'), and 1.2 mM MgCl₂, 200 μM deoxynucleotide triphosphates, and 1 unit of Taq polymerase (Abgene) were used in reactions cycled 35 times, denaturing at 96°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s. PCR products were run on 3% agarose gels containing 0.5 μg/ml ethidium bromide and visualized under a UV transilluminator. This reaction consistently resulted in one amplicon at ~150 bp (consistent with VEGF_148), and one amplicon at ~200 bp, consistent with VEGF_165b, VEGF_189, VEGF_206b and VEGF_206.

Sequencing of PCR Products. The band at ~150 bp was excised from the gel under UV transillumination, and the DNA was extracted using Qiaex (Qiagen). The DNA was then digested with appropriate enzymes and ligated into pBlueScriptKSI (Stratagene). Ligations were transformed into supercompetent XL-1 Blue Escherichia coli (Stratagene) and grown on ampicillin-resistant Luria broth agar plates. Colonies were amplified, and the plasmid DNA was purified using Qiagen columns. The DNA was then sequenced using T7 and T3 sequencing primers by fluorescent dideoxy termination sequencing (ABI370). Sequences were analyzed by automated fluorescent chromatography, and the sequence was confirmed against the chromatogram manually.

Cloning of Full-Length VEGF_165b. In four of the samples, the normal tissue had significantly more ~150-bp product than ~200-bp product. One of these samples was used to confirm the full length of the new isoform. The entire length of the product was amplified by RT-PCR using primers downstream of the original 3' UTR primer (V165X, 5' -AAT CTA GAC CTC TTC CTT CAT TTC AGG-3') engineered with an XhoI site and PCR conditions as described above. This resulted in production of amplicons of a variety of lengths (as described in Table 1). These were digested with KpnI and XhoI and ligated into pBlueScriptKSI (Stratagene). These were then screened by PCR using exon 7 and 3' UTR primers to reveal colonies that contained the novel splice variants.

Exon-specific PCR. cDNA was amplified using primers designed to detect exon 9-containing isoforms. One μM of a primer complementary to the last five bases of exon 7 and the whole of exon 9 (Exon 9, 5' -TCA GTC TTT CCT GGT GAG AGA TCT GCA-3'), 1 μM of primers complementary to exon 4

5'UTR Ex1 Ex2 Ex3 Ex4 Ex5 Ex6 Ex6b Ex7 Ex7b Ex8 3'UTR

VEGF_165b

VEGF_206

VEGF_189

VEGF_183

VEGF_165

VEGF_148

VEGF_145

VEGF_121

Ex9

V165K

7a

3'UTR

V165X

Fig. 2. Exon structure of VEGF. Exon 7 and 3' UTR primers were designed to amplify a 164-bp sequence if VEGF_165 was present, 133 bp if VEGF_148 was present, a 199-bp product if longer splice variants were present, and no band if shorter variants were present. V165K and V165X were designed to amplify to full-length product. VEGF_148b would result in a 663-bp product, VEGF_148a a 729-bp product, VEGF_145 an 801-bp product, and VEGF_206 a 852-bp product (see Table 1).
NOVEL INHIBITORY VEGF ISOFORM IN RENAL CANCER

Table 1. Expected PCR product size for V165K and V165SX primers for the different splicing variants of VEGF

<table>
<thead>
<tr>
<th>No. of nucleotides</th>
<th>121</th>
<th>145</th>
<th>148</th>
<th>165</th>
<th>183</th>
<th>189</th>
<th>206</th>
</tr>
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<tbody>
<tr>
<td>VEGF&lt;sub&gt;165&lt;/sub&gt;</td>
<td>597</td>
<td>669</td>
<td>694</td>
<td>729</td>
<td>783</td>
<td>801</td>
<td>852</td>
</tr>
<tr>
<td>VEGF&lt;sub&gt;165b&lt;/sub&gt;</td>
<td>531</td>
<td>603</td>
<td>628</td>
<td>663</td>
<td>717</td>
<td>735</td>
<td>786</td>
</tr>
</tbody>
</table>

Production of VEGF<sub>165b</sub>. Full-length VEGF<sub>165b</sub> or VEGF<sub>165</sub> (generated by PCR from tissue) was cloned into the expression vector pcDNA<sub>3</sub> using standard methodology and then transfected into HEK 293 cells, and a stable cell line was generated using a Geneticin selection. Confluent cells were incubated in basal M200 endothelial cell medium for 48 h, containing neither serum nor Geneticin, and the conditioned medium was assayed for VEGF<sub>165</sub> by Western blot. There was some VEGF<sub>165b</sub> and VEGF<sub>165</sub> in the conditioned medium that corresponded to published molecular weights for VEGF<sub>165b</sub> and VEGF<sub>165</sub>. This is probably because commercially available VEGF<sub>165</sub> is not glycosylated. VEGF<sub>165</sub> was the same molecular weight as VEGF<sub>165b</sub> and both isoforms correspond to previously published molecular weights for VEGF<sub>165b</sub> (21). This blot also confirms that most of the VEGF<sub>165</sub> and VEGF<sub>165b</sub> made in HEK cells had a slightly greater molecular weight than commercially available VEGF<sub>165</sub> (M<sub>r</sub> 23kDa compared with M<sub>r</sub> 18kDa). This is probably because commercially available VEGF<sub>165</sub> is not glycosylated.

Cell Proliferation Assays. Freshly isolated HUVECs incubated in 0.1% serum M200 overnight were incubated with either pcDNA3-CM, VEGF<sub>165b</sub>-CM (adjusted to 100 ng/ml VEGF<sub>165b</sub> with pcDNA3-CM), VEGF<sub>165</sub>-CM (adjusted to 100 ng/ml VEGF<sub>165</sub> with pcDNA3-CM), or a mixture of VEGF<sub>165b</sub>-CM and VEGF<sub>165</sub>-CM (adjusted to 100 ng/ml VEGF<sub>165b</sub> and 100 ng/ml VEGF<sub>165</sub> with pcDNA3-CM), and then 37 kBq of [<sup>3</sup>H]thymidine (Amersham Pharmacia) added. After 4 h, the cells were washed and trypsinized, cell number was counted on a hemocytometer, and radioactivity was measured on a beta counter (LKB-1217). Incorporation was calculated as counts/cell. Dose-response curves were carried out in a similar manner, except that VEGF<sub>165</sub>-b-CM was diluted with pcDNA3-CM, and 50 ng/ml VEGF<sub>165</sub> (Peprotech, NJ) added. Proliferation index was calculated as [<sup>3</sup>H]thymidine incorporation of VEGF<sub>165</sub>-treated cells compared with mean incorporation into cells with no VEGF<sub>165</sub> treatment.

Migration Assay. Migration assays were performed in a modified 24-well Boyden chamber containing collagen-coated polycarbonate filter inserts (8 μm pore; Millipore). The filters were placed in 24-well plates containing 0.5 ml/well of either: (a) VEGF<sub>165</sub>-CM containing 33 ng/ml VEGF<sub>165</sub>; (b) VEGF<sub>165b</sub>-CM containing 33 ng/ml VEGF<sub>165b</sub>; (c) VEGF<sub>165</sub>-CM and VEGF<sub>165b</sub>-CM (33 ng/ml of each isoform); or (d) pcDNA3-CM. HUVECs were suspended in serum-free medium, and 25,000 cells were added to the upper chamber of each well. The plate was incubated for 6 h to allow migration, medium was removed, and both chambers were washed with PBS (two times). 0.2 mg/ml thiazolyl blue (MTT) in medium was then added to both chambers and incubated for 3 h at 37°C. The medium was removed, and the chambers were washed with PBS (two times). Nonmigrated cell crystals in the upper chamber (stained blue) were removed with a cotton swab, which was placed in 1 ml of DMSO to dissolve the MTT product. Migratory cell crystals (on the underside of the insert) were also dissolved in MTT. The absorbance of soluble MTT was determined at a wavelength of 570 nm using a spectrophotometer. The percentage migration was then calculated from the intensity of the lower well as a percentage of the total intensity of both wells. Assays were run in sextuplet.

Pressure Myography. Third-order superior mesenteric arteries were dissected from 200- to 300-g male Wistar rats (sacrificed by stunning and cervical dislocation), and leak-free segments were mounted in an arteriograph at 80 mm Hg, in rat Ringer. Arteries were constricted with 0.6 μM phenylephrine (on the underside of the insert) were also dissolved in MTT. The samples were left overnight to permit complete solution of the product. The absorbance of soluble MTT was determined at a wavelength of 570 nm using a spectrophotometer. The percentage migration was then calculated from the intensity of the lower well as a percentage of the total intensity of both wells. Assays were run in sextuplet.
RESULTS

Tumor Tissue versus Normal Tissue

An amplicon consistent with VEGF*148 expression was generated by RT-PCR from mRNA extracted from normal human kidney samples. An example of the PCR products of four kidneys (benign and malignant tissues) is shown in Fig. 4a. This amplicon was detected in 17 of 18 non-malignant, histologically normal samples of human kidney but in a significantly lower proportion of malignant samples (4 of 18; Fig. 4b; \( P < 0.001; \) Fisher’s exact test). In addition, the median stage of the tumors of which PCR did not result in production of this amplicon was 3b (interquartile range, 2–4), whereas the median stage of the four tumors that did result in this amplicon after PCR was 2 (interquartile range, 1–2.25).

Fig. 4. a, PCR of cDNA reverse transcribed from mRNA extracted from four paired renal tissues. Bands were seen at \( \sim 150\) bp (VEGF*165b) in all four benign samples (B1–B4) and two of the malignant tissues (M3 and M4) but not in two malignant tissues (M1 and M2). Bands were cloned and sequenced from B1–B4 and M4. b, number of tissue samples with a band corresponding to VEGF*165b (■) or without VEGF*165b (□) in benign and malignant tissues.

Fig. 5. a, nucleotide sequence of VEGF*165 and VEGF*165b cDNA. The 66 bp downstream of exon 7 are missing from VEGF*165b. b, exon structure of the COOH-terminal end of VEGF*165 and VEGF*165b. The 3' UTR sequence of exon 8 contains a consensus intronic sequence for exon 9, a CT-rich region and a CAG immediately prior to the splice site. The nucleotide sequence results in an alternate 6-amino acid COOH terminus. Capital letters are open reading frames; lowercase, introns or 3' UTR (italics, VEGF*165; bold, VEGF*165b). c, predicted amino acid sequence of VEGF*165 compared with VEGF*165b. The 6 alternative amino acids result in a different COOH-terminal structure of the VEGF likely to affect receptor activation but not receptor binding or dimerization. The Cys is replaced with a Ser, and the COOH-terminal amino acids are a basic (underlined) and an acidic (italics) moiety instead of two basic ones. Therefore, the net charge on this end of the molecule will be altered (Genbank ref. no. AF430806).
Identity of the Isoform

Subsequent sequencing of this amplicon revealed an unexpected 3’ sequence (Fig. 5). This unexpected sequence was found in all seven PCR samples sequenced. The sequence indicated that the mRNA was not VEGF$_{148}$ but was spliced from the 3’ end of exon 7 into the 3’ untranslated region of VEGF$_{165}$ mRNA 44 bp downstream of the end of exon 8 (23). This splice site has the same first two nucleotides as exon 8 but a different 3’ sequence (Fig. 5a). We have termed this exon 9. The intronic region between exon 7 and exon 9 has an intronic consensus sequence of 5’-GT...CCAG-3’ and a high CT-rich region 6–24 bp prior to the 5’ end of exon 9 (Fig. 5b). PCR of the full-length product using primers V165K and V165X resulted in one strong band at 670 bp. Furthermore, nested PCR using the 3’ UTR and 7a primers described above resulted in a strong band at ∼130 bp, confirming that the full length was VEGF$_{165b}$. The full-length product was a 663-nucleotide sequence encoding a peptide 191 amino acids long. This peptide consisted of the same NH$_2$-terminal 185 amino acids as VEGF$_{165}$ (i.e., a 26-amino acid signal sequence followed by 159 amino acids corresponding to exons 1, 2, 3, 4, 5, and 7). However, the COOH-terminal 6 amino acids were not the same as exon 8 (Fig. 5c). The six amino acids that this new exon codes for are Ser-Leu-Thr-Arg-Lys-Asp (SLTRKD), followed by a stop codon, TGA. This splice variant would code for a mature 165-amino acid polypeptide with 96.4% identity with VEGF$_{165}$. We have termed this isoform VEGF$_{165b}$. This exact same COOH-terminal exon structure was seen in all clones sequenced that produced a PCR product of ∼130 bp with primers exon 7 and 3’ UTR.

Distribution of Expression of Exon 9 Containing Isoforms

mRNA from 16 different tissues was reverse transcribed and amplified using exon 7 and 3’ UTR primers. PCR products of lengths consistent with expression of exon 9 containing isoforms were clearly detected in umbilical cord, cerebrum, aorta, prostate, pituitary, lung, skeletal muscle, and placental tissue as well as kidney (Fig. 6a). Fainter bands were also seen in colon, skin, bladder, and spinal cord. We did not detect significant exon 9 containing isoforms in hypothalamus, inferior vena cava, or liver. Subsequent PCR using exon 9-specific primers confirmed the distribution of expression in the different tissues (Fig. 6b), but in this case, we did detect expression in liver, and expression in the pituitary was marginal. Interestingly, in aorta, prostate, and umbilical cord a slightly longer band was seen. It is not clear whether these are additional exon 9-containing isoforms such as VEGF$_{189b}$.

Functional Effects of VEGF$_{165b}$

Proliferation. To determine the functional effect of VEGF$_{165b}$, we measured endothelial cell proliferation by determination of [3H]thymidine incorporation/cell number during incubation with conditioned medium from transfected cells (Fig. 7a). The full-length cDNA generated by PCR was cloned into an expression vector (pcDNA3-VEGF$_{165b}$), as was full-length VEGF$_{165}$ (pcDNA3-VEGF$_{165}$), and each was transfected into HEK293 cells. The VEGF concentration of cell conditioned medium (VEGF$_{165b}$-CM), as determined by ELISA using pan VEGF antibodies, ranged from 80 to 400 ng/ml VEGF. CM from cells transfected with pcDNA3-VEGF$_{165b}$ (pcDNA3-VEGF$_{165b}$) and each was transfected into HEK293 cells. The VEGF concentration of cell conditioned medium (VEGF$_{165b}$-CM), as determined by ELISA using pan VEGF antibodies, ranged from 80 to 400 ng/ml VEGF. CM from cells transfected with pcDNA3-VEGF$_{165b}$ (pcDNA3-VEGF$_{165b}$) had a VEGF concentration of 100–260 ng/ml. Medium from cells transfected with pcDNA3-VEGF$_{165}$ (pcDNA3-VEGF$_{165}$-CM) had a VEGF concentration of 100–260 ng/ml. Medium from cells transfected with pcDNA3 alone (pcDNA3-CM) contained <62.5 pg/ml VEGF (the minimum detection limit of the ELISA). HUVECs were...
VEGF<sub>165</sub>b, there was a dose-dependent inhibition of [<sup>3</sup>H]thymidine incorporation stimulated by commercially available VEGF<sub>165</sub> (Fig. 7b), with a molar ratio IC<sub>50</sub> of 0.94 (i.e., equimolar inhibition). Additionally, VEGF<sub>165</sub>b-CM did not affect fibroblast growth factor-mediated proliferation (Fig. 7c).

**Migration.** To determine whether VEGF<sub>165</sub>b could inhibit other actions of VEGF<sub>165</sub>, associated with angiogenesis, we also measured the effect of VEGF<sub>165</sub>b on VEGF<sub>165</sub>-mediated migration of HUVECs (Fig. 8). We incubated HUVECs in VEGF<sub>165</sub>-CM (33 ng/ml VEGF<sub>165</sub>), and this resulted in a significant 24% ± 3% increase in migration of endothelial cells compared with pcDNA3-CM-alone (P < 0.01, ANOVA). VEGF<sub>165</sub>-CM containing 33 ng/ml VEGF<sub>165</sub>b did not stimulate migration (−3% ± 2.6% compared with pcDNA3-CM, not significant). Furthermore, there was no increase in migration when cells were incubated in a combination of both VEGF<sub>165</sub>b-CM and VEGF<sub>165</sub>-CM containing 33 ng/ml of each isoform (9.9 ± 5.8% compared with pcDNA3-CM). Therefore, VEGF<sub>165</sub>b did not stimulate migration and again significantly inhibited VEGF<sub>165</sub>-stimulated migration (P < 0.001, ANOVA).

**Vasodilatation.** We measured the effects of VEGF<sub>165</sub>b on vasodilatation to determine whether VEGF<sub>165</sub>b could inhibit the effects of VEGF<sub>165</sub> on intact vessels. Luminal perfusion of isolated pressurized rat mesenteric arteries in vitro with dialyzed pcDNA3-CM did not affect vessel diameter (Fig. 9a). Perfusion of the same arteries with dialyzed VEGF<sub>165</sub>b-CM (40 ng/ml) did not affect the diameter of the arteries either (Fig. 9a). Perfusion with dialyzed CM containing 20 ng/ml VEGF<sub>165</sub> resulted in significant vasodilatation (Fig. 9b), but this vasodilatation was abolished when perfused with VEGF<sub>165</sub>b-CM and VEGF<sub>165</sub> (40 ng/ml VEGF<sub>165</sub>b; 20 ng/ml VEGF<sub>165</sub>). Therefore, VEGF<sub>165</sub>b does not stimulate vasodilatation and inhibits VEGF<sub>165</sub>-mediated vasodilatation (Fig. 9b).

**DISCUSSION**

VEGF expression has previously been thought to be required for tumor growth. Although this is still evidently true, the data presented here suggest that regulation of splicing of VEGF may also be important in tumor development. We have discovered a novel endogenously produced VEGF mRNA from human kidney that appears to be down-regulated in tumors. It appears somewhat surprising that this isoform has not been described previously. However, the only method that would successfully distinguish between exon 9-containing isoforms and exon 8-containing isoforms is that of RT-PCR using primers that flank the exon 9-containing region and discrimination by high-reso-

![Fig. 7. Effect of VEGF<sub>165</sub>b on HUVEC proliferation. a. [<sup>3</sup>H]thymidine incorporation/ cell assessed in HUVECs incubated with CM without VEGF (pcDNA3), 100 ng/ml VEGF<sub>165</sub>, 100 ng/ml VEGF<sub>165</sub>b, or 100 ng/ml of both (VEGF<sub>165</sub>b + VEGF<sub>165</sub>), n = 3; bars, SE. P < 0.01, ANOVA. b, dose response: proliferation index measured in HUVECs treated with both isoforms compared with that treated with no VEGF<sub>165</sub>, n = 3; bars, SE. P = 0.001, ANOVA. c, effect of basic fibroblast growth factor (FGF) and VEGF on proliferation. [<sup>3</sup>H]thymidine incorporation in HUVECs incubated with pcDNA3-CM (□) or VEGF<sub>165</sub>b-CM (■) with 40 ng/ml VEGF<sub>165</sub> or 50 ng/ml bFGF, n = 3; bars, SE. P < 0.001, ANOVA, *, P < 0.05; **, P < 0.01; ***, P < 0.001 compared with VEGF<sub>165</sub>.

![Fig. 8. Effect of VEGF<sub>165</sub>b on migration of HUVECs. Cell migration was assessed in HUVECs incubated with pcDNA3-CM (<62.5 pg/ml VEGF), VEGF<sub>165</sub>-CM (33 ng/ml VEGF<sub>165</sub>), VEGF<sub>165</sub>b-CM (33 ng/ml VEGF<sub>165</sub>b), or a combination of both CM (33 ng/ml each VEGF isoform). P < 0.01, ANOVA, n = 6; bars, SE. *, P < 0.05; **, P < 0.01 compared with VEGF<sub>165</sub>.
lution gel electrophoresis. The vast majority of papers examining VEGF expression have used primers that are complementary either to the regions 5' to exon 9 or that produce products that would not distinguish VEGF 165 b from other isoforms such as VEGF 148 or VEGF 145 . Detection of mRNA using Northern blotting, RNase protection assay, or in situ hybridization would not result in a discrimination between VEGF 165 b and other isoforms of VEGF, because all other isoforms contain the exon 9 sequence in their 3' UTR. A search of the nucleotide database, however, provides interesting information on the conservation of this region. The entire 3' UTR from the end of exon 8 is 100% conserved between human and macaque. In other mammalian species, there is relatively good conservation of the supposed 3' UTR terminal to exon 8 (Fig. 10a). In fact, in the cow the mRNA has >95% identity in the 66 bases 3' to the stop codon of exon 8, and exon 9 is >90% identical. However, this identity breaks down immediately after the exon 9 stop codon (Fig. 10a). There is significantly less identity in the 22 bases after this stop codon (53%) than that in exon 9 (91%). This pattern is also evident in mouse, where the exon 9-containing sequence is 86% identical to human but only 23% identical in the 22 bp immediately after the exon 9 stop codon (Fig. 10a). Interestingly, the mouse sequence predicts an exon 9 of 7 amino acids of the sequence PLTGKTD, compared with SLTRKD in the human and macaque and RLTRKD in the cow. Therefore, four of six amino acids are conserved [XLTX(K/D)], and this appears to have been brought about by a double mutation, an adenosine insertion in the human at nucleotide 10 (mouse) and a cytosine to thymidine mutation at nucleotide 19 (mouse) that rescues the stop codon (Fig. 10a). This is indirect evidence for functional relevance of the splice site. Interestingly, it is only conserved in mammals, not in birds or fish (Fig. 10b).

There are a number of particularly interesting features of this new splice variant. The splice variant contains normal exons 1–5 and 7. Exon 1 encodes for the signal sequence; therefore, it is likely that this isoform is secreted (24). This was confirmed by our Western blot showing expression in CM. Exons 3 and 4 contain the dimerization domain (25) and receptor binding domains (26). These appear to be intact in this isoform, indicating that it is capable of homodimerization and potentially heterodimerization with other isoforms. More impor-
of the VEGF protein confers mitogenic activity. Digestion of VEGF121 with plasmin results in a 110-amino acid molecule that lacks exon 8 but contains exons 1–4 and most of exon 5. This results in a 110-amino acid VEGF that is less potent at increasing human umbilical vein endothelial cell number, both as a homodimer and as a heterodimer. However, the potency of VEGF in culture is not affected by the loss of exon 8. VEGF121, which has exons 1–5 and 8, has the same effect as VEGF145, indicating that it is not the loss of exon 8 but rather those amino acids coded for by exon 7 that specify the ability to increase cell number (in itself a measure of the balance between survival and proliferation). VEGF165b, which contains exon 7, should therefore have the same potency as VEGF165.

VEGF165b has an alternate exon instead of exon 8, however. Exon 9 encodes for 6 amino acids and therefore results in a protein of the same length as VEGF165, although the six amino acids are very different. Exon 8 encodes for Cys-Asp-Lys-Pro-Arg-Arg (CDKPRR). The cysteine forms a disulfide bond with Cys-146 in exon 7 (27). This results in the COOH terminus of VEGF165 being held close to the VEGF-R2 receptor binding domain in exon 4 in the three-dimensional structure of VEGF. In addition, the proline inserts a kink in the amino acid backbone of the molecule. Finally, the two terminal amino acids are highly positively charged arginine residues. Exon 9, on the other hand, codes for Ser-Leu-Thr-Arg-Lys-Asp (SLTRKD) and has lost the cysteine residue; therefore, the COOH terminus may not be held close to the receptor binding site. In addition, there is no proline kink, and there is no net charge on the terminal two amino acids. We have shown that this novel exon confers inhibitory activity upon the isoform. There are two obvious mechanisms by which this may occur. Either VEGF165 is able to bind to the VEGF receptors but not activate them, or it heterodimerizes with VEGF145 and prevents VEGF145-mediated stimulation of the receptors. VEGF165b contains all of the elements required for efficient dimerization: Cys-51 and Cys-60 in exon 3 (25); and receptor binding, for VEGF-R1 Asp63, Glu64, Glu67 in exon 3; for VEGF-R2 Arg82, Lys84. His 86 in exon 4 (26); and for neuropilin-1 Cys-136 to Cys-158 in exon 7 (28). This isoform substitutes exon 9 for exon 8 and therefore has no Cys-160, which normally binds to Cys-146 in exon 7 and will therefore be likely to affect folding and tertiary structure of the VEGF molecule.

It is particularly interesting that this isoform has been found in the kidney. The kidney, and in particular the glomerulus, has been shown to produce high levels of VEGF. This has always been assumed to be VEGF165 because it is detected by antibodies to VEGF165 isoform (29), in situ hybridization using probes to VEGF165 (30), PCR using primers specific to exon 8-containing isoforms (7), and so on. However, in all of these cases, the detection techniques used would not easily distinguish between VEGF165 and VEGF165b. The only reason we detected VEGF165b was that we were attempting to examine VEGF145 expression in this tissue and came across it serendipitously. Antibodies and in situ hybridization probes to VEGF165 may have detected VEGF165b inadvertently. It is interesting to note that there has been an outstanding question concerning the role of VEGF in the kidney, i.e., that despite high levels of VEGF produced by podocytes in the glomerulus, glomerular endothelial cells (which express VEGF receptors) are no more proliferative than other endothelial cells. Endothelial cell turnover in the glomerulus is low, as in other parts of the body, and there is no overt angiogenesis occurring. VEGF165b could explain this apparent paradox, high VEGF expression but low angiogenesis. Kidney tumors may grow in an environment in which VEGF is highly expressed under normal conditions, and yet angiogenesis is prevented. Therefore, tumors need to overcome some endogenous anti-angiogenic process to grow. If VEGF165b were to be inhibitory, then the down-regulation of VEGF165b by tumors that we have described here would be necessary for tumors to switch angiogenesis on in this tissue. Furthermore, the high production of VEGF by the glomerulus may actually be VEGF165b rather than VEGF165, and therefore this would explain why angiogenesis is not seen. The obvious next questions therefore are what is the relative abundance of VEGF145b and VEGF165 and what does VEGF165b do if it is not antiangiogenic? These questions have yet to be answered.

Finally, the fact that we detected exon 9-containing isoforms in a number of other tissues shows that these isoforms may be widely distributed in the body. Of particular note was the difference in expression between aorta and inferior vena cava and between pituitary and hypothalamus, because each of these pairs of tissues came from the same patients. It is also of particular interest because the pituitary gland has a large number of fenestrated capillaries (31), whereas the hypothalamus is considered to have a vasculature that forms a normal blood brain barrier. The glomerular capillaries are also fenestrated, and it may be that VEGF165b is necessary for formation of fenestrated capillaries, as has been shown for VEGF (32), but without inducing angiogenesis.

There are now >20 angiogenesis inhibitors undergoing clinical trials (2), many with encouraging results. However, most of these are potentially immunogenic (monoclonal anti-VEGF antibody for example) or are synthetic agents with potentially unpredictable side effects (e.g., thalidomide). Should functional studies confirm VEGF165b and its counterparts as inhibitory, their potential use as angiogenesis inhibitors would have the theoretical benefit of using molecules that appear to be expressed endogenously. We suggest therefore that the functional capacity of this new VEGF isoform requires further investigation.

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