VEGF<sub>121</sub>, VEGF<sub>165</sub> Overexpression Enhances Tumorigenicity in U251 MG but not in NG-1 Glioma Cells<sup>1</sup>

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a multifunctional cytokine with important roles in angiogenesis. VEGF is overexpressed in human cancers, including highly vascularized and infiltrative brain tumors. In our previous study of seven glioma cell lines, VEGF expression levels correlated with blood vessel density and tumorigenicity, and U251 MG and NG-1 cells were recognized as low-tumorigenic glioma cell lines. We hypothesized that low-tumorigenic cells can become highly tumorigenic when high levels of VEGF are expressed. To test this hypothesis, we constructed VEGF expression vectors containing 564 bp or 696 bp of VEGF<sub>121</sub> or VEGF<sub>165</sub> cDNA, respectively, and transfected them into U251 MG and NG-1 cells. In comparison to parental cells, the 20 VEGF-expressing clones examined had on average 8–10-fold more VEGF mRNA and 12–88-fold more secreted VEGF proteins. Four VEGF-overexpressing clones (U251 MG/V121-C2, U251 MG/V165-C3, NG-1/V121-C6, and NG-1/V165-C3) were selected for additional study. As VEGF production increased with population growth, U251 MG/V121-C2 and U251 MG/V165-C3 cells accumulated 47.9 and 22.0 ng of VEGF during a 5-day culture of 10<sup>6</sup> cells, a 313- and 144-fold overexpression when compared with that in parental U251 MG cells. NG-1/V121-C6 and NG-1/V165-C3 cells secreted 30.4 and 9.4 ng of VEGF, respectively, or 130- and 43-fold more than did the parental NG-1 cells. Subcutaneous implantation of the VEGF-overexpressing U251 MG cells into nude mice caused huge, soft hemorrhagic tumors to form, whereas controls maintained very small tumors. Intracranial implantation of the VEGF-overexpressing cell lines significantly shortened survival of the mice when compared with controls, and it caused formation of solid brain tumors with variable sized hemorrhages, whereas the controls had no apparent brain tumors. Tumorigenicity of U251 MG cells was synergized by co-overexpression of VEGF<sub>121</sub> and VEGF<sub>165</sub>. In addition, VEGF<sub>165</sub> seemed to be more potent to the brain endothelium than was VEGF<sub>121</sub>. More interestingly, except when an admixture of cells was implanted s.c., VEGF overexpression in NG-1 cells did not promote hemorrhagic tumor formation. These data suggested that a switch from a phenotype of low tumorigenicity to one of high tumorigenicity is possible when VEGF overexpression occurs, although other factors may also be required.

INTRODUCTION

VEGF is a multifunctional angiogenic cytokine that induces a series of protein phosphorylations, beginning with the autophosphorylation of its receptor tyrosine kinases (KDR/flk-1 and flt-1). VEGF increases influx of Ca<sup>2+</sup>, modulates effects of Ca<sup>2+</sup>-sensitive enzymes, and promotes expression of antiaiopotic proteins and serine proteases (1–3). Targeted disruption of the VEGF gene has indicated that VEGF is indispensable for new blood vessel formation (4) and for corpus luteum angiogenesis (5).

The human VEGF pre-mRNA is composed of eight polypeptide-coding exons that can be alternatively spliced to create five isoforms (6–8). Besides common regions (exon 1–5 plus exon 8) that become VEGF<sub>121</sub>, of all of the possible alternative-splicing products, inclusion of an additional exon 6 generates VEGF<sub>145</sub> (8). VEGF<sub>165</sub> has an additional exon 7, VEGF<sub>180</sub> has both exons (6a and 7), and VEGF<sub>206</sub> contains all of the exons (Fig. 1A). Although it is known that exon 7 contains heparin and a heparin-sulfate binding domain, and exon 6 possesses a second heparin-binding and extracellular matrix-binding domain, functions of the individual isoforms or the combination of isoforms are poorly understood. VEGF expression is tightly regulated in vivo (9–12), and deviation from normal levels of VEGF might be detrimental to the vasculature. A one-half reduction in VEGF gene dosage leads to severe vascular defects and early embryonic death in mice (4, 13). On the other hand, VEGF is locally overexpressed in many human diseases, including diabetic retinopathy, age-related macular degeneration, rheumatoid arthritis, and skin blister (1, 2). In experimental animals, intracerebral implantation of U87 MG cells expressing high levels of VEGF caused brain hemorrhage in as little as 60–90 h (14). Implantation of myoblasts expressing high levels of VEGF into the tibialis anterior muscle resulted in vasculogenesis and hemangioma after 24 days (15). Subcutaneous implantation of VEGF<sub>121</sub>- or VEGF<sub>165</sub>-expressing CHO cells into nude mice conferred a growth advantage but did not lead to malignant transformation or metastasis. Consequently, lesions formed by VEGF-expressing CHO cells were essentially benign and remained small (16). It was also reported in human melanoma SK-MEL-2 cells that VEGF overexpression increases tumor growth, angiogenesis, and experimental metastasis (17). In our previous report, we found that unlike basic fibroblast growth factor, production of VEGF correlated with angiogenesis and tumorigenicity in human glioma cell lines (18). Thus, we hypothesized that cancer cells with a low-tumorigenic phenotype, such as U251 MG and NG-1 cells, can become highly tumorigenic in certain stages of cancer development if conditions for VEGF overexpression are created. Unlike the previous literature, this report provides evidence not only that the switch occurs but also that it occurs only under specific circumstances and in specific cell lines. We detected a switch in U251 MG cells but not in NG-1 cells, suggesting that NG-1 cells gain negative factors or lack other positive angiogenic factors that are involved in blood vessel formation and tumor proliferation. We found that expression of EGFR, which in part controls the production of angiogenic factors (19), was lower in NG-1 cells than in U251 MG cells.

MATERIALS AND METHODS

Cell Lines and Cell Culture. U87 MG and U251 MG glioma cells were purchased from the American Type Culture Collection (Manassas, VA). NG-1 cells were derived from surgical specimens of human glioma and established in the Department of Neuro-Oncology at The University of Texas M. D. Anderson Cancer Center (20). Cells were cultured in a 1:1 mixture of DMEM and Ham’s F-12 medium, supplemented with 10% FCS, and incubated in a humidified atmosphere containing 5% CO<sub>2</sub> at 37°C.

VEGF Expression Vector Construction. Two oligomers, V389up (5'-cgagattacgCTCCGAAACCATGAACTTTC-3') and V840dn (5'-attctagcAGCTGTCTGCGATGATG-3'), were used to amplify VEGF by
PCR using a single-stranded cDNA template prepared from U87 MG cells. After digestion of the PCR products with EcoRI and XbaI, both a 564-bp and a 696-bp fragment were gel purified and ligated into EcoRI/XbaI sites of pCI-neo (Promega). The two resulting plasmids were named pCIneo-V121 and pCIneo-V165, and their insertion sequences were confirmed by automated DNA sequencing.

Stable Transfection and Single-Cell Colony Selection and Maintenance. NG-1 and U251 MG cells were transfected with 1 μg of purified plasmid DNA or 1 μg of vector in 1 μl of Lipofectamine (Life Technologies, Inc./Life Science Technologies, Inc., Gaithersburg, MD) in 1 ml of SFM. Medium containing geneticin (Life Technologies, Inc.), 200 μg/ml for NG-1 cells or 300 μg/ml for U251 MG cells, was used to select stable transfectants. Multiple single-cell colonies were selected, expanded, and maintained in the geneticin-containing medium until the experiments were performed. These colonies were cataloged as the NG-1 group (NG-1 parental, NG-1/pCIneo, NG-1/VEGF121, and NG-1/VEGF165 cells) and the U251 MG group (U251 MG parental, U251 MG/pCIneo, U251 MG/VEGF121, and U251 MG/VEGF165 cells, each followed by a C and a number to represent the clone number).

Southern and Northern Blot Analysis and VEGF Enzyme-linked Immunosorbent Assay. PCR products were separated by 6% PAGE and transferred to a Zeta-Probe GT Genomic Blotting Membrane (Bio-Rad, Hercules, CA) after the gel was boiled in 1× Tris-borate EDTA for 5 min. For VEGF mRNA analysis, 20 μg of total RNA isolated using the TRIzol reagent (Life Technologies, Inc.) was separated in a 0.7% agarose gel and transferred to the same type of membrane. The membranes were UV cross-linked and hybridized in rapid hybridization buffer (Amersham Life Science, Buckinghamshire, United Kingdom) with an unpurified 401-bp fragment of the VEGF common coding region that was labeled with 32P-dCTP using a random prime DNA labeling kit (Roche Diagnostic, Mannheim, Germany). Molecular analysis program (Bio-Rad) was used for image analyses.

Preparation of conditioned medium for VEGF ELISA was modified from our report described previously (18). Because we found that preconditioning for 6 h was unnecessary, this step was omitted. The number of seeded cells was increased to 10,000 cells/well.

In Vivo Tumorigenicity Tests. Tumorigenicity of the transfected cell colonies and parental cells was determined using s.c. injection and intracranial
implantation. Tumorigenicity tests using s.c. injection were performed twice for each group. The second test for the NG-1 group was conducted as described previously (18), where Matrigel (Collaborative Biomedical Products, Bedford, MA) was used to augment tumor growth in the nude mice. For the second test in the U251 MG group, cells were implanted on only one side of each mouse so that six mice were used per treatment. A mixture of cells with equal numbers of VEGF$_{121}$ and VEGF$_{165}$-producing cells (admix) was used for some experiments.

For intracranial implantation, only the U251 MG group was tested. The cells were prepared in a concentration of 1 $\times$ 10$^6$ cells/μl in SFM with 1 $\times$ antibiotics/antimycotics (Life Technologies, Inc.). Nude mice (23–27 g in weight) were first anesthetized by i.p. injection of ketamine (90–120 mg/kg) plus xylazine (5–10 mg/kg). Fully anesthetized, the animals were placed on a pile of gauze on the platform of the stereotactic apparatus and were secured. The surface area was sterilized with Betadine, and a skin incision was made using an 11-gauge sterile surgical blade. Two Q-tips were used to separate the incised skin, in which a hole was punched with a 20-gauge needle at the position of 3 mm north and 2 mm east of the bregma. Using a 26-gauge needle attached to a zero dead volume Hamilton syringe, 5 µl of the U251 MG cell group was slowly injected through the hole and into the mouse brain to a depth of 3.5 mm. To prevent cells from leaking out of the brain, a single injection, involving five discontinuous pushes and waits, took 10 min. Sterile clips were used to seal the skin. The implanted animals were examined twice a week. The first sign of animal sickness was a hunched posture, followed by emaciation, lethargy, or a moribund condition. In these situations, we received morbidity and mortality reports from institutional animal facility personnel. Because live brains were needed for histology and immunohistochemical analyses, we sacrificed sick animals when they weighed about 14–16 g. Animals were sacrificed by cervical dislocation. Brains of the sacrificed mice were dissected from the cranial cavity and fixed in 4% formaldehyde before paraffin embedding.

**Histology and Immunohistochemistry.** Hemorrhagic tumors or brain sections were examined by staining with H&E, anti-VEGF antibodies, and anti-CD31 antibodies. The detailed procedure we used for detecting VEGF expression was described previously (18). For detecting CD31, we followed the manufacturer’s (BioGenex, San Ramon, CA) procedures. To unmask antigen CD31, rehydrated tumor or brain sections were microwaved with antigen-retrieval solution AR 10 in a Trender Cooker (Nordic Ware, Minneapolis, MN) for 35 min.

**RESULTS**

**Endogenous and Exogenous VEGF Expression in Glioma Cells.** Various primer pairs were tested to determine the length of the 5’ and 3’ NCRs of single-stranded cDNA preparations from total RNA of glioma cell lines. The 5’ and 3’ NCRs were found to be ~80 bases and 170 bases (data not shown) using routine RT-PCR. By using the primer pair VAS389up and V840dn, which recognized the last 12 bases of the 5’ NCR and bases 89–111 of the 3’ NCR in VEGF mRNA, three products were amplified and resolved (Fig. 1B, left). They were hybridized to a 401-bp common coding region of a VEGF probe (Fig. 1B, right), and their sizes were calculated using the map shown in Fig. 1A. Together with DNA sequencing data (not shown), these data showed that glioma cells transcribe at least three VEGF mRNA isoforms, corresponding to VEGF$_{121}$, VEGF$_{165}$, and VEGF$_{185}$. Although Fig. 1B suggested that VEGF$_{121}$ is an abundant isoform in glioma cells, this could be the result of differences in RNA stability and RT-PCR efficiency among VEGF isoforms. This assumption is partially based on the Northern analysis in Fig. 1C (see Fig. 1D, first lane, left panel) where signals from VEGF$_{165}$ and VEGF$_{185}$ are stronger than the signal from VEGF$_{121}$.

VEGF$_{121}$ and VEGF$_{165}$ isoforms initially amplified from U87 MG cells were individually cloned into EcoRI/XbaI sites of pCIneo. Automated DNA sequencing confirmed no difference between the cloned VEGF sequences and previously reported VEGF sequences (6, 7). As predicted from our cloning map (Fig. 1C), two specific mRNAs, a 961-bp and a 1093-bp mRNA, were expressed in the transfected cells (Fig. 1D). Image analyses showed that VEGF mRNA levels were about 8–10 times higher in the transfected cell lines than in the parental cells. Three endogenous VEGF mRNAs were identified. Combined with RT-PCR data, these data suggested that the endogenous VEGF isoforms were VEGF$_{121}$, VEGF$_{165}$, and VEGF$_{185}$.

**High VEGF Expression in the NG-1 and U251 MG Cells Transfected with pCIneo-V121 or pCIneo-V165.** Several stably transfected colonies were randomly selected for evaluation of VEGF expression. VEGF concentrations in the conditioned medium were so high in most transfected clones that a 10–100 $\times$ dilution was necessary to avoid overloading in the VEGF ELISA.

Twenty single-cell colonies were examined for VEGF productivity. NG-1 cells transfected with pCIneo-V121 secreted about 3.7–7.8 ng of VEGF/10$^5$ cells within 24 h (Fig. 2A). The average amount of VEGF produced by these cells was 5.8 ng, ~25-fold more than that produced by parental cells. Although NG-1 clones transfected with pCIneo-V165 secreted less VEGF than did clones transfected with pCIneo-V121 (Fig. 2A), C1 and C3 clones expressed 12-fold more VEGF than did parental cells. After confirming the specific VEGF mRNA present in the cells, we selected NG-1/V121-C6 and NG-1/V165-C3 clones (Fig. 2A) for tumorigenicity studies. In U251 MG cells, transfection of pCIneo-V121 or pCIneo-V165 also increased secretion in examined clones (Fig. 2B). After Northern blot analysis, we similarly selected U251 MG/V121-C2 and U251 MG/V165-C3 for additional study. No clones examined from parental or vector-transfected cells expressed more VEGF than did the experimental populations (Fig. 2, A and B), suggesting that the vector did not interfere with host-cell VEGF regulation and that no VEGF-overexpressing clones were in the parental populations.

Before testing our hypothesis in animals, we examined the proliferation rates of selected clones in vitro and found that stable transfection of highly expressed VEGF did not promote or inhibit cell growth (data not shown). To assess the potential accumulation of VEGF at the site of injection in animals, we first examined VEGF production in the selected clones over 5 consecutive days in vitro (Fig. 2, C and D). A total of 30.4 ng and 9.4 ng of VEGF accumulated from 10$^4$ NG-1/V121-C6 and NG-1/V165-C3 clones, respectively, a 138- and 43-fold overexpression of VEGF when compared with parental cells (Fig. 2C). The 5-day accumulation of VEGF reached 47.9 ng and 22.0 ng in U251 MG/V121-C2 and U251 MG/V165-C3 cells, respectively (Fig. 2D), a 313- and 144-fold increase when compared with U251 MG cells. Interestingly, VEGF production increased with cell population growth. However, an effect of cell density on VEGF production was not apparent in parental or vector-transfected cells (Fig. 2, C and D), in which each accumulated ~0.2 ng of VEGF within 5 days.

**High VEGF Expression by U251 MG Cells but not NG-1 Cells Causes Hemorrhagic Tumors in s.c. Implantation Studies.** To test if low-tumorigenic cells can become high tumorigenic cells through VEGF overexpression, 1 $\times$ 10$^5$ cells from selected glioma cell lines were s.c. implanted into nude mice, and tumor growth was calculated. Fig. 3 demonstrates that individual or combined overexpression of the two VEGF isoforms (admix) in the low-tumorigenic U251 MG cells significantly boosted parental cell tumorigenicity. Tumor growth patterns (Fig. 3A) were very similar to VEGF production patterns in these cell lines (Fig. 2D), suggesting a positive correlation between VEGF production and tumorigenicity. U251 MG/V165-C3 cells seemed to be less tumorigenic than were U251 MG/V121-C2 cells, probably because of lower VEGF production (Fig. 2D). Admix tumors grew faster than tumors from the individual cell lines, suggesting a synergistic effect of VEGF$_{121}$ and VEGF$_{165}$ on certain periods of tumor progress. Some mice at day 27 (Fig. 3A) had borne tumors that...
exceeded the size limitation set by our institutional protocol, so we had to sacrifice the mice and dissect their tumors. Surprisingly, tumors formed from VEGF-overexpressing U251 MG cells were soft and easily broken when dissected (Fig. 4A), whereas tumors formed from parental cells were solid and small, or not evident, additionally indicating that U251 MG is a low-tumorigenic cell line (18). Several smaller hemorrhagic tumors formed in the mice implanted with pCIneo-transfected U251 MG cells; however, on average, they were much smaller than tumors from each of the three groups that overexpressed VEGF. The hemorrhagic tumors established a large complex blood vessel network that could be seen by the naked eye (Fig. 4B). Large amounts of RBCs surrounded by a wall of tumor cells were apparent (Fig. 4C). Although the size of these hemorrhagic tumors was variable, VEGF overexpression promoted tumor growth of the low-tumorigenic U251 MG cells by about 4–10-fold on average. These results were reproducible, because they were found in another set of s.c. implantation studies conducted in an earlier year (data not shown).
Hemorrhagic tumor sections were examined histochemically by antibodies against VEGF and CD31, an endothelium-specific marker. As expected, unlike tumors formed from parental cell implantation, every tumor derived from VEGF-transfected U251 MG cells stained positive for VEGF, and many variously sized blood vessels spread over the wall of the hemorrhage (Fig. 4, D and E). Taken together, our data suggested that pathological angiogenesis and constant bleeding of the tumor had occurred and that low-tumorigenic parental U251 MG cells became highly tumorigenic after VEGF overexpression.

Most interestingly, VEGF overexpression in NG-1 cells did not often produce hemorrhagic tumors. In the first of our two tumorigenicity studies, no Matrigel was used to enhance NG-1 tumor growth, because we assumed that overexpression of VEGF would be sufficient to promote host endothelium growth and, consequently, to form tumors. However, not a single NG-1 tumor was formed in this experiment (data not shown). Later, we mixed NG-1 cells with Matrigel and repeated the experiment where an additional cell line, NG-1/V121-C6 (Fig. 2A), was also tested for tumorigenicity. Except for two animals that received the admix injection, and were dead on the third day after implantation, all of the animals absorbed most of the liquid from the injected cell mixture so that the injected cell volume decreased substantially. Either very small tumors or no tumors were identified. A waiting time double than that of the U251 MG s.c. experiment was not sufficient to produce a single tumor larger than the volume we injected (Fig. 3B). Small pieces of the dissected tissues were sectioned and stained by H&E. We found that only one tissue that received NG-1 admix implantation contained a hemorrhagic structure (Fig. 4F). The rest of the dissected tissues were mostly mouse lymph nodes. Although VEGF expression was lower in the VEGF-transfected NG-1 cells than in VEGF-transfected U251 MG cells in general, VEGF production was higher in NG-1/V121-C6 cells than in U251 MG/V165-C3 cells (Fig. 2). Even so, no tumors were formed from implantation of NG-1/V121-C6 cells. In contrast, U251 MG/V165-C3 implantation resulted in large hemorrhagic tumors (Figs. 3A and 4A). These results suggest that unlike U251 MG cells, NG-1 cells lack certain positive factors or possess certain negative factors that are essential for their host blood vessel growth. EGFR levels were 36-fold lower in NG-1 cells than in U251 MG cells as determined by quantitative RT-PCR (Ref. 21; data not shown). Thus, EGFR could be one such factor.

High VEGF Expression by U251 MG Cells Implanted in Mouse Brains Causes Solid Tumors, Hemorrhages, and Shortened Survival. It is known that the endothelium in the brain differs from that in other parts of the body (22). The effect of s.c. implantation of
VEGF-transfected U251 MG cells on promotion of tumorigenicity and formation of hemorrhages was tested in an orthotopic xenograft mouse model. We wanted to determine whether $10^5$ VEGF$_{121}$- or VEGF$_{165}$-expressing U251 MG cells were sufficient to cause hemorrhages or solid tumors in mouse brains.

Overexpression of VEGF in U251 MG cells shortened the median survival of the mice (Fig. 5A). Eighty-nine days after injection with U251 MG/V165-C3 cells and 116 days after injection with U251 MG/V121-C2 cells, all of the mice reached the point for sacrifice; 60–80% of mice that received parental cell implantation were still alive. Although U251 MG/V165-C3 cells expressed less VEGF than did U251 MG/V121-C2 cells (Fig. 2B), mice receiving VEGF$_{165}$-overexpressing cells did not live as long (average, 70 days) as did mice receiving VEGF$_{121}$-overexpressing U251 MG cells (average, 85 days; Fig. 5A). This result suggests that VEGF$_{165}$ is more potent to the brain endothelium than is VEGF$_{121}$. The median survival for the U251 MG/pCIneo group was 104 days. At that point, half of the mice receiving parental cell injections were still alive.

Of the dissected brain sections examined by H&E staining no tumor growth was found in the U251 MG and U251 MG/pCIneo groups, except for one mouse from the U251 MG/pCIneo group that had a tumor outgrowth from the skull. In the U251 MG/V121-C2 and U251 MG/V165-C3 groups, large solid brain tumors were identified. In nearby areas, different size hemorrhages were found (Fig. 5, D and E), suggesting that it was much harder to increase blood supply through the brain endothelia than through the s.c. parts of the mouse body. Because VEGF-overexpressing NG-1 cells did not produce tumors in s.c. experiments, we assumed that they would not produce tumors in the tightly controlled brain environment and, thus, we did not perform those experiments.

**DISCUSSION**

The goal of this study is to investigate whether a low-tumorigenic cell line can be switched to a high tumorigenic through VEGF overexpression and to learn which isoform of two secreted VEGFs is more potent to this process. For this study, two glioma cell lines, U251 MG and NG-1, were selected, and clones exhibiting high VEGF expression were identified. The in vivo data presented here provided strong direct evidence that this switch is cell line dependent. The potency of isoform VEGF$_{121}$ and VEGF$_{165}$ to angiogenesis is tissue specific but synergistic.

It was reported that the implantation of VEGF-overexpressing primary myoblasts or U87 MG cells into the muscles or brains of nude mice led to hemangioma or hemorrhage formation (14, 15). In an earlier report, Ferrara et al. (16) found that VEGF expression did not promote transformation but conferred a growth advantage in vivo. The units used for describing VEGF overexpression and the cell numbers injected into a specific site differed greatly among sources, making data comparison difficult. To mimic an in vivo situation, we introduced a concept of 5-day VEGF accumulation (18). This was because angiogenesis requires chronic and sustained exposure to angiogenic factors, and the development of hemorrhage requires a threshold level of VEGF (14). Also, VEGF production is not consistent but increases as cell density increases (23). In addition, the onset of tumor cell-induced angiogenesis is 2–3 days, and the increase of tumor blood vessel formation is statistically significant 4–7 days after inoculation (24, 25).

Our findings suggest that VEGF overexpression alone may not be sufficient for certain cancer cells to generate solid or hemorrhagic tumors. It has been reported that neither the expression nor the exogenous administration of VEGF stimulates anchorage-dependent growth.
or anchorage-independent growth in CHO cells in vitro, but VEGF-
expressing CHO cells have the ability to proliferate s.c. and form
small solid tumors (16). However, in our study, VEGF overexpression
in NG-1 cells never conferred an advantage for in vivo proliferation
and never caused a single s.c. tumor. As observed in Fig. 2C, 107
VEGF-overexpressing NG-1 cells produced 9444–30,438 ng of
VEGF during the first 5 days of implantation. Daily averages were
higher than 200 ng, the threshold proposed by Cheng et al. (14) for
VEGF-overexpressing U87 MG cells to generate hemorrhages in nude
mouse brains. Our data suggest that other endothelial mitogenic
factors, such as VEGF-B, which forms heterodimers with VEGF and
is abundantly expressed in the heart and muscle but at low levels in
the brain (26), must be involved in solid or hemorrhagic tumor
formation. This could be one of the reasons why s.c. or i.p. implanta-
tion of primary myoblasts engineered for VEGF overexpression
causes hemorrhage (15, 27), whereas VEGF-overexpressing NG-1
cells in our study did not form tumors. A recent study found that a
transforming growth factor-α/epidermal growth factor receptor/EGFR auto-
crine pathway in part controls the production of angiogenic factors
and that treatment of an established xenograft GEO colon cancer
model with monoclonal antibody C225 (against EGFR) reduces tumor
growth (19). Besides low levels of EGFR expression in NG-1 cells, low levels of oncogene expression such as mutant K-ras
may be involved in NG-1 cells. It was reported that overexpression of VEGF121 in two mutant K-ras knockout cell lines, which were derived from two highly tumorigenic human colon
cancer cell lines, resulted in only weak restoration of tumorigenicity
(28, 29). This result suggests that VEGF overexpression alone in
certain cancer cells is not sufficient for a switch from a nontumori-
genic to a tumorigenic phenotype to occur. High expression levels of
angiogenic inhibitors such as maspin, a member of the serine protease
inhibitor (serpin) family, may also be involved in the low tumorige-
nicity of the NG-1 cells. High maspin expression is associated with
absence of lymph node metastasis and better rates of overall survival
in oral squamous cell carcinoma (30, 31).

On the other hand, we found that VEGF overexpression in low-
tumorigenic U251 MG cells promoted tumorigenicity. Evidence in-
cluded not only formation of large hemorrhagic s.c. tumors (Figs. 3A
and 4A) but also formation of solid tumors in the brain (Fig. 5, D and
E), probably because U251 MG cells expressed all of the factors
necessary for angiogenesis, but their expression levels were reduced
over years of passages. Thus, overexpression of a single positive
angiogenic factor such as VEGF may be sufficient to switch on an
original, highly tumorigenic phenotype, whereas this characteristic
may be absent in NG-1 cells. It is interesting to find differences in
gene expression patterns among glioma cell lines with significant
difference in tumorigenicity.

In the present study, we also observed that two VEGF isoforms
affected angiogenesis differently based on their spatial location.
VEGF121 was more potent to the s.c. endothelium (Fig. 3A) and
less potent to the brain (Fig. 5A) than VEGF165. The blood-brain barrier
and organ-specific differential expression of VEGF receptors could
have caused this difference. Neuruplin, one of the VEGF receptors, is
known to bind VEGF165 but not VEGF121 (3, 32). It is not known
whether brain endothelia express higher levels of neuruplin than do
endothelia from s.c. tissues, although it is known that 80% of VEGF
in the mouse brain is the VEGF164 isoform (homology of human
VEGF165) whereas in the skin, VEGF120 (homology of human
VEGF121) and VEGF164 expression levels are almost equal (33). The
presence of heparin and a heparin-sulfate binding domain in VEGF165
but not in VEGF121 (Fig. 1A) and the fact that brain is rich in
endothelium, which is the main site of heparin distribution (34), could
be another reason why VEGF165 is more potent than VEGF121 in the
brain. It is likely that the heparin-binding ability of VEGF165 provides
potential to influence the affinity of receptor binding, local concen-
tration, and stability of VEGF165. A number of studies have demon-
strated a great diversity of gene expression patterns from different
endothelia (22, 35). Similarly, we observed a synergistic effect of two
VEGF isoforms on angiogenesis (Figs. 3A and 4F). Three isoforms of
VEGF always coexist in different tissues (33). It has been demon-
strated in postnatal mice that the absence of both VEGF165 and
VEGF168 isoforms causes enlarged hearts, irregular heartbeatst, weak
heart contractions, and ischemic cardiomyopathy. Ultimately, the
mice that express VEGF120 only died of cardiac failure (36). How-
ever, it is very difficult to study whether VEGF isoforms differ in the
specificity and potency in angiogenesis in postnatal adults in vivo
because of the presence of endogenous VEGF isoforms. Our results
will help to elucidate further the functional differences between the
VEGF isoforms in various tissues.

Last, we found that VEGF-overexpressing U251 MG cell lines
might be useful for brain tumor studies. VEGF-overexpressing U87
MG cell lines grow and induce hemorrhage quickly, killing mice
within 2–3 days (14). Even with parental U87 MG cells, the average
survival of mice after implantation into the brain is ~3 weeks (37).
However, this is not long enough for drug studies in brain tumors. The
VEGF-overexpressing U251 MG cell lines we characterized induced
solid tumors and hemorrhages in mouse brains, but the mice lived ~80 days, which would give researchers more time to manage brain
tumor growth.

In conclusion, our results add a new dimension to understanding
the roles of VEGF overexpression in angiogenesis and tumorigenicity in
experimental and clinical settings. In experimental animals, tumor
growth is the ultimate result of guest/host interactions that involve
multiple gene function, in which a commitment of nutritional supply
from the host must be established. Otherwise, cancer cells will be
consigned to dormancy and will be eradicated, as seen after implanta-
tion of VEGF-overexpressing NG-1 cells. Likewise, in clinical set-
tings hypoxia, which up-regulates VEGF expression at the transcrip-
tional and posttranscriptional levels (9–12), may be established at a
certain stage of the interaction between a small malignant tumor mass
and its surrounding environment. A tumor with low tumorigenicity
such as a U251 MG cell-derived tumor may be switched to an
aggressive and metastatic tumor because of VEGF overexpression.
Thus, VEGF overexpression in cancer patients may become one
signal for malignant progression, but up-regulation of VEGF may not
be sufficient for promoting all cancers.

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