Expression of Vascular Endothelial Growth Factor Induces an Invasive Phenotype in Human Squamous Cell Carcinomas

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Inhibition of the vascular endothelial growth factor (VEGF) receptor Flk-1 has been shown to prevent invasion of experimental squamous cell carcinomas (SCC). To directly investigate the role of VEGF in tumor invasion, we stably transfected human SCC-13 cells, which are characterized by a noninvasive phenotype *in vivo*, with expression vectors containing murine VEGF₁₆₄ in sense (SCC/VEGF+) or antisense (SCC/VEGF-) orientation or with vector alone (SCC/vec). SCC/vec cells formed slowly growing, well-differentiated tumors with well-defined borders between tumor and stroma, after intradermal or subcutaneous injection. In contrast, SCC/VEGF+ tumors were characterized by rapid tumor growth, with small cell groups and single cells invading into the surrounding tissue, and by admixture of blood vessels and tumor cells in areas of tumor invasion. We detected an increase in tumor vessel density and size in VEGF-overexpressing tumors, resulting in a more than fourfold increase in total vascular areas. In contrast, SCC/VEGF- clones formed noninvasive, sharply circumscribed tumors with reduced vascular density. These findings demonstrate that selective VEGF overexpression was sufficient to induce tumor invasiveness, and they provide further evidence for an active role of the tumor stroma in cancer progression. (*Am J Pathol* 2000, 156:159-167)

The formation of new blood vessels is generally thought to be essential for tumors to grow beyond minimal size,^{1,2} providing oxygenation and nutrient perfusion as well as removal of waste products. Squamous cell carcinoma (SCC) of the skin, a malignant tumor of epidermal keratinocytes with destructive growth pattern and the ability to metastasize,³ is characterized by a richly vascularized stroma and has been shown to strongly express the

angiogenic factor vascular endothelial growth factor (VEGF), also known as vascular permeability factor.^{4,5} VEGF is a homodimeric, heparin-binding glycoprotein occurring in at least four isoforms of 121, 165, 189, and 201 amino acids, due to alternative splicing.^{6,7} VEGF binds to two type III tyrosine kinase receptors on vascular endothelial cells, Flt-1 and KDR/Flk-1.⁸⁻¹⁰ *In vivo*, VEGF enhances microvascular permeability¹¹ and angiogenesis,¹²⁻¹⁵ and VEGF is regarded as a major tumor angiogenesis factor.^{16,17} Blocking of VEGF function inhibited angiogenesis and suppressed tumor growth *in vivo*,¹⁸⁻²² and recently it was reported that antibody inhibition of the VEGF receptor Flk-1 prevented carcinoma cell invasion of malignant human keratinocyte xenotransplants.²³ However, the question remained unanswered whether VEGF expression in itself is sufficient to induce tumor invasion.²⁴

To address this question, we developed an *in vivo* xenotransplantation model for the intradermal tumor growth of human SCCs. We chose the human SCC-13 line²⁵ for our studies because of the relatively low endogenous VEGF expression and the high degree of differentiation and circumscribed growth pattern with lack of tumor invasiveness. SCC-13 cells were stably transfected with expression vectors containing murine VEGF (mVEGF)₁₆₄ in sense (SCC/VEGF+) or antisense (SCC/VEGF-) orientation or with vector alone (SCC/vec), and these cells were studied for up to 8 weeks after intradermal and subcutaneous xenotransplantation. We report here that selective VEGF overexpression in SCC-13 xenotransplants induced tumor invasion and also potently promoted tumor growth and angiogenesis.

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Materials and Methods

Cell Culture, Transfection, and Selection

The human SCC line SCC-13, kindly provided by Dr. James Rheinwald (Harvard Medical School, Boston, MA), was maintained in complete serum-free keratinocyte growth medium (KGM; Life Technologies Inc., Grand Island, NY). A 980-bp full-length mouse VEGF₁₆₄ complementary DNA (cDNA; GenBank accession number M95200²⁶) was cloned into a pCMV-NEO expression vector, which contains a cytomegalovirus enhancer-promoter and a neomycin/geneticin sulfate (G418) selection cassette composed of the Tn5 gene driven by the simian virus 40 promoter. VEGF sense- and antisense-oriented constructs were analyzed by restriction mapping and by direct sequencing using the Sanger dideoxy method.¹⁴ DNA transfections were performed by calcium phosphate precipitation as described previously,²⁶ using vector alone and sense and antisense VEGF expression constructs. At 48 hours after transfection, cells were split 1:3 into complete keratinocyte growth medium containing 600 μ g/ml G418 to select transfectants. Stably transfected SCC cell clones were expanded, and 10 clones for each construct were analyzed for VEGF messenger RNA (mRNA) expression and protein secretion. Three clones each with high expression of the transfected construct were further expanded and used for the xenotransplantation experiments described below.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was isolated from stable transfectants as described previously.²⁷ Northern blot analyses were performed using BioTrans nylon-supported membranes (ICN, Irvine, CA), according to the manufacturer's instructions. We used a 980-bp mouse VEGF₁₆₄ cDNA probe, isolated as described previously.^{15,26} This cDNA probe recognizes human and mouse VEGF mRNA.¹⁴ Endogenous human VEGF₁₆₅ mRNA yields two bands of 3.7 and 4.2 kb, whereas mVEGF₁₆₄ mRNAs encoded by the transfection vectors (sense and antisense VEGF) yield bands at 1.0–1.6 kb.¹⁴ A cDNA for β -actin (Clontech, Palo Alto, CA) was used as a probe to control for equal RNA loading, blotting, and hybridization. Hybridization probes were isolated cDNA fragments radiolabeled with a random-primed synthesis kit (MultiPrime; Amersham, Arlington Heights, IL). Blots were washed at high stringency (0.1 $\times$ SCC-1% sodium dodecyl sulfate at 65°C) and exposed on Kodak X-OMAT film.

Assays for VEGF Protein in Cell Culture Supernatants

Cultured cells were grown to confluence, the medium was changed, and cells were cultured for an additional 48 hours. Culture supernatants were then collected, cleared by centrifugation, and assayed for mVEGF levels by enzyme-linked immunosorbent assay. We used a commercially available enzyme-linked immunosorbent

assay kit to detect mouse VEGF (R&D Systems, Minneapolis, MN), according to the manufacturer's recommendations. After collection of culture supernatants, cells were trypsinized and counted, using a hemocytometer. Levels of mouse VEGF protein were calculated as nanograms of VEGF/10⁶ cells. In addition, we performed Western blot analyses of secreted mouse and human VEGF. Culture supernatants were applied to a column of heparin-Sepharose CL-6B (Amersham Pharmacia Biotech, Piscataway, NJ) at 4°C, and elution was carried out as previously described.²⁸ Samples were mixed with one-fourth volume of sample buffer (pH 7.5, containing 0.25 mol/L Tris, 5% sodium dodecyl sulfate, 1.25 mol/L sucrose) and heated to 100°C for 1 minute. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 10% acrylamide gels, using molecular weight calibrations as previously described.²⁸ Proteins were electrophoretically transferred to nitrocellulose paper (Bio-Rad, Hercules, CA) at 50 V for 24 hours, and, after several washes, immunolabeling was performed using either mouse anti-human VEGF monoclonal antibody 263 or a goat anti-mouse VEGF polyclonal antibody that showed a higher sensitivity for transfected mVEGF₁₆₄ (both obtained from R&D Systems).

Growth of Stably Transfected SCC-13 Cells in Immunodeficient Mice

Three individual clones of stably transfected SCC-13 cells were used for each construct (vector only, VEGF sense, VEGF antisense). In addition, the parental cell line was studied. Cells (2×10^6) were injected either intradermally or subcutaneously into immunodeficient BALB/c(nu/nu) mice (Taconic Farms, Germantown, NY). Each animal received one such injection in each flank, and each cell clone was injected into at least five mice. Tumor yields were determined as the percentage of persisting tumors of at least 1 mm³ tumor volume after 4 weeks. Tumor sizes were quantitated weekly for 8 weeks by using a digital caliper to measure the largest and smallest tumor diameters, and tumor sizes were calculated using the formula: volume = $\frac{4}{3} \times \pi \times (\frac{1}{2} \times \text{smaller diameter})^2 \times \frac{1}{2} \times \text{larger diameter}$.²⁹ Significances were calculated using the one-sided Student's *t*-test for unpaired samples. Animals were euthanized after 8 weeks, the back skin containing tumors was dissected and photographed, and tumors were harvested and processed for further analyses as described below. All animal experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Histology, *In Situ* Hybridization, and Immunohistochemistry

For routine histology, tumors were fixed in 4% paraformaldehyde-phosphate-buffered saline and processed and embedded in paraffin for hematoxylin and eosin staining as previously described.¹⁴ *In situ* hybridization of paraffin and frozen sections was performed as described

earlier,^{15,27} using pGEM or pBluescript II plasmids containing mouse VEGF, flt-1 (VEGFR-1), or flk-1 (VEGFR-2) cDNA fragments. The flt-1 and flk-1 clones were a kind gift from Clive Wood, Genetics Institute, Cambridge, MA. The flk-1 and flt-1 sequences were isolated by polymerase chain reaction from a mouse fetal thymus cDNA library.³⁰ The murine flk-1 transcription template was a 392-bp fragment encompassing amino acids 1 to 130 (nucleotides 268–660 of the flk-1 sequence described previously³¹), cloned into pGEM-T (Promega, Madison, WI). The sequence for murine flt-1 was obtained by degenerate polymerase chain reaction cloning of kinase domains, which resulted in a 640-bp cDNA fragment encoding the insert region from amino acid 832–1045 of a sequence described previously.³⁰ Transcription reactions were carried out by using a Riboprobe Gemini II kit (Promega) in the presence of [α -³⁵S]-UTP. Antisense (and control sense) probes were evaluated on alternate sections.

For immunohistochemistry, 6- μ m cryostat sections were stained with a rat anti-mouse platelet-endothelial cell adhesion molecule-1 (CD31) monoclonal antibody (Pharmingen, San Diego, CA) or with a rabbit anti-mouse collagen type IV polyclonal antibody (Biodesign International, Kennebunk, ME), using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Rat IgG1 (Pharmingen) was used as isotype-matched negative control in monoclonal antibody staining, and normal rabbit serum was used as control for polyclonal rabbit serum. To determine the degree of tumor-induced angiogenesis, cryostat sections of tumor xenografts were stained with an anti-mouse CD31 monoclonal antibody. Representative sections obtained from five tumors from each cell clone were analyzed, using a Nikon E-600 microscope (Nikon, Melville, NY). Images were captured with a Spot digital camera (Diagnostic Instruments, Sterling Heights, MI), and morphometric analyses were performed using the IP LAB software program (Scanalytics Inc, Fairfax, VA). Three different fields at $\times 60$ magnification were examined on each section, and the number of vessels per square millimeter, the size distribution, and the average and the total areas of tumor blood vessels were determined. The unpaired Student's *t*-test was used to analyze differences in the vessel areas.

Results

VEGF Expression in SCC-13 Cells Transfected with Sense or Antisense VEGF cDNA

SCC-13 cells were chosen for VEGF transfection and subsequent *in vivo* transplantation studies because of their high degree of differentiation and well-circumscribed growth pattern with lack of tumor cell invasion *in vivo*.²⁵ Moreover, our pilot studies had revealed low VEGF expression in SCC-13 cells *in vitro*, as compared with SCC-12, SCC-15, HaCaT, or A431 cells (data not shown). SCC-13 cells were stably transfected with the cDNAs encoding either sense (SCC/VEGF+) or antisense (SCC/VEGF–) orientations of the mouse 164-amino-acid VEGF

Figure 1. Highly increased VEGF expression in SCC-13 cells transfected with mVEGF₁₆₄ constructs. **A:** Northern blot of SCC-13 cell clones stably transfected with VEGF₁₆₄ in sense (S) or antisense (A) orientation or with vector control (C). Endogenous human VEGF was detected as a doublet of 4.2 and 3.7 kb, and transfected mVEGF transcripts were detected as a single 1.9-kb band. **B:** Western blot analysis of culture supernatants, using a goat anti-mouse VEGF polyclonal antibody, demonstrates potent secretion of transfected mVEGF by SCC/VEGF+ clones S13 and S17 but not by SCC/vec clones C11 and C13.

isoform under the control of a constitutive expression vector. As a control, other SCC-13 cells were transfected with vector alone without a VEGF insert (SCC/vec). We characterized VEGF expression in 10 stably transfected clones for each construct and chose three clones with strong expression of transfected mVEGF for further *in vivo* studies. As in the parental SCC-13 population, transfectants expressed low levels of endogenous human VEGF mRNA of 4.2 and 3.7 kb, whereas the vector-directed mVEGF mRNA (both sense and antisense) formed a readily distinguishable band at 1.9 kb (Figure 1A). SCC-13 cells transfected with vector alone (SCC/vec) expressed low levels of endogenous human VEGF mRNA but no detectable mVEGF mRNA. Three clones each with high expression of the transfection constructs were chosen for further analysis of secreted VEGF protein. Whereas no mVEGF was detectable in SCC/vec and SCC/VEGF– cell supernatants, between 18 and 23 ng/10⁶ cells of mouse VEGF were detected in SCC/VEGF+ cell supernatants (data not shown). Additional Western blot analyses of culture supernatants confirmed efficient VEGF secretion in SCC/VEGF+ clones, whereas little or no VEGF was detected in conditioned media harvested from SCC/vec clones (Figure 1B). No VEGF protein was detected in SCC/VEGF– clones.

Figure 2. A: VEGF overexpression (V+) significantly increased the percentage of tumors formed at injection sites after intradermal (I.D.) but not after subcutaneous (S.C.) application, as compared with control transfectants (vec). In contrast, tumor yields were decreased in VEGF-antisense transfectants (V-). Results are displayed as mean values $\pm$ SEM, obtained from three independent clones for each construct that were each injected into 8 to 10 sites. * $P < 0.05$; ** $P < 0.01$. B and C: Enhanced tumor growth of SCC/VEGF+ clones S13 and S17 (B) after intradermal injection and (C) after subcutaneous injection. Results are displayed as mean values of 10 tumors for each VEGF+ clone. A total of 14 tumors each, derived from 3 different clones, were evaluated for SCC/vec after I. D. and after S. C. application. No major variation between the different clones was observed. For SCC/VEGF- cells, five tumors could be evaluated after I.D. injection, and 12 tumors after S.C. injection. For the display of results obtained in SCC/vec and SCC/VEGF- tumors, data from three clones each were pooled.

Tumor Growth and Invasiveness of Transfected SCC-13 Cells in Immunodeficient Mice

Three clones for each construct (SCC/vec, SCC/VEGF+, SCC/VEGF-), as well as the parental cell line, were injected in duplicate intradermally into five mice each. In a separate experiment, three clones each of sense- versus antisense-transfected cells were injected subcutaneously into 4–5 nude mice each. Tumor growth was monitored weekly by measurements of the longest and shortest diameter of tumors for a total of 8 weeks. After intradermal injection, VEGF-overexpressing SCC-13 cells were characterized by significantly increased tumor yields ($P < 0.05$). Of SCC/VEGF+ transplants, 79% developed into tumors, as compared with 50% for SCC/vec clones (Figure 2A). In contrast, tumor yields of SCC/VEGF- cells were significantly reduced ($P < 0.01$; Figure 2A). After subcutaneous injection, however, tumor yields of control-transfected clones were higher (64%) than after intradermal application and were not significantly different from SCC/VEGF+ (80%) or SCC/VEGF- (55%) clones. However, the growth rate of established SCC/VEGF+ tumors was dramatically higher than that of control tumors in both intradermal and subcutaneous locations. After intradermal injection, SCC/VEGF+ cells formed significantly larger tumors (average of 262 mm² after 8 weeks, compared with 4 mm² for controls) than SCC/vec or SCC/VEGF- cells (Figure 2B and Figure 3, a–c). Similar results were found after subcutaneous injection (Figure 2C). Results obtained with the parental cell line were identical to those obtained with SCC/vec clones (data not shown). SCC/vec cells formed well-differentiated SCCs with a clearly demarcated border to the surrounding tumor stroma (Figure 3, d and g) and without detectable tumor invasion. In contrast, SCC/VEGF+ tumors were less well differentiated and were characterized by an irregular infiltrative edge with small cell groups and single cells invading into the surrounding stroma (Figure 3, e and h). The few SCC/VEGF- trans-

plants that developed into tumors showed only minimal growth with formation of well-circumscribed tumor nodules (Figure 3, f and i).

Expression of VEGF and Its Receptors in SCC-13 Tumors

To confirm that VEGF sense or antisense mRNA expression was maintained in tumor cells after transplantation into the skin of nude mice, we performed *in situ* hybridizations after 8 weeks of tumor growth. The VEGF riboprobe used for these hybridizations recognizes all isoforms of human and mouse VEGF mRNA.¹⁴ These studies confirmed that strong VEGF mRNA expression was maintained in SCC/VEGF+ tumors (Figure 4, a and b) whereas only weak VEGF mRNA expression was detected in control SCC/vec tumors (Figure 4, c and d) and in SCC/VEGF- tumors (Figure 4, g and h). Importantly, SCC/VEGF- tumors maintained high expression of the VEGF antisense mRNA, as detected by *in situ* hybridization with a VEGF sense riboprobe (Figure 4, e and f). In accordance with previously reported findings in a variety of malignant human and murine tumors, strong expression of flk-1 and flt-1 mRNA was detected in tumor vessels of SCC/VEGF+ xenotransplants (Figure 5, b, d, f, and h), whereas only low expression levels were detected in SCC/vec (Figure 5, a, c, e, and g) and SCC/VEGF- transplants.

Increased Tumor Angiogenesis and Admixture of Angiogenic Vessels in VEGF-Overexpressing Tumors

Immunohistochemical analysis of tissue sections stained for collagen IV, a basement membrane component, and for platelet-endothelial cell adhesion molecule-1 (CD31), an endothelial junction molecule,³² demonstrated an al-

Figure 3. Highly enhanced tumor growth, angiogenesis, and invasiveness of intradermally applied SCC cells transfected to overexpress VEGF (**b, e, h, i, o**), as compared with SCC cells transfected with vector only (**a, d, g, k, n**) or with a VEGF-antisense expression construct (**c, f, i, m, p**). **a–c:** SCC xenotransplants after 8 weeks in vivo; photographed from the underside of the skin. Small, avascular SCC/vec tumor (**a**) and SCC/VEGF[–] tumor (**c**), as compared with large and richly vascularized SCC/VEGF⁺ tumor (**b**). Original magnification, $\times 2$. **d–f:** H&E stains show small, rounded, well-delineated control SCC/vec tumors (**d, g**) and SCC/VEGF[–] tumors (**f, i**), in contrast to a large SCC/VEGF⁺ tumor (**e**) that is characterized by invasion of tumor cells into the desmoplastic vascular stroma (**h**), including single-cell invasion. Original magnifications, $\times 30$ (**d–f**) and $\times 120$ (**g–i**). **k–m:** Immunohistochemical staining for collagen type IV demonstrates blood vessels at the well-demarcated edge of SCC/vec control tumors (**k**) and SCC/VEGF[–] tumors (**m**). In contrast, VEGF-overexpressing tumors showed an intimate admixture of blood vessels and tumor (**l**). Original magnification, $\times 120$. **n–p:** Immunohistochemical staining of the endothelial junction molecule CD31 confirms the presence of blood vessels surrounding control (**n**) and SCC/VEGF[–] tumors (**p**), and the ingrowth of blood vessels into SCC/VEGF⁺ tumors (**o**). Original magnification, $\times 120$.

most continuous layer of blood vessels surrounding SCC/vec and SCC/VEGF[–] tumors (Figure 3, k, m, n, and p). These tumor vessels were focally dilated; however, ingrowth of vessels into the SCC/vec or SCC/VEGF[–] tu-

mors was not observed. In contrast, VEGF-overexpressing SCC/VEGF⁺ tumors showed a considerable admixture of blood vessels and tumor cells, most prominently in areas of tumor invasion (Figure 3, l and o) and

Figure 4. In situ hybridization with a VEGF antisense probe demonstrates strong expression of VEGF mRNA in 8-week-old xenotransplants of SCC/VEGF+ cells (a, b), as compared with vector-only-transfected control cells (c, d) and SCC/VEGF- cells (g, h). Hybridization with a VEGF sense riboprobe shows strong expression of VEGF antisense mRNA in SCC/VEGF- xenotransplants (e, f). Bright-field (a, c, e, g) and dark-field (b, d, f, h) microscopy; original magnification, $\times 30$.

a significantly increased density of tumor vessels (71.7 ± 13.6 vessels/ mm^2), as compared with 39.4 ± 4.5 vessels/ mm^2 in control SCC/vec tumors (Figure 6A). In contrast, the vessel density was significantly reduced to 26.0 ± 5.6 vessels/ mm^2 in SCC/VEGF- tumors (Figure 6A). Interestingly, the average vessel size in SCC/VEGF+ tumors was greatly increased to $814 \pm 90 \mu\text{m}^2$, as compared with $403 \pm 52 \mu\text{m}^2$ in SCC/vec control tumors and $272 \pm 40 \mu\text{m}^2$ in SCC/VEGF- tumors (Figure 6B). This was mainly due to an almost twofold increase of the percentage of vessels larger than $500 \mu\text{m}^2$ in SCC/VEGF+ tumors (18.2% versus 9.3% in SCC/vec), as well as of grossly dilated vessels larger than $1000 \mu\text{m}^2$ (12.6% versus 6.5%; Figure 6D). No vessels larger than $500 \mu\text{m}^2$ were detected in SCC/VEGF- tumors (Figure 6D). It is important that, when the parameters vessel density and average vessel size were combined to calculate total vascular areas, a much more pronounced increase in tumor angiogenesis was detected in VEGF-overexpressing tumors. Although vessels accounted for $1.05\% \pm 0.18\%$ of the total tumor area in SCC/vec tumors (Figure 6C), a more than fivefold increase was observed in SCC/VEGF+

tumors ($6.05\% \pm 0.99\%$). These data suggest total vascular area measurements as a more sensitive parameter than vascular density for the evaluation of tumor angiogenesis.

Discussion

Growth of solid tumors is dependent on the induction of new blood vessels.³³ VEGF has been shown to be a major tumor angiogenesis factor (for review, see^{16,34}), and several lines of experimental evidence support this concept.^{14,35,36} Recently, Skobe et al demonstrated that blocking the VEGF receptor Fek-1 inhibited tumor invasion of malignant human epidermal HaCaT keratinocytes growing as xenotransplants in immunodeficient mice.²³ The authors concluded that VEGF-induced angiogenesis was required for the induction of tumor invasion. However, the question remained unanswered whether increased VEGF expression itself might be sufficient to induce tumor invasion.²⁴ The data presented here pro-

Figure 5. In situ hybridization with antisense flk-1 (a–d) and flt-1 (e–h) riboprobes reveals marked upregulation of flk-1 and flt-1 mRNA expression in 8-week-old SCC/VEGF+ tumors (b, d, f, h), as compared with little or no expression in control SCC/vec tumors (a, c, e, g). Bright-field (a, b, e, f) and dark-field (c, d, g, h) microscopy; magnification, $\times 120$.

vide strong direct evidence that selective overexpression of VEGF can indeed induce an invasive tumor phenotype.

We chose the established human SCC line SCC-13 for our studies because SCC-13 xenotransplants form well-differentiated, noninvasive tumors after subcutaneous xenotransplantation into immunodeficient mice.²⁵ Therefore, we established stable SCC-13 transfectants with selective overexpression of mVEGF₁₆₄. To enable an even more direct comparison between high and low

VEGF expression by SCC-13 xenotransplants, we also established stable transfectants that overexpressed a VEGF₁₆₄ antisense construct. Strong expression of the transfected VEGF sense or antisense overexpression constructs was confirmed in stably transfected SCC-13 clones by Northern hybridizations, and Western blot analyses revealed a potent induction of mouse VEGF secretion in SCC/VEGF+ cells. Overexpression of VEGF led to a significantly enhanced rate of SCC tumor formation after intradermal tumor cell application, as compared with control-transfected cells. In contrast, tumor cells overexpressing the VEGF gene in antisense orientation showed significantly reduced tumor yields. These data suggest that VEGF-induced endothelial-cell activation of host vessels plays an important role in establishing the initial environmental milieu for successful growth and survival of the transplanted tumor clones in the dermis. They are in accordance with a previous report demonstrating that overexpression of mVEGF₁₆₄ by human malignant melanoma cells led to increased metastatic efficiency after intravenous injection of tumor cells into immunodeficient mice, as judged by the number of lung metastases.¹⁴ However, after application of tumor cells into the more richly vascularized subcutaneous space, control-transfected SCC clones showed higher tumor yields than after intradermal injection, and no significantly altered tumor yields were detected in clones overexpressing VEGF or a VEGF antisense construct. These findings reveal an important role of the stromal tumor microenvironment for the rate of successful tumor growth and suggest, furthermore, that the initial growth advantage provided by high VEGF secretion may be of less importance in already richly vascularized tissues.

Importantly, VEGF-overexpressing SCC-13 clones showed dramatically enhanced growth of established tumors in both intradermal and subcutaneous locations, as compared with controls transfected with vector without insert only. Results obtained with the parental cell line were identical to those obtained with SCC/vec clones, excluding any negative effects of cell transfection and subsequent cell selection on the tumor growth capacity of stably transfected cells. Eight weeks after transplantation, SCC/VEGF+ clones had formed tumors of more than

Figure 6. Morphometric analysis of tumor vessels by computer-assisted image analysis. **A:** Increased vessel density as determined by the number of CD31-positive vessels per square millimeter in SCC/VEGF+ tumors (V+), as compared with SCC/vec (vec) or SCC/VEGF- (V-). Bars represent mean values $\pm$ SEM. **B:** Average vessel area (square micrometers) was increased in SCC/VEGF+ tumors. **C:** Highly increased percentage of vessel area per total area in SCC/VEGF+ tumors. **D:** Increased proportion of larger vessels ($> 500 \mu\text{m}^2$ and $> 1000 \mu\text{m}^2$ respectively), in SCC/VEGF+ tumors. Significance levels of differences between SCC/VEGF+ and SCC/vec (unpaired Student's *t*-test): ***P* < 0.01; ****P* < 0.001.

60-fold the size of SCC/vec or SCC/VEGF- clones. To the best of our knowledge, this is the first time that an extremely slowly growing tumor with a high degree of differentiation has been investigated for the effects of induced VEGF expression. The VEGF-mediated increase of tumor growth was much more prominent than in previously reported xenotransplant models using highly malignant tumor cell lines.¹⁴ It is of interest that SCC/VEGF- xenotransplants maintained tumors of approximately 1 to 2 mm in diameter but were unable to grow beyond this minimal size.

Histological analysis revealed that control SCC/vec xenotransplants formed well-differentiated, rounded tumors without apparent tumor invasion. Tumors comprised multilayered epithelium with occasional mitoses and a clear demarcation of the border between tumor cells and surrounding tumor stroma. In marked contrast, VEGF-overexpressing SCC/VEGF+ xenotransplants showed malignant growth behavior with an irregular tumor stroma border and invasion of small nests of tumor cells and single tumor cells into the surrounding tissue. These findings confirm our original hypothesis that selective overexpression of VEGF enables tumor invasion, and they extend the findings by Skobe et al that an anti-flk-1 antibody inhibited tumor invasion.²³ The mechanisms by which VEGF mediates the induction of tumor invasiveness are presently unknown. However, it is conceivable that VEGF released by tumor cells induces release of matrix metalloproteinases by endothelial cells that, in turn, might help to create an environment that is permissive to tumor cell invasion. Indeed, induction of matrix metalloproteinases by VEGF has been demonstrated in human endothelial cells.³⁷ Alternatively, VEGF-stimulated tumor vessels might release activators of matrix metalloproteinase expression by tumor cells themselves. Recently, inhibition of the $\alpha v\beta 3$ integrin on angiogenic blood vessels was shown to inhibit invasiveness of experimental breast carcinomas.³⁸ Combined with our previously reported findings that VEGF potentially induced $\alpha v\beta 3$ expression in skin dermal microvascular endothelial cells,³⁹ these results suggest a potential role of integrins and their receptors in mediating VEGF-induced tumor invasion. In addition, VEGF-induced vascular hyperpermeability, leading to extravasation of plasma proteins and formation of a fibrin-rich stroma,¹⁷ may further facilitate ingrowth of fibroblasts and blood vessels to form a tumor stroma submissive for tumor invasion.

In accordance with previous reports on the induction of tumor vessels by VEGF,¹⁴ we detected a significantly increased density of tumor vessels in SCC/VEGF+ xenotransplants. Whereas tumor vessels in control tumors were found to merely surround the well-differentiated tumors, VEGF-overexpressing SCCs were characterized by an intimate admixture of vessels and tumor cells. It is of interest that measurement of total vascular areas as a percentage of tumor area occupied by vessel provided a much more sensitive parameter (a more than fivefold increase in SCC/VEGF+ tumors) than vessel density or average vessel size to detect VEGF-induced tumor angiogenesis. In conclusion, our results demonstrate that selective overexpression of the angiogenesis factor

VEGF in highly differentiated SCCs is sufficient to induce tumor invasiveness, in addition to promotion of tumor growth and angiogenesis. Our studies also provide direct evidence for an active role of the tumor stroma in cancer progression.

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