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# Differential Involvement of Vascular Endothelial Growth Factor in the Survival of Hypoxic Colon Cancer Cells

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## Abstract

**The recent approval of bevacizumab (Avastin), a humanized anti-vascular endothelial growth factor (VEGF) monoclonal antibody, in combination with chemotherapy for the treatment of patients with metastatic colorectal cancer, has provided proof of principle of the efficacy of antiangiogenic strategies for cancer therapy. The activity of bevacizumab is primarily attributed to its ability to inhibit endothelial cell survival. Whether anti-VEGF strategies may also have a direct effect on cancer cell survival is poorly understood. We show that serum-starved colon cancer cells differentially respond to autocrine production of VEGF with the induction of hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) and survival under hypoxic conditions. Inhibition of VEGF or VEGF receptor 2 (VEGFR2)/KDR, but not VEGFR1/Flt-1, was sufficient to abrogate VEGF-mediated induction of HIF-1 $\alpha$  and survival in sensitive HCT116, but not in resistant HT29, colon cancer cells. These results provide evidence that a VEGF/KDR/HIF-1 $\alpha$  autocrine loop differentially mediates survival of hypoxic colon cancer cells, and they suggest that colon cancer cells may be intrinsically sensitive or resistant to anti-VEGF strategies, which may determine the therapeutic efficacy of bevacizumab. [Cancer Res 2008;68(1):285–91]**

## Introduction

Bevacizumab (Avastin), a humanized anti-vascular endothelial growth factor (VEGF) monoclonal antibody, has been recently approved for the treatment of patients with metastatic colorectal cancer in combination with chemotherapy (5-fluorouracil, leucovorin, and irinotecan), providing proof of principle that antiangiogenic strategies may be effective for the treatment of human cancers (1). Addition of bevacizumab to chemotherapy increased overall survival by  $\sim$ 5 months in patients with metastatic colorectal cancer (1). However, which patients may benefit from treatment with bevacizumab is unclear (2). To explain the increased therapeutic efficacy of combination strategies containing anti-VEGF antibodies, several hypotheses have been proposed, including inhibition of new blood vessel formation or “normalization” of the dysfunctional tumor vasculature (3, 4). Whether anti-VEGF strategies may have a direct effect on cancer cell survival is not known.

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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VEGF-A is a major angiogenic factor secreted under hypoxic conditions by both cancer cells and stromal infiltrating cells (5). VEGF-A binds to two tyrosine kinase receptors on endothelial cells: VEGF receptor-1 (VEGFR1/Flt-1), whose function remains poorly defined, and VEGFR2 (KDR), which mediates proliferation and survival of endothelial cell (6). Expression of VEGFRs has also been shown in nonendothelial cells, including hematopoietic stem cells and monocytes, and more recently in a variety of tumor types (7, 8). However, the function of VEGFRs on cancer cells is poorly understood. Expression of Flt-1 in colon cancer cells has been recently associated with induction of cell motility and invasion (9). In contrast, the expression and function of KDR in colon cancer cells, if any, has not been shown.

Most solid tumors develop regions of low oxygen tension because of a tissue imbalance between oxygen supply and consumption (10). Hypoxic cancer cells are more invasive and metastatic, resistant to apoptosis and ultimately to chemotherapy and radiation therapy (11). Hypoxia inducible factor 1 (HIF-1), a master regulator of the hypoxic response in mammalian cells, may act as a survival factor for cancer cells by inducing the expression of genes that mediate angiogenesis, including but not limited to VEGF-A, tumor metabolism, and metastasis (12). Failure to eliminate the hypoxic fraction of solid tumors is eventually associated with poor prognosis. Hence, the identification of survival pathways used by hypoxic cancer cells is important for the potential therapeutic implications, and strategies to target hypoxic cell signaling are being actively pursued (13).

We show that colon cancer cells differentially express a functional VEGF/KDR/HIF-1 $\alpha$  autocrine loop that mediates survival under hypoxic conditions. Importantly, bevacizumab induced apoptosis of hypoxic HCT116 cells, which express a functional KDR but not HT29. These results show that (a) VEGF mediates survival of hypoxic HCT116 colon cancer cells in a HIF-1 $\alpha$ -dependent fashion, (b) colon cancer cells can differentially express a functional KDR receptor, and (c) the presence of a functional response to VEGF may be associated with, and potentially predict for, efficacy of anti-VEGF therapies in colon cancer patients.

## Materials and Methods

**Cell culture and reagents.** HT29 and HCT116 cells were maintained in RPMI containing 5% fetal bovine serum (FBS; Cambrex BioScience), and 2 mmol/L glutamine at 37°C in a humidified incubator containing 21% O<sub>2</sub>, 5% CO<sub>2</sub> in air (called normoxia). Experiments under hypoxic conditions (1% O<sub>2</sub>) were performed in the hypoxic workstation Invivo<sub>2</sub> 400 (Biotrace International). Neutralizing antibodies against human insulin-like growth factor (IGF-I), VEGF, basic fibroblast growth factor (bFGF), platelet-derived growth factor-BB (PDGF-BB), and EGF and human recombinant VEGF-A were purchased from R&D Systems.

**Flow cytometry analysis.** To evaluate cell surface expression of VEGFR2/KDR, cells were stained with specific PE-conjugated antibodies

(R&D Systems) in 0.5% of bovine serum albumin for 1 h at 4°C, washed, and immediately analyzed by flow cytometry (FACScan, Becton Dickinson). Samples were gated on a dot plot showing forward scatter and side scatter to exclude cell debris not within normal cell size. Gated cells were plotted on a histogram plot, showing the background staining overlay with the sample staining. Annexin V staining was performed using the Annexin V FITC apoptosis kit (BD Bioscience) according to the manufacturer's instructions. Gated cells were plotted on a dot plot showing Annexin V staining and propidium iodide staining.

**Immunoblotting and immunoprecipitation.** Cells were lysed in radioimmunoprecipitation assay buffer, and typically, 100- $\mu$ g protein was separated in a 4% to 20% Tris-glycine gel (Invitrogen) and transferred on a polyvinylidene difluoride membrane (Invitrogen), as previously described (14). The following antibodies were used: anti-HIF-1 $\alpha$  (BD Biosciences), anti-NRP1, anti Flt-1, KDR, PLC $\gamma$  (Santa Cruz Biotechnology),  $\beta$ -actin (Chemicon), phosphorylated extracellular signal-regulated kinase 1/2 (ERK1/2) and total ERK1/2 (Cell Signaling), and anti-phosphorylated tyrosine (Upstate). VEGFR2/KDR was immunoprecipitated from 500  $\mu$ g of cell lysates by incubation with 10  $\mu$ L of anti-KDR antibody (Santa Cruz Biotechnology) for 1.5 h at 4°C followed by addition of 20  $\mu$ L protein A/G agarose (Santa Cruz Biotechnology) overnight at 4°C. The mixture was centrifuged at 12,000 $\times$ *g* for 20 min; pellet was mixed with 40  $\mu$ L of 2 $\times$  Laemmli buffer, boiled for 5 min, and subjected to SDS-PAGE and Western blotting as previously described (14).

**Transient transfection.** Specific small interfering RNA (siRNA) oligonucleotides targeting VEGF (ID 42868), Flt-1 (ID 190, 191), KDR (ID 121,122), and neuropilin 1 (ID 114375) were obtained from Ambion. HT29 and HCT116 were transfected using Oligofectamine (Invitrogen) according to the manufacturer's instruction. Briefly, siRNA complexes were added to subconfluent cells and then incubated in serum-free condition for 4 h at 37°C. Medium was then changed to RPMI with 5% FBS, and cells were incubated for an additional 48 h before treatment, as indicated.

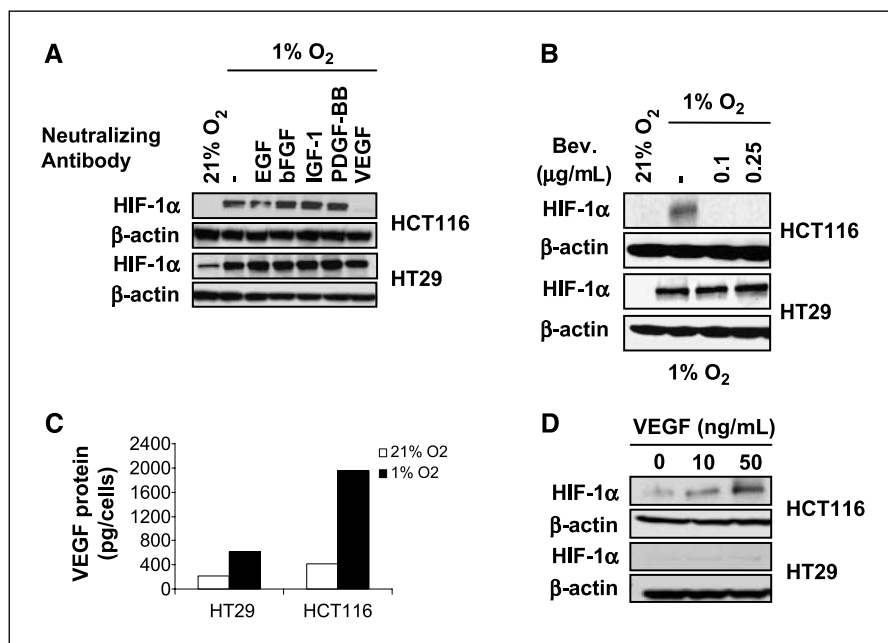
**Real-time PCR.** Total cellular RNA was isolated using RNA minikit (Qiagen). One microgram of total RNA was used to perform reverse transcription-PCR using RT-PCR kit (PE Biosystems) according to the manufacturer's instruction. Real-time PCR was performed using an ABI-Prism 7700 Sequence Detector (Applied Biosystems) as previously described (15). Primers specific for KDR mRNA were obtained from Applied Biosystems (sequences are available upon request). Detection of 18S rRNA, used as internal control, was performed using premixed reagents from Applied Biosystems.

**VEGF ELISA.** Total levels of VEGF-A protein were measured on cell-free supernatant using human VEGF-A Quantikine ELISA kit (R&D Systems).

## Results

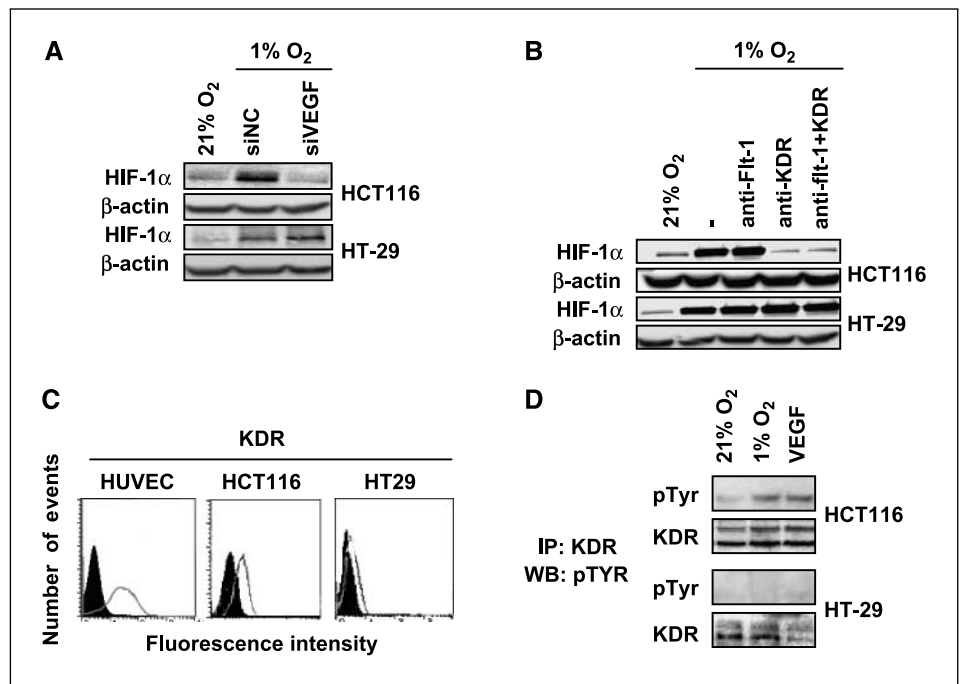
**VEGF differentially regulates hypoxic induction of HIF-1 $\alpha$  in colon cancer cells.** To investigate the involvement, if any, of autocrine production of growth factors in the hypoxic induction of HIF-1 $\alpha$  in cancer cells, we screened a panel of 30 different cancer cell lines, corresponding to the most common tumor types. Cells were cultured, in the absence of exogenous growth factors, under normoxic or hypoxic conditions for 18 h, in the absence or presence of neutralizing antibodies targeting growth factors that are frequently dysregulated in human cancers, including EGF, bFGF, IGF-I, PDGF, and VEGF. Interestingly, we found that distinct growth factors were implicated in the hypoxic induction of HIF-1 $\alpha$  in different tumor types and within the same tissue type (Supplementary Fig. S1 and data not shown), consistent with cell type-specific genetic alterations and aberrant signaling pathways.

We focused our attention on colon cancer cell lines, in which we found that anti-VEGF antibodies significantly inhibited hypoxic induction of HIF-1 $\alpha$  in HCT116, HCT-15, SW620, and HCC2998, but not in HT29, COLO-205, and KM-12 (Fig. 1A; and Supplementary Fig. S1). The differential response of colon cancer cell lines to anti-VEGF antibodies was of interest because bevacizumab is currently used for the treatment of patients with metastatic colorectal cancer. HIF-1 $\alpha$  was expressed at low or undetectable levels in normoxic HCT116 and HT29 and was not affected by addition of anti-VEGF antibodies (data not shown). Neutralizing anti-VEGF antibodies (Fig. 1A) or bevacizumab (Fig. 1B), but not antibodies against EGF, bFGF, IGF-I, or PDGF, completely abrogated hypoxic induction of HIF-1 $\alpha$  protein in HCT116 cells, consistent with autocrine production of VEGF in these cells. In contrast, neither anti-VEGF antibodies (Fig. 1A) nor bevacizumab (Fig. 1B) had any effect on HIF-1 $\alpha$  accumulation in HT29. To confirm the induction of VEGF protein by hypoxia, HCT116 and HT29 were cultured under normoxic or hypoxic conditions, and the levels of VEGF protein were measured by ELISA. Hypoxia induced 3-fold and



**Figure 1.** Anti-VEGF antibodies inhibit hypoxic induction of HIF-1 $\alpha$  in HCT116, but not HT29. **A**, HCT116 and HT29 cells were serum-starved for 24 h. Cells were then incubated for an additional 18 h under normoxic (21% O<sub>2</sub>) or hypoxic (1% O<sub>2</sub>) conditions in the absence or presence of the indicated neutralizing antibodies: anti-EGF (50 ng/mL), anti-bFGF (1  $\mu$ g/mL), anti-IGF-1 (10  $\mu$ g/mL), anti-PDGF-BB (100 ng/mL), and anti-VEGF (50 ng/mL). HIF-1 $\alpha$  protein accumulation was assessed by immunoblotting.  $\beta$ -Actin is shown as loading control. **B**, cells were cultured as described above under normoxic or hypoxic conditions in the absence or presence of increasing concentrations of bevacizumab (*Bev*), and levels of HIF-1 $\alpha$  and  $\beta$ -actin were assessed by Western blot. **C**, cells were cultured as described above and incubated under normoxic or hypoxic conditions for 18 h. Levels of VEGF protein in the supernatants were measured by a commercially available ELISA kit. **D**, cells were cultured as described above in the absence or presence of the indicated concentrations of recombinant VEGF for 18 h and levels of HIF-1 $\alpha$  and  $\beta$ -actin were assessed by Western blot.

**Figure 2.** HCT116 cells, but not HT29, express a functional KDR. **A**, HCT116 and HT29 were transfected with siNC or siVEGF. At 48 h after transfection, cells were serum-starved for 24 h and incubated under normoxic or hypoxic conditions for 16 h. Levels of HIF-1 $\alpha$  and  $\beta$ -actin were assessed by Western blot. **B**, HCT116 and HT29 cells were serum-starved for 24 h. Cells were then incubated under normoxia or hypoxia in the absence or presence of neutralizing antibodies against Flt-1 (50 ng/mL) or KDR (50 ng/mL), alone or in combination for 18 h. HIF-1 $\alpha$  protein accumulation was assessed by immunoblotting.  $\beta$ -Actin is shown as loading control. **C**, surface expression of KDR in HUVEC, HCT116, and HT29 cells cultured under normoxic conditions was analyzed by flow cytometry using a specific anti-human KDR antibody. *Dark area*, cells stained with an isotype-matched control antibody; *white area*, KDR-positive cells. Data are from one representative experiment of three performed. **D**, cells were cultured under normoxia in the absence or presence of recombinant VEGF (50 nmol/L) for 15 min or under hypoxic conditions for 18 h. Cell lysates were immunoprecipitated with anti-KDR antibodies, and immunoblotting was performed using anti-KDR or anti-phosphorylated Tyr antibodies.



5-fold higher levels of VEGF protein in HT29 and HCT116, respectively, relative to normoxic cells (Fig. 1C). However, hypoxia induced much higher levels of VEGF protein in HCT116 (1.8 ng/mL) relative to HT29 (0.6 ng/mL) cells. To rule out the possibility that lower levels of secreted VEGF might account for the lack of response in HT29, cells were treated with increasing concentrations of VEGF (up to 50 ng/mL) under normoxic conditions, and the levels of HIF-1 $\alpha$  protein were measured. VEGF induced HIF-1 $\alpha$  protein accumulation in a dose-dependent fashion in HCT116, but not in HT29 (Fig. 1D).

To further validate the involvement of VEGF-A and to rule out the possibility that intracellular (16, 17), rather than secreted, VEGF may still be active in HT29, cells were transfected with siRNA negative control (siNC) or targeting VEGF-A (siVEGF), and hypoxic induction of HIF-1 $\alpha$  was measured. siVEGF, but not siNC, completely inhibited hypoxic induction of HIF-1 $\alpha$  in HCT116, but had no effect in HT29 (Fig. 2A), demonstrating that autocrine production of secreted VEGF is involved in the hypoxic induction of HIF-1 $\alpha$  in HCT116, but not HT29, and revealing an intrinsic differential responsiveness of colon cancer cells to VEGF.

**KDR, but not Flt-1, mediates VEGF-dependent induction of HIF-1 $\alpha$  in HCT116, but not HT29, hypoxic cells.** To address which receptor was required for the VEGF-dependent autocrine induction of HIF-1 $\alpha$  in colon cancer cells, HCT116 and HT29 cells were cultured under normoxic or hypoxic conditions in the absence or presence of neutralizing antibodies against Flt-1 or KDR, and HIF-1 $\alpha$  levels were measured. Anti-KDR, but not anti-Flt-1, antibodies completely abolished hypoxic induction of HIF-1 $\alpha$  in HCT116 cells (Fig. 2B), consistent with VEGF-dependent activation of VEGFR2/KDR. In contrast, neither anti-KDR nor anti-Flt-1 antibodies affected hypoxic induction of HIF-1 $\alpha$  in HT29 (Fig. 2B).

The expression, if any, of KDR in colon cancer cells is controversial. Flow cytometry analysis revealed low, but detectable, levels of KDR surface expression, relative to human endothelial cell, in HCT116 and little, if any, in HT29 (Fig. 2C). Consistent with this result, KDR mRNA, measured by real-time PCR, was present in

HCT116, but at significantly lower levels relative to human umbilical vascular endothelial cell (HUVEC) and was barely detectable in HT29 (Supplementary Fig. S2). To investigate KDR functional activation, HCT116 and HT29 cells were treated under normoxic conditions in the absence or presence of recombinant human VEGF or under hypoxic conditions. KDR was immunoprecipitated and Western blot was performed with anti-phosphotyrosine antibodies. Both hypoxia and VEGF induced phosphorylation of KDR, relative to untreated normoxic cells, in HCT116, but not HT29, cells, consistent with the presence of a functional receptor in HCT116 (Fig. 2D).

Next, HCT116 and HT29 were transiently transfected with either siNC or targeting two distinct sequences of Flt-1 and KDR, respectively, and hypoxic levels of HIF-1 $\alpha$  were measured (Fig. 3A). Levels of Flt-1 and KDR expression were decreased by >90% in HCT116 transfected with the corresponding siRNA. Notably, siRNA targeting KDR, but not Flt-1, completely inhibited hypoxic induction of HIF-1 $\alpha$  expression relative to siNC, consistent with data obtained using anti-KDR antibodies. In contrast, neither siRNA was able to affect HIF-1 $\alpha$  expression in HT29 (Fig. 3B). These results are consistent with low, yet functional, levels of KDR expression in HCT116 cells, but not in HT29 cells.

Neuropilin-1 is a component of KDR, which increases the affinity of the receptor for VEGF in endothelial cells (18). We tested the expression of neuropilin-1 in HCT116 and HT29 cells cultured under normoxic or hypoxic conditions. Neuropilin-1 was expressed at much higher levels in HCT116 relative to HT29 and was not modulated by hypoxia (Fig. 3C). To test whether neuropilin-1 was functionally involved in the autocrine induction of HIF-1 $\alpha$  by VEGF, HCT116 and HT29 cells were transiently transfected with siNC or targeting neuropilin-1 (siNRP). siNRP significantly inhibited hypoxic induction of HIF-1 $\alpha$  expression in HCT116, but not HT29, cells (Fig. 3D), consistent with the involvement of neuropilin in KDR functional activation. Similar results were obtained with a second siRNA targeting a distinct sequence of neuropilin-1 (data not shown).



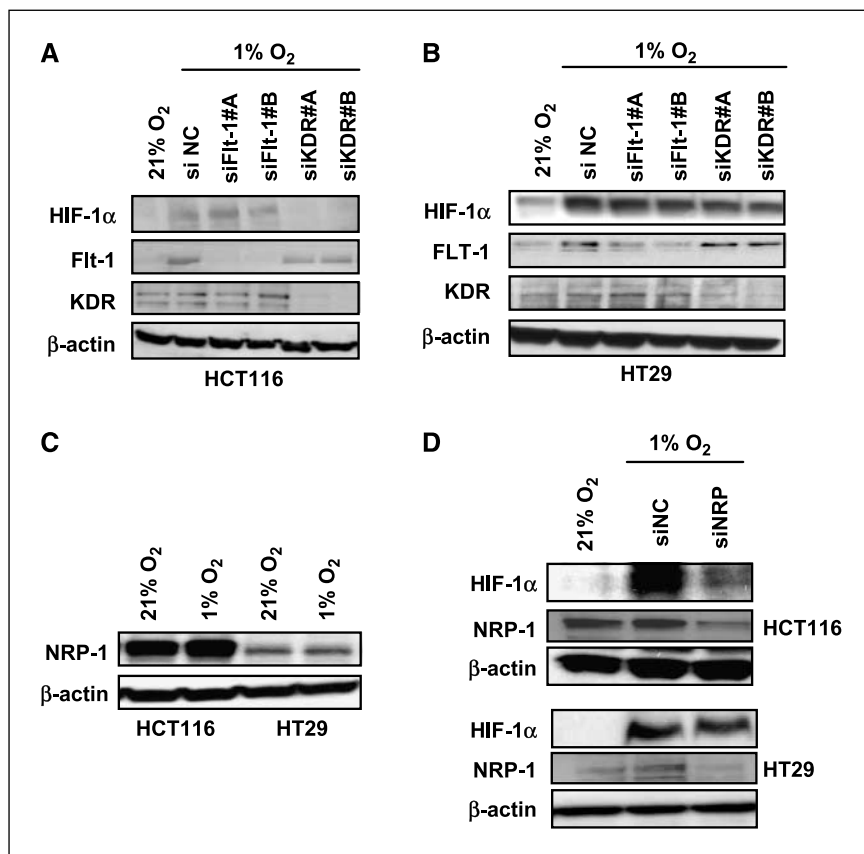
**Autocrine production of VEGF mediates survival of HCT116, but not HT29, cells under hypoxic conditions.** As hypoxic cancer cells may be resistant to apoptosis, we hypothesized that VEGF might be involved in mediating survival of hypoxic colon cancer cells expressing a functional KDR. HCT116 and HT29 were cultured under normoxic or hypoxic conditions in the absence or presence of either bevacizumab or control IgG, and the percentage of apoptotic cells was measured by Annexin V staining. A lower percentage of apoptotic HCT116 cells was observed under hypoxic relative to normoxic conditions (19% versus 30%; Fig. 4A), consistent with hypoxia-dependent autocrine production of survival factor(s) and the previously reported induction of apoptosis in HCT116 after serum withdrawal (19). Addition of bevacizumab, but not a control IgG, increased the number of hypoxic apoptotic cells to 29%, comparable with levels observed in normoxic cells (30%). More importantly, anti-KDR, but not anti-Flt-1, antibodies increased the percentage of apoptotic cells from 21%, in the presence of a control IgG, to 35% (Fig. 4A). These results show that the effects of VEGF on survival of hypoxic HCT116 cells are mediated by KDR, and they suggest that other VEGF isoforms, which are not neutralized by bevacizumab, may contribute to survival of hypoxic HCT116 by a KDR-dependent mechanism (20). In contrast, slightly higher levels of apoptosis were detected in HT29 cultured under hypoxic relative to normoxic conditions (18% versus 12%), and none of the antibodies used affected survival of hypoxic HT29 cells (Fig. 4A).

To further validate the involvement of a VEGF/KDR autocrine loop in the survival of hypoxic cells, HCT116 were transiently transfected with siNC or siVEGF, Flt-1 (siFlt-1), or KDR (siKDR),

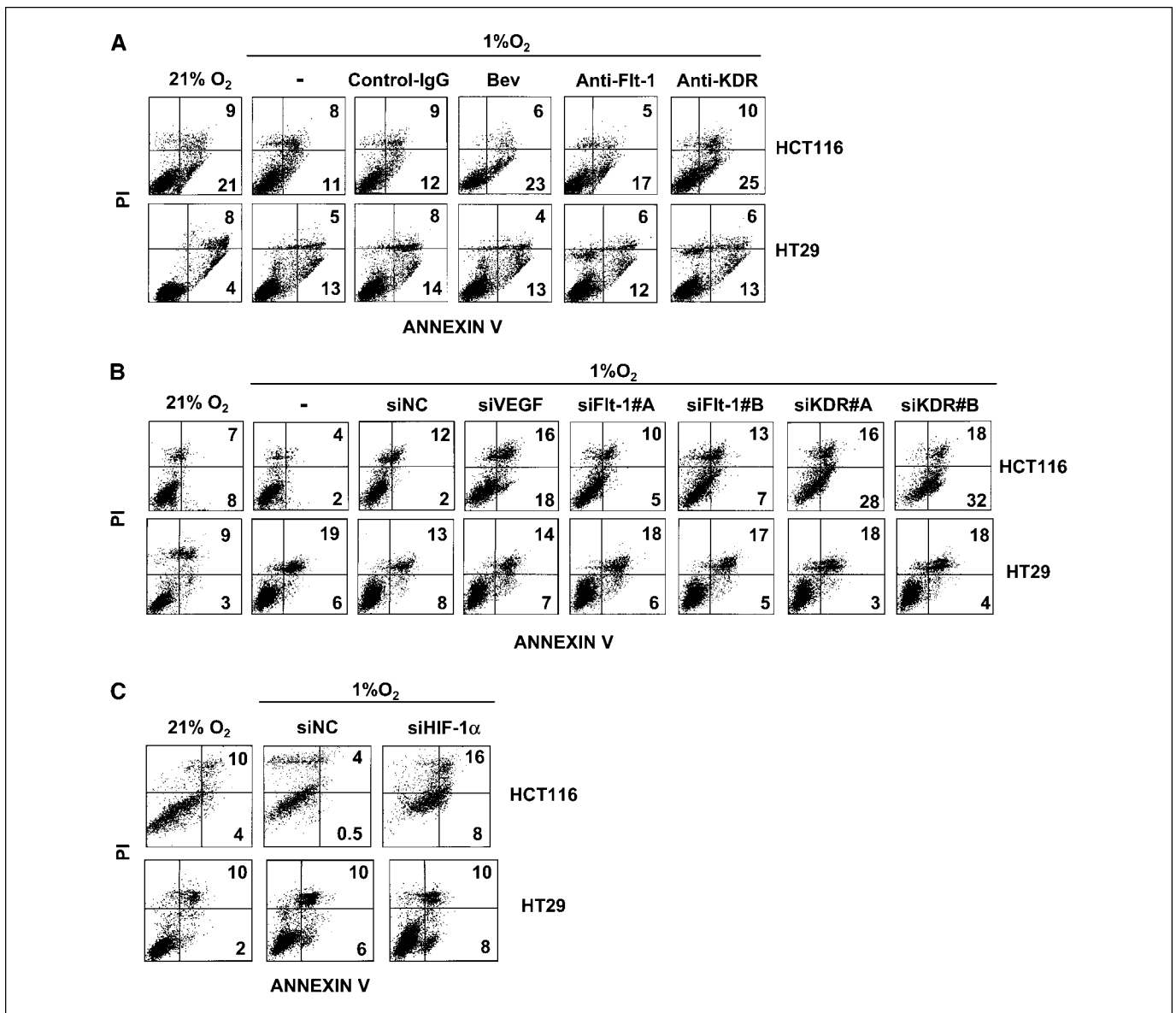
respectively. As shown in Fig. 4B, siVEGF increased apoptosis of hypoxic cells to 34%, relative to 14% in the presence of siNC. In addition, siKDR consistently increased the percentage of apoptotic cells from 14% (siNC) to 44% and 50%, respectively, using two siRNA targeting distinct sequences (Fig. 4B). In contrast, two distinct siRNA targeting Flt-1 had no effect on survival of hypoxic HCT116 cells and neither siRNA had any effect on survival of HT29 (Fig. 4B). Anti-VEGF antibodies, siVEGF, or siKDR also increased the levels of poly(ADP-ribose) polymerase (PARP) cleavage in hypoxic HCT116, relative to control IgG or siNC, providing further evidence of induction of apoptosis under these conditions (data not shown). To address if HIF-1 $\alpha$  was involved in the VEGF-dependent survival effect, HCT116 and HT29 cells were transfected with siNC or siRNA targeting HIF-1 $\alpha$  and cultured under normoxic or hypoxic conditions. siRNA targeting HIF-1 $\alpha$  increased the percentage of apoptosis in hypoxic HCT116, but not HT29, cells to 24%, relative to 4.5% in the presence of siNC (Fig. 4C).

Overall, these data show that VEGF and HIF-1 $\alpha$  mediate survival of hypoxic colon cancer cells, and they suggest that colon cancer cells may be differentially sensitive to induction of apoptosis upon inhibition of VEGF activity.

**VEGF mediates survival of HCT116 cells via a PLC $\gamma$ /ERK-dependent pathway.** PLC $\gamma$  is phosphorylated in a KDR-dependent fashion and may activate the ras/raf pathway in endothelial cells (21, 22). To test whether PLC $\gamma$  was activated under our experimental conditions, HCT116 cells were cultured under normoxic or hypoxic conditions in the absence or presence of neutralizing antibodies targeting VEGF, KDR, or Flt-1 and tyrosine phosphorylation of PLC $\gamma$  was measured in cell lysates. Hypoxia



**Figure 3.** KDR mediates autocrine induction of HIF-1 $\alpha$  by VEGF in HCT116, but not HT29, cells. *A and B*, HCT116 (*A*) or HT29 (*B*) cells were transfected with siNC or targeting two distinct regions of Flt-1 (siFlt1#A and siFlt1#B) or KDR (siKDR#A and siKDR#B), respectively. At 48 h after transfection, cells were serum-starved for 24 h and then incubated under hypoxia for 18 h. Normoxic nontransfected cells (*lane 1*). Levels of HIF-1 $\alpha$ , Flt-1, and KDR were measured by Western blot.  $\beta$ -Actin is shown as loading control. *C*, HCT116 and HT29 were serum-starved and then were incubated under normoxic or hypoxic conditions for 18 h, and levels of neuropilin 1 (*NRP-1*) were assessed by immunoblotting.  $\beta$ -Actin is shown as loading control. *D*, HCT116 and HT29 were transfected with siNC or siNRP. At 48 h after transfection, cells were serum-starved for 24 h and incubated under hypoxia for 24 h. Normoxic nontransfected cells (*lane 1*). Levels of HIF-1 $\alpha$  and neuropilin 1 were measured by Western blot.  $\beta$ -Actin is shown as loading control.



**Figure 4.** Autocrine secretion of VEGF mediates KDR-dependent survival of HCT116, but not HT29, cells. *A*, HCT116 and HT29 cells were serum-starved in RPMI for 24 h. Cells were then incubated under normoxia or hypoxia in the absence or presence of bevacizumab or neutralizing antibodies against Flt-1 or KDR or isotype-matched IgG for 24 h. Percentage of apoptotic cells was measured by flow cytometry analysis of Annexin V versus propidium iodide–positive cells. Percentage of Annexin V–positive cells (*bottom*, propidium iodide–negative; *top*, propidium iodide–positive) from one representative experiment. *B*, HCT116 and HT29 cells were either nontransfected or transfected with siNC, siVEGF, Flt-1 (siFlt-1#A and siFlt-1#B) or KDR (siKDR#A and siKDR#B). At 48 h after transfection, cells were serum-starved for 24 h and then incubated under normoxic or hypoxic conditions for an additional 24 h. Apoptosis was measured by Annexin V staining. Percentage of Annexin V–positive cells from one representative experiment. *C*, HCT116 and HT29 cells were transfected as described above with siNC or siRNA targeting HIF-1 $\alpha$  (*siHIF-1 $\alpha$* ) and incubated under hypoxia for 24 h. Normoxic nontransfected cells are also shown.

induced phosphorylation of PLC $\gamma$  in HCT116, relative to normoxic cells, which was almost completely abrogated by addition of neutralizing antibodies against either VEGF or KDR, but not Flt-1, consistent with a functional VEGF/KDR pathway (Fig. 5A). In contrast, PLC $\gamma$  phosphorylation was not detectable in HT29 cells cultured under hypoxia or in the presence of VEGF (data not shown).

Next, we tested the phosphorylation of ERK1/2 in HCT116 cells cultured under normoxia or hypoxia in the absence or presence of PLC $\gamma$  (U73122) or ERK (PD98059) inhibitors. We found that ERK was activated under hypoxic conditions and was inhibited by both PD98059 and U73122, indicating that ERK1/2 activation is

downstream of PLC $\gamma$  (Fig. 5B). Notably, inhibition of PLC $\gamma$  or ERK1/2 also significantly affected hypoxic induction of HIF-1 $\alpha$ , suggesting that the same signaling pathways mediate survival and HIF1 $\alpha$  expression in HCT116 cells (Fig. 5C). Indeed, inhibition of PLC $\gamma$  or ERK1/2 increased the number of apoptotic cells to 30% and 58%, respectively, relative to hypoxic cells (12%; Fig. 5D). Consistent with these results, PARP cleavage was observed in normoxic but not in hypoxic HCT116 cells and was induced in hypoxic cells treated with U73122 or PD98059 (data not shown).

In summary, our data show that a VEGF/KDR/PLC $\gamma$ /ERK1/2 pathway is activated by hypoxic HCT116 cells to induce HIF-1 $\alpha$  expression and survival under hypoxic stress conditions.

## Discussion

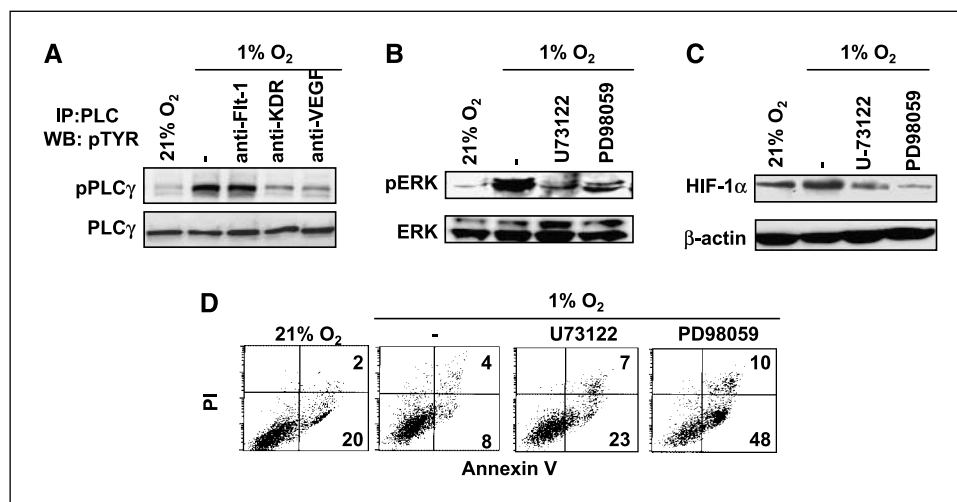
We recently identified an autocrine survival loop involving FGF-2 and HIF-1 $\alpha$  in hypoxic human endothelial cells (14). By culturing colon cancer cells under hypoxic conditions in the absence of exogenous growth factors, which mimics nutrient and oxygen deprivation found in solid tumors, we have discovered that anti-VEGF-A antibodies, including bevacizumab, significantly inhibited hypoxic induction of HIF-1 $\alpha$  in HCT116, HCT15, SW620, and HCC2998, but not HT29, KM12, and COLO205. More importantly, inhibition of VEGF or VEGF signaling blocked survival of hypoxic HCT116, but not HT29, cells. These results are consistent with (a) the potential involvement of VEGF in mediating survival of hypoxic colon cancer cells and (b) a differential responsiveness of hypoxic colon cancer cells to VEGF and its inhibition.

The involvement of VEGF in mediating survival of hypoxic colon cancer cells was surprising because VEGF is thought to be primarily a survival factor for endothelial cells (23). Recently, however, evidence has been provided that VEGF may also act on cancer cells that express receptors for VEGF, including Flt-1, KDR, and flt-4 (7, 8, 24). In nonendothelial cells, Flt-1 has been shown to mediate migration of human monocytes and, more recently, cell motility and invasion of colon cancer cells lines (9). The expression and function of KDR has been shown in hematopoietic stem cells and in hematologic malignancies (7, 8). However, the functional role of KDR, if any, in solid tumors remains elusive. We found that anti-KDR antibodies or siRNA targeting KDR, but not Flt-1, significantly inhibited hypoxic induction of HIF-1 $\alpha$  and survival in HCT116, but not HT29. These results raised the possibility that HCT116 expressed a functional KDR receptor. Analysis of the expression and function of KDR in HCT116 and HT29 cells was overall consistent with low, yet functional, levels of KDR in HCT116, but not HT29. Accordingly, phosphorylation of KDR, an indication of its functional activation, was only detectable in HCT116 and not in HT29.

The relatively low, yet functional, levels of KDR expression in HCT116 is consistent with two possibilities: the first is that very low levels of KDR surface receptor are expressed on the majority of cells, which would account for the response of HCT116 cells to anti-VEGF antibodies; alternatively, only a subset of cells expresses a functional receptor and is sensitive to VEGF inhibition, which is more consistent with the pattern of KDR surface expression that we observed. Conversely, the presence of an autocrine survival loop in a subset of cells expressing a functional KDR would render them more resistant to chemotherapy-induced apoptosis under hypoxic stressed conditions, which may contribute to treatment failure. Interestingly, expression of VEGFR(s) has been shown in stem cell-like populations, and evidence has been provided that hypoxia may be a favorable environmental condition for the maintenance of the stem cell phenotype (25, 26).

The lack of a functional VEGF/KDR signaling pathway in HT29 may confer intrinsic resistance to anti-VEGF therapies. The possibility that intracellular, rather than secreted, VEGF might be signaling in HT29 was ruled out by using siRNA targeting VEGF or KDR, which failed to show any effect on hypoxic induction of HIF-1 $\alpha$  or survival in HT29. It is possible that the overall low levels of expression of both ligand (VEGF) and receptor (KDR) or coreceptor, e.g., neuropilin, in HT29 might account for the lack of functional activity. Neuropilin-1, a coreceptor of KDR that increases the affinity for VEGF, is expressed in both endothelial cells and cancer cell lines (27, 28) and plays a crucial role in VEGF signaling. Indeed, we found that neuropilin-1 was expressed at higher levels in HCT116, relative to HT29, and was functionally implicated in the hypoxic induction of HIF-1 $\alpha$  in HCT116.

HIF-1 $\alpha$  may act as a survival factor in a tumor type-specific fashion, and strategies to inhibit HIF are being developed (13). Our data indicate that PLC $\gamma$  and ERK1/2 are involved in VEGF-dependent induction of HIF-1 $\alpha$  and survival of hypoxic HCT116



**Figure 5.** A PLC $\gamma$ -dependent pathway mediates autocrine induction of HIF-1 $\alpha$  and survival by VEGF. **A**, HCT116 cells were serum-starved for 24 h. Cells were then cultured under normoxic or hypoxic conditions in the absence or presence of neutralizing antibodies against Flt-1, KDR or VEGF for 18 h. Cell lysates were immunoprecipitated with anti-PLC $\gamma$  antibodies, and immunoblotting was performed with anti-PLC $\gamma$  or anti-phosphorylated tyrosine antibodies. **B**, HCT116 cells were cultured as described above under normoxic or hypoxic conditions in the absence or presence of inhibitors of PLC $\gamma$  (U73122, 10  $\mu$ M) or ERK (PD98059, 50  $\mu$ M) for 24 h. Levels of total and phosphorylated ERK were measured by immunoblotting. **C**, HCT116 cells were cultured as above in the absence or presence of U73122 or PD98059 under normoxic or hypoxic conditions for 18 h. Levels of HIF-1 $\alpha$  were measured by Western blot.  $\beta$ -Actin is shown as loading control. **D**, HCT116 cells were cultured under normoxic or hypoxic conditions in the absence or presence of inhibitors of PLC $\gamma$  (U73122, 10  $\mu$ M) or mitogen-activated protein kinase (PD98059, 50  $\mu$ M), and apoptosis was measured by Annexin V staining. Percentage of Annexin V-positive cells from one representative experiment.

cells. PLC $\gamma$  is phosphorylated in a KDR-dependent fashion and may activate the ras/raf pathway in endothelial cells (21, 22). Consistent with this observation, we found that ERK1/2 was phosphorylated in a PLC $\gamma$ -dependent fashion in hypoxic HCT116 cells and was required for both HIF-1 $\alpha$  expression and cell survival. These results indicate that PLC $\gamma$  may represent a novel target for the inhibition of survival of hypoxic colon cancer cells, and they suggest that small molecules targeting signaling pathways frequently dysregulated in human cancers may effectively block HIF-1 $\alpha$  and survival of hypoxic cells in a cell type-specific fashion.

The recent approval of bevacizumab for the treatment of metastatic colorectal cancer in combination with chemotherapy has provided evidence that antiangiogenic strategies may be effective in human cancers and has generated enthusiasm for their clinical application. However, the exact mechanism by which bevacizumab increases the efficacy of chemotherapy remains poorly understood. Mechanisms implicating inhibition of new blood vessel formation, normalization of existing vasculature, or inhibition of circulating endothelial precursor cells have been suggested (3, 4). However, a direct effect of anti-VEGF antibodies on survival of colon cancer cells has not been implicated. Our results show that colon cancer cells may be intrinsically vulnerable or resistant to VEGF inhibition. Taking HCT116 and HT29 as paradigms of distinct functional phenotypes of colon cancer cells, we speculate that efficacy of anti-VEGF therapies may be, at least in part, determined by the intrinsic sensitivity of colon cancer cells to

inhibition of VEGF-dependent survival pathways. Interestingly, we found that addition of bevacizumab to 5-fluorouracil increases the percentage of apoptotic cells in VEGF-sensitive (HCT116) but not resistant (HT29) colon cancer cells (data not shown). The limited survival benefit of colorectal cancer patients treated with bevacizumab suggests that only a subset may benefit from the addition of anti-VEGF antibodies to conventional chemotherapy. Ongoing efforts to identify predictive biomarkers of response to anti-VEGF therapies are predominantly, if not exclusively, focused on endothelial cells and the vasculature of solid tumors. Our results suggest that, in addition to the effect on endothelial cells, the intrinsic sensitivity or resistance of hypoxic cancer cells to VEGF inhibition may contribute to the efficacy of anti-VEGF therapies for human cancers.

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