Hypoxia-Inducible Factor-1-Independent Regulation of Vascular Endothelial Growth Factor by Hypoxia in Colon Cancer

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ABSTRACT

The induction of vascular endothelial growth factor (VEGF) is an essential feature of tumor angiogenesis, and the hypoxia-inducible factor-1 (HIF-1) transcription factor is known to be a key mediator of this process. In colon cancer, the frequently mutated K-ras oncogene also can regulate VEGF expression, but the role that K-ras may play in hypoxia is unknown. Hypoxia induced VEGF promoter activity, mRNA, and protein levels in colon cancer cells. Although HIF-1α was induced by hypoxia, VEGF reporter constructs with selectively mutated hypoxia-response elements remained responsive to hypoxia. In addition, “knockdown” of HIF-1α by RNA interference only minimally inhibited the hypoxic induction of VEGF. A region of the VEGF promoter between −420 and −90 bp mediated this HIF-independent induction by hypoxia. The introduction of K-rasVal12 augmented the hypoxic induction of VEGF, and this was observed in wild-type and HIF-1α knockdown colon cancer cells. Thus, VEGF may be induced by hypoxia through HIF-dependent and HIF-independent pathways, and K-ras also can induce VEGF in hypoxia independent of HIF-1. These findings suggest the existence of multiple mechanisms regulating the hypoxic induction of VEGF in colon cancer.

INTRODUCTION

Rapidly growing tumors routinely outstrip their supply of oxygen and nutrients, and the induction of new blood vessels is critical to sustain neoplastic proliferation (1). New blood vessels can be stimulated to grow when factors that promote angiogenesis are up-regulated or those that inhibit angiogenesis are down-regulated. Vascular endothelial growth factor (VEGF) is a key proangiogenic factor, and therapeutic approaches that inhibit VEGF in human malignancies now are in clinical trials (2, 3). Interestingly, VEGF is overexpressed not only in advanced colon cancers but also in premalignant colonic adenomas (4). The Wnt and K-ras signaling pathways are activated frequently during early stages of colonic carcinogenesis, and we demonstrated previously their role in the regulation of VEGF expression (5). Although VEGF protein levels and blood vessel counts are similar in adenomas and nonmetastatic malignancies, their levels are significantly higher in metastatic colorectal tumors (6). The additional genetic and environmental factors that may contribute to this enhanced VEGF expression in advanced tumors are not defined fully.

One environmental factor that is likely to play a role is hypoxia. Most solid tumors develop regions of low oxygen tension because of an imbalance in oxygen supply and consumption. Clinical and experimental evidence suggests that tumor hypoxia is associated with a more aggressive phenotype (7). Hypoxia is a potent stimulator of VEGF expression, and this induction is thought to be mediated primarily through hypoxia-inducible factor-1 (HIF-1; Ref. 8). HIF-1 is a heterodimeric basic helix-loop-helix transcription factor composed of two subunits, HIF-1α and HIF-1β (ARNT). HIF-1α is the key regulatory component because it is degraded rapidly in normoxic conditions but stabilized and activated during hypoxia (9, 10). The HIF-1 complex recognizes a consensus hypoxia response element (HRE) in the promoter of a broad range of target genes (11). HIF-1α is overexpressed in most human malignancies (12), and HIF-1α expression levels correlate with tumor progression and aggressive behavior (11). Therapies targeting HIF-1α consequently are considered to be a potential approach for patients with various types of cancer (13, 14).

There is a large body of evidence demonstrating a pivotal role for HIF-1α in tumor growth and angiogenesis through its trans-activation of hypoxia-inducible genes (15). However, it is unlikely that the cellular response to hypoxia is mediated solely through HIF-1α. Cells derived from HIF-1α knockout embryos demonstrate a significant, albeit reduced, induction of VEGF in response to hypoxia, suggesting that HIF-1α may not be the only transcriptional activator of VEGF (16, 17). Although post-transcriptional stabilization of mRNA also contributes to the hypoxic induction of VEGF (18), it has been shown that the VEGF promoter can be induced by hypoxia when canonical HREs are mutated or deleted in human cancer cell lines (8, 19). These findings imply the existence of alternative transcriptional mechanisms that do not depend on HIF-1α in the hypoxic regulation of VEGF.

It has been demonstrated previously that oncogenic K-ras regulates VEGF transcription in colon cancer (5, 20). However, the role that this pathway may play specifically in states of hypoxia is not well defined. Other signaling molecules that can be activated by hypoxia include Src (21), e-Jun (22), nuclear factor κB (23), and cyclooxygenase-2 (24). A number of groups have demonstrated a role for H-ras signaling in hypoxia through regulation of HIF-1α phosphorylation, but there have been no studies of the more relevant K-ras isoform in hypoxic conditions in colon cancer (16, 25, 26).

The present study sought to characterize the molecular mechanisms that might regulate VEGF expression in colon cancer during hypoxia. In particular, we were interested in the roles of HIF-1α and the K-ras pathway that is critical in normoxia. Although HIF-1α is up-regulated in colon cancer by hypoxia, it does not appear to be necessary for the induction of VEGF. In contrast, the hypoxic induction of VEGF appears to depend almost entirely on HIF-1α in pancreatic and hepatocellular cancer cells. VEGF promoter deletion studies have identified a 330-bp region that mediates this HIF-1α-independent response. Activated K-ras also can enhance VEGF gene transcription in hypoxia. This enhancement of VEGF expression by K-ras is not mediated through HIF-1α, indicating that the functional repertoire of oncogenic K-ras mutations in colon cancer can be expanded to include the HIF-independent regulation of angiogenesis.

MATERIALS AND METHODS

Plasmid Constructions. Human VEGF promoter luciferase constructs were prepared as described previously (5). A series of 5′-deletion constructs—2.8-kb-VEGF-luc, 2.3-kb-VEGF-luc, 1.9-kb-VEGF-luc, 1.5-kb-VEGF-luc, and 1.0-kb-VEGF-luc—contained VEGF promoter sequences from −1811 bp to +1036 bp, −1278 bp to +1036 bp, −850 bp to +1036 bp, −420 bp to +1036 bp, and −90 bp to +1036 bp relative to the transcription initiation site, respectively (27). Site-directed mutagenesis was performed using the 2.3-kbVEGF-luc construct, which contained two key HREs (8). One or both of these HREs (5′-TACGTTGG >5′-TAAAAAGG at −975 bp and 5′-TACGTGGC >5′-TAAAAAGG at −306 bp; Ref. 8) were mutated selectively, and

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they are designated mHiF-975/VEGF-luc, mHiF-306/VEGF-luc, and mHiF-975/306/VEGF-luc. The introduced mutations were confirmed by DNA sequencing. An HRE reporter plasmid (HRE-luc) was generated by subcloning four copies of the canonical HRE (5′CCACATGGCATACTCGTTGCTCAG- CAGCTCTCCTCTCCTCCTATGCA) into pGL3-basic vector (Promega, Madison, WI). Dr. Ramnik Xavier provided the pGFP-K-ras<sup>Val12</sup> expression vector (28).

**Cell Culture.** Caco2, ColoHSR, HT-29, DLD-1, HepG2, and Panc-1 cell lines (all from American Type Culture Collection, Manassas, VA) were maintained in recommended growth media with 10–20% fetal bovine serum (Cellgro; Mediatech, Herndon, VA) supplemented with 2% penicillin/streptomycin (BioWhittaker, Rockland, ME). Hypoxic conditions were achieved by culturing cells in a sealed hypoxia chamber (Billups-Rothenberg, Del Mar, CA) after flushing with 1% O<sub>2</sub>, 3% CO<sub>2</sub>, and 96% N<sub>2</sub>. To minimize the effect of serum growth factors, the cell culture medium was switched to serum-free UltraCulture (BioWhittaker) before the cells were subjected to hypoxia.

**Transfections and Reporter Assays.** Transient transfections were performed using the cationic lipid Lipofectamine 2000 (Life Technologies, Inc., Rockville, MD) according to the manufacturer’s specifications. All of these experiments were performed in 24-well tissue culture plates with cells plated to reach 50–60% confluence on the day of transfection. Transfection efficiency in Caco2 cells averaged between 50–60%, as measured by coexpression of green fluorescent protein. Cells were allowed to recover in regular culture medium for 18–20 h after transfection, switched to UltraCulture medium, and then exposed to normoxia or hypoxia for 24 h before luciferase reporter assays were performed.

VEGF-luciferase reporter constructs of 0.4–0.6 μg were cotransfected with 2 ng of pRLCMV (Promega) as a control. pRL-null (gift of Dr. Daniel Tenen), a promoter-less Renilla construct, was used when cells were cotransfected with a K-ras expression vector because Ras has been shown to induce the pRL-CMV plasmid (29). As indicated, 0.2–0.6 μg of expression vector were cotransfected, and the total amount of transfected DNA was kept constant by adding corresponding empty plasmid. Luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega). Experiments were performed in duplicate wells a minimum of three times. The relative luciferase activity (mean ± SE) was calculated as Firefly luciferase activity/Renilla luciferase activity. The level of hypoxic induction was calculated as the ratio of the relative luciferase activity in hypoxia to that in normoxia.

**siRNA Preparation.** The pSUPER vector that expresses short hairpin small interfering RNA (siRNA) under the control of the polymerase-III H1-RNA promoter was used after inserting pairs of annealed DNA oligonucleotides between the BgIII and HindIII restriction sites according to the manufacturer’s protocol (Oligoengine, Seattle, WA; Ref. 30). Two different 19-nucleotide (nt) sequences derived from human HIF-1α mRNA (U22243; bp 1470–1489 and bp 2192–2211) were selected, and these HiF-1α-specific inserts were designed to include sequences in sense and antisense orientations, separated by a 9-nt spacer. The 64-nt oligos are described below: HIF-1α/siRNA1470 forward oligo, 5′-gatcccaATCCAGAATCACTTAAATTGATTGATTGTTAAGAA3′; HIF-1α/siRNA1470 reverse oligo, 5′-agctttttccaaaaATCCAGAATCACTTAAATTGATTGATTGTTAAGAA3′. These siRNA constructs are termed HIF-1α/siRNA1470 and HIF-1α/siRNA2192, respectively.

**Northern Blot Analysis.** Total RNA was prepared using TRIzol reagent (Life Technologies, Inc.). Fifteen μg of total RNA were analyzed using a random prime-labeled 400-bp human VEGF cDNA (5) or 2.5-kb Glut-1 cDNA (31), and 18S rRNA was used as a loading control. For hypoxia studies, RNA was harvested from cells after 8–12 h of incubation in 1% O<sub>2</sub>. The mRNA levels were quantified using Image J software (http://rsb.info.nih.gov/ij/index.html) and normalized to that of 18S rRNA.

**ELISA.** VEGF levels of media supernatant and cellular extracts were measured. Medium was collected after 24 h of normoxic or hypoxic conditions and centrifuged to remove floating cells. VEGF protein levels were assayed using a human VEGF-specific ELISA (Quantikine; R&D Systems, Minneapolis, MN) normalized to total protein content measured by the Bradford assay (Bio-Rad, Hercules, CA).

**Western Blot Analysis.** Protein lysates were harvested from cells subjected to normoxia or hypoxia for 8 h. The 8-h time point was selected after preliminary studies revealed maximal induction of HIF-1α at 8 h that persisted for 24 h (data not shown). Cells were lysed in chilled lysis buffer [50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP40, 1 mM Na3VO4, and 1 mM NaF supplemented with Complete Mini Protease Inhibitor tablets (Roche Molecular Biochemicals, Mannheim, Germany). Twenty to 80 μg of protein extracts were resolved on a 7% NuPAGE Tris-Acetate polyacrylamide gel (Life Technologies, Inc.) and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The blots were probed with HIF-1α (Transduction Laboratories, Lexington, KY; 1:250), HIF-2α (Novus Biologicals, Littleton, CO; 1:250), or β-actin antibody (Sigma; 1 μg/ml). Immunoreactive proteins were visualized using the Western Lighting Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Boston, MA).

Dephosphorylation assays of HiF-1α were performed using whole cell extracts. Cells lysates were incubated with 200 units λ-phosphatase (New England Biolabs, Beverly, MA) for 30 min at 37°C.

**Statistical Analysis.** Statistical differences were analyzed by the Student’s t test, and P values < 0.05 were considered statistically significant.

**RESULTS**

**Induction of VEGF by Hypoxia in Colon Cancer Cells.** To determine the effect of hypoxia on VEGF gene expression in colon cancer, cultured cell lines were exposed to 1% O<sub>2</sub> for 12 h. VEGF mRNA levels were measured by Northern blot analysis. The colon cancer cell lines Caco2, HT-29, ColoHSR, and DLD-1 expressed VEGF in normoxic conditions (21% O<sub>2</sub>). When switched to a hypoxic environment, endogenous VEGF mRNA levels were strongly up-regulated, as shown in Fig. 1A. The induction ranged from 2.2- to 3.8-fold when compared with basal levels in normoxia. A VEGF-specific ELISA confirmed that hypoxia also up-regulated VEGF protein levels. VEGF cellular protein levels increased 78% in Caco2, 125% in ColoHSR, and 105% in HT-29 cells (data not shown).

Hypoxia may up-regulate VEGF through either transcriptional or post-transcriptional mechanisms (18, 19). To confirm that this observed effect was transcriptional, VEGF promoter-luciferase reporter constructs were used. The full-length 2.8-kb VEGF promoter construct demonstrated a 3- to 6-fold induction of luciferase activity in Caco2 and ColoHSR cells (Fig. 1B), consistent with the Northern blot findings.

**Induction of HIF-1α by Hypoxia in Colon Cancer Cells.** It is well established that HIF-1α is induced by hypoxia in many cell types, and it trans-activates a broad range of genes involved in the cellular hypoxic response (11). To determine the role of HiF-1α in colon cancer, immunoblot analysis was first performed. In Caco2 and HT-29 cells, HIF-1α protein was induced strongly by hypoxia (Fig. 1C). In normoxia, HIF-1α expression was seen in HT-29 but not in Caco2 cells, but phosphorylated and unphosphorylated forms of HIF-1α were detected in hypoxic conditions in both cell lines. HIF-2α is a factor closely related to HIF-1α and also can play a role in the transcriptional response to hypoxia (32, 33). HIF-2α was not expressed in HT-29 cells. In Caco2 cells, faint expression of HIF-2α was identified, and this was only minimally up-regulated in hypoxia (data not shown). Thus, HIF-2α is unlikely to play an important role in hypoxia in these cells.

**Mutagenesis of HREs of the VEGF Promoter.** We sought to determine the role of HIF-1α in the hypoxic regulation of VEGF in colon cancer. As demonstrated previously, a VEGF promoter construct displayed hypoxic up-regulation commensurate to the induction seen by Northern blot analysis (Fig. 1B). There are two consensus HREs at −975 bp (5′-TACGGG) and −306 bp (5′-TACGTTGG)
in the VEGF promoter (8). Site-directed mutagenesis was performed to selectively alter these sites in the 2.3-kbVEGF-luc construct, either individually or in combination. Surprisingly, there was only a modest 19\% inhibition of the hypoxic induction of VEGF in Caco2 cells when the consensus HRE at −975 bp was mutated (Fig. 2A). In addition, disruption of a second potential HRE at −306 bp failed to block the hypoxic induction. The mHIF-975VEGF-luc construct also displayed similar up-regulation in ColoHSR colon cancer cells (Fig. 2B). In contrast, control studies in a hepatocellular (HepG2) and pancreatic (Panc-1) cell line demonstrated nearly complete suppression of the hypoxic induction of VEGF transcription when the consensus HIF-1 binding site at −975 bp was mutated (Fig. 2B).

These findings suggested that in colon cancer cells, HIF-1α may not be the only mediator of the hypoxic up-regulation of VEGF. To formally exclude the possibility that HIF-1α might bind to the VEGF promoter at a previously unrecognized site, we expressed a constitutively active HIF-1α in which the proline at amino acid position 564 in the pVHL binding motif was mutated to alanine (HIF-1α/PS64A; Ref. 34). This prevents degradation of HIF-1α in normoxic conditions. Overexpression of HIF-1α/PS64A in normoxia strongly up-regulated an HRE reporter in Caco2 cells (Fig. 3). The wild-type 2.3-kbVEGF-luc construct also was induced, but a mutation of the HRE at −975 bp rendered the VEGF promoter unresponsive to HIF-1α/PS64A, indicating that this is the only element through which HIF-1α regulates VEGF expression. Because this mutant construct remained responsive to hypoxia (Fig. 2A), alternative mechanisms independent of HIF-1α also must mediate the hypoxic induction of VEGF in colon cancer cells. HIF-2α is unlikely to serve this function because HIF-2α trans-activates the VEGF promoter through the same response elements as HIF-1α.

Inhibition of HIF-1α in Colon Cancer Cells. To gain additional insight into the role of HIF-1α in VEGF transcription, loss-of-function studies were performed using a siRNA approach. The siRNA pSUPER vector system was used to knock down HIF-1α in Caco2 cells (30). Two different siRNA constructs, HIF-1α/siRNA1470 and HIF-1α/siRNA2192, were cotransfected with either a consensus HRE-luc reporter or the 2.3-kbVEGF-luc reporter. As shown in Fig. 4, both siRNA constructs dramatically reduced HRE-luc reporter activity in hypoxia, demonstrating their potent silencing effects. In contrast, inhibition of HIF-1α resulted in only a 19–22\% reduction in VEGF promoter activity in hypoxia.

We then established Caco2 cells stably expressing HIF-1α/siRNA1470 (Caco2-HIF-kd1470) and HIF-1α/siRNA2192 (Caco2-HIF-kd2192). The hypoxic induction of HIF-1α protein was inhibited completely in Caco2-HIF-kd1470 and Caco2-HIF-kd2192 cells when compared with control transfected cells, Caco2-empty (Fig. 5A). Silencing of HIF-1α also was confirmed by an HRE-luc reporter assay (data not shown). Consistent with the previous transient transfection studies shown in Fig. 4, hypoxic induction of VEGF mRNA, promoter activity, and protein was observed (Fig. 5, B–D). This effect appeared to be VEGF specific because knockdown of HIF-1α in these cells almost inhibited completely the induction of a distinct hypoxia-inducible gene, GLUT-1 (Fig. 5B). It should be noted that induction of VEGF mRNA levels by hypoxia was reduced 13\%; VEGF promoter activity was reduced 30\%; and VEGF protein levels decreased 24\% when HIF-1α was knocked down, indicating that HIF-1α does have a functional role in the induction of VEGF. Nevertheless, these findings demonstrate that the role of HIF-1α in the hypoxic induction of VEGF transcription in colon cancer is modest and that alternative HIF-1α-independent mechanisms may predominate.

To confirm that this effect was not unique to Caco2 cells, DLD-1 colon cancer cells stably expressing both HIF-1α siRNA constructs also were generated (Fig. 6A). Northern blot studies again demonstrated that hypoxic induction of VEGF persisted even in the absence of HIF-1α. In contrast, expression of a distinct hypoxia-inducible gene, GLUT-1, was suppressed when HIF-1α was knocked down (Fig. 6B).

Identification of a Regulatory Region of the VEGF Promoter Responsive to Hypoxia. To identify the region of the VEGF promoter that might mediate HIF-independent expression during hypoxia in colon cancer cells, serial 5Δ-deletion constructs were used (Fig. 7). The full-length VEGF promoter construct was induced strongly by hypoxia. Stepwise deletion of sequences from −1811 to −420 bp did not significantly attenuate the hypoxic response. Of note, deletion of the consensus HRE at −975 bp failed to block the hypoxic induction, consistent with previous studies in which this HRE was mutated selectively (Fig. 2A). However, a dramatic reduction of the hypoxic
induction of VEGF was observed when the promoter region between −420 bp and −90 bp was deleted. Hypoxia also strongly up-regulated the 1.5-kb-VEGF-luc but not the 1.0-kb-VEGF-luc construct in ColoHSR and DLD-1 cells (data not shown). There is a potential HRE at −306 bp (8). However, previous studies that selectively mutated this element demonstrated that it does not mediate hypoxic induction (Fig. 2A). Other motifs within this region include a T-cell factor (TCF) binding element, CCAAT box, and GC-rich sequences that resemble Sp1 and AP2 sites. None of these motifs has been recognized previously to activate gene expression in hypoxic conditions. These results collectively suggest the existence of a unique HRE between −420 bp and −90 bp of the VEGF promoter.

**Effect of K-ras Signaling on Hypoxic Induction of VEGF.** The K-ras oncogene frequently is mutated in colorectal cancer and has been demonstrated previously to regulate VEGF expression in normoxic conditions (5, 35). In addition, H-ras signaling has been shown to regulate HIF-1α through phosphorylation (26, 36). We were curious to determine whether K-ras regulates the hypoxic induction of VEGF through HIF-1α. We first confirmed the effect of K-ras on the

**Fig. 3.** Constitutively active hypoxia-inducible factor-1α (HIF-1α; HIF-1α/P564A) fails to up-regulate the vascular endothelial growth factor (VEGF) promoter with a mutated hypoxia response element (HRE) at −975 bp. Reporter constructs containing either the VEGF promoter or four tandem copies of a canonical HRE (HRE-luc) were cotransfected with HIF-1α/P564A into Caco2 cells. pRL-CMV was used as an internal control. After transfection, cells were incubated in normoxia for 48 h, and dual luciferase assays were performed as described in "Materials and Methods." The results are displayed as fold induction by HIF-1α/P564A. Mean values from three independent transfections are shown. pRES, empty vector.

**Fig. 4.** Silencing of hypoxia-inducible factor-1α (HIF-1α) does not block the hypoxic induction of the vascular endothelial growth factor (VEGF) promoter. The HIF-1α small interfering RNA (siRNA) constructs were cotransfected with either a control hypoxia response element (HRE)-luciferase (HRE-luc) or a 2.3-kb VEGF promoter luciferase construct. Cells then were switched to hypoxic conditions for 24 h before luciferase activity was measured. The first bar (pSUPER control) represents the fold induction of the HRE or the VEGF reporter by hypoxia, and the subsequent bars indicate the hypoxic induction when HIF-1α is silenced by either the HIF-1α/siRNA1470 or HIF-1α/siRNA2192 constructs. Mean values from three independent transfections are shown.

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hypoxic regulation of VEGF. Mutant K-ras Val12 was expressed in Caco2 cells, and VEGF mRNA levels in normoxia and hypoxia were measured by Northern blot analysis (Fig. 8A). K-ras Val12 induced VEGF mRNA levels 1.8-fold in normoxia, and hypoxia induced VEGF mRNA levels 3.1-fold, but the combination up-regulated VEGF almost 6-fold, suggesting that oncogenic K-ras interacts with hypoxia to synergistically regulate VEGF. This enhancement also was observed with a VEGF promoter construct (Fig. 8B), indicating that this interaction between K-ras and hypoxia was transcriptional.

To determine whether this effect of K-ras Val12 was mediated through HIF-1α, we examined the effect of K-ras Val12 on HIF-1α expression levels and its phosphorylation status under hypoxic conditions. As shown in Fig. 8C, K-ras Val12 did not regulate either the levels or the phosphorylation of HIF-1α. In addition, studies were performed using a VEGF reporter construct selectively mutated at the HRE at −975 bp or −306 bp. As described earlier, the mHIF-975VEGFLuc and mHIF-306VEGFLuc constructs are induced by hypoxia in Caco2 cells (Fig. 2A). This induction was enhanced strongly by K-ras Val12 (Fig. 8B). Of note, there was a reduction in the activity of the mHIF-975VEGFLuc construct in the presence of hypoxia and K-ras Val12 (P < 0.05). Nevertheless, this synergistic response was substantial and is consistent with previous results demonstrating a modest contribution by HIF-1α in hypoxia. Control studies indicated that K-ras did not augment the hypoxic induction of an HRE reporter plasmid, confirming that K-ras does not enhance VEGF expression through HIF-1 (Fig. 8B). Finally, we tested Caco2 cells stably expressing siRNA against HIF-1α (Caco2-HIF-kd1470). Expression of K-ras Val12 in these cells also strongly enhanced the hypoxic up-regulation of VEGF promoter activity (Fig. 8D). Thus, oncogenic K-ras can up-regulate VEGF in an HIF-1-independent manner.

**DISCUSSION**

Angiogenesis is a critical step in the pathogenesis of human tumors, and VEGF has been established as one of the key growth factors mediating this process. We have demonstrated previously the roles of the K-ras and Wnt signaling pathways in the regulation of VEGF in early colon neoplasia (5). As tumors progress to more advanced stages of malignancy, levels of VEGF continue to increase. The current study sought to define molecular pathways that might regulate this enhanced production of VEGF. In particular, we focused on the role of hypoxia and have identified an HIF-1-independent pathway that is an important regulator of the hypoxic expression of VEGF in colon cancer.

Hypoxia commonly develops as tumors enlarge and progress to malignancy. In addition to the well-described response mediated by the HIF-1α transcription factor, there are other pathways that can be activated. Src has been demonstrated to be important in several tumor types, including colon cancer (21, 37). Transcription factors, including nuclear factor-kB (24) and cyclic AMP-responsive element binding protein (38), are activated by hypoxia. Cyclooxygenase-2, a strong inducer of VEGF (39), also can be up-regulated by hypoxia (24, 40). Despite the pivotal role of cyclooxygenase-2 in the pathogenesis of colon cancer, we could not establish a specific function for cyclooxygenase-2 in the hypoxic induction of VEGF (data not shown).
HIF-1 has been established as a critical factor in the cellular response to hypoxia, in part through in vivo studies that selectively inactivate either the HIF-1α or HIF-1β (ARNT) subunit (16, 17, 41, 42). In cultured HIF-1α/− embryonic stem (ES) cells, a transient up-regulation of VEGF mRNA in hypoxia was observed, but by 16 h, this up-regulation had disappeared (17, 43, 44). Curiously, total VEGF mRNA levels were elevated in embryos deficient in HIF-1α when compared with wild-type controls (41). HIF-1α/− ES cells implanted into nude mice can display tumor-like growth (45). In these “tumors,” VEGF levels are reduced but not absent, and there is decreased vascularization (43, 44). These findings collectively indicate a key role for HIF-1 in the hypoxic regulation of VEGF. However, it should be noted that the inhibition of VEGF expression is not absolute in HIF-1α/− cells, and hypoxic induction can be detected. One possible explanation may be enhanced VEGF mRNA stabilization, but a contribution from VEGF gene transcription cannot be ruled out (17). Another important consideration is that there are likely to be cell-specific differences in the hypoxic regulation of VEGF. Specifically, ES cells may not reflect the behavior of true tumor cells derived from epithelial tissues. The absence of mutations in specific oncogenes and tumor suppressor genes in these ES cell tumor models limits their ability to recapitulate human tumors. For example, ARNT-deficient hepatoma cells display persistent hypoxic induction of VEGF mRNA, indicating that pathways independent of HIF-1 may regulate hypoxia-responsive genes, including VEGF, in epithelial-derived cancer cells (42).

HIF-1α is induced by hypoxia in colon cancer cells. HRE reporter activity was stimulated by hypoxia, indicating that HIF-1α is functional in these cells. However, when HIF-1α was silenced by RNA interference, a significant up-regulation of VEGF in response to hypoxia persisted. Thus, there is likely to be an alternative pathway that also can mediate hypoxic induction of VEGF. A recent report in which HIF-1α was knocked down by siRNA duplex oligonucleotides in HCT116 colon cancer cells demonstrated an inhibitory effect on VEGF (46). Possible explanations for this discrepancy may be different methods of HIF-1α silencing used or intrinsic differences in the cell lines studied. Our data demonstrated that hypoxia strongly up-regulates VEGF promoter activity, mRNA, and protein when HIF-1α is silenced by siRNA in transient and stable conditions.

HIF-2α is an HIF-1α homologue that also can dimerize with ARNT. HIF-2α gene transcription can be up-regulated in HIF-1α null cells (17). The HIF-2 protein complex binds to the same consensus element as HIF-1. Hypoxia only weakly induced HIF-2α expression in our colon cancer cells, suggesting only a minor contribution by HIF-2. More importantly, mutagenesis of the HREs in the VEGF promoter failed to block hypoxic induction. This excludes a role for HIF-2 and HIF-1. In addition, HIF-2α protein levels were not increased in HIF-1α knockdown colon cancer cells (data not shown). Thus, HIF-2α is not likely to account for the HIF-1-independent regulation of VEGF in colon cancer cells.

Interestingly, this HIF-1-independent regulation of VEGF may be cell specific because the mHIF-975VEGF-luc construct was unresponsive to hypoxia in hepatocellular and pancreatic cancer cell lines tested. This observation indicates that HIF-1 may be the primary mediator of the hypoxic regulation of VEGF in some cell types, but that other cell types (e.g., colonic) possess alternative secondary mechanisms.

The HIF-independent induction of VEGF in hypoxic conditions may be mediated by a regulatory region of the VEGF promoter between −420 and −90 bp. There is a potential HRE at −306 bp, but this element does not activate the VEGF promoter (8). Other motifs within this regulatory region include a TCF binding site, a CCAAT box, and several GC-rich sequences that bear partial homology to Sp1 and AP2 consensus sites. None of these elements has been established.
This suggests that oncogenic signaling pathways can regulate angiogenesis in states of hypoxia. Furthermore, these findings raise the possibility that there may be therapeutic targets in addition to HIF-1 that could block hypoxia-induced responses in cancer.

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