Tumorigenesis and Neoplastic Progression

Overexpression of Vascular Endothelial Growth Factor 189 in Breast Cancer Cells Leads to Delayed Tumor Uptake with Dilated Intratumoral Vessels

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Vascular endothelial growth factor (VEGF) is essential for breast cancer progression and is a relevant target in anti-angiogenesis. Although VEGF121 and VEGF165, the fully or partially secreted isoforms, respectively, have been the focus of intense studies, the role of the cell-associated VEGF189 isoform is not understood. To clarify the contribution of VEGF189 to human mammary carcinogenesis, we established several clones of MDA-MB-231 cells stably overexpressing VEGF189 (V189) and VEGF165 (V165). V189 and V165 clones increased tumor growth and angiogenesis *in vivo*. Remarkably, V165 induced the most rapid tumor uptake, whereas V189 increased vasodilation. *In vitro* overexpression of VEGF165 and VEGF189 increases the proliferation and chemokinesis of these cancer cells. Interestingly, overexpression of VEGF189 increased cell adhesion on fibronectin (1.9-fold) and vitronectin (1.6-fold), as compared to VEGF165, through $\alpha 5 \beta 1$ and $\alpha v \beta 5$ integrins. Using the BIACore system we demonstrated for the first time that VEGF189 binds directly to neuropilin-1, which is strongly expressed in MDA-MB-231 cells. In contrast, VEGF-R2 was not significantly expressed and VEGF-R1 was expressed at low level. Our *in vitro* results suggest an autocrine effect of VEGF189 on breast cancer cells, probably through neuropilin-1. In conclusion, our data indicate that VEGF189 participates in mammary tumor growth through both angiogenesis and nonangiogenic functions. Whether VEGF189 overexpression is correlated to prognosis in human breast tumors remains to be established. (Am J Pathol 2008, 172:167–178; DOI: 10.2353/ajpath.2008.070181)

Tumor growth requires the establishment and remodeling of the vascular system, involving paracrine signaling between various growth factors and endothelial receptors.¹ Vascular endothelial growth factor (VEGF) is a key regulator of developmental, physiological, and pathological neovascularization (angiogenesis), especially involved in tumor growth.² Understanding the functions and properties of VEGF, which exists as several isoforms, is an approach to tumor growth control.

The VEGF gene codes for several spliced variants³ containing 121, 145, 165, 189, and 206 amino acids in human and one amino acid shorter in mice. Depending of the presence of genomic exons 6 and 7, these isoforms are either secreted as soluble forms (VEGF121 and VEGF165) or remain cell- or matrix-associated (VEGF189, VEGF206, and partially VEGF165).^{4–7} VEGF121 and VEGF165, which are considered as the most abundant isoforms, have been the focus of intense studies. In contrast, the role of cell-associated VEGF189 isoform in tumor growth and vascularization is not understood. *In vitro*, VEGF189 can be cleaved into shorter bioactive forms by proteases such as plasmin,⁵ urokinase plasminogen activator,⁷ and MMP-3.⁸ VEGF isoforms have different binding affinities for the VEGF receptors, VEGF-R1 (Flt-1), VEGF-R2 (KDR/Flk-1), and for neuropilin-1 (NRP-1)^{4,9,10} a receptor of the semaphorin receptor family. VEGF165 binds to VEGF-R1, VEGF-R2, and NRP-1, whereas VEGF189 has been shown to bind preferentially to VEGF-R1 unless cleaved by proteases.^{5,7} The interaction between VEGF and their endothelial receptors stimulates receptor-associated kinase activity and initiates signaling pathways leading to angiogenesis.^{2,11} Recently, VEGF189 has been shown to stimulate proliferation and migration of endothelial cells.¹² Heparin-binding VEGFs have also been shown to

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be involved in vascular branching complexity at the earliest stages of angiogenic invasion in several organs.¹³

Although VEGF189 is usually considered as present in low amounts, several studies have reported important differences in the organ-specific expression pattern of VEGF^{14,15} during development¹⁵ and reproduction.¹⁶ VEGF189 is the major isoform expressed in the human lung and is associated with the maturation of the alveolar epithelium.^{15,16} Both VEGF164 and VEGF188 predominate in the mouse heart and liver.¹⁵ Mice that lack the 164 to 188 isoforms, but express the VEGF120 isoform, exhibit impaired myocardial angiogenesis and ischemic cardiomyopathy.¹⁷ VEGF164/164 mice are healthy and have normal retinal angiogenesis, whereas VEGF120/120 mice exhibit severe defects in vascular outgrowth and VEGF188/188 mice display impaired arterial development.¹⁸ Furthermore, we have reported previously the increase of VEGF189 expression in human endometrial cells during the secretory phase of the menstrual cycle and during early gestation,¹⁹ also suggesting that this isoform plays a role in physiological vascular remodeling during the reproductive process.

In breast cancers, VEGF165 and VEGF121 have been shown to accelerate breast tumor development.^{20–23} In contrast, the role of VEGF189 in breast cancer progression and angiogenesis has never been investigated. In certain cancers, the expression of the VEGF165 or VEGF189 isoform has been associated with differences in tumor growth.^{24–26} An increase of cell-associated VEGF189 expression has been observed in lung and colon cancers and in glioblastomas.^{16,24–26} VEGF189 is related to poorer prognosis in lung cancer and osteosarcoma.^{26–29} Xenografts of VEGF189-overexpressing colon cancer cells grew more slowly than those of VEGF165-overexpressing cells.³⁰ VEGF188-expressing mouse fibrosarcoma, although hypervascularized, was not associated with tumor growth,³¹ and melanoma cells transfected with VEGF189 remained nontumorigenic and dormant.^{25,31} These results emphasize a complex role of VEGF189 in tumoral development.

In this study, we evaluated the role of VEGF189 in the progression of human breast cell carcinoma. For this purpose, we generated stable human breast carcinoma cells (MDA-MB-231) overexpressing VEGF165 or VEGF189 isoforms. The effect of VEGF189 expression on angiogenic potential and tumor cell behavior were determined both *in vitro* and *in vivo*. Interestingly, we demonstrate that VEGF189 binds to NRP-1, a specific property demonstrated so far for VEGF165. For the first time, our findings show that VEGF189, as well as VEGF165, contributes to breast cancer progression and angiogenesis. Furthermore, we provide evidence that their effects are mediated through different paracrine and autocrine mechanisms.

Materials and Methods

Cell Culture

Human breast cancer cells, MDA-MB-231 (American Type Culture Collection, Molsheim, France), were routinely main-

tained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 100 µg/µl sodium pyruvate. The anti- $\alpha_5\beta_1$, anti- $\alpha_v\beta_3$, anti- $\alpha_v\beta_5$ antibodies were purchased from AbCys (Paris, France).

Human umbilical vein endothelial cells (HUVECs) were isolated with collagenase I.³² Cells were cultured on 0.2% gelatin-coated dishes in Medium 199 (M199) supplemented with 20% FBS, 2 mmol/L glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 15 mmol/L of HEPES, and 0.4% endothelial cell growth supplement (Promocell, Heidelberg, Germany). Second passage cells and a mixture of three cords were used for all experiments. All cell culture reagents were from Life Technologies Inc. (Cergy Pontoise, France).

Generation of Stable Clones Overexpressing VEGF Isoforms

The clones pUC-VEGF165 and pUC-VEGF189 were initially obtained from Dr. J. Abraham (Scios-Nova, Mountain View, CA). The cDNA fragment corresponding to VEGF165 or VEGF189 gene was subcloned into a bicistronic eukaryotic expression vector, pRCEN, containing the neomycin resistance gene as a selective marker, as previously described.⁷ Stable transfections of MDA-MB-231 cells were performed using the Fugene 6 reagent according to manufacturer's instructions (Roche Diagnostics, Meylan, France).³³ Parental cells were also transfected with pRCEN vector (control plasmid). The transfected cells were selected with G418 antibiotic (1 mg/ml, Life Technologies) in DMEM-10% FBS for 6 to 8 weeks. Stable transfected clones were isolated and maintained in DMEM-10% FBS in the presence of 500 µg/ml of G418.

RNA Extraction and Reverse Transcription

Seventy percent confluent MDA-MB-231 cells were cultured in DMEM-10% FBS. RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Cergy Pontoise, France). Reverse transcription of 1 µg of RNA was performed using 200 U of Superscript II RNase H reverse transcriptase with random hexamers (Invitrogen).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Transcript quantification for VEGF was performed using TaqMan technology (LightCycler 2.0, Roche) and standard curve quantification method using the LightCycler software 3-1, according to described techniques. Briefly, cDNAs for VEGF189 and VEGF165 were prepared from total RNA, amplified by RT-PCR, and cloned using TOPO II TA cloning kit (Invitrogen). A standard curve for each transcript was generated using serial dilutions of cloned products ranging from 1 to 10⁹ molecules/µl. The copy number of unknown samples was calculated by setting

their PCR cycle number to the standard curve. To correct for differences in both RNA quality and quantity between samples, the expression levels of interest transcripts were normalized to the housekeeping $\beta 2$ -microglobulin gene transcripts. The following primers and probes (Eurogentec, Angers, France) were used: VEGF189-antisense, 5'-CCACAGGGAACGCTTAGGAC-3'; VEGF165-antisense, 5'-GCTTCTCCGCTCTGAGCA-3'; VEGF sense 5'-AGCAAGACAAGAAAAAATCAGTTCGAGGAAA-3'.

Transcript quantification of VEGF receptors, VEGF-R1, VEGF-R2, and NRP-1 was performed as previously described³⁴ using the TATA box-binding protein (TBP) as the endogenous RNA control, and each sample was normalized on the basis of its TBP content. Results are expressed as N-fold differences in target gene expression relative to TBP gene expression.

Preparation of Conditioned Media and Cell Extracts

Cells (5×10^5) were plated and grown in DMEM in Petri dishes until subconfluence was reached. Medium was changed, and cells were incubated in DMEM medium supplemented with heparin (50 μ g/ml; Sigma, Steinheim, Germany) for 24 hours to release cell-associated VEGF. Conditioned media (CM) were collected and concentrated using Centrprep (Amicon, Bedford, MA). Total cell extracts were obtained after cell lysis. Protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL). The concentration of cell culture medium was performed before Western blot and analysis of biological activity (endothelial cell proliferation assay).

VEGF Immunoassay

VEGF concentrations were measured in cell culture supernatant from heparin-treated cells using a standard enzyme-linked immunosorbent assay (ELISA) protocol (Quantikine human VEGF; R&D Systems, Minneapolis, MN). All data were normalized to cell protein content obtained after cell lysis. Results were expressed as ng VEGF/mg cell protein. Each sample was measured in duplicate.

Western Blot Analysis

Protein samples (40 μ g from CM) in Laemmli buffer were loaded onto a 12.5% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis under reducing conditions and then transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). VEGF was revealed after incubation with rabbit anti-human VEGF-A antibody (1:200; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) at room temperature for 1 hour, followed by a 1-hour incubation with horseradish peroxidase-conjugated mouse anti-rabbit antibody (1:10,000; DAKO, Glostrup, Denmark). Visualization was performed with ECL reagent (Amersham Biosciences, Orsay, France).¹⁹

Endothelial Cell Proliferation Assay

HUVECs were seeded into gelatin-coated 96-well plates (5×10^3 /well) and maintained in M199 containing 1% FBS for 24 hours. VEGF-induced proliferation was measured by adding CM from heparin-treated clones to HUVECs for 72 hours. Cell proliferation was measured using the XTT assay (Roche Diagnostics), as previously described.¹² Results were normalized to data obtained with HUVECs incubated with CM from control-vector transfected cells. Recombinant human VEGF165 (R&D Systems) and VEGF189⁷ were used as controls. The experiments were performed three times in triplicate.

Determination of MDA-MB-231 Cell Growth

Cell Counting

Cells were plated (10^5 cells into 6-cm Petri dishes) in DMEM containing 10% FBS. Cell counts were performed every 24 hours in duplicate. The doubling time of the various clones was calculated using the formula $T_c = 0.37/\log A/A_0$, where A is the cell number at time T of proliferation, and A_0 is the cell number at an initial time point T_c .³⁵ Each clone was tested in triplicate in three different experiments.

XTT Assay

Cells were plated into 96-well plates (5×10^3 /well) in DMEM without serum at 37°C, 5% CO₂. Metabolic activity was analyzed using the XTT assay for 72 hours. In some experiments, cells were incubated with anti-human VEGF-A neutralizing antibody at 10 μ g/ml (R&D Systems).

Cell Adhesion Assay

Ninety-six-well plates were coated for 2 hours at 37°C with vitronectin [10 μ g/ml in phosphate-buffered saline (PBS)] or fibronectin (30 μ g/ml in PBS) (Sigma-Aldrich). Negative controls consisted of 2% BSA-coated wells. Cell adhesion assay was performed as described.³⁶ Cells (30,000 cells/well) were allowed to adhere to the substrate for 1 hour at 37°C. Unattached cells were removed by gently washing with PBS and 1% BSA, then adherent cells were rinsed, fixed with paraformaldehyde (4%), and quantified using crystal violet staining (0.1%). The optical density was measured at 540 nm. To determine the role of the integrin in cell adhesion to fibronectin or vitronectin, the cells were pretreated with anti- $\alpha_5\beta_1$ (MAB1969, 10 μ g/ml), anti- $\alpha_v\beta_3$ (VMA1976, 10 μ g/ml), anti- $\alpha_v\beta_5$ antibody (VMA1961, 10 μ g/ml), or a control IgG for 30 minutes at 4°C before adhesion to fibronectin- or vitronectin-coated dishes.

Wound Healing Assay

Confluent cell cultures were grown on 24-well plates in DMEM-10% FBS. Scratches were made with a tip of a

micropipette, and cells were maintained in serum-free DMEM for 48 hours. Chemokinesis analysis was performed as previously described¹² by counting the number of cells that had moved across the starting line under a microscope (DMRB; Leica Microsystems, Rueil-Malmaison, France). Three fields were analyzed for each well at $\times 5$ amplification, and each experiment was performed three times in triplicate.

In Vivo Tumorigenesis

All procedures involving animals were conducted in accordance with the care and use guidelines of the Central Institute for Experimental Animals. Subconfluent cells (5×10^6 cells/200 μ l PBS) were injected subcutaneously in the flank of 6-week-old female SCID mice (Charles River, Wilmington, UK). After a palpable tumor was observed, tumor volume (mm^3) was measured with a caliper every 2 days according to the formula: volume = $(a^2 \times b) \times \pi/6$,³⁷ where a and b were the smallest and largest tumor diameters, respectively. The latency period is defined as the time required observing tumors larger than 100 mm^3 . At termination of the study, all tumors were fixed overnight in 4% paraformaldehyde. Tissue was paraffin-embedded and subsequently sliced into 5- μ m sections according to the standard protocol for histological (H&E) and immunohistochemical analysis.

Immunohistochemistry

VEGF and Isolectin Staining

Five- μ m-thick sections were deparaffinized in xylene substitute and rehydrated in PBS. Demasking of antigen was performed with citrate buffer (pH 6.5) for 20 minutes at 99°C, followed by the block of endogenous peroxidase activity (3% hydrogen peroxide for 10 minutes). Immunostaining for VEGF was performed as previously described.³⁸ Sections were incubated with blocking serum in PBS containing 5% BSA, followed by incubation with rabbit anti-human VEGF-A antibody (1:200 dilution in PBS/0.01% Triton X-100; Santa Cruz Biotechnologies) for 1 hour, followed by incubation with a biotinylated goat secondary anti-rabbit antibody (1:200 dilution; Vector Laboratories, Burlingame, CA). For visualization of endothelial cells, sections were incubated with GSL-1 isolectin B4 (1:50 dilution, Vector Laboratories) for 1 hour followed by incubation with goat antibody to GSL-1 isolectin B4 (diluted in 1:400, Vector Laboratories) for 30 minutes, and with biotinylated rabbit anti-goat immunoglobulins (diluted in 1:400, DAKO) for 20 minutes. Sections were finally incubated with streptavidin-biotin-peroxidase complex (LSAB+ kit, DAKO) for 10 minutes, stained in 3,3'-diaminobenzidine tetrahydrochloride, and counterstained with Mayer hematoxylin (Sigma).

Measurement of Microvessel Density and Vascular Area

The quantification of angiogenesis was performed by measuring microvessel density and area on sections of

the whole tumor; three sections were analyzed in five distinct visual fields (magnification $\times 40$), using a point-counting grid. The most representative tumor zones were studied in each case. Results of vessel density were expressed as mean ($\pm$ SD) of vessel number per mm^2 . The intratumor vessel area was expressed as the ratio of determined counts to total points of grid according to the method of Weibel and colleagues,³⁹ using the following equation $S = Pv/(Pt \times n) \times 100$, where Pv = number of grid points over endothelial cells or lumen of microvessels, Pt = number of points in a grid (265 points corresponding to 1.06 mm^2), and n = number of fields counted.

Surface Plasmon Resonance Spectroscopy

Analyses were performed at 25°C with the BIAcore 2000 system (BIAcore AB, Uppsala, Sweden) using HBS-EP buffer [10 mmol/L HEPES, pH 7.4, containing 150 mmol/L NaCl, 3 mmol/L ethylenediaminetetraacetic acid, and 0.005% (v/v) Surfactant P20]. Recombinant NRP-1 in 10 mmol/L sodium acetate buffer (pH 5.0) was covalently coupled to a CM5 chip using an amine coupling kit (BIAcore) according to the manufacturer's instructions. Regeneration of the chip surface was achieved by running 5 μ l of HCl, 100 mmol/L, and NaOH, 100 mmol/L, through the flow cell at 30 μ l/minute. VEGF165 or VEGF189 solutions were perfused over the immobilized NRP-1 at a flow rate of 20 μ l/minute, and the resonance changes were recorded. The sensorgram of the immobilized NRP-1 surface was subtracted by that of the control surface, and the data thus obtained were analyzed by nonlinear curve fitting of the Langmuir binding isotherm with BIAevaluation software (BIAcore).

Statistical Analysis

One-way analysis of variance analysis with Tukey's multiple comparison posttest was used to test differences between three or more groups. Significance is presented as $*P < 0.05$. The terms $n = x$ is used to indicate the number of independent experiments (x) performed.

Results

Generation of MDA-MB-231 Cells Stably Expressing VEGF189 or VEGF165 Protein

To compare the impact of VEGF189 and VEGF165 overexpression on mammary tumor progression, we generated MDA-MB-231 cells stably transfected with VEGF165 or VEGF189 cDNA (referred as V165 and V189, respectively). MDA-MB-231 cells stably transfected with control vector were referred as control vector clones (cV). We analyzed by quantitative real-time PCR the expression of transcripts using oligonucleotide primers of isoform-specific sequences. Twenty-four MDA-MB-231 clones expressing VEGF165, twenty-nine MDA-MB-231 clones expressing VEGF189, and six clones expressing endogenous VEGF, only, were analyzed. Four clones of

Table 1. Characterization of VEGF165- and VEGF189-Overexpressing MDA-MB-231 Cells

	Clone number	VEGF165	VEGF189	Western blot
Control vector	12	10	3	+/-
	14	11	6	+/-
MDA-V189	9	20	231	+
	13	9	121	++
	25	19	123	++
	30	8	81	+
	32	9	8	+
MDA-V165	1	178	0.15	+
	18	1191	0.16	ND
	35	1048	0.12	ND
	42	2211	0.15	++
	54	2013	0.18	ND

Expression of VEGF mRNA and protein was assessed by real-time PCR and Western blot analysis in VEGF165-transfected cells (MDA-V165), VEGF189-transfected cells (MDA-V189), and vector-transfected cells (control vector). Copy quantification numbers of VEGF transcripts, described in Materials and Methods, are presented as number of copies of target gene per 10^3 copies of $\beta 2$ -microglobulin. Western blot analysis was performed as described in Figure 1. Results are those of representative clones. The clones (V165-42, V165-54, and V189-13, V189-25) with the highest levels of VEGF mRNA and protein (++) were chosen for further experiments. +/-, + indicate very low level or low levels of VEGF detection; ND, not determined.

V165 and four clones of V189 expressed VEGF transcripts at higher levels than each specific isoform in parental cells or control clones (Table 1). Among the VEGF-expressing cell clones, clones V189-13 and V189-25, V165-42 and V165-54, were selected according to VEGF189 or VEGF165 expression at mRNA (Table 1) and protein levels (Figure 1). Control vector-transfected cells,

Figure 1. Analysis of VEGF expression in selected clones. MDA-MB-231 clones, transfected with vectors expressing various VEGF isoforms, were treated with heparin (50 μ g/ml) for 24 hours. Proteins from the conditioned medium were analyzed using Western blot analysis (A) and ELISA immunoassay (B). A: Proteins (40 μ g from CM) were analyzed by immunoblotting using anti-human VEGF-A antibody. rVEGF165 and rVEGF189 (25 ng) were used as controls and ran at 24 kDa or 28 kDa, respectively. Five experiments were performed with similar results. B: VEGF proteins from the conditioned media (triplicate samples) were measured using an ELISA kit. Results are expressed as fold induction of the VEGF secretion in selected clones versus parental cells MDA-MB-231. Values represent means $\pm$ SD of a representative experiment of five experiments performed in triplicate.

Figure 2. Endothelial cell proliferation by VEGF189 and VEGF165 produced by selected MDA-MB-231 cells. HUVECs (5×10^3 /well) were incubated with the conditioned medium of selected V165 and V189 clones, as described in Materials and Methods; the proliferation assay was performed using the XTT assay. Results are expressed as fold induction of the endothelial cell proliferation of selected clones versus control clones ($\pm$ SD). Positive controls are rVEGF165 and rVEGF189 proteins (10 ng/ml). This figure shows a representative result of three independent experiments performed in triplicate.

cV-12 and cV-14, were used as controls. The presence of cell-associated VEGF189 was assayed in the culture supernatants, after the addition of heparin (50 μ g/ml) to the culture dishes of the V189-transfected cells (see Materials and Methods). Using Western blotting (Figure 1A), the expression of a VEGF 24-kDa isoform was detected in clone V165, and of a VEGF 28-kDa isoform was detected in clone V189, whereas low levels of VEGF were detected in control clones or in MDA-MB-231 parental cells. Heparin treatment resulted in a significant increase of VEGF secretion into the conditioned medium by VEGF165- and V189-overexpressing clones (30 to 60 ng/mg protein, using ELISA assay, corresponding to 3.5- to 2.5-fold induction), as compared to parental cells (Figure 1B).

The biological activity of secreted VEGF has been analyzed using an endothelial cell proliferation assay (Figure 2). Human recombinant purified VEGF189 or VEGF165 increased HUVEC proliferation, as previously described.¹² As shown in Figure 2, the conditioned medium of VEGF-overexpressing clones (V165 and V189) increased the proliferation of HUVECs (1.5- to 2-fold), as compared to that of control vector clones. These results suggest that this endothelial effect is probably attributable to VEGF secreted by V165 and V189 clones.

VEGF189 Overexpression Promotes *In Vivo* Primary Tumor Growth

The effects of overexpression of VEGF189 or VEGF165 on tumor growth were investigated by using *in vivo* subcutaneous transplantation into mice of V189 or V165 clones parallel to cV cells. Preliminary experiments performed in athymic nude mice with different V189 clones (V189-13, V189-25, and V189-32) showed that mice that received V189 clones developed tumors (50%) more rapidly than mice that received control cells (data not shown). To optimize tumor uptake, we then transplanted V165 and V189 transfectants subcutaneously into SCID

Figure 3. Growth curves of MDA-overexpressing VEGF189 and VEGF165 tumors induced in SCID mice. Cells (5×10^6) were subcutaneously injected in SCID mice as described in Materials and Methods. **A:** Tumors were generated from V189-13 or V165-42 clone, or cV cells. Tumor diameters were measured every 2 days. Results are expressed as the mean of tumor volumes $\pm$ SEM. Data represent two different experiments ($n = 9$). **Single asterisk** indicates statistical significance ($P < 0.05$) when comparing tumor growth of V189-13 with tumor control. **Double asterisks** indicate statistical significance when comparing tumor growth of V189-13 with V165-42. **B:** Tumors were generated from V189-13, V189-25, or V165-42, V165-54 clones, or cV cells. Data represent an experiment using four animals in each group (mean tumor value $\pm$ SEM). **A** and **B** are performed using different groups of animals. Differences in tumor uptake between V165 and V189 clones were observed in **A** and **B** (5 and 3 days, respectively).

mice. In this case, 100% of SCID mice developed tumors with VEGF transfectants. As shown in Figure 3A, VEGF189 transfectant showed significantly enhanced growth rate, as compared to vector transfectants ($n = 9$, $P < 0.05$), and reached a volume of 800 mm³ in ~26 days. In addition, V165 clones showed the most rapid tumor uptake, as compared to V189 clones (Figure 3A). This result was observed with all selected clones (V189-13 and V189-25, as compared to V165-42 and V165-54) in several independent experiments (Figure 3, A and B), indicating that VEGF189 and VEGF165 induce different tumor uptake but similar tumor growth.

Overexpression of VEGF189 Enhances MDA-MB-231 Tumor Vascularization

Because we showed that VEGF189 affects angiogenesis *in vitro*,¹² we decided to investigate the ability of selected V189 clones to modulate *in vivo* angiogenesis. Hematoxylin and eosin (H&E)-stained sections of the tumors revealed the presence of tumor cells and numerous vessels in both V189 and V165 tumors (Figure 4, top). These tumors strongly express VEGF protein (Figure 4, middle). Immunostaining of sections with GSL1 isolectin B4, a

specific marker for endothelial cells, revealed that VEGF-overexpressing tumors were highly vascularized, as compared to control tumors (Figure 4, bottom). Morphometric analysis showed that vascular density was significantly increased in both cases (15.1 ± 2.1 and 13.1 ± 3.7 vessel number per mm² for V189-13 and V165-42, respectively), as compared to control tumors (5.5 ± 1.8 for control vector). VEGF189 overexpression led to a great number of large size vessels as compared to V165 tumors and deduced from analysis of vessel area [Figure 4, A (bottom) and B] ($P < 0.05$). Interestingly, some vessels also showed numerous shape irregularities, within the same and different areas, suggesting a heterogeneous vascular network.

VEGF189 Enhances MDA-MB-231 Cell Proliferation and Chemokinesis

Recently, *in vivo* and *in vitro* studies have suggested that VEGF165 has autocrine functions in breast cancer cells, especially in cell proliferation, survival, and migration.^{22,40,41} We then analyzed the possible autocrine effects of VEGF189 on tumor cell behavior, such as proliferation, chemokinesis, and adhesion of VEGF-expressing clones.

The growth characteristics of selected V189 and V165 clones was first examined *in vitro* by cell counting. The doubling time of V189-13 and V165-42 clones was similar (25.4 ± 1.3 and 22.7 ± 1 hours, respectively); however, these clones grew significantly faster ($P < 0.05$) than control vector transfectants or parental cells (doubling time 30.6 ± 3 and 32.6 ± 2.2 hours, respectively) (not shown). As illustrated in Figure 5A, the overexpression of VEGF189 significantly increased the cell number using the XTT test (1.7-fold induction, $P < 0.05$), as compared to control cells. This effect, which was also observed with VEGF165, was significantly inhibited in the presence of anti-VEGF antibodies (Figure 5A), showing the specificity of this proliferative effect. Using the wound healing assay (Figure 5B), we showed a significant increase in the chemokinesis for V165 and V189 clones (2- and 2.3-fold induction, respectively; $P < 0.05$), compared to control clones. Altogether, these results indicate a direct link between VEGF overexpression and the increase of human mammary cell line proliferation and chemokinesis.

VEGF189 Overexpression Is Associated with an Increase of Cell Adhesion

We then compared the adhesive capacity of V189, V165, and cV clones using different extracellular matrix proteins. As shown in Figure 6A, cell adhesion on fibronectin and vitronectin was significantly increased for V189 clones (1.9- to 1.6-fold induction, respectively; $P < 0.05$), in comparison with cV clones ($P < 0.05$). Cell adhesion of V189 clones on collagen IV was also increased (not shown). In contrast, cell adhesion of V165 clones was not significantly increased (Figure 6A).

Figure 4. Representative illustrations of tumor sections labeled with anti-VEGF and a marker of endothelial cells. Tumors were developed in SCID mice after injection of clones as described in Figure 3. The tumor vascularization was analyzed when all tumors reached 800 mm³. **A:** Histology of tumors (V189-13, V165-42, and cV) by H&E staining (**top**). Immunohistochemistry revealed high expression of VEGF in V165-42 and V189-13 tumors (**middle**). Immunostaining using a GSL-1 isolectin B4 antibody identified the endothelial cells (**bottom**). **Arrows** indicate blood vessels. **B:** Microvascular area quantification performed on tumor sections after labeling of vessels with an antibody against isolectin B4. Microvascular areas ($\pm$ SD) represents the fraction of the total tissue occupied by the wall or/and the lumen using computer-assisted image analysis, as described in Materials and Methods.³⁹ A significant increase ($*P < 0.05$ versus control, and $*P < 0.05$ versus V165-42) of the microvascular area was observed in the V189-13 clone. Scale bars = 10 μ m.

We then analyzed whether increased adhesion of V189 clones could involve an integrin-mediated pathway. Integrins are a large family of adhesion molecules, primarily responsible for the attachment to extracellular matrix components. It has been proposed that matrix-bound VEGF promotes endothelial cell adhesion through an integrin-dependent pathway.⁴² Using RT-PCR analysis, fluorescence-activated cell sorting analysis, or immunofluorescence, we first determined the expression of integrins α_2 , α_5 , β_1 , $\alpha_v\beta_3$, and $\alpha_v\beta_5$ in VEGF-overexpressing MDA-MB-231 clones (data not shown).⁴³ We observed that overexpression of VEGF189 did not affect the expression pattern of $\alpha_5\beta_1$ and α_v integrins (unpublished experiments).³⁰ To identify the integrin receptors contributing to the attachment of V189 clones to the different extracellular matrix (fibronectin and vitronectin), adhesion assays were performed in the presence of neutralizing antibodies. As shown in Figure 6B, neutralizing antibodies against $\alpha_5\beta_1$ significantly inhibited the adhesion of VEGF189-overexpressing clones to fibronectin ($P < 0.01$). In contrast, the adhesion to fibronectin was not significantly reduced in the presence of neutralizing antibodies to $\alpha_v\beta_5$, $\alpha_v\beta_3$, as well as β_1 alone (not shown). Furthermore, the adhesion of V189 to vitronectin was significantly reduced by anti- $\alpha_v\beta_5$ ($P < 0.05$) but not by anti- $\alpha_v\beta_3$ antibodies (Figure 6C). Thus, the overexpression of VEGF189 increased tumor adhesion to fibronectin and vitronectin and this adhesion is mediated through $\alpha_5\beta_1$ and $\alpha_v\beta_5$, respectively.

VEGF189 Binds to NRP-1

In a preliminary attempt to characterize further the mechanisms of autocrine VEGF189 function, we analyzed the presence of VEGF receptors in the tumor cells and the binding of VEGF189 to NRP-1 *in vitro*. First, *in vitro* experiments using heparin treatment indicate that VEGF189 is associated to the cell-surface of V189 clones (see Materials and Methods). The expression of VEGF receptors in V189 or V165 clones was analyzed using quantitative RT-PCR and Western blot analysis. NRP-1 was strongly expressed in all MDA-MB-231 clones similar to HUVECs (Figure 7A). In contrast, VEGF-R1 and VEGF-R2 were expressed at low level in these cells and in parental cells as previously described.^{40,41} Furthermore, we observed that the overexpression of VEGF did not change significantly the level of NRP-1 expression.

Because the binding of VEGF165 to NRP-1 has been described and shown to be involved in tumor cell behavior,^{10,41} we tested if VEGF189, which possesses the same NRP-1 binding sequence, could also bind NRP-1. The binding assay of VEGF189 to NRP-1 was performed using a BIAcore sensor approach, as described in Materials and Methods. Interestingly, as shown in Figure 7B, VEGF189 bound to NRP-1-conjugated sensor chip in a dose-dependent manner in a direct binding assay. VEGF165 also bound to an NRP-1-conjugated sensor chip with fast kinetics. In contrast, VEGF121 did not bind to NRP-1 sensor chip, as previously described.⁴⁴

Figure 5. *In vitro* behavior of VEGF-overexpressing breast cancer MDA-MB-231 cells. **A:** Cell proliferation assay. Cells (5×10^3 /well) were grown in DMEM for 72 hours and proliferation was measured using the XTT assay. Results were normalized to control vector-transfected cells ($\pm$ SEM), $*P < 0.05$. In some experiments, anti-VEGF antibody ($10 \mu\text{g/ml}$) was added. $**P < 0.05$ statistical difference observed in the presence or absence of anti-VEGF antibody. **B:** Chemokinesis assay, as described in Materials and Methods. Results were normalized to control vector-transfected cells (fold induction $\pm$ SEM). Three independent experiments were performed.

Discussion

The existence of multiple isoforms exhibiting different binding affinities toward heparin sulfate and different extracellular localization raised the possibility that individual isoforms may have different functions on different aspects of tumor growth. VEGF189 is more strongly associated with the cell membrane than other isoforms of human VEGF (VEGF121, VEGF165). The role of this isoform in breast cancer has remained elusive. We reported earlier that VEGF189 may improve proliferation and migration of endothelial cells.¹² In this study, we investigated the potential role of VEGF189 in breast tumor growth using tumor cells that overexpress this isoform. Our findings demonstrate for the first time that VEGF189-overexpressing MDA-MB-231 cells (V189 clones) induce the development of highly vascularized breast tumors when injected subcutaneously into nude or SCID mice and promote autocrine stimulation of tumor cells (proliferation, chemokinesis, and adhesion). Remarkably, we observed differences in tumor uptake and angiogenesis of V189 clones *in vivo*, as well as differences in the ca-

Figure 6. Cell adhesion of VEGF-overexpressing breast cancer MDA-MB-231 cells. **A:** The cell suspension (3×10^4 cells) was incubated for 2 hours at 37°C on 96-well plates coated with fibronectin ($30 \mu\text{g/ml}$) or vitronectin ($10 \mu\text{g/ml}$). Fixed adherent cells were quantified by crystal violet staining. All experiments ($n = 10$) were run in triplicate, $*P < 0.05$. Data are represented as fold induction between VEGF-overexpressing clones versus control vector clones ($\pm$ SEM). **B:** Cells were preincubated with anti- $\alpha_5\beta_1$ antibody ($10 \mu\text{g/ml}$) or a control IgG ($10 \mu\text{g/ml}$) for 30 minutes before adhesion to fibronectin. Data are represented by means $\pm$ SEM ($n = 3$). **C:** Cells were preincubated with anti- $\alpha_v\beta_3$ antibody ($10 \mu\text{g/ml}$), anti- $\alpha_v\beta_5$ antibody ($10 \mu\text{g/ml}$), or a control IgG, for 30 minutes before adhesion to vitronectin. Data are represented by means $\pm$ SEM ($n = 3$). $*V189$ clones versus control vector, $P < 0.05$; $**V189$ clones with or without $\alpha_5\beta_1$ (**B**) or $\alpha_v\beta_5$ (**C**) antibodies, $P < 0.05$.

Figure 7. Analysis of VEGF receptor expression and VEGF189 binding. **A:** Quantitative RT-PCR analysis of VEGF-R1, VEGF-R2, and NRP-1 transcripts expressed in the VEGF-overexpressing clones, V165-42 and V189-13. Copy numbers were calculated from standard curves, and data were normalized to TBP gene expression. HUVECs were used as controls. **B:** Binding of VEGF189, VEGF165, and VEGF121 to NRP-1 BIAcore-conjugated sensor chip was analyzed as described in Materials and Methods.

capacity of tumor cell adhesion *in vitro*, as compared to V165 clones.

VEGF189 Increases the Tumor Growth of MDA-MB-231 Cells *In Vivo*

This study provides the first evidence that overexpression of VEGF189 isoform by human breast carcinoma cells confers a growth advantage *in vivo*. The overexpression of VEGF165 in MDA-MB-231 cells significantly increases mammary tumor growth, as previously described in MCF-7 cells⁴⁵ or in a transgenic mouse model.²² However, we observed a difference in the tumor uptake between the two VEGF-overexpressing clones, the latency period being increased in V189 clones, compared to V165 clones. These results suggest an isoform-dependent tumor implantation, in

agreement with previous data obtained in VEGF189 overexpressing colon carcinoma.³⁰ The reason for the difference in V189 tumor uptake is unknown but suggests a possible association with biochemical properties of VEGF189, ie, a cell surface- and extracellular matrix-anchored protein.

VEGF189 Induces Vascularization of the Tumor Xenografts

Our results show that overexpression of VEGF189 in MDA-MB-231 cells significantly increases tumor angiogenesis through an increase of vascular density, as observed for VEGF165. These data extend previous *in vitro* and *in vivo* studies showing that recombinant VEGF189 increases vascular permeability.¹⁹ An increase of stromal blood vessels in xenografts of VEGF189 transfectants was also observed with the colon cancer cell line SW-480³⁰ and with fibrosarcomas.³¹ These results suggest that VEGF189 produced by carcinoma cells can stimulate angiogenesis by acting in a paracrine manner on vicinal endothelium. Interestingly, an increased vascular area associated with large and dilated vessels was observed coincidentally with VEGF189 overexpression in breast tumors, suggesting an increased blood flow in the tumors. This vasodilation effect was more pronounced in V189 than in V165 tumors, a finding that was previously observed in another angiogenic model (Matrigel).¹² Hypervascularization and vasodilation in V189 breast tumors might arise from the biodistribution of the VEGF189 protein, ie, its ability to be sequestered locally by binding to the cell surface or extracellular matrix, leading to high local VEGF concentrations that may be required for the maintenance of vascular beds.^{15,19,31} The cleavage of this isoform^{5,7,8} may also influence the rate of growth factor release and the formation of a concentration gradient, which might provide different effects on vascular cells. Recent findings indicate that a specific cohort of MMP can cleave VEGF189⁸ and directly process the release of shorter fragments that generate enlarged vessels. Enhanced tumor angiogenesis with abnormally large vascular lumina was also described using overexpression of NRP-1⁴⁶ and MT-4MMP.⁴⁷ Currently, the mechanisms that regulate this fundamental aspect of vascular development are still unclear.^{48,49} Electron microscopic and biochemical studies are in progress to analyze the vascular differences between V189 and V165 tumors.

VEGF Overexpression Modifies Breast Carcinoma Cell Behavior

Our *in vitro* data indicate that the anchored VEGF189 isoform modulates autocrine function, in addition to promoting angiogenesis. The overexpression of VEGF189 increases the proliferation, chemotaxis, and adhesion of breast cancer cells. VEGF189-overexpressing cells are more chemokinetic than parental cells, as deduced from wound-healing experiments. We used this assay, rather

than the classical Boyden chamber assay performed with a VEGF chemotaxis gradient, because our cells secrete high amounts of VEGF. Earlier findings suggest that VEGF165 can sustain proliferation, survival, and migration of breast carcinoma independently of angiogenesis, by stimulating autocrine signaling in these cells.^{22,40,50,51} However, the mechanisms are not well understood. In contrast to endothelial cells, VEGF-R1 and VEGF-R2, are expressed at very low levels in MDA-MB-231 cells (this study).¹⁰ NRP-1 is widely expressed in tumor cells.^{10,50} Several studies have revealed that NRP-1 can function as a key receptor for the VEGF165-induced autocrine effects in different cancer cells, including MDA-MB-231 breast cancer cells,^{40,52,53} consistent with the positive role of NRP-1 in tumorigenesis.⁵⁴ Our findings using V165 and VEGF189 clones suggest an implication of NRP-1 in the proliferation and chemotaxis signaling by acting as a receptor for both isoforms.

The new finding that VEGF189 overexpression increases the proliferation and chemotaxis of breast cancer cells suggests that VEGF189 also modulates autocrine cell function. The mechanisms remain to be analyzed. However, we demonstrate for the first time that VEGF189 binds to NRP-1, using surface plasmon resonance experiment (BIAcore biosensor assay). Preliminary experiments performed in different experimental conditions (0.2 to 10 nmol/L of VEGF) suggest a higher affinity of VEGF189 toward NRP-1, when compared to VEGF165. The binding of the VEGF165 isoform, but not of the VEGF121 isoform, to NRP-1 was also observed in this study, in agreement with previous data.^{10,44,55,56} These findings are consistent with the presence in VEGF189 and VEGF165 of the basic sequence encoded by exon 7, which interacts with NRP-1.¹⁰ Although preliminary, our findings using V189 clones support the idea that VEGF189 could modulate some of the autocrine functions of breast carcinoma cells through NRP-1. Experiments are in progress to inhibit NRP-1 expression in our clones, using a Sh RNA approach. Preliminary results also indicate the implication of NRP-1 in the MAP kinase signaling of VEGF189. Further studies are in progress to determine the mechanisms of autocrine VEGF189 function.

VEGF 189 Increases the Adhesion of Breast Cancer Cells

Our data indicate for the first time an increase of the adhesion of VEGF189-overexpressing MDA-MB-231 cells to extracellular matrix (fibronectin, vitronectin), as compared to V165 and cV clones. Extracellular matrix-bound VEGF has been previously described to promote adhesion of endothelial cells with a higher efficiency of VEGF189 as compared to VEGF165, through an integrin-dependent (β_1 and $\alpha_v\beta_3$) pathway.⁴² MDA-MB-231 cells and VEGF-overexpressing clones exhibit relatively high levels of integrins $\alpha_2\beta_1$, $\alpha_5\beta_1$, as well as $\alpha_v\beta_5$ and lesser amount of $\alpha_v\beta_3$ (two integrins for vitronectin) (unpublished data).^{37,43} Our experiments with integrin-blocking antibodies demonstrate that the integrins $\alpha_5\beta_1$ and $\alpha_v\beta_5$ are involved in VEGF189-induced adhesion to fibronectin

and vitronectin, respectively. The vitronectin receptor $\alpha_v\beta_3$ does not appear to influence the adhesion of VEGF-overexpressing MDA-MB-231 on vitronectin, in contrast to endothelial cells. Because overexpression of VEGF189 did not affect the expression pattern of $\alpha_5\beta_1$ and α_v , the increased adhesive activity of VEGF189 to fibronectin and vitronectin could be associated with activation of the key integrins and with their reorganization at the cell surface.⁵⁷

In conclusion, our work provides new insights into the role of VEGF189 in breast cancer. VEGF189 seems to have a different action *in vivo*, as compared to VEGF165, concerning the latency period in tumor growth and vascular pattern. In addition, we showed that VEGF189 and VEGF165 increase the proliferation and chemokinesis of mammary tumoral cells. We noticed that VEGF189 overexpression increases cell adhesion through specific integrins. Further experiments are in progress to analyze the role of VEGF189 in breast cancer cell survival. Recent findings have raised the possibility that blocking NRP-1 in tumors can increase the anti-VEGF effects, and that NRP-1 is required for vascular remodeling.^{46,54} The binding of VEGF189 to this receptor further supports the investigations to elucidate its specific contribution in tumor development. The relationships between the expression of cell-bound isoform VEGF189 and the clinicopathological features in human breast tumors remain to be established.

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