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Advances in Brief

Vascular Endothelial Growth Factor Is Essential for Initial but not Continued *in Vivo* Growth of Human Breast Carcinoma Cells

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Abstract

In this study, we used a self-contained tetracycline-regulated retroviral vector system to elucidate the role of vascular endothelial growth factor (VEGF) in controlling s.c. growth of human T-47D breast carcinoma cells. VEGF expression was tightly regulated by this system, both *in vitro* and in nude mouse xenografts. A 2.4-fold increase in tumor volume was associated with VEGF overexpression. Tumor growth was almost completely inhibited when VEGF was suppressed from the time of T-47D cell inoculation, and a 6-fold reduction in tumor volume was observed when VEGF suppression was started in 175-mm^3 tumors. However, no growth inhibition was observed when VEGF suppression was started in 820-mm^3 tumors. In these tumors, basic fibroblast growth factor and transforming growth factor α RNA expression was detected after VEGF was switched off. These findings demonstrate that VEGF is critical for the initial s.c. growth of T-47D breast carcinoma cells, whereas other angiogenic factors can compensate for the loss of VEGF after the tumors have reached a certain size.

Introduction

It is now widely recognized that angiogenesis is an essential step for solid tumor growth (1). Tumor vascular density has been shown to be an independent prognostic marker in several types of human tumors, including breast carcinoma (2). VEGF,[3] also known as VPF, is a strong endothelial cell mitogen and has been shown to increase the permeability of microvessels to 50,000-fold that of histamine (3). The effects of VEGF are mediated through two distinct high-affinity endothelial cell surface receptors, flt-1 and KDR/Flk-1, both of which are type III tyrosine kinase receptors (4). Increased expression of VEGF has been reported in several types of human tumors and was shown to correlate with poor prognosis (5, 6). In animal models, antisense VEGF and monoclonal antibody to VEGF inhibited VEGF tumor growth (7–10), whereas overexpression of VEGF enhanced tumor growth (11–13). The Tet system is a novel drug-regulated gene expression system that represents an important tool for evaluating the effects of a given gene on cells (14). We used a modified version of the Tet system in which the two components have been organized within the same vector (15). This allows overexpression of the gene under study in the absence of tetracycline and decrease in the basal expression of the gene by apparent antisense inhibition in the presence of tetracycline. Here, we show for the first time that the relevance of VEGF for *in vivo* growth of human breast carcinoma cells is dependent on the size of the tumor. When the tumors have reached a certain size, VEGF is not essential for supporting tumor growth and other angiogenic factors, such as bFGF and TGF-α, can substitute adequately for VEGF.

Materials and Methods

Tetracycline-regulated Retroviral Vector. The parent PBSTR-1 retroviral vector was provided generously by Dr. S. A. Reeves (Massachusetts General Hospital, Boston, MA). It is a modified version of the previously described Tet system (14, 15). Briefly, the PBSTR-1 vector contains both components of the Tet system. The regulator unit, containing the tetracycline-controlled transactivator (*tTA*) gene, is under the transcriptional control of internal SV40 promoter, and the response unit is under the transcriptional control of a minimal cytomegalovirus promoter, containing seven tet operators (tet O). The response unit is inserted into the vector in an antisense orientation relative to the regulator unit. A multiple cloning site was inserted downstream of the response unit. The puromycin (*puro*) resistance gene is present under the transcriptional control of the 5′ retroviral LTR. Human VEGF cDNA (a generous gift from Dr. M. Shibuya, University of Tokyo, Tokyo, Japan), was cloned into the multicloning site of the PBSTR-1 vector, at a *Bam*HI site, forming Tet-VEGF, and the sequence was confirmed. The Tet-VEGF construct is shown in Fig. 1.

Cell Culture. The human T-47D breast carcinoma cell line and BING retrovirus amphotrophic packaging cell line were purchased from the American Type Culture Collection (Rockville, MD) and grown in their respective American Type Culture Collection-recommended media. The transduction of Tet-VEGF vector into BING packaging cells was performed in the presence of tetracycline with some modifications, as described previously (16). For control, the PBSTR-1 vector alone was transduced into BING cells (Tet-vector). T-47D cells were infected with the supernatants containing the Tet-VEGF or the Tet-vector retrovirus and cloned under puromycin selection (2 μg/ml) in the presence of tetracycline (1 μg/ml) for 14 days. Puromycin-resistant T-47D clones were expanded and assayed for the background levels of VEGF. Subconfluent monolayers of the cloned cell lines were rinsed with PBS and incubated in serum-free medium for 24 h in the presence of tetracycline (1 μg/ml). Culture supernatants were collected, the cell debris was removed by centrifugation, and VEGF level was measured using an ELISA kit (R&D Systems, Minneapolis, MN). Among the Tet-VEGF clones, the one with the lowest level in the presence of tetracycline was chosen for further examination.

***In Vitro* Proliferation Assay.** *In vitro* proliferation was determined by measuring the conversion of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium to formazan (Promega, Forward, WI) by cells cultured in 96-well plates in the presence or absence of tetracycline. The absorbance at 490 nm represents conversion to formazan, which is directly proportional to the number of living cells in culture. Absorbance was read with an ELISA plate recorder ($n = 6$ per group).

Growth of VEGF-Transduced Human T-47D Breast Carcinoma Cells *in Vivo*. The effect of VEGF stimulation and suppression on tumor growth was studied in nude mice. For s.c. growth, it was necessary to coinject the human T-47D breast carcinoma cells with Matrigel (Becton Dickinson Labware, Bedford, MA). First, 50 μl of Matrigel were injected s.c. into the flanks of 6-week-old female NIH nude mice, followed by inoculation of the Tet-VEGF or Tet-vector-transduced T-47D cells (5×10^5 cells/100 μl of PBS) at

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[3] The abbreviations used are: VEGF, vascular endothelial growth factor; VPF, vascular permeability factor; TGF, transforming growth factor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; RT-PCR, reverse transcription-PCR; Tet, tetracycline-controlled transactivator response promoter.

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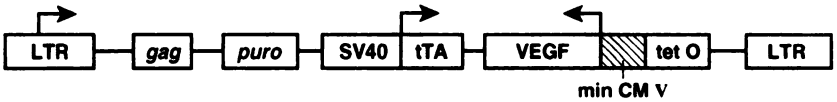

Fig. 1. VEGF containing Tet-regulated retroviral vector. The vector is composed of the regulator unit, containing the *tTA* gene. The regulator unit is under the transcriptional control of SV40 promoter, and the response unit is under the control of a minimal cytomegalovirus promoter (*min CMV*) containing tet operators (*tet O*). The response unit is inserted into the retroviral vector in an antisense orientation, relative to the regulator unit. The puromycin resistance gene (*puro*) is under the transcriptional control of the 5′ retroviral long terminal repeat (*LTR*). Human VEGF cDNA was cloned into the multicloning site of the response unit. *Arrows* indicate the transcription units and the direction of transcription.

the same site. Of the 45 mice included in the study, 35 were inoculated with the Tet-VEGF-transduced T-47D cells and 10 with the Tet-vector control cells. The 35 mice were divided into four groups. The first group received tetracycline in the drinking water (1 mg/ml) throughout the experiment to suppress VEGF. The remaining 25 mice were given normal drinking water to allow overexpression of VEGF. At day 21, when the average tumor volume was 175 mm^3, a group of eight Tet-VEGF mice was started on tetracycline, and on day 28, another group of seven mice with an average tumor volume of 820 mm^3 was started on tetracycline. The remaining 10 mice continued on normal drinking water. Ten mice that were injected with T-47 cells carrying Tet-vector alone served as control. The s.c. tumors were measured twice a week using a caliper, and the tumor volumes were calculated. To evaluate whether VEGF expression was regulated appropriately by tetracycline *in vivo*, three mice from each group were euthanized on day 32 and the tumors were pooled for analysis of VEGF RNA and protein expression. After euthanasia, the tumors were split in half and fixed in formalin for histological examination or snap frozen in liquid nitrogen for RNA isolation and protein analysis.

RNA and Protein Expression Analysis. VEGF protein concentration was measured in culture supernatants and tumor lysates. Tumor lysates were prepared, and the samples were equalized for protein concentration prior to measuring VEGF levels by ELISA. VEGF RNA expression *in vivo* was examined by Northern blot analysis. Briefly, total RNA was extracted from the s.c. tumors, and aliquots (15 μg) were electrophoresed in 1% agarose-formaldehyde gels, transferred to nylon membrane (Schleicher & Schuell, Keene, NH), and hybridized with 1×10^6 cpm/ml of ^{32}P-labeled human cDNA template VEGF antisense riboprobe. The same membrane was then hybridized with a β-actin probe to normalize differences in RNA loading. RT-PCR was used for evaluating RNA expression of bFGF, PDGF, and TGF-α and -β in the s.c. tumors. Primer sequences of each of these factors were published previously (17). RT-PCR was performed at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 30 cycles. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

Statistical Analysis. Tetracycline-mediated effect on VEGF production *in vitro* was analyzed by one-way ANOVA. Significant differences were determined by Tukey's multiple comparison test ($P < 0.05$).

Results

Control of VEGF Expression *in Vitro* Using a Tetracycline-Regulated Retroviral Vector. Tet-VEGF was transduced into human T-47D breast mammary carcinoma cells, which constitutively express low levels of VEGF. In the absence of tetracycline, up-regulation of VEGF gene expression resulted in a 5.6-fold increase of the secreted amount of VEGF protein over a period of 24 h, compared to the Tet-vector control (Fig. 2*A*). In the presence of tetracycline (1 μg/ml) in the culture medium, VEGF production was suppressed 60% compared to the control level by apparent antisense inhibition. The tight regulation of VEGF expression by tetracycline was demonstrated further when, upon its addition to the culture medium at 24 h, the production of VEGF decreased drastically and increased when tetracycline was excluded from the medium (Fig. 2*A*).

The *in vitro* replication rate was not different between the Tet-VEGF- and Tet-vector-transduced T-47D breast carcinoma cells and was not affected by the presence or absence of tetracycline (Fig. 2*B*).

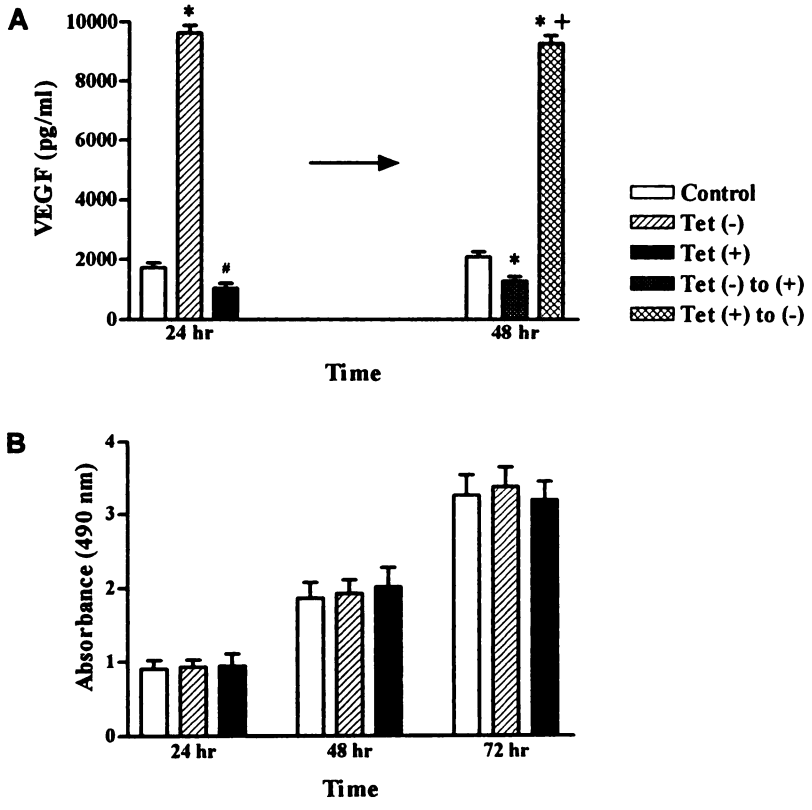

Fig. 2. *A*, tetracycline-mediated effect on VEGF production *in vitro*. *Left*, VEGF levels in 24-h culture supernatants of T-47 breast carcinoma cells transduced with Tet-vector alone (*Control*) or Tet-VEGF in the absence [*Tet (−)*] and presence [*Tet (+)*] of tetracycline. *Right*, the tetracycline-mediated effect on VEGF production by the same cells was reversed (*arrow*) 24 h after tetracycline was either added [*Tet (−) to (+)*] or removed [*Tet (+) to (−)*] from the culture supernatants. *, significantly different from control ($P < 0.05$); #, significantly different from Tet (−) ($P < 0.001$); +, significantly different from Tet (−) to (+) ($P < 0.001$). *B*, *in vitro* proliferation rate of the T-47D cells transduced with Tet-vector alone (*control*) or Tet-VEGF in the absence [*Tet (−)*] or presence [*Tet (+)*] of tetracycline. *Key* shown in *A* applies to *B* as well. Data are means; *bars*, SE.

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Effect of VEGF Overexpression and Suppression on s.c. Tumor Growth. To examine the effect of modulation of VEGF expression on tumor growth, the Tet-VEGF- and Tet-vector-transduced T-47D breast carcinoma cells were coinjected s.c. with Matrigel into the flanks of nude mice. Tumor growth was slow in all of the experimental groups during the first 18 days (Fig. 3*A*). No further tumor expansion was noted in the group on tetracycline-mediated VEGF suppression. The group overexpressing VEGF showed evidence of accelerated tumor growth on day 21 and the Tet-vector control group 3 days later. The difference in tumor volumes between the VEGF-overexpressing cells and the Tet-vector control groups ranged from 2-fold on day 21 to 2.4-fold at the end of the experiment on day 44 (Fig. 3*A*). When tetracycline-mediated VEGF suppression was started on day 21 in tumors with an average volume of 175 mm^3, designated as "small" tumors, the growth rate slowed drastically, resulting in a 6-fold reduction in the average tumor volume, compared to the group overexpressing VEGF throughout the experiment (Fig. 3*B*). Interestingly, no inhibition of tumor growth was observed when VEGF suppression was started on day 28, after the tumors had reached an average volume of 820 mm^3 ("large" tumors). Tetracycline by itself in the drinking water did not affect tumor growth or health status of the mice. Consequently, the tumor volumes of the two groups were combined and are shown as one control group in Fig. 3*A*.

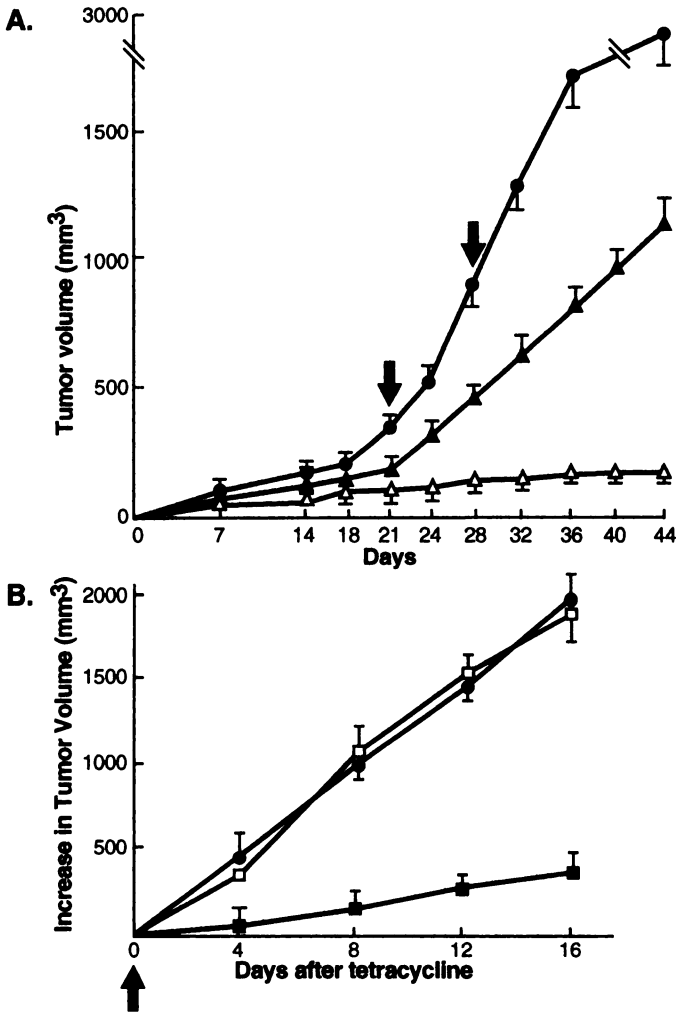

Fig. 3. The growth effect of tetracycline-mediated suppression of VEGF is dependent on tumor size. *A*, T-47D breast carcinoma cells carrying the Tet-VEGF construct were injected s.c. into nude mice. Half of the mice received tetracycline in the drinking water (△), and the other half did not (●). T-47D cells carrying Tet-vector alone served as control (▲). Tumor volumes were determined by tridimensional caliper measurements at indicated time points. *B*, the effect of VEGF suppression in established tumors is shown as a separate growth curve. Tetracycline was added to the drinking water of two groups of mice, one with an average tumor volume of 180 mm^3, designated as small tumors (■), and another group of mice with an average tumor volume of 820 mm^3, designated as large tumors (●). Tumor growth was monitored for a period of 16 days and compared to the group that overexpressed VEGF throughout the entire experimental period (□). *Arrows*, time points when administration of tetracycline to the small and large tumors was started. Data shown are means; *bars*, SE (n = 10).

Histological examination of H&E-stained sections of xenografted tumors, which were removed at the end of the experiment, revealed increased necrosis and fibrous tumor stroma associated with VEGF overexpression but no evidence of edema. There was mild mononuclear inflammatory infiltrate surrounding the tumors in all of the groups. The number of mitotic figures was 30% higher in the VEGF-overexpressing group. The number of proliferating cell nuclear antigen-positive cells was variable with areas of very high proliferative activity in both groups. Sections of the minute tumors from the group that received tetracycline during the entire experimental period showed a few nests of viable tumor cells but no evidence of proliferative activity. A study has been initiated to characterize further the tumor vasculature and the morphological changes accompanied by modulation of VEGF expression in this model.

RNA Expression of VEGF and Other Angiogenic Factors in s.c. Tumors. Northern blot analysis was performed on RNA from s.c. tumors removed from three mice in each group, which were euthanized on day 32. As observed *in vitro*, VEGF was tightly regulated *in vivo*. When compared to the tumors derived from the Tet-vector control-transduced T-47D cells, there was a marked VEGF RNA overexpression in the Tet-VEGF tumors of the mice that were never given tetracycline in the drinking water and suppression in the tumors of the mice that were on tetracycline during the entire experimental period (Fig. 4*A*, *left*, *Lanes 1–3*). VEGF suppression was also observed in the large tumors of the mice that were placed on tetracycline on day 28 (Fig. 4*A*, *left*, *Lane 4*). VEGF was suppressed to the same extent in the small tumors (data not shown). The VEGF protein levels reflected closely the RNA expression, observed by Northern blot analysis (Fig. 4*A*, *right*).

In an attempt to elucidate why the growth of large tumors was not halted after VEGF production was suppressed, the tumors were screened for other angiogenic factors, including bFGF, PDGF, TGF-α, and TGF-β, using RT-PCR. As shown in Fig. 4*B*, RNA expression of bFGF and TGF-α was switched on 4 days after the suppression of VEGF, whereas these angiogenic factors were not detected in the tumors of the same size overexpressing VEGF during the entire experimental period. In the small tumors however, tetracycline-mediated suppression of VEGF did not result in increased expression of the angiogenic factors listed above (data not shown).

Discussion

The present study revealed that VEGF was critical during the initial stages of s.c. growth of human breast carcinoma cells in nude mice but was not needed for continued growth after the tumors had reached a certain size. The role of VEGF as a major angiogenic factor is well established from a number of reports on human malignancies and experimental models (18). We demonstrated previously, using paired samples of human breast carcinomas and the adjacent nonneoplastic tissues, that VEGF RNA expression was elevated significantly in the carcinomas, whereas bFGF and TGF-α and TGF-β were expressed variably (19).

The Tet system used in the present study has both the response and regulator units inserted into the same retroviral vector (15). The major advantage of using this system *in vivo* is that gene expression is tightly regulated by tetracycline in the drinking water and can be efficiently

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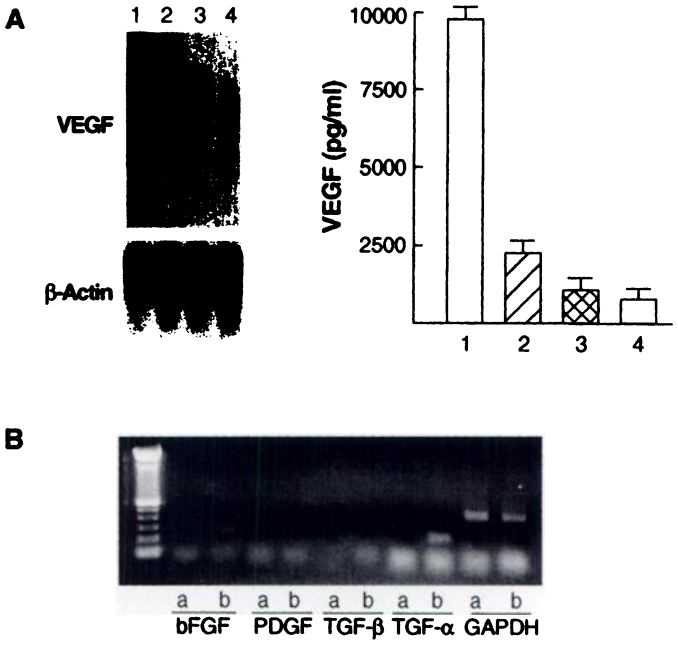

Fig. 4. Expression of VEGF and other angiogenic factors, derived from Tet-VEGF and Tet-vector-transduced T-47D cells. *A, left,* Northern blot analysis of VEGF RNA expression in tumors with: overexpression of VEGF throughout the experiment (*Lane 1*); Tet-vector control (*Lane 2*); suppression of VEGF throughout the experiment (*Lane 3*); suppression of VEGF started in large tumors (820 mm^3; *Lane 4*). The blot was hybridized with antisense VEGF riboprobe, followed by a β-actin probe to normalize differences in RNA loading. *A, right,* VEGF protein concentration measured in tumor homogenates by ELISA. The tumor samples are organized in the same order as the RNA samples in *A, left.* Data shown are as means; *bars,* SE (n = 3). *B,* induction of bFGF and TGF-α in large tumors. RNA expression of bFGF, PDGF, TGF-β, and TGF-α was examined by RT-PCR in the large (820 mm^3) tumors with and without tetracycline-mediated VEGF suppression. Results are shown from tumors overexpressing VEGF throughout the experiment (*a*) and from large tumors in which VEGF was suppressed after they reached a volume of 820 mm^3 (*b*). Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

switched on and off in the same tumor. Our results showing VEGF-dependent modulation of tumor growth are consistent with reports by others on increased tumorigenicity following transfection of VEGF cDNA (11–13) and suppressed tumor growth as a result of antisense or antibody-mediated inhibition of VEGF (7–10). One of the significant features of the Tet system used here is that the basal expression of the response unit is decreased by apparent antisense inhibition. Accordingly, we observed a 60% decrease in VEGF protein production when tetracycline was present in the culture medium. This level of inhibition is comparable to that accomplished through the use of a eukaryotic expression vector carrying an antisense VEGF cDNA (8, 10).

When analyzing s.c. growth of the T-47D breast carcinoma cells in nude mice, we observed an initial lag period of approximately 18 days, during which tumor growth was slow, regardless of the level of VEGF production. The relevance of VEGF for tumor growth was clearly apparent in the tetracycline-treated mice, which failed to develop tumors beyond a few mm. In the VEGF-overexpressing group however, accelerated tumor growth was first noted at day 21 and in the Tet-vector control at day 24. Interestingly, the rate of tumor expansion was almost identical for these two groups for the remainder of the experimental period with 2–2.4-fold larger tumors in the VEGF-overexpressing group. This implies that the VEGF-mediated growth effects mainly took place during the first 3 weeks, which raises an important question of the role of VEGF at different stages of tumor growth. VEGF was recently reported to act as an autocrine growth factor on AIDS Kaposi's sarcoma cells (20), but our study as well as others on glioma, melanoma, and MCF-7 breast carcinoma cell lines did not show any effect of VEGF overexpression on *in vitro* growth (8, 12, 13). VEGF is also known as VPF (3, 21). As such, it causes a rapid microvascular permeability, allowing plasma proteins and other macromolecules to escape into the extracellular matrix. VEGF can thus provide an enriched growth environment for the breast carcinoma cells, especially in an ectopic location. This enrichment may entail both nutrients and growth factors leaking out of the blood vessels and the development of a stromal network. Our histological findings of substantially increased stroma in the VEGF-overexpressing tumors is in keeping with the concept that VEGF plays an important role in promoting stromal formation through its function as VPF. Examination of the H&E-stained sections showed that both the VEGF-overexpressing and the control tumors were well vascularized, although the VEGF-overexpressing tumors displayed more extensive necrosis, as well as a slightly (30%) higher number of mitotic figures. Additional studies will be needed to characterize the morphological differences induced by VEGF overexpression.

The most intriguing observation of this study was that VEGF was not necessary for growth after the s.c. tumors had reached a certain size. Suppression of VEGF had no effect on the expansion of the large tumors, although it resulted in marked inhibition of the small tumors. This may at least in part be explained by the data showing up-regulation of bFGF and TGF-α in the large tumors following suppression of VEGF. It would therefore appear that bFGF and TGF-α and perhaps other as-yet-unidentified angiogenic factors adequately compensate for the loss of VEGF. The question arises why bFGF and TGF-α were only detected after the tumors reached a certain size. We propose that a sufficient amount of stroma must be generated to support the expression of angiogenic factors, such as bFGF and TGF-α. This reiterates the importance of VEGF as a permeability factor for promoting stromal formation, although its function as a stimulator of angiogenesis is clearly important also for sustaining the events leading to the development of mature stroma and supporting tumor growth. It is widely accepted that tumor stroma is vital for providing pathways for neovessels and serving as a reservoir for growth factors and other macromolecules. Human breast carcinomas generally possess abundant fibrous stroma, which commonly occupies most of the tumor mass. In the context of our findings, it will be important to dissect out the VEGF-mediated biological effects on the early tumor development. We conclude from the present model of xenografted human breast carcinoma cells in nude mice that VEGF is critical for tumorigenesis and that its vascular permeabilization effect is likely to be critical for generating an enriched milieu for tumor growth.

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