

# Hypoxia and Acidosis Independently Up-Regulate Vascular Endothelial Growth Factor Transcription in Brain Tumors *in Vivo*<sup>1</sup>

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## Abstract

Hypoxia and acidosis are hallmarks of tumors as well as critical determinants of response to treatments. They can upregulate vascular endothelial growth factor (VEGF) *in vitro*. However, the relationship between tissue oxygen partial pressure (pO<sub>2</sub>)/pH and VEGF transcription *in vivo* is not known. Thus, we developed a novel *in vivo* microscopy technique to simultaneously measure VEGF promoter activity, pO<sub>2</sub>, and pH. To monitor VEGF expression *in vivo*, we engineered human glioma cells that express green fluorescent protein (GFP) under the control of the VEGF promoter. These cells were implanted into the cranial windows in severe combined immunodeficient mice, and VEGF promoter activity was assessed by GFP imaging. Tissue pO<sub>2</sub> and pH were determined by phosphorescence quenching microscopy and ratio imaging microscopy, respectively. These techniques have allowed us to show, for the first time, that VEGF transcription in brain tumors is independently regulated by the tissue pO<sub>2</sub> and pH. One week after tumor implantation, significant angiogenesis was observed, with increased GFP fluorescence throughout the tumor. Under hypoxic or neutral pH conditions, VEGF-promoter activity increased, with a decrease in pO<sub>2</sub> and independent of pH. Under low pH or oxygenated conditions, VEGF-promoter activity increased, with a decrease in pH and independent of pO<sub>2</sub>. In agreement with the *in vivo* findings, both hypoxia and acidic pH induced VEGF expression in these cells *in vitro* and showed no additive effect for combined hypoxia and low pH. These results suggest that VEGF transcription in brain tumors is regulated by both tissue pO<sub>2</sub> and pH via distinct pathways.

## Introduction

Hypoxia and acidosis are common characteristics of solid tumors (1–5). Both pO<sub>2</sub><sup>3</sup> and pH are important determinants of tumor growth, metabolism, and response to conventional and novel therapeutics (1, 4–6). VEGF is one of the most potent angiogenic factors. VEGF is expressed in a wide variety of tumors and is correlated with angiogenesis, tumor growth, invasion, metastasis, and prognosis (7). Hypoxia upregulates VEGF via HIF in various cells *in vitro* (8). Earlier studies, such as VEGF expression in the perinecrotic region or the center of multicellular tumor spheroids, suggest that hypoxia upregulates VEGF *in vivo* (7). However, we and others found a lack of spatial correlation between hypoxia and VEGF expression (9, 10). Compared with pO<sub>2</sub>, VEGF regulation by pH is not well understood. Acidic pH can induce VEGF expression in cultured vascular endothelial cells and tumor cells (11, 12). However, the effect of low pH

on VEGF expression *in vivo* is not known. In this study, we determined the relationship between tissue pO<sub>2</sub>/pH and VEGF expression *in vivo* using novel, noninvasive imaging techniques. To monitor VEGF expression *in vivo*, we engineered human glioma cells that express GFP under the control of the VEGF promoter, and we monitored VEGF-promoter activity by GFP imaging (13, 14). Tissue pO<sub>2</sub> and pH were determined by phosphorescence quenching microscopy and ratio imaging microscopy, respectively (3). These techniques have allowed us to show, for the first time, that VEGF transcription in brain tumors is independently regulated by the tissue pO<sub>2</sub> and pH.

## Materials and Methods

**Tumors.** Human tumor cell lines U87 MG, MU89, and LS174T, were maintained in DMEM supplemented with 10% FBS at 37°C in a humidified CO<sub>2</sub> atmosphere. For hypoxia and acidic pH studies, serum-free medium (BioWhittaker, Rockland, ME) was used. The medium's pH was adjusted with 20 mM 2-(*N*-morpholino) ethane-sulfonic acid, and 20 mM Tris (hydroxymethyl) aminomethane and 1% O<sub>2</sub>-5% CO<sub>2</sub>-balance N<sub>2</sub> was used for hypoxia.

**Northern Blot Analysis.** Messenger RNA was extracted using the Fast-Track mRNA isolation kit (Invitrogen, San Diego, CA). Messenger RNA (2 μg/lane) was fractionated on a 1.0% denaturing formaldehyde/agarose gel, electrotransferred at 0.6 amp to GeneScreen nylon membrane (DuPont Col, Boston, MA), and UV cross-linked with a UV-Stratalinker 1800 (Stratagene, La Jolla, CA). VEGF and the β-actin cDNA probe were synthesized by PCR using primers for VEGF: 5'-TCC GGA TCC ATG AAC TTT C-3', and 5'-TGG CTC ACC GCC TTG GCT-3'; and for β-actin: 5'-TGT ATG CCT CTG GTC GTA CC-3', and 5'-CAA CGT CAC ACT TCA TGA TGG-3'. The GFP gene (600 bp) was obtained as a Hind III-Not I fragment from the VEGF-GFP construct described previously (13). The cDNA probes were radiolabeled with the use of the random-prime labeling technique with [<sup>32</sup>P]dCTP (15).

**ELISA.** The VEGF protein level in cultured medium was analyzed using the Quantikine VEGF ELISA kit (R&D Systems, Minneapolis, MN) following the manufacturer's protocol.

**Introduction of GFP Vectors into Tumor Cells.** The generation of VEGF-GFP construct was described previously (13). A 25-μg linearized VEGF-GFP construct was introduced into U87 MG cells by electroporation. The DNA construct was mixed with 10<sup>6</sup> cells in PBS and incubated on ice for 10 min. Then, the cells were electroporated with 400 V, 25 μF pulse (Gene Pulser; Bio-Rad Laboratories, Hercules, CA). After electroporation, the cells were incubated on ice for 10 minutes and thereafter plated at a density of 10<sup>5</sup> cells/100-mm dish in culture medium with 10% FBS. After 48 h, the medium was supplied with puromycin at a concentration of 1 μg/ml, and stable clones were isolated after a 10-day selection.

**FACS Analysis.** U87-VC2 cells were prepared for cytometry by trypsinization, washing in PBS, and fixation with 2% formaldehyde. The fluorescence profile in the scatter gate corresponding to viable tumor cell was analyzed by Coulter Epics flow cytometer (EPICS XL-MCL; Miami, FL).

**Luciferase Reporter Gene Assays.** The VEGF promoter (13) was subcloned into the peak12 luciferase reporter gene vector. LS174T, MU89, and U87 MG cells (3 × 10<sup>5</sup>) were plated in 10-cm-diameter culture dishes 24 h before transfection. The cells were cotransfected with 20 μg of the VEGF-firefly luciferase construct and 2 μg of pRL-TK/plate using the calcium

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<sup>3</sup> The abbreviations used are: pO<sub>2</sub>, oxygen partial pressure; VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; GFP, green fluorescent protein; FBS, fetal bovine serum; CCD, charged coupled device; SCID, severe combined immunodeficient; BCECF, 2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein; NFκB, nuclear factor κB.

phosphate method. pRL-TK, obtained from Promega (Madison, WI) contains the Herpes simplex virus thymidine kinase promoter region upstream of *Renilla luciferase* and was used as an internal control for transfection efficiency. Sixteen h later, the plates were washed three times with PBS and incubated for 6 h in MEM containing 10% FBS and then subdivided and plated into two 38-mm-diameter dishes, which were incubated until the cultures became confluent. Then the cells were exposed for 6 h to neutral or acidic pH conditions. Cell lysates were prepared using the Dual Luciferase Assay System (Promega). The light intensity was measured on 20  $\mu$ l of cell lysates using a luminometer (Turner Designs, Sunnyvale, CA).

**Cranial Window.** The cranial window was implanted in SCID mice 8–10 weeks of age bred and maintained in the defined flora animal facility in Edwin L. Steele Laboratory (Boston, MA), as described (16). Seven to 10 days later, a small piece (1 mm in diameter) of U87-VC2 tumor was implanted in the center of the window. For intravital microscopy, the animals were anesthetized and put on a polycarbonate plate with the head fixed.

**Intravital Microscopy Work Station.** The workstation consisted of an upright microscope (Zeiss Axioplan; Oberkochen, Germany) equipped with transillumination and fluorescence epi-illumination, a flashlamp excitation device (EG&G, Salem, MA), two independent outlet ports, two separate eye-piece units, a motorized X-Y stage with a  $\pm 1.0$ - $\mu$ m lateral resolution (Burleigh Instruments, Fishers, NY), a set of optical filters, a motor-controlled filter wheel, an intensified CCD camera (C2400-88; Hamamatsu, Bridgewater, NJ), a video monitor (Sony, Montvale, NJ), a photomultiplier tube (model 9203B; Products for Research, Inc., Danvers, MA), a dual-trace digital oscilloscope (model TDS-320; Tektronix, Beaverton, OR), a video recorder (SVO-9500MD; Sony), and a frame-grabber board (Data Translation, Marlboro, MA) for image digitization on a PC computer (Compaq, Houston, TX).

**VEGF Promoter Activity via GFP Imaging.** GFP fluorescence intensities (509 nm) in U87-VC2 tumors were imaged through the intensified CCD camera port with excitation at 488 nm. An optimal configuration with a sampling depth of  $\leq 25$   $\mu$ m, an adequate signal:noise ratio, and a lateral spatial resolution of  $5 \times 5$   $\mu$ m<sup>2</sup> was obtained using a 400- $\mu$ m pinhole in the light excitation pathway and a  $\times 40$  water-immersion objective (3). Tumor locations was selected and stored for subsequent profile measurements using the computer-assisted X-Y stage controller. Tissue autofluorescence levels were determined by the imaging of U87 MG tumors. By imaging known concentrations of recombinant GFP protein (EGFP; Clontech, Palo Alto, CA) in capillary tubes, the GFP calibration curve was obtained and used for the calculation of instantaneous GFP concentrations from GFP fluorescence intensities.

**High-resolution Interstitial pH Measurements.** Fluorescence ratio imaging microscopy of pH, its implementation, application to thick tissues, and calibration, were performed as described (3, 17). The cell-impermeant form of the pH-sensitive fluorochrome BCECF (0.7 mg/kg i.v.; Molecular Probes, Eugene, OR) was used. Emission intensities (570 nm) were imaged through the CCD camera port with sequential excitations at 440 and 495 nm. The X-Y stage was cycled through the same locations used for GFP measurements (3).

**Interstitial pO<sub>2</sub> Measurements.** Tissue pO<sub>2</sub> was measured based on the O<sub>2</sub>-dependent phosphorescence quenching of albumin-bound palladium meso-tetra (4-carboxyphenyl) porphyrin (60 mg/kg; Harvard Apparatus, Holliston, MA), as described (3). The phosphorescence signal resulting from flashlamp excitation (540 nm) of the tissue was detected at  $\geq 630$  nm using the photomultiplier tube and averaged on the oscilloscope before computer storage. The phosphorescence decay data were converted to pO<sub>2</sub> values according to a standard calibration method (3, 18). The X-Y stage was cycled through the same tumor locations used for the VEGF-GFP and pH measurements.

**Statistical Analysis.** The relationship between tissue pO<sub>2</sub>/pH and VEGF-promoter activity in U87-VC2 tumors was analyzed by linear regression using StatView (SAS Institute, Inc., Cary, NC).  $P < 0.05$  is considered to be statistically significant.

## Results and Discussion

**Acidic pH Induces VEGF mRNA and Protein in Various Tumor Cell Lines.** Acidic pH is a common characteristic of solid tumors, although its cause and consequences are still not clearly understood (2). It is reported to induce various angiogenic molecules

such as basic fibroblast growth factor, interleukin 8, nitric oxide, and VEGF in cultured cells (11, 15, 19). First, we confirmed that acidic extracellular pH induces VEGF mRNA expression and protein synthesis in various tumor cell lines *in vitro* by Northern blot and ELISA, respectively. We examined three different types of human tumors, LS174T colon adenocarcinoma, Mu89 melanoma, and U87 MG glioma. All three human tumor lines showed increased VEGF expression under acidic pH culture conditions in a pH-dependent manner (Fig. 1A). Increased VEGF level became apparent 2 h after the acidic pH exposure (Fig. 1B). Accumulation of VEGF protein in acidic culture media became significant 8 h after the low-pH treatment (Fig. 1C and 1D). In agreement with human tumor lines such as pancreas, ovarian, colon and prostate carcinoma (12) and vascular endothelial cells (11), we found increased VEGF expression by acidic extracellular pH in all three different tumor lines tested.

**VEGF Promoter Construct Responds to Hypoxia and Acidic pH.** A prerequisite to measure the spatial correlation between tissue pO<sub>2</sub>, pH, and VEGF promoter activity *in vivo* is the establishment of a technique that allows us to monitor these three parameters simultaneously and noninvasively. To accomplish this, we engineered a GFP construct driven by the VEGF promoter (13). Once this gene construct is stably transfected into the cells, VEGF promoter activity can be visualized as a fluorescence of GFP. We transfected the VEGF-GFP construct into U87 MG glioma by electroporation and selected stable clones. When grown *in vivo*, these clones emitted bright green fluorescence (Fig. 2A). To validate the system, we tested GFP inducibility by hypoxia (1% O<sub>2</sub>) in the brightest clone, U87-VC2. U87-VC2 cells were cultured in either normoxia or 1% oxygen. The fluorescence of

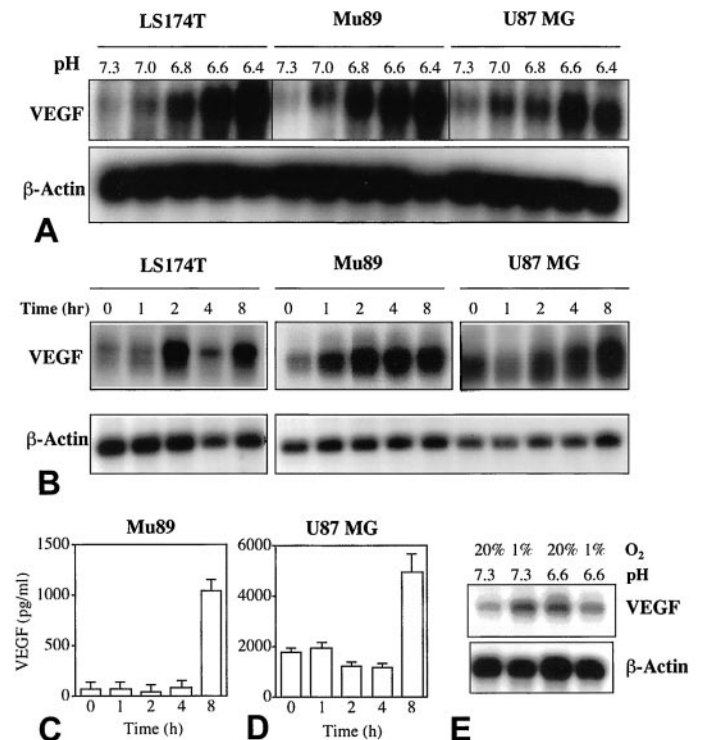


Fig. 1. Induction of VEGF expression by acidic pH and hypoxia. A, Northern blot for VEGF mRNA in three human tumor cell lines 6 h after exposure to different pHs. Acidic pH upregulates VEGF transcription in all three cell lines tested in a pH-dependent manner. B, time-course change in VEGF mRNA in three tumor lines after exposure to pH 6.6. VEGF expression became apparent by 2 h after the acidic pH culture. C and D, VEGF protein level in culture medium of Mu89 (C) and U87 MG (D) tumor cells after the exposure to pH 6.6. VEGF protein level became significantly high by 8 h after the acidic pH culture. E, effect of low pH and hypoxia on VEGF mRNA expression. Both acidic pH (pH 6.6) and hypoxia (1% O<sub>2</sub>) increases the VEGF mRNA level; however, there was no additional increase in the VEGF mRNA level by combination of hypoxia and acidic pH.

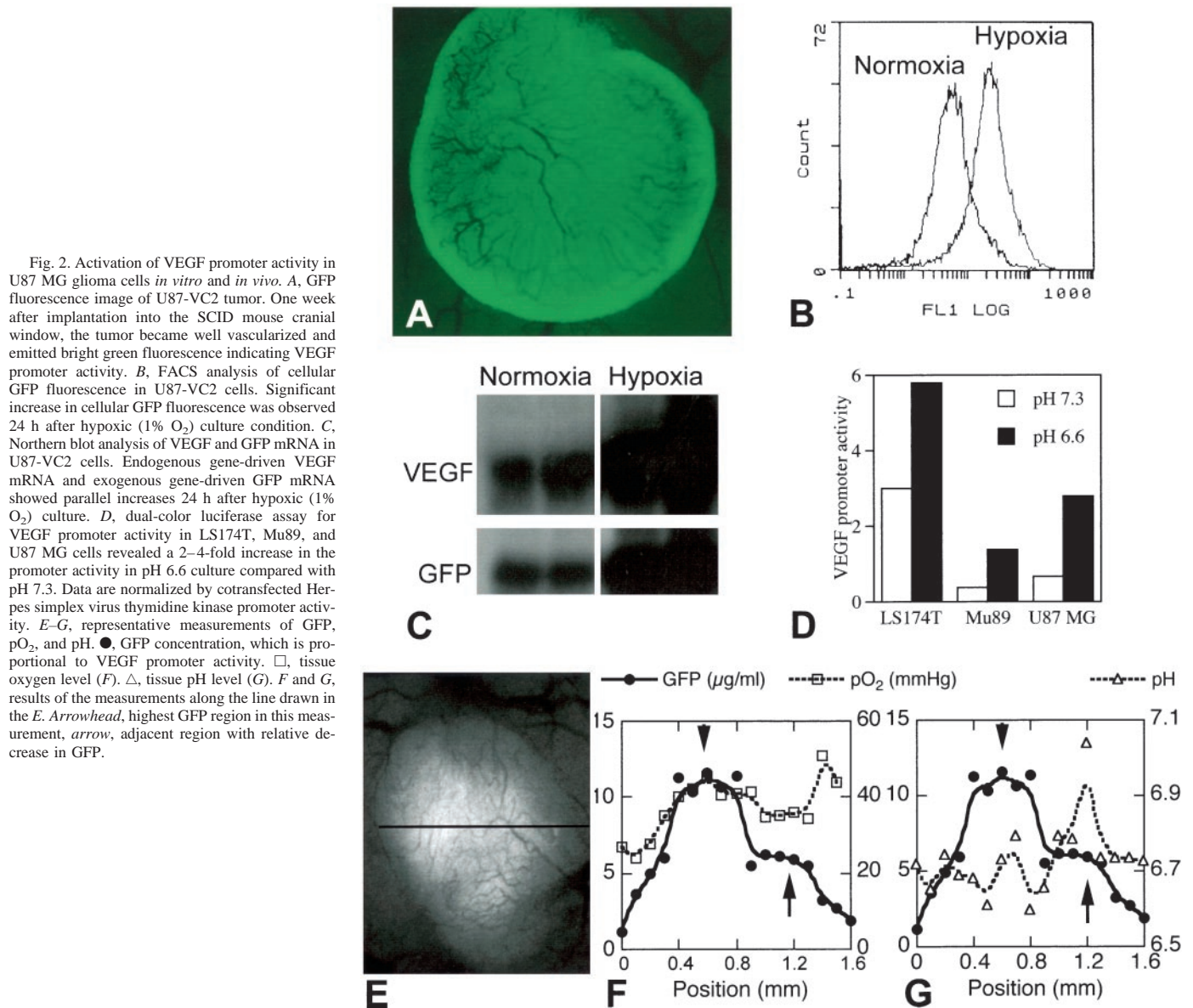


Fig. 2. Activation of VEGF promoter activity in U87 MG glioma cells *in vitro* and *in vivo*. **A**, GFP fluorescence image of U87-VC2 tumor. One week after implantation into the SCID mouse cranial window, the tumor became well vascularized and emitted bright green fluorescence indicating VEGF promoter activity. **B**, FACS analysis of cellular GFP fluorescence in U87-VC2 cells. Significant increase in cellular GFP fluorescence was observed 24 h after hypoxic (1% O<sub>2</sub>) culture condition. **C**, Northern blot analysis of VEGF and GFP mRNA in U87-VC2 cells. Endogenous gene-driven VEGF mRNA and exogenous gene-driven GFP mRNA showed parallel increases 24 h after hypoxic (1% O<sub>2</sub>) culture. **D**, dual-color luciferase assay for VEGF promoter activity in LS174T, Mu89, and U87 MG cells revealed a 2–4-fold increase in the promoter activity in pH 6.6 culture compared with pH 7.3. Data are normalized by cotransfected Herpes simplex virus thymidine kinase promoter activity. **E–G**, representative measurements of GFP, pO<sub>2</sub>, and pH. ●, GFP concentration, which is proportional to VEGF promoter activity. □, tissue oxygen level (**F**). △, tissue pH level (**G**). **F** and **G**, results of the measurements along the line drawn in the **E**. Arrowhead, highest GFP region in this measurement, arrow, adjacent region with relative decrease in GFP.

individual cells was significantly increased by hypoxia, as demonstrated by the fluorescence-activated cell sorter profile (Fig. 2B). We also determined mRNA level of *GFP* (exogenous construct) and *VEGF* (endogenous gene) in U87-VC2 cells by Northern blot analysis. Both GFP and VEGF mRNA levels showed parallel increases by hypoxia (Fig. 2C). VEGF promoter activity was also induced by acidic pH as determined by transient gene transfer and dual color luciferase assay (Fig. 2D). Thus, we confirmed that tumors bearing the *VEGF-GFP* construct can be used to assess VEGF promoter activity *in vivo*.

**Acidic pH Region Shows Increased VEGF Promoter Activity *in Vivo*.** On the basis of *in vitro* results, we used U87-VC2 tumors grown in SCID mouse cranial windows. The cranial window provides an orthotopic microenvironment for glioma. Seven to 8 days after implantation, tumors were well vascularized, became approximately one-half the size of the window, and were brightly fluorescent (Fig. 2, A and E). GFP fluorescence in U87-VC2 tumor cells was visualized by fluorescence microscopy, and GFP fluorescence intensity was translated into instantaneous GFP concentration using a calibration curve generated from various known quantities of recombinant GFP. To measure pH and pO<sub>2</sub> profiles with high spatial resolution, we used

two noninvasive optical techniques: fluorescence ratio imaging microscopy, using pH sensitive BCECF, and phosphorescence-quenching microscopy, using oxygen sensitive porphyrine probe, respectively (3). The partial confocal effect was obtained using a pinhole in the light path. A computer-assisted stage controller allowed us to repeat measurements in the same region for multiple parameters. Using these techniques, we succeeded in quantifying GFP fluorescence intensity, pO<sub>2</sub>, and pH with high spatial resolution.

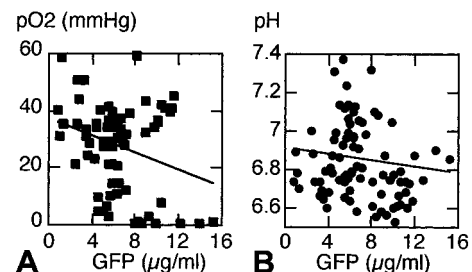


Fig. 3. Overall relationship between VEGF promoter activity and tissue oxygen or pH level. GFP concentration inversely correlated with tissue pO<sub>2</sub> ( $P = 0.014$ ; A) but not with pH ( $P = 0.33$ ; B).

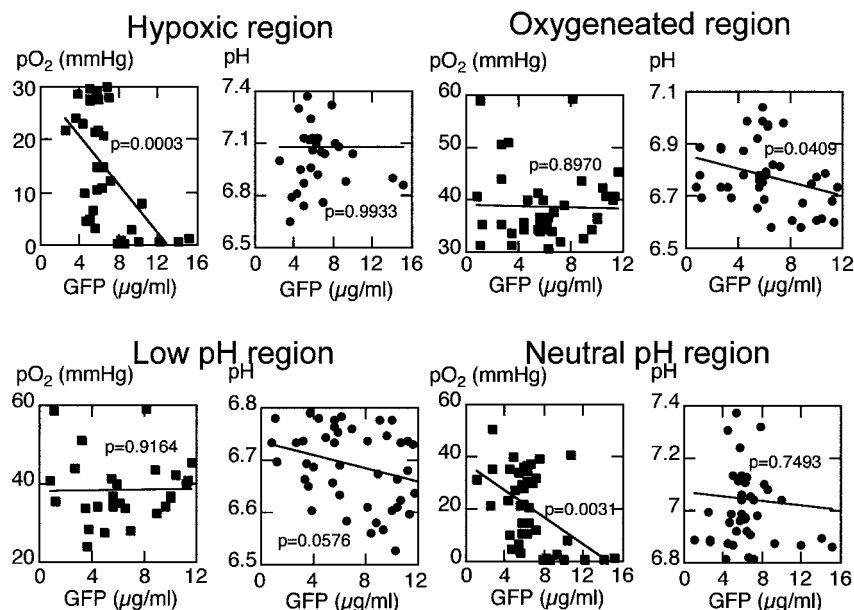


Fig. 4. Regional relationship between VEGF promoter activity, tissue pO<sub>2</sub>, and extracellular pH. The data presented in Fig. 3 was divided further into either hypoxic (*top left*, pO<sub>2</sub> < 30 mmHg) or oxygenated (*top right*, pO<sub>2</sub> > 30 mmHg) region and either low pH (*bottom left*, pH < 6.8) or neutral pH (*bottom right*, pH > 6.8) region. Each data set was analyzed using linear regression analysis. Significant inverse correlation was found between tissue pO<sub>2</sub> and VEGF promoter activity in hypoxic and neutral pH regions. Tissue pH level tends to relate with VEGF promoter activity in oxygenated and low pH regions.

Fig. 2E shows representative measurements in one of the U87-VC2 tumors. We scanned across the tumor in 100- $\mu$ m increments (Fig. 2E). First, we measured GFP fluorescence intensity and background fluorescence for pH measurement. Then, we injected the BCECF probe and measured fluorescence excited by pH-sensitive and -insensitive wavelengths. Finally, we injected the porphyrin probe and measured phosphorescence decay after strobe-light excitation for pO<sub>2</sub> measurement. The accuracy of the stage controller is 0.1  $\mu$ m. Thus, we can observe exactly the same location for three different measurements: GFP, pO<sub>2</sub>, and pH. Contrary to widely accepted hypotheses, the region with strongest GFP signal (*arrowhead*) in this tumor was not hypoxic (Fig. 2F). In fact, this tumor was relatively well oxygenated throughout the measurement track, ranging from 20 to 60 mmHg. On the other hand, the strong GFP region showed low tissue pH (Fig. 2G). The adjacent region (*arrow*) showed a relative decrease in GFP and an increase in pH with no change in pO<sub>2</sub>. These results suggest that acidic pH induces VEGF promoter activity *in vivo*. However, GFP, pO<sub>2</sub>, and pH profiles were heterogeneous within the tumor as well as between the tumors.

**Under Hypoxic or Neutral pH Conditions, VEGF Promoter Activity Increased with a Decrease in pO<sub>2</sub> and Independent of pH.** To determine the relationship between VEGF promoter activity and tissue oxygen and/or pH level, we analyzed all measurements (93 regions in eight tracks in five animals) using linear regression. There was relatively weak but statistically significant correlation between the tissue oxygen level and VEGF promoter activity (Fig. 3A). On the other hand, there was no correlation between tissue pH and VEGF promoter activity (Fig. 3B). Median tissue pO<sub>2</sub> was 32.3 mmHg, and median pH was 6.79.

Although both hypoxia and acidic pH are frequently observed in solid tumors, intravital measurements with high spatial resolution showed no correlation between pO<sub>2</sub> and pH in individual regions (3). Furthermore, recent studies showed a lack of correlation between tissue oxygen/redox status and VEGF expression (9, 10). To understand the lack of overall correlation between VEGF promoter and pO<sub>2</sub>/pH, we divided the data into four groups: hypoxic (pO<sub>2</sub> < 30 mmHg), oxygenated (pO<sub>2</sub> > 30 mmHg), low pH (pH < 6.8), and neutral pH (pH > 6.8). This allowed comparison of VEGF promoter activity under each of these conditions. In the hypoxic region, tissue pO<sub>2</sub> was inversely correlated with VEGF promoter activity

( $P = 0.0003$ ), whereas tissue pH showed no correlation with VEGF promoter activity (Fig. 4, *top left*). On the other hand, there was significant inverse correlation between tissue pH and VEGF promoter activity ( $P = 0.041$ ) in the relatively oxygenated region and no correlation between tissue pO<sub>2</sub> and VEGF promoter activity in this region (Fig. 4, *top right*).

**Under Low pH or Oxygenated Conditions, VEGF Promoter Activity Increased with a Decrease in pH and Independent of pO<sub>2</sub>.** In the low pH region, there was a tendency of higher VEGF promoter activity with lower pH ( $P = 0.058$ ) and no correlation between VEGF promoter activity and pO<sub>2</sub> (Fig. 4, *bottom left*). On the other hand, there was a significant inverse correlation ( $P = 0.003$ ) between tissue pO<sub>2</sub> and VEGF promoter activity in neutral pH regions, and no correlation between tissue pH and VEGF promoter activity in this region (Fig. 4, *bottom right*). Hypoxia and acidic pH seemed to induce VEGF expression in tumor cells via distinct pathways.

**No Additive Effect of Acidic pH and Hypoxia in VEGF Expression *in Vitro*.** We confirmed the lack of synergism by combining hypoxia and acidic pH using an *in vitro* system. Although both hypoxia (1% O<sub>2</sub>) and acidic culture media (pH 6.6) induced VEGF expression, there was no additional increase in VEGF mRNA when we combined hypoxia and acidic pH (Fig. 1E). Hypoxia increases HIF 1 $\alpha$  protein stability and transcriptional activity (8). Under hypoxic conditions, HIF-1 heterodimer complex binds to the hypoxia response element in the VEGF promoter and induces its transcription. On the other hand, acidic pH does not increase HIF-1 $\alpha$  or its binding activity to the hypoxia response element (15). Acidic pH induces IL-8 (15) and inducible nitric oxide synthase (19) via NF $\kappa$ B, and NF $\kappa$ B mediates murine VEGF up-regulation (20). Shi *et al.* reported that transient exposure of acidic pH increases NF $\kappa$ B binding activity to the VEGF gene in human pancreatic carcinoma cells (12). Thus, the mechanisms of VEGF upregulation by hypoxia and acidic pH are different.

Because tumor microenvironment is heterogeneous, capability of VEGF induction by both hypoxia and acidic pH may potentiate tumor growth by recruiting blood vessels more effectively. In other words, tumor can be more aggressive if either hypoxia or acidic pH induces genes such as VEGF. Tumors consist of not only neoplastic cells but also non-neoplastic host stromal cells. We have shown that host stromal cells express significant amounts of VEGF in the tumors grown in dorsal skin chambers (13, 21). However, stromal cell VEGF

promoter activity was not apparent when we grew U87 MG tumors in the cranial window of the VEGF-GFP transgenic mice (data not shown). In fact, there are not many stromal cells in this tumor when grown in the cranium (22).

It is not clear why there is no additive effect on VEGF promoter activity with the combination of hypoxia and acidic pH. Most tumor cells maintain intracellular pH at a neutral level despite low extracellular pH (23). However, tumor cells may not be able to maintain transmembrane proton gradient under hypoxic and acidic conditions because of severe nutrient/energy deficiency. Thus, intracellular pH decreases and, subsequently, the cells undergo apoptosis (24). Hypoxia also induces apoptosis via HIF-1 $\alpha$  (14, 25, 26). The same microenvironmental stress can induce both pro- and antitumor events. A single stress may predominantly induce survival factors, whereas multiple stresses may lead to cell death. Additional studies are warranted because understanding of these mechanisms will provide tumor-specific treatment strategies.

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