HIF-1α-mediated Up-Regulation of Vascular Endothelial Growth Factor, Independent of Basic Fibroblast Growth Factor, Is Important in the Switch to the Angiogenic Phenotype during Early Tumorigenesis

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Abstract

The switch to the angiogenic phenotype represents a critical checkpoint during tumor progression. The acquisition of new capillary vessels provides newly vascularized tumor nodules with a distinct biological advantage over their avascular counterparts by conferring upon them the ability to expand and develop both locally and metastatically. To identify the molecules and mechanisms underlying this rate-limiting step in successful tumorigenesis, we have developed an in vivo tumor model that reproducibly recapitulates the angiogenic switch. Using this model, we have analyzed vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hypoxia-inducible factor-1a (HIF-1α) expression and activity in both avascular and vascular growth phases of the tumor. A significantly higher level of VEGF protein was detected in avascular tumor nodules compared with vascular nodules. As avascular tumors became vascularized, VEGF levels decreased ~10-fold. In contrast, bFGF levels were not elevated in avascular nodules but rather were detected at levels ~2 times higher in vascular nodules compared with the avascular tumor nodules. Given that VEGF is transcriptionally regulated by HIF-1α, immunohistochemical studies of chondrosarcoma nodules were conducted and revealed that the nuclear translocation of HIF-1α was detected exclusively in avascular tumor nodules. This study implicates HIF-1α-mediated up-regulation of VEGF but not bFGF in the switch to the angiogenic phenotype during tumorigenesis.

Introduction

Angiogenesis, the process of new capillary formation from the preexisting vasculature, is required for successful tumor growth and metastasis. During tumorigenesis, neoplastic lesions first undergo an avascular growth phase to a size not much greater than a few millimeters in diameter (1, 2). This first stage in tumor expansion is followed by a second event that distinguishes a growing tumor from one that is dormant. This event, the switch from avascular to vascular phenotypes, “the angiogenic switch,” initiates a cascade of events that results in the hallmark expansion of tumor volume and subsequent metastasis. The new vasculature acquired during this period provides a mechanism by which an in situ tumor lesion can circumvent the critical limitations of the oxygen diffusion distance and restrictions to nutrient exchange.

Although much is now known about the positive and negative regulators of established angiogenesis, relatively little is known or understood about the mechanisms and molecules associated with what has been called a rate-limiting event in tumor progression. To specifically study the onset of neovascularization, we have recently developed a novel in vivo model that recapitulates the angiogenic program during tumor progression from avascular tumor lesions, through the acquisition of the angiogenic phenotype, to exponential growth of the tumor (3). Using this model of Swarm rat chondrosarcoma, preangiogenic and angiogenic tumor nodules can be harvested and analyzed biologically, and by using molecular strategies, used to identify molecules and events that correlate with the angiogenic switch.

It has been proposed that the angiogenic switch is regulated by the net balance of angiogenic stimulators and inhibitors (4). Reasoning that molecules that are operative during progression of a tumor through the angiogenic program during tumorigenesis might drive the earliest stage of the program, i.e., the angiogenic switch, we have used the chondrosarcoma model to study the roles of the angiogenic mitogens bFGF and VEGF in this early stage of tumor progression, when tumors switch to the angiogenic phenotype and enter the rapid growth phase. Here, we report data that supports a key role for hypoxia-induced VEGF, but not bFGF, at the onset of angiogenesis during early tumor development in vivo. Continued identification and characterization of the molecular mechanisms underlying the angiogenic switch during early tumorigenesis has the potential to provide insights into developing strategies for early cancer treatment, diagnosis, and perhaps prevention.

Materials and Methods

In Vivo Tumor Model. Rat chondrosarcoma nodules representing distinct preangiogenic and angiogenic tumor lesions were obtained as reported previously (3). Briefly, Swarm rat chondrosarcoma tumors were harvested after s.c. growth in male Sprague Dawley rats. These tumors were propagated by the injection of 0.1 ml of 6 × 106 cells s.c. into both hips of each rat (100–120 g). After tumor harvest, tumor cell suspensions were prepared by mincing chondrosarcoma tissue in Ringer’s lactate solution, passing it through a steel siev and through 20–30-gauge needles consecutively. The tumor cell suspension was adjusted to 6 × 105 cells/ml, and 1 ml of this suspension was injected into an air sac created by s.c. injection of 25 ml of air into the sacral region of the back of male Sprague Dawley rats (100 g). By day 10–12, tumor nodules, either avascular or vascular, appeared in the air sac. Tumor nodules were harvested under a stereomicroscope.

Capillary EC Proliferation Assay. Capillary EC proliferation was analyzed as reported previously (5, 6). Bovine capillary ECs were the kind gift of Dr. Judah Folkman and Catherine Butterfield (Children’s Hospital, Boston, MA). ECs were maintained in DMEM (JRH Biosciences, Lenexa, KS) supplemented with 10% FCS (HyClone, Logan, UT), 1% glutamine penicillin-streptomycin, and 3 ng/ml bFGF (Scios Nova, Mountain View, CA). On day 1, ECs were trypsinized, resuspended in DMEM with 5% FCS, and plated on gelatin-coated 96-well culture plates at 2000 cells/well. On day 2, medium was changed, and PBS-dialyzed tissue extracts were loaded into wells. Control wells received equivalent amount of PBS or PBS containing 1 ng/ml bFGF as controls. Cells were then incubated for 72 h in 10% CO2. On day 5, each well was rinsed with 200 μl of Ca2+- and Mg2+-free PBS and incubated with 100 μM of Cu2+.
μl of 0.1 M acetate buffer (pH 5.5) containing 0.1% Triton X-100 and 10 μM of p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) for 2 h. After incubation, 10 μl of 1 N sodium hydroxide was added to each well, and the absorbance at 410 nm was determined using an ELISA microplate reader. Results were verified by cell counting assays using a Coulter counter (5, 6). To neutralize the bioactivity of the mitogens of interest, monospecific immunonutralizing antibodies were added to culture medium with tissue extracts on day 2 using the same amount of normal IgG from the same species as control.

**Protein Extraction and ELISA Assay.** Rat chondrosarcoma or rat xiphoid cartilage (control) samples were minced and extracted by stirring in extraction buffer composed of 2 M NaCl, 0.02 M HEPES, and 0.02% NaN₃ at a ratio of 1:8 (tissue weight:buffer volume) for 24 h as reported previously (7). Tissue extracts were centrifuged at 1000 × g for 10 min and dialyzed against Dulbecco’s PBS buffer overnight. Protein concentrations were determined with the Bio-Rad protein assay (Bio-Rad, Hercules, CA) according to manufacturer’s instructions. To determine concentrations of VEGF or bFGF in the tissue extracts, samples were tested in duplicate using a VEGF or bFGF ELISA assay kit (R&D Systems, Minneapolis, MN).

**RT-PCR, DNA Subcloning, and Sequencing.** Total RNA was purified from tissues using a Qiagen RNeasy Mini kit according to the manufacturer’s instructions (Qiagen, Valencia, CA). RNA concentrations were determined by UV spectrophotometry, and reverse transcriptions were conducted using 1 μg of total RNA for each sample with the First Strand cDNA Synthesis kit (Boehringer Mannheim, Indianapolis, IN). cDNA was normalized using PCR with β-actin as an internal control. To detect VEGF and HIF-1α, PCR reactions were performed using 10 μl of each primer, 0.2 mM of each deoxynucleotide triphosphate, and 1 unit of Taq DNA polymerase in a PTC-100 Cycler (MJ Research, Waltham, MA). Primers used for detecting VEGF were designed based on the rat VEGF sequence (GenBank Accession no. M32167) [forward 5'-ACC AGC GCA GCT ATT GCC GT, reverse 5’-CAC CGC CTT GCC TTC TGCA CA]. The HIF-1α primers were based on the rat HIF-1α sequence (GenBank Accession no. AF057308), [forward 5'-ACA GTG GTA CTC ACA GTC GG, reverse 5’-CCC TGC ACA TTC AGG TTT CTG GCT].

To confirm the identity of the fragments obtained by PCR as being specific VEGF isoforms, the PCR products were subcloned into pCR3 plasmids using the TA-clone kit (Invitrogen, San Diego, CA). The plasmid DNA was purified with a Qiagen miniprep kit (Qiagen, Valencia, CA) and sequenced.

**Histology and Immunohistochemistry.** For paraffin sections, tissues were fixed overnight in 10% buffered formalin or Carnoy’s fixative and processed by standard histological procedures. Alternatively, for frozen sections, tissues were fixed in 10% buffered formalin, washed in PBS, embedded in OCT freezing medium (Tissue Tek; Sakura Finetek, Torrance, CA) and sectioned in the cryostat. For immunohistochemistry, tissue sections were incubated with normal goat serum to block nonspecific antibody binding sites. The sections were then incubated with a mouse monoclonal antibody against HIF-1α (Novus Biologicals, Littleton, CO) overnight at 4°C. After rinsing with PBS three times, the sections were incubated with biotin-conjugated goat antimouse IgG (Sigma Chemical Co.) for 1 h at room temperature. After rinsing, the sections were incubated with avidin-biotin-peroxidase complex by using the VECTASTIN elite ABC kit (Vector Laboratories, Burlingame, CA). The final brown precipitate was developed using a 3,3'-diaminobenzidine kit (Vector Laboratories).

**Results**

**VEGF and bFGF are the Major Capillary EC Mitogens in Chondrosarcoma.** Growth factors that are expressed and secreted by tumor cells play a crucial role in tumor angiogenesis. To determine whether chondrosarcoma tumors produce specific mitogens that stimulate capillary EC proliferation, we first studied the mitogenic activities of chondrosarcoma by using a standard EC proliferation assay. Tumor extracts stimulated EC proliferation in a dose-dependent manner in comparison with controls (Fig. 1A). An ~2.5-fold increase of EC proliferation was observed when 20 μl (40 μg of total protein) of the extracts were applied to each well in 96-well plates.

To identify the specific mitogens that stimulated EC proliferation, a number of neutralizing antibodies were tested in the EC proliferation assays. As shown in Fig. 1B, addition of an anti-bFGF neutralizing antibody (purified IgG; R&D Systems) resulted in a maximal 70% suppression of EC proliferation induced by 40 μg of tumor extract. An anti-VEGF neutralizing antibody (antigen-affinity purified IgG; R&D Systems) was able to suppress up to 30% of EC proliferation induced by 40 μg of tumor extracts (Fig. 1C). When a combination of bFGF and VEGF antibodies were added to each well, a complete suppression of EC proliferation was achieved, demonstrating that bFGF and VEGF are the major growth factors responsible for EC proliferation in this tumor system.

**VEGF and bFGF Expression in Preangiogenic and Angiogenic Tumor Nodules in Vivo.** After determining that bFGF and VEGF are the major EC mitogens in parental chondrosarcoma, we then asked whether bFGF and VEGF levels change during the time in which the tumor nodules become angiogenic. Avascular and vascular tumor nodules were isolated from air sacs, and protein was extracted from both nodule types. After protein concentrations were normalized, bFGF and VEGF levels were measured by ELISA. As shown in Fig. 2A, vascular nodules contained ~1.7 times more bFGF as did the avascular nodules, suggesting an up-regulation of bFGF expression in the transition from preangiogenic to angiogenic stages in this system. In striking contrast, we detected a high level of VEGF in avascular...
and was normalized by PCR using sequence. cDNA was synthesized by reverse transcription of total on exon 5 and a reverse primer based on exon 8 of rat VEGF COOH terminus (8). Therefore, we designed a forward primer based nodules, vascular nodules, and xiphoid cartilage (Fig. 2). In contrast, VEGF164 and VEGF120 is significantly elevated in avascular nodules in comparison to that in vascular nodules (Fig. 2B). Avascular nodules contained ~11-fold more VEGF protein than did vascular nodules. This ratio was consistently observed in three independent experiments.

To determine which VEGF isoforms were involved in the angiogenic switch in this model, we examined mRNA expression of VEGF in avascular and vascular nodules as well as in control xiphoid cartilage by RT-PCR. Previous studies have shown that all of the VEGF isoforms possess exons 1–5 at the NH2 terminus and exon 8 at COOH terminus (8). Therefore, we designed a forward primer based on exon 5 and a reverse primer based on exon 8 of rat VEGF sequence. cDNA was synthesized by reverse transcription of total RNA from avascular, vascular tumor nodules, and xiphoid cartilage and was normalized by PCR using β-actin as an internal control. Two prominent VEGF transcripts were detected by RT-PCR in avascular nodules, vascular nodules, and xiphoid cartilage (Fig. 2C). These bands migrated at molecular weights consistent with their identification as rat VEGF164 and VEGF120 based on the published rat VEGF sequence data. These two VEGF fragments from the PCR reactions were cloned and sequenced. Sequence analysis showed that these two bands correspond to VEGF164 and VEGF120 isoforms, respectively. On the basis of these data, we conclude that VEGF164 and VEGF120 are the two major VEGF isoforms expressed in this tumor.

Comparative RT-PCR demonstrated that the expression of both VEGF164 and VEGF120 is significantly elevated in avascular nodules in comparison to that in vascular nodules (Fig. 2C). In contrast, vascular nodules and cartilage express similar levels of mRNA for both these VEGF isoforms. Hence, two VEGF isoforms, VEGF164 and VEGF120, are up-regulated at the mRNA level during the avascular stage of early tumorigenesis in this animal model.

We next analyzed VEGF expression levels during the course of chondrosarcoma s.c. growth in vivo. As shown in Fig. 3A, chondrosarcoma tumors grew slowly during the first 5 days after the s.c. injection of 6 \times 10^5 cells. From day 7, tumor growth accelerated and reached a rapid growth phase by day 12. On day 5, blood vessels began to appear exclusively on the tumor capsules and had invaded the tumor mass by day 7. VEGF levels were monitored during this time period and are shown in Fig. 3B. A relatively high level of VEGF (6.4 pg/µg) was detected on day 1 (24 h after tumor cell inoculation), and its level peaked on day 3 in tumors. VEGF levels decreased significantly to 4.2 pg/µg on day 5 when angiogenesis was initiated in the tumor nodules and decreased additionally when tumors became more vascularized after day 7. By day 12, when the tumors were undergoing rapid growth, the VEGF level was as low as 1.1 pg/µg. Therefore, VEGF levels showed a >10-fold decrease during the period of time (day 3 versus day 12) when the tumors were becoming vascularized (angiogenic). This ~10-fold decrease reflects that observed in VEGF levels as the avascular nodules became vascularized.

**Nuclear Translocation of HIF-1α in Avascular Tumor Nodules.** Because VEGF expression is regulated by hypoxia, we next studied the expression of HIF-1α, a key regulatory gene involved in oxygen hemostasis. We first analyzed the expression of this transcription factor by RT-PCR. There was no detectable difference in HIF-1α gene expression between the avascular and vascular tumor nodules as analyzed by RT-PCR (data not shown). Given its role as a transcription factor, we next analyzed HIF-1α expression in tumor nodules by immunohistochemistry using a monospecific antibody against HIF-1α. Using this approach we detected HIF-1α in most of the tumor cells in avascular nodules (Fig. 4A). In contrast, HIF-1α was rarely detected in the tumor cells of vascular nodules (Fig. 4B). Most strikingly, HIF-1α staining was localized to the nucleus of many tumor cells in avascular tumor nodules (Fig. 4A), and this nuclear

Fig. 2. Comparison of bFGF and VEGF levels in avascular and vascular chondrosarcoma tumor nodules. A, concentration of bFGF in vascular nodules is 1.7 times greater than in avascular nodules. B, in contrast, VEGF levels are 11-fold higher in avascular nodules than in vascular nodules. Data are representative of three independent experiments. C, VEGF mRNA levels in tumor nodules and control cartilage. RT-PCR revealed mRNAs of two VEGF isoforms corresponding to VEGF120 and VEGF164. PCR amplification was normalized using β-actin as an internal control.

Fig. 3. VEGF expression during s.c. tumor growth. Chondrosarcoma cells (6 \times 10^5) were injected s.c. into both hips. Tumors were dissected on days indicated. Tumor weights were measured (A), and VEGF levels in tumor extracts were determined using ELISA (B). Initially, high VEGF levels were detected in avascular nodules (day 3). VEGF levels decreased significantly as tumor nodules became vascularized (day 5).
translocation of HIF-1α was not detected in tumor cells of vascular nodules (Fig. 4B). These results are consistent with those reported previously, that HIF-1α is primarily regulated at the post-translational level (9).

**Discussion**

Much of the emphasis of the work in the field of tumor angiogenesis in the past 25 years has focused on the identification of the molecules that stimulate or inhibit the already established vasculature of that tumor. In contrast, very little is known today about the identity of the molecules or mechanisms underlying one of the earliest and most critical events in tumor progression: the switch to the angiogenic phenotype. Identification of the activators and suppressors of the angiogenic switch can lead to the development of therapeutics and complementary diagnostics that could be used clinically to detect a cancer and to therapeutically intervene at the earliest stages of tumor development to suppress its progression through the angiogenic checkpoint, the passage of which is required for exponential local growth and which is permissive for distant metastasis.

A major reason for the lack of progress in understanding the process through which the angiogenic switch occurs is the limitation imposed by the lack of in vivo models that reproducibly recapitulate this event. We have developed a model to study the switch to the angiogenic phenotype, which is based on the progression of chondrosarcoma, a tumor of cartilage (3). In this study we have determined the roles that the angiogenic mitogens VEGF and bFGF play in the acquisition of the new vasculature of the tumor. The results of this research suggest a sequence of events leading to the triggering of the angiogenic switch in which the helix-loop-helix transcription factor HIF-1α is translocated to the nucleus of avascular chondrosarcoma cells, up-regulating VEGF, activating the angiogenic switch, and inducing neovascularization. Once the trigger has been activated, VEGF levels decrease >10-fold. However, in striking contrast during this same window of time, bFGF levels show an opposite profile with their levels increasing >2-fold in the vascular tumor nodules. The latter result is not surprising given that it was from chondrosarcoma that bFGF was originally purified and identified (10). It is interesting to note that this kinetic profile of EC mitogens during the angiogenic switch is consistent with a previous report of the role of these mitogens in a model of established s.c. growth of T-47D breast carcinoma cells in vivo in which it was shown that VEGF is essential for the initial but not continued in vivo growth of these breast carcinoma cells (11).

Much is known about the role of hypoxia in established tumors and their vasculature in contrast with the relatively little amount of information available regarding hypoxia during the angiogenic switch. For example, blood flow rates have been shown to decrease with increasing tumor size (12), and hypoxia-induced VEGF up-regulation often exists in tumor regions near necrotic areas as revealed by in situ hybridization (13, 14). This inadequate blood supply attributable to a rapid expansion of tumor lesions causes hypoxia in a variety of tumors, in turn activating a variety of genes (15) and inducing resistance of tumor cells to cancer therapies such as chemotherapy and radiation. In fact, tumor hypoxia has been shown to be an adverse prognostic factor for a variety of cancer types (reviewed in Ref. 16), and tumor growth has been shown to be inhibited by disruption of hypoxia-inducible transcription (17).

HIF-1α is a principle transcription factor that regulates cellular responses to physiological and pathological hypoxia (9). Under normoxic conditions, HIF-1α protein is rapidly degraded presumably via the ubiquitin-proteasome pathway such that very low levels of protein can be detected in the cytoplasm. However, HIF-1α protein significantly accumulates in response to hypoxia (18). HIF-1α then dimerizes with aryl hydrocarbon receptor nuclear translocator and translocates to the nucleus where it can activate hypoxia-sensitive genes by binding to their promoter/enhancer regions (9).

It is now widely appreciated that VEGF expression is mediated by HIF-1α during hypoxia. The VEGF gene contains a number of HIF-1α binding sites at its regulatory region, and HIF-1α is able to activate the VEGF promoter (19, 20). Deletion of the HIF-1α gene or disruption of HIF-1α transcription results in the lack of VEGF secretion by tumor cells, suppresses angiogenesis, and dramatically retards solid tumor growth (17, 21). Therefore, our finding in this study that VEGF is highly expressed in the avascular tumor nodules is consistent with the immunohistochemical data presented in Fig. 4, which documents the importance of the translocation of HIF-1α to the nucleus of the avascular chondrosarcoma chondrocytes. These results are also consistent with the in vitro observation that VEGF is up-regulated in inner (hypoxic) layers of glioma spheroids (22). It may be that rapid cell proliferation of the tumor cells creates an increasingly hypoxic state in the avascular tumor nodules, which becomes more severe as the nodules approach 2–3 mm in diameter. Additional growth of these avascular nodules much beyond this size requires neovascularization.

We propose that the hypoxia-HIF-1α-VEGF axis functions as an early, upstream activator of the angiogenic switch in this tumor model. As avascular tumors grow, their mass reaches the limits of the oxygen diffusion distance, which induces the hypoxic state. The increasing hypoxia results in the accumulation and nuclear translocation of HIF-1α in the tumor cells of the avascular nodules. HIF-1α activates VEGF transcription, resulting in a >10-fold increase of VEGF protein in the avascular tumor nodules, which ultimately recruits new capillaries to the tumor nodules. Because HIF-1α is overexpressed in most human cancers (23), this sequence that triggers the onset of the angiogenic switch may apply to many types of human cancer as well. Once vascularized, oxygen levels rise to normoxic levels, resulting in the decrease of VEGF detected in the vascular tumor nodules. During this same time period, the angiogenic mitogen bFGF increases ~2-fold during the angiogenic switch, perhaps serving to maintain the new vasculature post-angiogenic switch. In a different model of the angiogenic
switch during carcinogenesis of pancreatic islets in transgenic mice, both VEGF and acidic FGF, unlike in the chondrosarcoma model, are expressed constitutively in normal islet β-cells of control mice and in all stages of the islet carcinogenesis pathway (24). In this model, VEGF is released from extracellular stores by MMP-9, an event which increases the bioavailability of the angiogenic mitogen, thereby activating angiogenesis (25).

The results of the current study suggest that the hypoxia-HIF-1α-VEGF axis may represent potentially important therapeutic intervention sites for very early cancer treatment. More specifically, they suggest that the use of combination therapies that target individual elements in the axis, including HIF-1α, VEGF, and MMPs, may provide a powerful and early approach to prevent tumor progression by blocking the angiogenic checkpoint, that critical stage in tumor development during which an avascular tumor acquires its own vasculature and begins its exponential growth through tumor progression and malignancy. Intervention at this earliest of checkpoints would provide an early treatment opportunity that is not currently available and might be applicable to the host of non-neoplastic diseases that are also characterized by dysregulated angiogenesis.

References

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