Mapping of the Vascular Endothelial Growth Factor-producing Hypoxic Growth in Multicellular Tumor Spheroids Using a Hypoxia-specific Marker


ABSTRACT

We have investigated the hypoxia-inducibility of vascular endothelial growth factor (VEGF) in multicellular tumor spheroids of HT29 cells using a monoclonal antibody to a fluorinated bioreductive drug, EF5 [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide], a chemical probe for hypoxia. We have shown that VEGF expression is predominantly localized in interior spheroid cells that are sufficiently hypoxic to bioreductively activate the 2-nitroimidazole and produce immunologically detectable adducts of the EF5 compound. Northern blotting analyses demonstrated that VEGF165 is the predominant form of VEGF produced by HT29 cells and that the phorbol ester 12-O-tetradecanoylphorbol-13-acetate did not induce VEGF expression. This study demonstrates that VEGF expression is up-regulated in response to hypoxia and in the microenvironments found in human multicellular tumor spheroids. This investigation also illustrates the utility of the EF5 binding in multicellular tumor spheroids as a means of studying the expression and regulation of hypoxia-inducible genes.

INTRODUCTION

Angiogenesis plays a crucial role in both normal and pathological processes, including embryonic and fetal organ development, wound healing, diabetic retinopathy, and tumor progression (1–4). Tumor angiogenesis is regulated by both positive and negative modulators released from tumor cells or host cells recruited to the area (5). A number of polypeptides are known to be angiogenic in vivo (4). In particular, VEGF, also known as vascular permeability factor, acts directly on endothelial cells to promote angiogenesis in vivo (6, 7). A monoclonal antibody specific for VEGF has been shown to suppress the growth of some human tumors in vivo (8).

VEGF is a disulfide-bonded homodimeric protein that is expressed as four isoforms because of alternative mRNA splicing (9–11). The isoforms, VEGF206, VEGF189, VEGF165, and VEGF121, are composed of monomers of 206, 189, 165, and 121 amino acids in length, respectively (9–11). VEGF121 and VEGF165 are soluble proteins while VEGF189 and VEGF206 are bound to extracellular heparin-containing proteoglycans (12). Substantial variation in the expression pattern of VEGF has been reported (11, 13). Although the specific biological significance of each protein is not known, VEGF206 is rare and VEGF165 is the most abundant isoform (11).

VEGF expression is induced under hypoxic conditions in vitro (6). The mRNA levels of VEGF increase dramatically when cell cultures are exposed to hypoxia, and return to background levels when the cells are transferred to normal oxygen tensions (6). However, direct evidence for the induced expression of VEGF under hypoxic conditions in vivo has not been demonstrated. In situ hybridization experiments have shown specific expression of VEGF mRNA in the periphery of the necrotic (and presumably hypoxic) regions of glioblastoma tumors (6). These experiments have also shown that capillary bundles are preferentially clustered alongside VEGF-producing cells in the tumor.

Because of the complex structure of primary tumors, it is often difficult to distinguish hypoxia-induced gene expression from the effects of necrosis or other tumor microenvironments. In this study, we have used multicellular tumor spheroids of a human colon carcinoma cell line and a new hypoxia-specific bioreductive compound to demonstrate a direct relationship between hypoxia and the enhanced expression of VEGF. Spheroids are three-dimensional tissue structures that are grown in suspension cultures or on nonadhesive surfaces (14). They represent homogeneous cellular populations that can be grown to different sizes with or without necrotic centers. An antibody to a fluorinated bioreductive compound, EF5 [2-(2-nitro-1H-imidazol-1-yl)-N-(2,2,3,3,3-pentafluoropropyl)acetamide], was used as a positive control to detect hypoxic cells (15). EF5 is a nitroimidazole that can be biochemically reduced resulting in a product (or products) that bind covalently to cellular macromolecules. This process is progressively inhibited by increasing oxygen tensions. Therefore, the intensity of the EF5 binding signal can be used to estimate the degree of hypoxia present in a tissue. EF5 can be used to detect areas of very low oxygen as long as the hypoxic signal remains above that arising from background adduct formation (15). Factors that might contribute to oxygen-independent binding include the presence of high levels of two electron reductases such as DT-diaphorase [NADPH:quinone oxidoreductase] and the intracellular accumulation of EF5 because of limited excretion (16). Although EF5 presents some of the problems associated with the aerobic binding of other 2-nitroimidazoles (16), fluorescent antibody studies with ELK3.51-Cy3 have correlated localized fluorescent fluorescence within tumor and spheroid sections with absolute measures of oxygen tensions (17, 18). ELK3.51-Cy3 has been used to quantify EF5 adduct formation in EMT6 spheroids and to show that EF5 binding is directly related to radiobiological hypoxia (17). EF5 also demonstrates uniform oxygen-dependent binding in cell lines previously shown to exhibit heterogeneous binding of the 2-nitroimidazole misonidazole (15).

ELK3.51-Cy3 is a fluorochrome-conjugated monoclonal antibody to the drug adducts. This single protein detection system has several advantages including: (a) minimizing the size of the reporter molecules; (b) allowing the optimization of binding; (c) providing a detection technique which should give a signal directly proportional to the amount of antibody present; and (d) allowing the use of flow cytometry to analyze the distribution of signal within cells of a heterogeneous population.

The results described in this article demonstrate that VEGF mRNA expression was induced under hypoxic expression in human HT29 colon carcinoma cells and that expression was largely restricted to the regions that bound the reduced EF5 molecule. The results of an independent study using the spheroid model has recently been reported showing...
unstained sections of the spheroids photographed under phase-contrast (A and B) and VEGF antibody (C and D) and with ELK3-5 I (E and F) after EF5 treatment. The diameter, 105 mm; height, 205 mm). The spinner flasks are designed such that grown in the same medium in dishes coated with 2% Difco agar (prepared in and collected by centrifugation. To grow spheroids, cells (—5 X 10^5) were supplemented with 10% iron-fortified fetal bovine serum and grown to confluency at 37°C in 5% CO2. EMT6 mouse mammary carcinoma cells were seeded onto 10-cm in diameter tissue culture dishes in DMEM medium (19).

**EXPRESSION OF VEGF IN SPHEROIDS**

**MATERIALS AND METHODS**

**Monolayer and Spheroid Cultures.** HT29 cells (~2.5 X 10^5) were seeded onto 10-cm in diameter tissue culture dishes in DMEM medium supplemented with 10% iron-fortified fetal bovine serum and grown to confluence at 37°C in 5% CO2. EMT6 mouse mammary carcinoma cells were grown in BME medium containing 10% FCS. Cells were treated with trypsin and collected by centrifugation. To grow spheroids, cells (~5 X 10^5) were grown in the same medium in dishes coated with 2% Difco agar (prepared in medium without serum) and incubated at 37°C in 5% CO2. After 4 days, small spheroids (100–200 μm in diameter) were separated from single cells by sedimentation and placed in 300 ml medium in 500-ml spinner flasks (base diameter, 105 mm; height, 205 mm). The spinner flasks are designed such that gaseous exchange between the medium and gas phase is facilitated and gaseous equilibrium between the two phases can be attained quickly. The magnetic spinners in the flasks were spun at 100 rpm in a 37°C warm room, and the medium was changed every 48 h. The growth of the spheroids was estimated by measuring the diameter of 30 spheroids using a graticule in an inverted microscope.

**Hypoxia Chamber Protocol.** Hypoxia experiments were performed according to a protocol established in our laboratory (20). Cells in Petri dishes were placed inside specially designed chambers attached to a 5% CO2-N2 manifold on a vacuum line. The chambers were bathed in a circulating water bath kept at 37°C. Atmospheric oxygen was extracted at room temperature over 2 h by 10 cycles of pumping to a fixed pressure followed by filling with 5% CO2-N2 (pO2 < 0.02%). The end of the pumping cycles was chosen as O-h hypoxia. The oxygen concentration within the manifold and the chambers was measured with a polarographic oxygen electrode (Controls Katharobic, Gulph Mills, PA) attached to a test chamber. The chambers were sealed by closing the valves to the manifold, and the cells were incubated for various times in a 37°C incubator. Spheroids in the spinner flasks were gassed with 5%CO2-N2 and incubated at 37°C for indicated time periods.

**Immunohistochemistry.** HT29 spheroids were fixed in Streck Tissue Fixative (Streck Laboratories, Inc., Omaha, NE) for 4 h at room temperature. Fixation was followed by 6 h in 15% sucrose in PBS at 4°C. Spheroids were embedded in OCT compound (Miles, Elkhart, IN) and immediately frozen in liquid nitrogen. Cryostat sections were cut at 10–12 μm, placed on Fisher Plus slides (Fisher Scientific, Pittsburgh, PA), and stored in air-tight boxes at ~80°C until use. The sections were allowed to return to room temperature before opening the boxes. EMT6 spheroid sections were fixed in ~20°C acetone for 10 min and air dried. All sections were blocked in 5% HS in PBS for 15 min at room temperature. A polyclonal rabbit antihuman IgG antibody raised against a peptide fragment (amino acids 4–24) of human VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody. This antibody (100 μl/sec) dilute to a final concentration of 5 μg/ml in PBS-HS was added to the tissue sections, and the slides were incubated at room temperature for 2 h. The slides were then washed three times in PBS-HS and incubated with FITC-conjugated antirabbit serum diluted in PBS-HS for 2 h at room temperature (170 μl/sec). For EF5 experiments, the monoclonal antibody ELK3-51 conjugated with the indocarbocyanine dye molecule Cy3 (30 mg/ml in PBS-HS) was added (100 μl/sec) and incubated for 6 h at 4°C. After incubation, the slides were washed three times with PBS-HS, mounted in 2% n-propyl gallate in 70% glycerol-0.03 M Tris (pH 9.0), and examined with a Zeiss fluorescence microscope.

**RNA Analysis.** Total cellular RNA was purified by using the CsCl gradient method as described previously (21). The RNA species (15 μl/lane) were separated on a denaturing formaldehyde-agarose gel, transferred to a nylon membrane, and probed with a 32P-labeled ~0.6-kb cDNA fragment of VEGF165 (obtained from Dr. Amato Giaccia, Stanford University, Palo Alto, CA). The hybridization reaction was carried out at 60°C overnight, and the filter was washed in 0.1% SDS-0.1 X SSC at 65°C for 1 h. Densitometry was performed by using a Lynx 4000 image analyzer (Applied Imaging, Santa Clara, CA).

**Fig. 1.** Immunohistochemical staining of EMT6 spheroids with a polyclonal anti-VEGF antibody (C and D) and with ELK3-51 (E and F) after EF5 treatment. The unstained sections of the spheroids photographed under phase-contrast (A and B) and dark-field conditions (G and H) are also shown. Bar, 100 μm.

**Fig. 2.** Expression of VEGF mRNA in HT29 monolayer cells treated with TPA or grown under aerobic, hypoxic, or hypoxic/reoxygenation conditions. Cells were incubated in 5% CO2-air for 0 (Lane 1), 6 (Lane 2), and 24 h (Lane 3), treated with TPA for 0.5 (Lane 4) and 2 h (Lane 5), and incubated under hypoxic conditions for 0, 2, 4, 6, 8, and 24 h (Lanes 6–11, respectively) or after reoxygenation for 4 h (Lane 12). Zero-h hypoxia refers to the end of the 10 pumping cycles, which takes about 2 h. Total cellular RNA, purified on CsCl gradients, was separated by gel electrophoresis and transferred onto a nylon membrane. The blot was hybridized to a 32P-labeled probe prepared from a cDNA fragment of VEGF165 as described in the text.
Fig. 3. Immunohistochemical staining and in situ hybridization of HT29 spheroids with a monoclonal anti-EF5 antibody (ELK3-51) or a VEGF-specific RNA probe. Spheroids were grown in 5% CO2-air as described in the text. For immunohistochemical studies, spheroids were treated with 100 μM EF5 for 3 h prior to treatment with the ELK3-51 antibody. a, section from a large spheroid (~1000 μm in diameter) grown under aerobic conditions and stained with ELK3-51; b, in situ hybridization of a serial section from a; c, bright-field view of the spheroid section in b; d, section from a small spheroid (~400 μm in diameter) exposed to hypoxia for 24 h, stained with ELK3-51; e, in situ hybridization of a serial section from d; f, bright-field view of the spheroid section in e; g, section from a hypoxic small spheroid hybridized with “sense” VEGF probe; h, in situ hybridization of a large hypoxic spheroid; i, bright-field view of the section in h. Bars, 100 μm.

In Situ Hybridization. HT29 spheroids were fixed in Histochoice (Ameresco, Inc., Solon, OH) for 4 h at room temperature. Cryostat sections were cut at 8–10 μm, placed on Fisher Plus slides (Fisher Scientific), and stored in air-tight boxes at −80°C until use. The in situ hybridization method of Wilcox et al. (22) was used. Frozen slides were thawed at room temperature and fixed in 4% formaldehyde for 10 min. After the sections were washed in 0.5 X SSC, they were treated with proteinase K (5 μg/ml) for 10 min at room temperature. Prehybridizations were performed in 50% formamide, 0.3 M NaCl, 20 mM Tris (pH 8.0), 5 mM EDTA, 1X Denhardt’s solution, 10% dextran sulfate, and 10 mM DTT at 55°C for 3 h. The 35S-labeled VEGF riboprobes (sense and antisense) were prepared by using the Riboprobe Gemini System II (Promega, Madison, WI). Labeled probes (~0.6 × 10^6 counts/section) were added and incubated at 55°C overnight. Sections were washed with 2X SSC, 10 mM β-mercaptoethanol, and 1 mM EDTA, and then treated with 20 μg/ml RNase A for 30 min at room temperature. This procedure was followed by a high stringency wash for 2 h in 0.1X SSC, 10 mM β-mercaptoethanol, and 1 mM EDTA at 55°C and two more washes with 0.5X SSC. Sections were quickly dehydrated with ethanol, vacuum dried, and subjected to autoradiography.

RESULTS

Selective Visualization of Hypoxic Cells and VEGF-producing Cells in Multicellular Tumor Spheroids by Immunohistochemistry.

We had previously demonstrated that hypoxic cells in the central region of multicellular tumor spheroids of EMT6 mouse mammary carcinoma cells could be readily visualized by immunohistochemistry after the spheroids were treated with EF5 (15). To examine the spatial distribution of VEGF-producing cells with respect to the anti-EF5 antibody staining pattern, EMT6 spheroids were grown in the presence of 100 μM EF5 for 3 h under aerobic conditions, and harvested and sectioned for immunohistochemical studies. Fig. 1 shows the immunostaining results for EMT6 spheroids grown to 800 μm and 1400 μm in diameter. In the larger spheroids, VEGF protein was detected in the inner cells (Fig. 1, D) where the highest binding of EF5 was present (Fig. 1, F). Relatively, lower levels of VEGF protein (Fig. 1, C) and EF5 binding (Fig. 1, F) were detected in the core regions of smaller spheroids. No necrosis was detected in the 800-μm diameter spheroid while a necrotic center was present in the larger spheroid (Fig. 1, A and G versus B and H). These findings illustrated that the anti-VEGF antibody binding pattern closely parallels that of the anti-EF5 antibody binding profile in mouse EMT6 spheroids.

VEGF Expression in Human HT29 Cells Exposed to Hypoxia.

To determine whether the VEGF expression and EF5 adduct in human spheroid cells were similar to those observed in mouse EMT6 spheroids, we investigated the expression of VEGF in human HT29 colon
carcinoma cells. First, we examined VEGF mRNA expression in HT29 monolayer cells incubated under hypoxic culture conditions for various time periods. Cells grown under 5% CO₂-air were used as controls. In this experiment, we also examined the effects of reoxygenation and treatment with the phorbol ester TPA on VEGF mRNA levels. As shown in Fig. 2, exposure of cells to hypoxia resulted in significant accumulations of VEGF mRNA during a 2–24-h period (Fig. 2, compare Lanes 1–3 to Lanes 6–11). The maximum increase (~4.5-fold greater than in air) was attained after 2–4 h of hypoxia and was sustained for at least 24 h. Reoxygenation for a period of 4 h resulted in a significant drop in the VEGF mRNA level (Fig. 2, Lane 12). Treatment with TPA had no effect on VEGF mRNA expression (Fig. 2, Lanes 4 and 5).

The major VEGF mRNA species that accumulated under hypoxia had a molecular weight of ~4.0 kb, similar to that reported for VEGF₁₆₅ (10). Two other mRNA species with molecular weights of about 5.5 and 2.0 kb also hybridized with the VEGF probe. These two mRNA species appeared as minor bands and were present in only the hypoxia-exposed samples.

**Colocalization of VEGF Expression and Hypoxic Regions of HT29 Spheroids.** HT29 spheroids were exposed to 100 μM EF5 in spinner flasks for 3 h under both aerobic (5% CO₂-air) and hypoxic (5% CO₂-N₂) conditions. They were harvested and sectioned for immunohistochemical staining and in situ hybridization studies. Fig. 3 shows regions of high binding of EF5 and VEGF mRNA expression in HT29 spheroids grown under aerobic or hypoxic conditions. In large (~1000 μm in diameter) aerobic spheroids, the EF5 staining pattern was mainly localized in the cells in the spheroid interior around the necrotic center (Fig. 3a) as seen in the phase photograph (Fig. 3c). in situ hybridization of a serial section of the same spheroid using a VEGF-specific antisense probe showed that VEGF mRNA levels were also significantly higher in cells of the inner core than those located in the outer layers (Fig. 3b). In small (~400 μm in diameter) hypoxic spheroids, EF5 binding was detected throughout the spheroids (Fig. 3d). Consistent with these EF5 binding results, the in situ hybridization experiments showed high levels of VEGF mRNA present throughout the spheroids (Fig. 3e). In large hypoxic spheroids, high levels of VEGF mRNA were produced by cells in the peripheral layers as well as those in the core region around the necrotic region (Fig. 3h). Hybridization with the control sense probe showed only background levels of binding (Fig. 3g). No necrosis was detected in the small spheroids (Fig. 3f).

Immunohistochemical studies using a polyclonal anti-VEGF antibody showed that VEGF protein was present throughout the small hypoxic spheroids (data not shown). In larger spheroids (~1000 μm in diameter), VEGF protein was predominantly detected in cells in the inner regions around the necrotic center (Fig. 4, b and c) where the highest EF5 binding was present (Fig. 4a). No binding was detected when spheroids were immunostained with the secondary antibody alone (Fig. 4d).

The results presented above support the general conclusion that VEGF protein expression colocalized with sites of EF5 binding and thus areas of hypoxia in human HT29 cell spheroids. However, based on our results with EMT6 and HT29 cells it appears that the levels and pattern of VEGF expression may depend on the particular cell line under study.

**DISCUSSION**

The generally accepted assumption that VEGF expression is induced under hypoxia in vivo has been predominantly based on in vitro correlations between the accumulation of VEGF mRNA and low oxygen tensions in various cell lines (6, 7). In this study, we have been able to demonstrate that the expression of VEGF mRNA in multicellular tumor spheroids of human HT29 cells correlated with areas of tissue hypoxia. The spheroid cells that produced the highest levels of VEGF mRNA were located in the regions where the highest binding of the hypoxia-specific marker EF5 was detected.

In an independent study, Shweiki et al. (19) demonstrated that VEGF mRNA expression was up-regulated in the inner layers of rat...
glioma spheroids. These authors provided indirect evidence that the inner layers were composed of hypoxic cells because reoxygenation relieved induced VEGF expression. They also demonstrated that VEGF expression was independently induced by glucose deprivation and that glucose-starved cells were unable to induce VEGF expression in response to hypoxia (19).

The use of multicellular tumor spheroids and EF5 in this study has established that induced VEGF expression can be separated from the effects of necrosis or cellular heterogeneity present in solid tumors. Multicellular tumor spheroids represent genetically homogeneous cell populations containing gradients of glucose, oxygen, and pH (14). The use of EF5 as a specific marker for hypoxic tissues mapped the hypoxic regions of large spheroids to the inner cell layers surrounding the necrotic center. This molecule and its specific monoclonal antibody have previously been used to discriminate hypoxic from aerobic tissue (15). It is worth noting that detectable EF5 binding and VEGF expression probably have different oxygen dependencies. At the concentrations used in these experiments, EF5 is nontoxic and is less sensitive than other bioeducative compounds to oxygen-independent mechanisms of activation (15).

Based on Northern blot analysis, it appears that the predominant VEGF isoform produced by HT29 cells is VEGF165 (Fig. 2). The major mRNA species produced by these cells had a molecular weight of \(\sim 4.0\) kb, similar in size to that reported for VEGF165 (10). Of the two minor mRNA bands detected using the Northern blotting analysis, the higher molecular weight species may have been the mRNA for VEGF189 (10). The \(\sim 2.0\)-kb band could have been a partial RNA degradation product or a different functional form of VEGF transcript.

It has been demonstrated that phorbol ester activators of protein kinase C also induce expression of the VEGF gene (10, 23). A synergistic effect of mutant p53 and protein kinase C on VEGF expression has recently been demonstrated (24). In these studies, cells carrying mutant p53 expressed higher levels of VEGF than those expressing the wild-type p53 gene when treated with TPA. Although HT29 cells contain a mutated form of the p53 gene (25), we were unable to demonstrate that TPA induces VEGF mRNA accumulation in this cell line. It is possible that the process of TPA induction of VEGF requires steps that are nonfunctional in HT29 cells.

Accumulating evidence suggests that genes regulated by hypoxic stress are important participants in malignant progression and in the response of solid tumors to anticancer therapies. In this study, we have demonstrated that VEGF expression correlates with hypoxia in multicellular tumor spheroids of a human cell line. Combined with EF5 as a hypoxia-specific marker, spheroids can provide a useful in vitro tumor model for studies of expression and regulation of hypoxia-inducible genes.

REFERENCES


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