A New ELISA for Use in a 3-ELISA System to Assess Concentrations of VEGF Splice Variants and VEGF_{110} in Ovarian Cancer Tumors

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BACKGROUND: Vascular endothelial growth factor (VEGF), which affects tumor angiogenesis, is expressed as different splice variants, including the major isoforms VEGF_{165} and VEGF_{121}, and can be cleaved by plasmin to generate VEGF_{110}. The amount of VEGF_{121} and VEGF_{110} in biological samples has not been well studied.

METHODS: We developed an ELISA that detects VEGF_{165} and VEGF_{121} equally, but does not detect VEGF_{110}. We used this ELISA together with 2 other ELISAs, one detecting VEGF_{165} and the other detecting VEGF_{165}, VEGF_{121}, and VEGF_{110} equally, to assess the concentrations of VEGF_{121} and VEGF_{110} in ovarian cancer tumors.

RESULTS: The median concentrations in ovarian cancer tumor lysates were 0.61 (range <0.055–74) fmol/mg protein for VEGF_{165}, 1.4 (range <0.20–500) fmol/mg protein for VEGF_{165} plus VEGF_{121}, and 2.3 (range <0.079–520) fmol/mg protein for total VEGF including VEGF_{110} (n = 248). VEGF concentrations measured by the 3 ELISAs were highly correlated (r = 0.91–0.94). Median estimated VEGF_{121} and VEGF_{110} concentrations were 0.77 and 0.58 fmol/mg protein, respectively. In lysates with measurable VEGF_{165} and total VEGF concentrations, mean VEGF_{165} was approximately 31% (SD 23%) of the total VEGF (n = 217). In contrast, VEGF_{165} constituted approximately half of the total circulating VEGF.

CONCLUSION: VEGF_{165}, VEGF_{121}, and VEGF_{110} may be present at significant amounts in ovarian cancer tumors.

Vascular endothelial growth factor (VEGF), a homodimeric glycoprotein, regulates blood vessel formation during development and pathological angiogenesis (1, 2). VEGF interacts with 2 receptor tyrosine kinases, VEGFR-1 and VEGFR-2. Increased VEGF concentrations were found to correlate with a poor prognosis in cancer (3–8). The human VEGF gene has eight exons. Alternative RNA splicing generates isoforms containing 121, 145, 165, 183, 189, and 206 amino acids. These splice variants have different solubilities based on their affinities for heparin. VEGF_{165} is partially soluble, whereas VEGF_{121} is freely soluble. The extracellular matrix–bound VEGF can be released by plasmin cleavage to generate the VEGF_{110} fragment, consisting of amino acids 1–110, a process that may be an important mechanism to locally regulate the bioavailability of VEGF (1, 9–11). The exact roles of these different VEGF molecules remain unclear.

The relative abundance of different VEGF splice variants was previously evaluated by RNA analysis (12, 13). VEGF_{121} and VEGF_{165} were the dominant variants expressed in cancer, including breast and ovarian cancer. Previously, we measured VEGF_{165} [using VEGF_{165–206} ELISA (14)] and total VEGF [using VEGF_{110–206} ELISA, formerly named VEGF_{121–206} ELISA (5)] in breast cancer tumor lysates. VEGF_{165} constituted approximately 36% of the total VEGF (5). The amount of VEGF_{121} and VEGF_{110} could not be determined because antibodies specific to VEGF_{121} (consisting of amino acids 1–115 and 160–165) were unavailable, and antibodies to VEGF_{110} also bound VEGF_{165} and VEGF_{121}. To distinguish VEGF_{121} from VEGF_{110}, we developed a new ELISA that uses monoclonal antibody 5C3 as coat. 5C3 likely binds near amino acids 1–110, a process that may be an important mechanism to locally regulate the bioavailability of VEGF (1, 9–11). The exact roles of these different VEGF molecules remain unclear.

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matrix metalloproteinase-cleaved VEGF fragments with amino acids additional to 1–110, previously observed in the ascites of ovarian cancer patients (16). The VEGF_{110–206} ELISA detects all 5 VEGF molecules equally (Fig. 1C) and is expected to quantify the molarity of total VEGF. The amount of VEGF_{121} (which may include fragments with amino acids additional to 1–110) can be obtained by subtracting the amount of VEGF detected by the VEGF_{110–206} ELISA from that detected by the new ELISA. The amount of VEGF_{110} (which may include fragments with amino acids fewer than 1–110) can be obtained by subtracting the amount of VEGF detected by the new ELISA from that detected by the VEGF_{110–206} ELISA.

The new ELISA was performed in 96-well plates (MaxiSorp, Nunc) coated with 1 mg/L 5C3 in 50 mmol/L carbonate buffer, pH 9.6, overnight at 4 °C. The plates were blocked with 5 g/L BSA in PBS for 1 h at room temperature. VEGF_{165} calibrators (Genentech, 0.10–13.4 pmol/L in 2-fold serial dilutions) and samples in 5 g/L BSA, 0.5 ml/L polysorbate 20, 5 mmol/L EDTA, 2.5 g/L CHAPS, 2 g/L bovine γ-globulin (Sigma), and 0.35 mol/L NaCl in PBS were added and incubated for 2 h. The plates were washed with 0.5 ml/L polysorbate 20 in PBS. Bound VEGF was detected with biotinylated A4.6.1 followed by horseradish peroxidase–conjugated streptavidin (GE Healthcare) and 3,3′,5,5′-tetramethyl benzidine (Kirkegaard & Perry Laboratories) as the substrate. Absorbance was read at 450 nm. The detection limit (3 SD above the mean of the zero calibrator) was 0.026 pmol/L (1.0 ng/L VEGF_{165}) in buffer. For quantification, we...
considered only values ≥0.10 pmol/L (8 SD above the mean of the zero calibrator, n = 15), which was equivalent to a quantification limit of 1.0 pmol/L in samples, because a minimum dilution of 1:10 was used for tumor lysates (owing to low sample volumes) and for plasma samples (owing to matrix effects). The recovery of VEGF165 (0.10 –13.4 pmol/L) added into 10% normal human EDTA-plasma was 77%–100%. With the 0.31 and 1.5 pmol/L controls (1:10 diluted normal human EDTA-plasma with VEGF165 added), interassay CVs were 6.6% and 2.6%, respectively, and intraassay CVs were 2.4% and 1.4%, respectively (n = 15).

The 3 ELISAs use nonglycosylated VEGF165 produced in Escherichia coli as the calibrator; however, they quantify glycosylated VEGF equally. We measured recombinant glycosylated VEGF165 in the conditioned media of 6 stable clones of transfected Chinese hamster ovary cells and obtained similar concentrations (within 10%) in the 3 ELISAs (data not shown). All 3 ELISAs detect VEGF (VEGF-A) specifically. VEGF-B, VEGF-C, and VEGF-D (R&D Systems) at 1.3 nmol/L gave only background signals. The reproducibility of VEGF110–206 ELISA was evaluated using the 0.080 and 1.0 pmol/L controls (1:10 diluted normal human EDTA plasma with VEGF165 added). Intraassay CVs were 18% and 9.5%, respectively, and intraassay CVs were 14% and 6.5%, respectively (n = 34). The 3 ELISAs showed good linearity; the mean CVs of the corrected values from 3–4 3-fold serial dilutions of tumor lysates were 7.6% (SD 4.8%) (n = 82), 10.2% (SD 4.2%) (n = 33), and 7.3% (SD 4.8%) (n = 64), and from 3–4 2-fold serial dilutions of plasma samples were 10.2% (SD 8.9%) (n = 27), 7.4% (SD 2.2%) (n = 4), and 8.7% (SD 3.6%) (n = 8) in the VEGF165–206, VEGF121–206, and VEGF110–206 ELISAs, respectively.

We measured VEGF in ovarian cancer tumor lysates, collected at the University of Munich, Klinikum Grosshadern, under institutional review board approval (5). Total protein concentrations were determined by the Lowry method, using a protein standard containing human albumin and γ-globulin (Sigma, Germany) as the calibrator. Median concentrations (n = 248) were 0.61, 1.4, and 2.3 fmol/mg protein in the VEGF165–206, VEGF121–206, and VEGF110–206 ELISAs, respectively (Table 1). VEGF concentrations measured by the 3 ELISAs correlated well (r = 0.91, 0.94, and 0.91 for VEGF165–206 vs VEGF110–206, VEGF121–206 vs VEGF110–206, and VEGF165–206 vs VEGF121–206, respectively, on the log-log scale) (Fig. 1, D–F). Median estimated VEGF121 and VEGF110 concentrations were 0.77 and 0.58 fmol/mg protein, respectively. The mean relative amount of VEGF110 was approximately 57% (SD 21%, n = 34), the mean relative amount of VEGF165 was approximately 31% (SD 23%), n = 217, Table 1) was lower at higher total VEGF concentrations (38% (SD 17%) for the quarter of samples with the lowest VEGF concentrations and 12% (SD 6%) for the quarter of samples with the highest VEGF concentrations). Tumors with higher VEGF concentrations may have more plasmin activity; however, further studies are needed (17).

To rule out that the 37 °C incubation steps used in the VEGF165–206 ELISA caused additional protease digestion of VEGF165, we measured selected samples in a corresponding colorimetric ELISA, with room temperature incubation steps, and obtained similar VEGF concentrations (within 10%, n = 7). The neutralizing antibody, A4.6.1, was used in all 3 ELISAs (see Fig. 1 in the online Data Supplement). A4.6.1 is the parent murine antibody of Bevacizumab, which was approved for cancer treatment (18). Soluble VEGFR-1 was previously found in breast cancer tumor lysates (19). We added VEGF165 into 6 ovarian cancer tumor lysates to evaluate recovery in the 3 ELISAs. We measured the added VEGF165 at expected concentrations (within 20%) (data not shown) with no indication of VEGFR-1 interference.

Because normal tissues were not available for comparison with tumor tissues, we measured circulating VEGF concentrations in the 3 ELISAs. EDTA-plasma samples were collected from coronary artery disease patients or healthy individuals at the Hennepin County Medical Center, Minneapolis, (n = 298) and St Thomas’ Hospital, London, (n = 89, except 38 samples from heparin therapy patients were collected in tubes without EDTA) under institutional review board approval. VEGF was measurable in only 18% of the samples in the least sensitive VEGF121–206 ELISA. The mean relative amount of VEGF165 was approximately 57% (SD 33%, n = 77) and 57% (SD 21%, n = 46) of the total VEGF in the 2 sample sets, respectively (see Table 1 in the online Data Supplement). These percentages were higher than those found in the ovarian cancer tumor lysates (P < 0.0001 using Dunnett’s test, JMP software, SAS Institute). We also used the 3 ELISAs to measure VEGF in conditioned media (containing 3.6–32 pmol/L total VEGF) of ovarian and breast cancer cell lines and obtained similar VEGF165 and VEGF121 percentages to those determined previously by RNA analysis (12) (see Table 2 in the online Data Supplement). We affinity-purified VEGF from conditioned medium (containing approximately 150 pmol/L VEGF165 and 89 pmol/L VEGF121) of human rhabdomyosarcoma A673 cells. VEGF165 and VEGF121 presence was confirmed by protein blotting analysis (see Fig. 2 in the online Data Supplement).

In conclusion, our data indicate for the first time that VEGF110 may be present at significant concentrations in ovarian cancer tumors. Because VEGF165 constitutes less than half of the total VEGF, it may be necessary to inhibit VEGF121 and VEGF110 along with VEGF165 (20) to effectively treat ovarian cancer. VEGF
concentrations measured by the 3 ELISAs correlated well, suggesting that a similar correlation exists between prognosis and VEGF concentrations. This 3-ELISA system is a useful tool to assess the concentrations of VEGF splice variants and VEGF110 in biological samples.

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