A Hypoxia-Driven Vascular Endothelial Growth Factor/Flt1 Autocrine Loop Interacts with Hypoxia-Inducible Factor-1α through Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase 1/2 Pathway in Neuroblastoma

Bikul Das,[1,3] Herman Yeger,[2,4] Rika Tsuchida,[1] Risa Torkin,[2] Matthew F.W. Gee,[5] Paul S. Thorner,[2,4] Masabumi Shibuya,[6] David Malkin,[1,3,5] and Sylvain Baruchel[1,3]

[1]New Agent and Innovative Therapy Program, Division of Hematology and Oncology, Department of Pediatrics and [2]Department of Pediatric Laboratory Medicine and Pathobiology, Hospital for Sick Children; [3]Institute of Medical Science, [4]Department of Laboratory Medicine and Pathobiology, and [5]Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada; and [6]Department of Genetics, Institute of Medical Sciences, University of Tokyo, Japan

Abstract

Flt1, an "fms-like tyrosine kinase" receptor, has been suggested to play an active role in vascular endothelial growth factor (VEGF)–mediated autocrine signaling of tumor growth and angiogenesis. Here, we used a neuroblastoma model to investigate the role of VEGF/Flt1 signaling in hypoxia-mediated tumor cell survival, drug resistance, and *in vivo* angiogenesis. SK-N-BE(2), a highly malignant neuroblastoma cell line resistant to hypoxia-induced apoptosis expresses active Flt1 but lacks VEGFR2 expression. We found that 24-hour hypoxia (<0.1% O_2) alone (no serum deprivation) showed sustained activation of extracellular signal-regulated kinase 1/2 (ERK1/2) associated with bcl-2 up-regulation and resistance to etoposide-induced (5 μmol/L) apoptosis. Treatment with anti-VEGF and anti-Flt1 antibodies inhibited ERK1/2 activation, down-regulated bcl-2, and reversed the hypoxia-mediated drug resistance to etoposide. Similar results were obtained with U0126 and ursolic acid, specific and nonspecific inhibitors of ERK1/2, respectively. We confirmed the protective role of Flt1 receptor by small interfering RNA knockout and Flt1 overexpression studies. Subsequently, we found that inhibition of VEGF/Flt1 autocrine signaling led to reduced hypoxia-inducible factor-1α (HIF-1α) phosphorylation. Furthermore, the reduced phosphorylation was associated with down-regulation of basic fibroblast growth factor, a downstream target of the HIF-1α and VEGF pathways. Our findings suggested an expanded autocrine loop between VEGF/Flt1 signaling and HIF-1α. We investigated the angiogenic activity of the loop in an *in vivo* Matrigel plug assay. The hypoxia-treated conditioned medium induced a strong angiogenic response, as well as the cooption of surrounding vessels into the plugs; ursolic acid inhibited the angiogenesis process. We also found that three other Flt1-expressing neuroblastoma cell lines show hypoxia-mediated drug resistance to etoposide, melphalan, doxorubicin, and cyclophosphamide. Taken together, we conclude that a hypoxia-driven VEGF/Flt1 autocrine loop interacts with HIF-1α through a mitogen-activated protein kinase/ERK1/2 pathway in neuroblastoma. The interaction, in the form of an autocrine loop, is required for the hypoxia-driven cell survival, drug resistance, and angiogenesis in neuroblastoma. (Cancer Res 2005; 65(16): 7267-75)

Introduction

Tumor hypoxia is associated with metastasis, angiogenesis, and resistance to radiation and some chemotherapeutic agents. In tumor cells, hypoxia-responsive pathways modulate well-orchestrated signaling mechanisms that drive cell survival, drug resistance, and angiogenesis (1, 2).

One of the principal regulators of hypoxia-responsive pathways is the hypoxia-inducible factor (HIF-1), which plays a pivotal role in hypoxia-mediated angiogenesis, migration, and invasion. HIF-1 is a transcription factor comprised of α and β subunits, the former being regulated by oxygen concentration. In a hypoxic environment, HIF-1α is activated by phosphorylation and then binds to the HIF-1α-binding site of putative target genes (2). HIF-1α activates expression of a number of genes related to growth and survival that include insulin-like growth factor-2 (IGF-2) and transforming-growth factor-β (TGF-β). IGF-2 and TGF-β bind to the IGF receptor (IGFIR) and epidermal growth-factor receptor (EGFR), respectively, to activate signaling cascades that lead to HIF-1α expression. This initiates a HIF-1α autocrine loop, which may be involved in tumor cell survival and growth in a hypoxic environment (3). HIF-1α also induces vascular endothelial growth factor (VEGF) and its receptor Flt1 (Fms-like receptor 1 or VEGFR1; refs. 2, 3). Recent investigations suggest that Flt1 may play an active role in VEGF-mediated autocrine growth and survival of a diverse group of tumors (4–13). It is possible that similar to TGF-β/EGFR and HIF-1α autocrine loop, VEGF/Flt1 may also form an autocrine loop with HIF-1α. Because VEGF is a highly potent growth factor involved in tumor growth and angiogenesis (14), such an autocrine VEGF/Flt1 and HIF-1α loop may contribute to cell survival, drug resistance, and angiogenesis during hypoxia.

Neuroblastoma is a childhood tumor of neural crest origin in which expression of VEGF and its receptors (Flt1 and VEGFR2) is correlated with high-grade biology (15). Autocrine VEGF/Flt1 activity was reported in a neuroblastoma cell line SK-N-BE, derived from a stage IV neuroblastoma patient (15). We found that the SK-N-BE(2) cell line, which is derived from the same patient after relapse, is resistant to cisplatin compared with several other neuroblastoma cell lines (16). We also found that SK-N-BE(2) cells

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

Requests for reprints: Sylvain Baruchel, New Agent and Innovative Therapy Program, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G1X8. Phone: 416-813-7795; Fax: 416-813-5327; E-mail: sylvain.baruchel@sickkids.on.ca.

doi:10.1158/0008-5472.CAN-04-4575

were resistant to hypoxia-mediated apoptosis.[7] Jogi et al. reported that hypoxia-treated SK-N-BE(2) cells markedly up-regulated VEGF and HIF-1α and showed accelerated xenograft growth compared with normoxia-treated cells (17). These findings suggest that SK-N-BE(2) is a suitable cell line to study the autocrine VEGF/Flt1 pathway during hypoxia. Therefore, we developed a SK-N-BE(2) model of hypoxia-mediated *in vitro* drug resistance and *in vivo* angiogenesis to study VEGF/Flt1 autocrine signaling and its relation to HIF-1α activity.

Here we report that VEGF/Flt1 autocrine signaling is required for the hypoxia-mediated sustained activation of extracellular signal-regulated kinase 1/2 (ERK1/2), and subsequent survival, drug resistance, and *in vivo* angiogenesis. Inhibition of VEGF or ERK1/2 reduced HIF-1α activity. Taken together, these findings implicate the self-regulated survival pathway of the VEGF/Flt1 autocrine loop as an important component of the HIF-1α mediated hypoxia-responsive pathway.

Materials and Methods

Cell culture and induction of hypoxia. The human neuroblastoma cell line, SK-N-BE(2) was obtained from American Type Culture Collection (Manassas, VA). NUB-7 cell line was developed and characterized in our laboratory(18). IMR-5 was kindly provided by Dr. Meredith Irwin (Division of Hematology and Oncology, The Hospital for Sick Children); NGP and the LAN15N cell lines were kindly provided by Dr. David Kaplan (Cancer Research Program, Sickkids). Other neuroblastoma cell lines were obtained from American Type Culture Collection. Neuroblastoma cell lines were maintained in α-MEM (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum and antibiotics, in a humidified atmosphere of 5% CO_2 at 37°C. Radiobiological hypoxic conditions (<0.1% O_2) were established in a sealed chamber using the BBL GasPak Plus anaerobic system envelopes with a palladium catalyst (Becton Dickinson, Cockeysville, MD) as previously described (19, 20). Moderate hypoxic conditions (2% O_2) were established in a hypoxia incubator (Forma Scientific, Inc., Marietta, OH) where N_2 was used to compensate for the reduced O_2 level.

Reagents and drugs. Cisplatin [*cis*-platinum (II) diamine dichloride], etoposide, doxorubicin, vinblastine, melphalan, and ursolic acid were obtained from Sigma (St. Louis, MO). Alamar blue reagent for the cytotoxic assay was obtained from Biosource International (Camarillo, CA). Caspase-3/7 substrate, *N*-acetyl-DEVD-7-amino-4-methylcoumarin (DEVD-AMC) peptide was obtained from BIOMOL Research Laboratories (Plymouth Meeting, PA). Recombinant human $VEGF_{165}$ ($rhVEGF_{165}$), nonspecific goat IgG, and a neutralizing antibody against VEGF and Flt1 (anti-human mouse monoclonal antibody) were purchased from R&D Systems (Minneapolis, MN). Source of other reagents, antibodies, and kits are noted along with the assay descriptions.

Reverse transcriptase-PCR for vascular endothelial growth factor–related receptors. Total RNA was isolated from neuroblastoma cell lines using Trizol (Invitrogen, Burlington, Ontario, Canada), and first-strand cDNA was synthesized using reverse transcriptase M-MLV (Promega, Madison, WI) followed by PCR using a TGradient Thermocycler (Whatman Biometra, Goettingen, Germany) with the following cycling conditions: 35 cycles of 94°C for 60 seconds, 52°C for 60 seconds, and 72°C for 60 seconds for Flt1, NRP-1, NRP-2, and VEGFR3. Thirty-five cycles of 94°C for 30 seconds, 56.0°C for 30 seconds, 72°C for 55 seconds for VEGFR2. Two rhabdomyosarcoma cell lines RH4 and RH28 were used as a positive control for Flt1 and VEGFR2, respectively, as reported previously (21). HeLa cell line was used as a negative control. The quality of the RNA was evaluated by reverse transcriptase-PCR (RT-PCR) for glyceraldehyde-3-phosphate dehydrogenase. Primers were used as previously described (22).

Alamar blue cytotoxicity assay. The assay was done as previously described (16, 23). Briefly, cells grown in 24-multiwell plates (Costar, Cambridge, MA) were treated with each drug and incubated with 10% Alamar blue solution. Relative fluorescence units (RFU) were obtained using the Gemini Spectra MAX microplate reader (emission, 540 nm; excitation, 590 nm; cutoff, 570 nm). Cell survival was expressed as the percent of RFU normalized to cells grown in vehicle (DMSO or ethanol). Experiments were done in triplicates.

Cellular caspase-3/7 assay. The caspase-3/7 activity assay was done as previously described (24) with minor modifications (25).

Western blotting. Protein extracts were obtained using the cell lysis buffer (Cell Signaling Technology, Beverly, MA) mixed with protease inhibitor cocktail (Mini Pill, Roche, Nutley, NJ). For whole cell lysate preparation and HIF-1α detection, additional chemicals were used as described (26). Fifty micrograms of the clear protein lysate was resolved on 8% to 12% SDS-PAGE gels and transferred to 0.45-μm nitrocellulose membranes. The blocked membranes were incubated overnight with the following antibodies: bcl-2, Bax, XIAP, Survivin (Cell Signaling Technology); human HIF-1α (BD Transduction Laboratory, San Diego, CA); β-actin (Sigma); active mitogen-activated protein kinase (MAPK)/ERK1/2, ERK1/2 (Promega); Flt1 (Chemicon International, Temecula, CA); basic fibroblast growth factor (bFGF), phospho-tyrosine kinase, and vinculin (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoblotting was detected by enhanced chemiluminescence (Amersham Bioscience, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. Densitometry study was done using the TotalLab software (Amersham Scientific, Oakville, Ontario, Canada).

Membrane protein isolation and immunoprecipitation. We used a crude membrane preparation technique (27) to detect the phosphorylated form of Flt1 membrane receptor as previously described (28).

Immunohistochemistry. Immunohistochemistry was done using a Ventana diaminobenzidine kit (Ventana Medical Systems, Tucson, AZ) in an automated stainer according to manufacturer's instructions. Flt1 antibody (Santa Cruz Biotechnology) was used in 1:100 dilution.

Human vascular endothelial growth factor immunoassay. The assay was done as described (29). Briefly, after each treatment, the medium was collected and VEGF was measured by quantitative sandwich enzyme immunoassay technique using a Quantikine kit (R&D Systems) according to the manufacturer's instructions and normalized to cell number ($ng/10^6$ cells) using the trypan blue exclusion method of viable cell counting.

Small interfering RNA. All the small interfering RNA (siRNA) reagents including transfection reagents were obtained from Dharmacon Research, Inc. (Chicago, IL). The Flt1 siRNA SMARTpool reagent was used to knockdown Flt1 expression in neuroblastoma cells. *In vitro* transfection was done by using the DharmaFECT transfection reagent. Nontargeting siRNAs (siCONTROL) was used as a siRNA control. Forty percent confluent cells in 24-well plates were treated with 100 nmol/L siRNA, according to manufacturer's instructions, and the hypoxia experiments were conducted 48 hours after transfection.

Flt1 overexpression. The VEGFR-1 expression vector, human full-length VEGFR-1 cDNA in BCMGS neo vector (30) and pcDNA3/GFP plasmid were used to transfect the IMR-5 cell line using LipofectAMINE 2000 transfection reagent (Invitrogen Canada, Inc., Burlington, Ontario, Canada).

***In vivo* angiogenesis assessment by the Matrigel plug assay.** Tumor angiogenesis was quantified in a Matrigel plug by the FITC-dextran perfusion method as previously described (31) with some modifications. Briefly, the conditioned medium (200 μL) of normoxia/hypoxia-treated cells was mixed with 600 μL aliquot of Matrigel (BD Bioscience, San Jose, CA) and immediately injected s.c. into both flanks of 6- to 8-week-old female BALB/c nude mice (Charles River Laboratory, Montreal, Canada). On day 10, 0.25 mL of 25 mg/mL FITC-dextran (Sigma) in PBS was injected, and an hour later, blood samples (300 μL) and Matrigel plugs were collected. The right flank plugs were homogenized, and the supernatant read for fluorescence as described (31). Angiogenic response was expressed as a ratio of Matrigel plug fluorescence/plasma fluorescence. The left flank plugs

[7] B. Das, unpublished data.

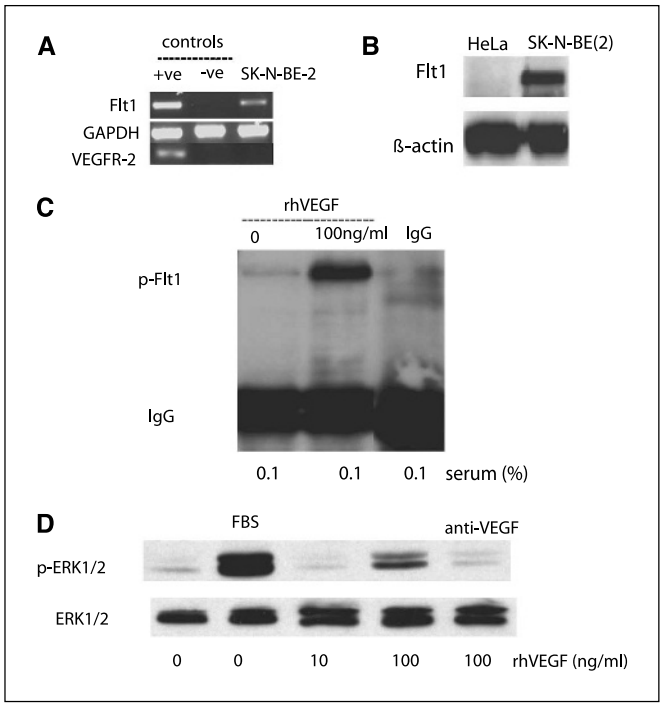

Figure 1. VEGF-Flt1 signaling is active in SK-N-BE(2) cells. *A,* RT-PCR and Western blot analysis show presence of Flt1 and absence of VEGFR2 in SK-N-BE(2) cells. RH4 (a rhabdomyosarcoma cell line) and HeLa extracts served as positive and negative controls, respectively. RH28 (another rhabdomyosarcoma cell line) served as positive control for VEGFR2 RNA. *B,* Western blot shows that Flt1 protein is expressed. HeLa cell extract was used as negative control and β-actin was used as a loading control. *C,* immunoprecipitation of membrane protein shows that rhVEGF treatment induces Flt1 phosphorylation. Cells were serum starved for 24 hours and rhVEGF was added. After 10 minutes of incubation, cells were lysed and the crude membrane protein lysates (250 μg) were immunoprecipitated using a phosphotyrosine kinase antibody and immunoblotted against Flt1 antibody. IgG was used as a loading control. *D,* activation of ERK1/2 by rhVEGF is inhibited by a neutralizing antibody against VEGF. SK-N-BE(2) cells were serum starved for 24 hours followed by rhVEGF$_{165}$ (10-100 ng/mL) treatment for 10 minutes. One group of cells was initially treated with a neutralizing anti-VEGF antibody (100 ng/mL) 4 hours before the rhVEGF treatment. Cell lysates (50 μg) were immunoblotted for p-ERK1/2.

were fixed in formalin, paraffin embedded, and 5-μm sections stained with H&E for histology. Experiments were conducted according to the CCAC guidelines and approved by the Hospital for Sick Children's Animal Research Committee.

Statistical analysis. The results of the Alamar blue assay were analyzed by nonlinear regression analysis. The results of Caspase 3/7, VEGF and Matrigel-FITC assays were analyzed using Student's *t* test. Values were expressed as mean ± SE unless otherwise specified. Statistical significance was set at $P < 0.05$.

Results

Vascular endothelial growth factor/Flt1 autocrine pathway is active in SK-N-BE(2) cells. A RT-PCR study reported that SK-N-BE cells [the parent cell line of SK-N-BE(2)] express Flt1 receptor (15). We did RT-PCR and immunoprecipitation to investigate the presence of active VEGF receptors in the SK-N-BE(2) cell line. Flt1 receptor is expressed, and upon rhVEGF treatment, the receptor is autophosphorylated (Fig. 1*A-C*). VEGF-Flt1 autocrine signaling has been found to activate the MAPK/ERK pathway (32, 33). We showed that activation of ERK1/2 by rhVEGF is inhibited by a neutralizing antibody against VEGF (Fig. 1*D*). The RT-PCR analysis also confirmed that VEGFR2 (Fig. 1*A*) is absent in SK-N-BE(2) cells.

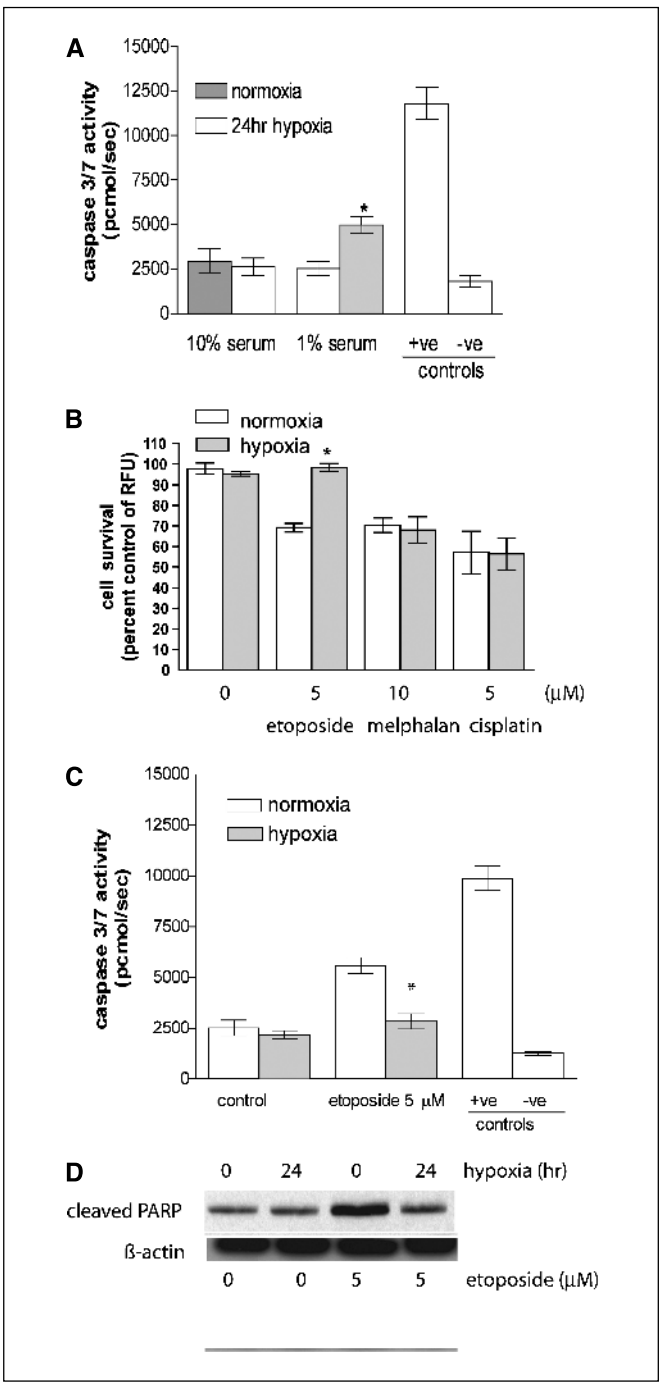

Figure 2. An *in vitro* model of hypoxia-induced drug resistance in SK-N-BE(2) cells. SK-N-BE(2) cells were grown in 1% to 10% serum-containing medium and treated with 24 hours of hypoxia (<0.1% O_2) and subsequently incubated in normoxia for the next 24 hours (media were not changed; fresh serum-in-media was added to make up to 10% serum). The following data represent the results of two experiments done in triplicates. *Bars,* SE. *A,* hypoxia does not induce apoptosis to cells grown in 10% serum. Cells were lysed and incubated with caspase-3/7 substrate as described in Materials and Methods. Staurosporine 10 μmol/L and Z-vad-fmk were used as positive and negative controls, respectively. *B,* hypoxia treatment reduced 5 μmol/L etoposide-induced toxicity by 26% ($P = 0.004$); cells grown in 24-hour hypoxia and 10% serum were treated with etoposide, melphalan, and cisplatin for the next 24 hours under normoxic condition. *C,* cells treated as in (*B*) were analyzed for caspase-3/7 activity. Hypoxia reduced 5 μmol/L etoposide-induced caspase-3/7 activity by 40% ($P < 0.05$). *D,* cells treated in (*C*) were immunoblotted for cleaved poly(ADP-ribose) polymerase (*PARP*) and β-actin (as a loading control). Hypoxia reduced etoposide-induced cleavage of poly(ADP-ribose) polymerase. *, $P < 0.05$.

However, Neuropilin-1, Neuropilin-2, and VEGFR3 mRNA were present (data not shown).

An *in vitro* model of hypoxia-induced drug resistance to study vascular endothelial growth factor/Flt1 system. To evaluate the role of VEGF/Flt1 autocrine signaling in hypoxia response pathway, we first developed an *in vitro* model of hypoxia-induced drug resistance, where cells were exposed to hypoxia (<0.1% or 2% O_2) in the presence of serum to avoid glucose deprivation or cell cycle inhibition. We found that SK-N-BE(2) cells treated with 24 hours of severe hypoxia (<0.1% O_2 with 10% serum) did not experience cytotoxicity/cell cycle arrest (data not shown) or apoptosis (Fig. 2*A*). We then treated the hypoxic cells with three cytotoxic drugs, etoposide, melphalan, and cisplatin for the subsequent 24 hours under normoxia. Hypoxia treatment reduced the etoposide (5 μmol/L) cytotoxicity by 26% (P = 0.004; Fig. 2*B*) and caspase-3/7 activity by 40% (P < 0.05; Fig. 2*C*). Western blot analysis of cleaved poly(ADP-ribose) polymerase confirmed the etoposide resistance in hypoxia (Fig. 2*D*). Because antiapoptotic proteins XIAP (X-linked inhibitor of apoptosis) and survivin inhibit caspase-3/7 (34) and etoposide-mediated caspase-3/7 activity (35), respectively, we examined the possible up-regulation of these two proteins during hypoxia. The expression of XIAP and survivin did not change significantly in normoxia versus hypoxia (data not shown).

Moderate hypoxia (2% hypoxia) of 24 hours duration without serum deprivation failed to show drug resistance (data not shown). In addition, we did not find hypoxia-induced resistance against cisplatin and melphalan (Fig. 2*B*). Nonetheless, our results suggest that severe hypoxia alone could induce resistance to etoposide-induced apoptosis independent of glucose deprivation or cell cycle arrest (data not shown). Hence, we used this model of hypoxia-induced resistance to apoptosis to investigate the VEGF/Flt1 autocrine system during hypoxia.

Vascular endothelial growth factor/Flt1 system is involved in hypoxia-mediated cell survival and drug resistance. Etoposide induces apoptosis in the HepG2 cell line (a hepatocellular carcinoma cell line) through bcl-2/Bax-dependent mechanisms (36). Erler et al. found that hypoxia (0.5% O_2) can induce resistance against etoposide by down-regulating bcl-2/Bax ratio in the HepG2 cell line (37). Beierle et al. found that $VEGF_{165}$ up-regulates bcl-2 protein levels in neuroblastoma resulting in resistance to tumor necrosis factor-α–mediated apoptosis (38). Hypoxia up-regulates HIF-1α, which in turn up-regulates VEGF and Flt1 (2). Therefore, we hypothesized that HIF-1α-mediated induction of VEGF/Flt1 autocrine signaling led to bcl-2 up-regulation and subsequent survival and etoposide resistance during hypoxia. To investigate this possibility, SK-N-BE(2) cells were treated with the neutralizing anti-VEGF antibody and hypoxia followed by exposure to etoposide. The antibody treatment completely reversed the hypoxia-mediated drug resistance (Fig. 3*A*). In addition, anti-VEGF antibody treatment reduced survival of SK-N-BE(2) cells during hypoxia by 28% (P = 0.008; Fig. 3*A*). We then investigated the bcl-2/Bax ratio in normoxia versus hypoxia. Bcl-2 protein level was significantly up-regulated in hypoxia versus normoxia, whereas the level of Bax protein did not change significantly. Densitometry revealed a several fold increase of bcl-2/Bax ratio in hypoxia versus normoxia (Fig. 3*B*). The anti-VEGF treatment significantly down-regulated bcl-2 level under hypoxia (Fig. 3*C*). We also confirmed that hypoxia treatment induced HIF-1α and up-regulated Flt1 (data not shown). The level of $VEGF_{165}$ in the conditioned medium of hypoxia-treated

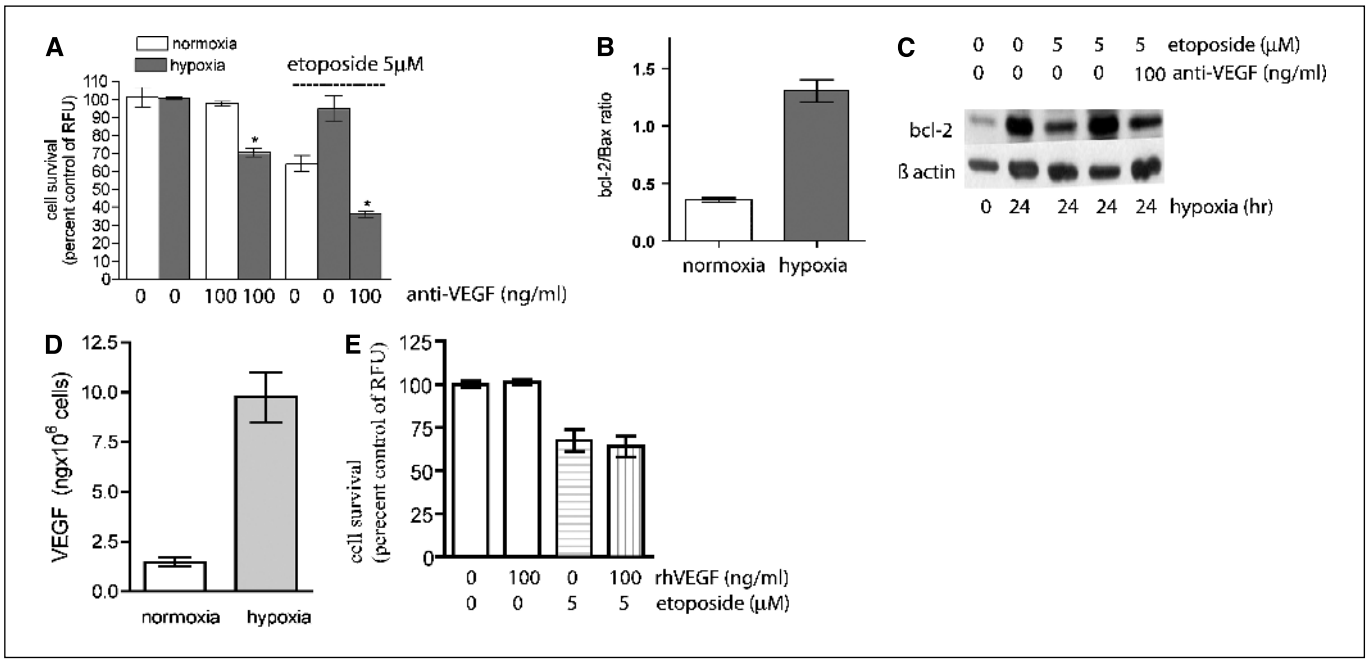

Figure 3. VEGF/Flt1 system is involved in hypoxia-mediated resistance. *A,* anti-VEGF antibody treatment significantly reversed the hypoxia-mediated etoposide resistance and increased the hypoxia-induced cytotoxicity. SK-N-BE(2) cells were treated with 100 ng/mL of monoclonal anti-VEGF antibody and incubated under 24 hours of hypoxia. Subsequently, cells were treated with 5 μmol/L etoposide for the next 24 hours under normoxic condition. Cytotoxicity was measured by Alamar blue assay. *B,* hypoxia significantly increased the bcl-2/Bax ratio compared with normoxia (P < 0.05). Cell lysates of SK-N-BE(2) grown in 24 hours of hypoxia were immunoblotted for bcl-2, Bax, and β-actin (as a loading control). Densitometry study was done using the TotalLab software (Amersham Scientific). *C,* anti-VEGF antibody treatment significantly reduced hypoxia-induced up-regulation of bcl-2 in the etoposide treatment group. *D,* hypoxia up-regulated $VEGF_{165}$ secretion by >7-fold (P < 0.05). $VEGF_{165}$ secretions were measured in the conditioned medium of 24-hour hypoxia versus normoxia treated cells. *E,* rhVEGF treatment did not influence cell proliferation or etoposide-induced cytotoxicity. SK-N-BE(2) cells were treated with 100 ng/mL of rhVEGF and incubated in normoxia for 24 hours. Subsequently, cells were treated with 5 μmol/L etoposide for the next 24 hours.

cells increased by >7-fold reaching ~10 ng/million cells/mL (Fig. 3*D*). Because SK-N-BE(2) cells expressed VEGR3 and NRP-1, we used Flt1 siRNA knockdown to investigate the exclusive role of Flt1 in VEGF-mediated survival and drug resistance during hypoxia. siRNA knockdown of Flt1 resulted in severe toxicity during hypoxia (64% increase of toxicity; $P < 0.0126$; Supplementary Fig. S1*A-C*). We then investigated the hypoxia-mediated resistance against etoposide in a Flt1-overexpressing IMR-5 cell line. Flt1-overexpressed cells were resistant to hypoxia-induced toxicity (46% reduction of toxicity compared with non-Flt1 transfected cells; $P < 0.03$). Flt1 overexpression resulted in 35% reduction of etoposide toxicity during hypoxia ($P < 0.05$; Supplementary Fig. 1*D-E*).

The above results suggest that hypoxia up-regulates VEGF/Flt1 signaling leading to bcl-2 up-regulation and subsequent cellular survival during hypoxia. Hence, treatment with rhVEGF should recapitulate the hypoxia-mediated bcl-2 up-regulation and drug resistance. SK-N-BE(2) cells were treated with rhVEGF (100 ng/million cells/mL) for 24 hours followed by etoposide treatment. rhVEGF treatment failed to induce resistance against etoposide-induced cytotoxicity (Fig. 3*E*). Moreover, 24 hours of rhVEGF treatment did not change bcl-2 levels (data not shown). We then increased the duration of rhVEGF treatment for 2 to 3 days but failed to see changes in bcl-2 expression or etoposide-induced toxicity (data not shown). These results suggest that VEGF/Flt1 signaling alone was not sufficient to induce hypoxia-mediated up-regulation of bcl-2 and resistance against etoposide.

A hypoxia-driven vascular endothelial growth factor/Flt1 autocrine loop that sustains extracellular signal-regulated kinase 1/2 activity during hypoxia. Our findings that hypoxia alone and not rhVEGF was able to up-regulate bcl-2 in SK-N-BE(2) cells suggest differential signaling of VEGF/Flt1 in normoxia versus hypoxia. Recently, hypoxia and/or rhVEGF treatment has been found to up-regulate bcl-2 by activating MAPK/ERK1/2 pathway in serum-deprived hepatocellular carcinoma cells (39). Sustained activation of MAPK/ERK has been suggested to play an important role in cell survival (40). Yokoi et al. reported hypoxia-induced sustained activation of ERK1/2 in a pancreatic cell line where hypoxia induced resistance against gemcitabine (1). We investigated the activation of phospho-ERK1/2 (p-ERK1/2) by rhVEGF or hypoxia. rhVEGF treatment resulted in a transient activation of p-ERK1/2 that lasted for <10 minutes (Fig. 4*A*), whereas hypoxia resulted in a sustained increase of p-ERK1/2 levels that reached its peak within the first 3 hours of hypoxia and then continued for >6 hours after hypoxia (Fig. 4*B*). In addition, anti-VEGF antibody markedly reduced hypoxia-mediated ERK1/2 activation (Fig. 4*B*). To further investigate the role of sustained ERK1/2 activation on bcl-2 up-regulation and drug resistance, we used a specific inhibitor of MAPK/ERK, U0126, and a nonspecific inhibitor, ursolic acid, a dietary triterpenoid found to inhibit ERK1/2 (41). Both U0126 and ursolic acid inhibited hypoxia-mediated sustained ERK1/2 activation, reduced the cellular survival during hypoxia, and reversed the hypoxia-induced drug resistance in a dose-dependent

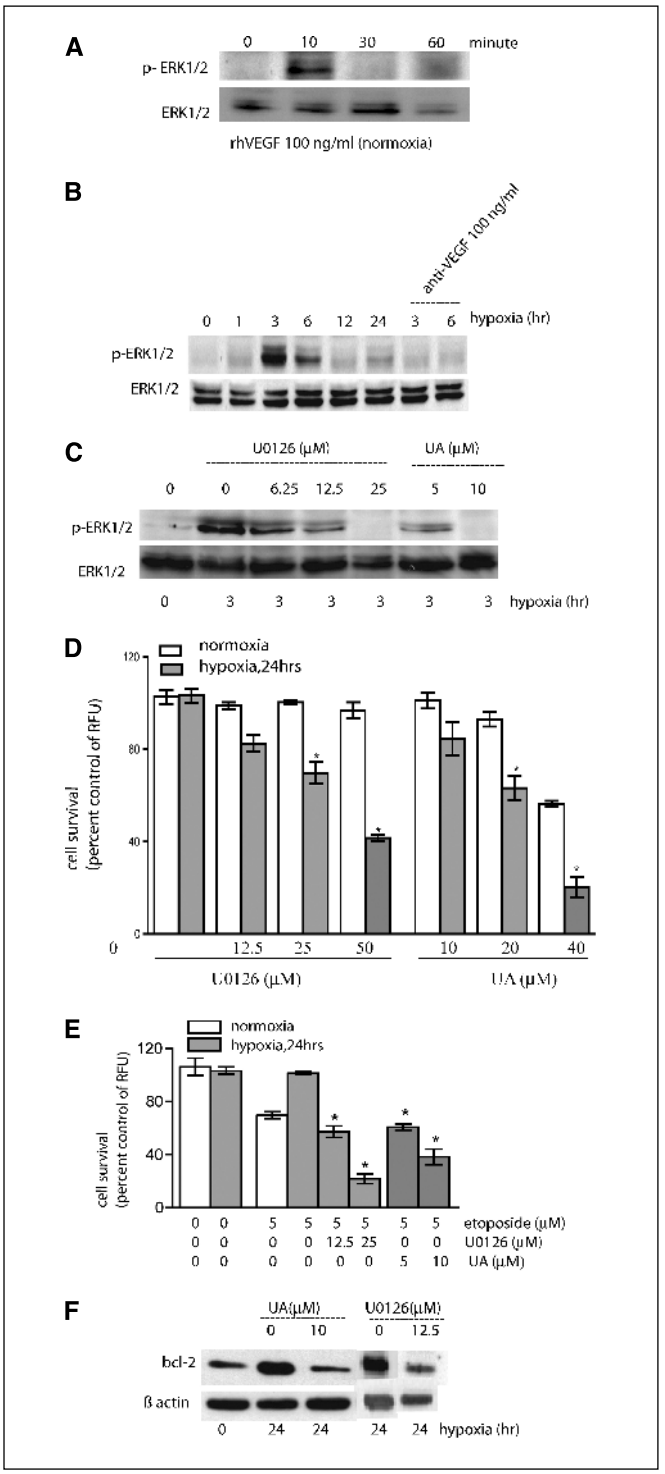

Figure 4. Sustained ERK1/2 activation is involved in HIF-1α activity and bcl-2 up-regulation. SK-N-BE(2) cells grown in 10% serum were treated with either rhVEGF (100 ng/mL) or hypoxia for 1 to 24 hours and cells were lysed and immunoblotted against active MAPK/ERK1/2 antibody. *A,* rhVEGF treatment induced transient activation of ERK1/2. The active ERK1/2 returned to its basal level within the first 30 minutes of treatment. *B,* hypoxia activated ERK1/2 for >6 hours, with the peak activity reached at 3 hours after hypoxia. Anti-VEGF antibody inhibited the sustained activation ERK 1/2. *C,* ERK 1/2 inhibitors U0126 (specific) and ursolic acid (*UA*; nonspecific) reduced hypoxia-mediated MAPK/ERK activation. *D,* U0126 and ursolic acid treatment led to marked cytotoxicity of hypoxia-treated cells. SK-N-BE(2) cells grown in 10% serum were treated with U0126 or ursolic acid and 24 hours of hypoxia. Cytotoxicity was measured by Alamar blue assay. *E,* U0126 and ursolic acid reversed hypoxia-induced resistance against etoposide. Cells grown in 24 hours of hypoxia were subsequently treated with 5 μmol/L etoposide and further incubated for another 24 hours under normoxia. U0126 and ursolic acid significantly reversed the hypoxia-mediated resistance to etoposide-induced cytoxicity as measured by Alamar blue assay. *F,* U0126 and ursolic acid reduced bcl-2 up-regulation during hypoxia. Cells were treated with U0126 and ursolic acid followed by exposure to 24 hours of hypoxia. Cell lysates were obtained and probed for bcl-2 protein. β-Actin served as a loading control.

manner (Fig. 4*C-E*). Furthermore, both U0126 and ursolic acid reduced the hypoxia-induced up-regulation of bcl-2 protein (Fig. 4*F*). Our findings on ursolic acid are interesting as it is a dietary compound with potential antitumor therapeutic applications.

Vascular endothelial growth factor/Flt1-induced sustain extracellular signal-regulated kinase 1/2 activation regulates hypoxia-inducible factor-1α activity. We found that anti-VEGF antibody treatment led to reduction of hypoxia-mediated bcl-2 up-regulation (Fig. 3*C*) and ERK1/2 phosphorylation (Fig. 4*B*). We also found that anti-Flt1 antibody treatment reduces hypoxia-mediated ERK1/2 phosphorylation (data not shown). In addition, we found that hypoxia up-regulated HIF-1α, Flt1, and VEGF (Fig. 3*D*). These results suggest that HIF-1α up-regulated VEGF/Flt1 signaling leading to sustain activation of ERK1/2. Interestingly, ERK1/2 has been found to directly phosphorylate the COOH-terminal domain of HIF-1α, the latter being required for the transcriptional activity of HIF-1α (26). Therefore, we investigated the HIF-1α phosphorylation status in the presence of ERK1/2 inhibitors, ursolic acid and U0126, by Western blots as described previously (26). As expected, the ursolic acid and U0126 treatment groups showed marked reduction of HIF-1α phosphorylation (Fig. 5*A*). Because HIF-1α activation is upstream of VEGF and Flt1 up-regulation, the HIF-1α phosphorylation should be independent of anti-VEGF treatment. However, we found that anti-VEGF antibody treatment also reduced HIF-1α phosphorylation (Fig. 5*A*). This result suggests that the VEGF/Flt1 autocrine loop may regulate HIF-1α activity. Therefore, we hypothesized that during the initial phase of hypoxia, VEGF/Flt1 signaling activates ERK1/2 that activates HIF-1α resulting in increased activity of VEGF/Flt1, eventually forming an autocrine loop (Fig. 6). In such a case, inhibition of ERK1/2 should lead to the down-regulation of VEGF. As expected, the ursolic acid treatment group showed marked reduction of VEGF secretion (Fig. 5*B*). Most importantly, ursolic acid did not inhibit the basal VEGF secretion, suggesting that ursolic acid–mediated inhibition of VEGF secretion was directly related to HIF-1α-related induction of VEGF secretion. To further study the loop (Fig. 6), we investigated the expression of bFGF, a potent angiogenic growth factor and one of the important downstream products of both HIF-1α and VEGF systems (42, 43). We found that hypoxia induced an increase in bFGF protein level, which was down-regulated by ursolic acid (Fig. 5*C*). The normoxia level of bFGF remained unaffected by ursolic acid treatment (data not shown). These results suggest that during hypoxia, VEGF/Flt1 autocrine system may cooperate with HIF-1α activity in an autocrine loop that up-regulates important survival and angiogenic factors such as bcl-2 (Fig. 3*B-C*) and b-FGF (Fig. 5*C*), respectively. To analyze the potential angiogenic effects of up-regulated VEGF/Flt1 and HIF-1α autocrine loop *in vivo*, we applied a murine Matrigel plug assay to hypoxia-treated conditioned media (details in Materials and Methods). As expected, hypoxia markedly increased *in vivo* Matrigel plug perfusion compared with normoxic medium (Fig. 7*A*). Interestingly, microscopic examination of the plugs revealed numerous host capillaries spreading into the plug (Fig. 7*C*). In its initial stage of progression, a tumor mass remains in a severely hypoxic microenvironment, where, as in the early stage of wound healing, new capillaries sprout from adjacent vessels to invade the tumor mass, a process called cooption (44). Therefore, we did H&E and Factor VIII staining of the Matrigel plugs to study vessel cooption. In the H&E staining, the plugs containing normoxic-conditioned medium showed an intact margin and no cooption (Fig. 7*E*), whereas the plugs containing

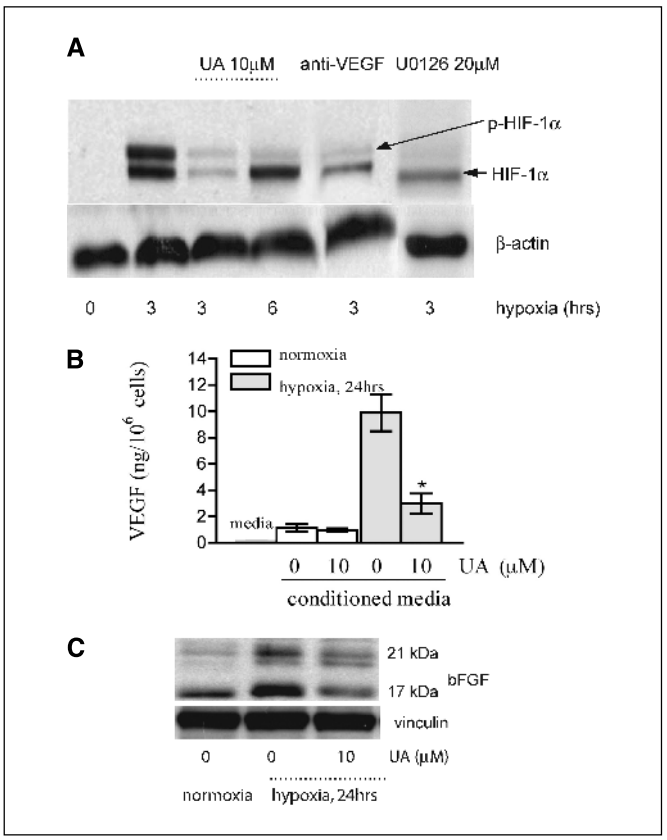

Figure 5. VEGF/Flt1 autocrine loop interacts with HIF-1α. *A,* SK-N-BE(2) cells treated cells with ursolic acid (*UA*), anti-VEGF antibody, and U0126 were subjected to 3 to 6 hours of hypoxia. After termination of hypoxia, cells were immediately washed with cold buffer containing 1 mmol/L sodium orthovandate and subjected to lysis in whole cell lysate buffer containing a cocktail of phosphatase inhibitors (Materials and Methods). HIF-1α was detected by Western blot analysis. U0126, ursolic acid, and anti-VEGF antibody inhibited the phosphorylation of HIF-1α. β-Actin was used as a loading control. *B,* SK-N-BE(2) cells were incubated in 24 hours of hypoxia in the presence or absence of ursolic acid 10 μmol/L, and the conditioned media used to measure VEGF secretion by an ELISA method. Ursolic acid 10 μmol/L inhibited hypoxia-induced $VEGF_{165}$ secretion. The level of VEGF was normalized to cell numbers by counting the cells by trypan blue exclusion dye method. *C,* hypoxia up-regulated bFGF expression; ursolic acid 10 μmol/L down-regulated it. Cells grown in a similar condition like (*B*) were lysed and probed for bFGF protein using Western blot analysis. Vinculin was used as a loading control.

hypoxic-conditioned medium showed a broken margin and invasion of numerous capillary-like structures deep into the plugs (Fig. 7*F*). Factor VIII staining confirmed the presence of endothelial cells lining these capillaries (data not shown). The ursolic acid–treated group showed dramatic reduction of capillary infiltration into the plug (Fig. 7*D* and *G*) and a dramatic reduction (>80% reduction) in the perfusion (Fig. 7*A*).

Evidence of vascular endothelial growth factor/Flt1 autocrine loop in other neuroblastoma cell lines. SK-N-BE(2) is a post-chemotherapy cell line expressing mutant p53. Because tumor hypoxia may select p53 mutant cells (45), it was possible that the VEGF/Flt1 and HIF-1α autocrine loop may be a p53-dependent phenomenon. In addition, we did not find hypoxia-induced resistance against cisplatin and melphalan in the SK-N-BE(2) cell line. Therefore, we investigated hypoxia-mediated survival in 15 neuroblastoma cell lines. We found that only five cell lines (NBL-S, NUB-7, SH-5YSY, SHEP, and SKNDZ) other than SK-N-BE-2 could tolerate 24 hours of hypoxia (0.1% oxygen). RT-PCR reveals that all

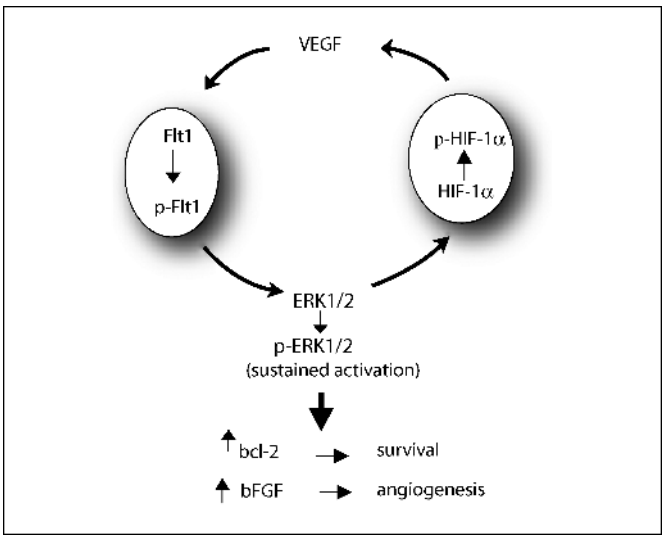

Figure 6. A schematic diagram of VEGF/Flt1-HIF-1α autocrine loop. In this loop, VEGF/Flt1 first activates ERK1/2 during the initial period of hypoxia, ERK1/2 in turn activates HIF-1α leading to the up-regulation of VEGF, and Flt1, which further stimulates the ERK1/2 system.

these lines except SHEP express Flt1 receptor (Supplementary Fig. 2*A*). We investigated hypoxia-mediated drug resistance in NBL-S, NUB-7, and SH-5YSY. The results are summarized in a table (Supplementary Table 1). All three cell lines show relative resistance to cylcophosphamide (4-hydroxy cyclophosphamide), doxorubicin, melphalan, and etoposide. To investigate the role of a VEGF/Flt1 autocrine system, NBL-S and NUB-7, both expressing wild-type p53 were treated with anti-Flt1 antibody. The antibody treatment significantly reduced survival during hypoxia by >40% ($P < 0.05$; data not shown). To investigate whether Flt1 expression is a common feature of neuroblastoma, we examined 10 primary neuroblastoma cases for Flt1 expression by immunohistochemistry. Five of 10 tumors expressed Flt1 (Supplementary Fig. 2*B*).

Discussion

Flt1, a "fms-like tyrosine kinase" receptor was initially thought to play the role of a decoy receptor of VEGF (14). However, subsequent research suggested that Flt1 may play an active role in VEGF-mediated autocrine signaling of tumor growth and invasion (5, 9, 12, 13). Here we report a hypoxia-driven VEGF/Flt1 autocrine signaling and its interaction with HIF-1α in neuroblastoma.

VEGF/VEGFRs signaling have been primarily studied in endothelial cells. In this context, VEGFR2 has been defined as the dominant signaling receptor for VEGF-induced mitogenesis and vascular permeability because of its strong tyrosine kinase activity. In contrast, Flt1 has weak tyrosine kinase activity, yet a 10-fold higher binding affinity for VEGF. This led to the suggestion that Flt1 acts as a decoy receptor, capable of being a positive or negative regulator of angiogenesis, through its ability to sequester VEGF from signaling through VEGFR2 (14). However, recent studies of Flt1 in several tumor cells, including prostate, colon, pancreas, glioblastoma, lymphoma, leukemia, and mesothelioma suggest that VEGF/Flt1 signaling has autocrine survival activity (4–13). In several colon carcinoma cell lines, autocrine VEGF/Flt1 promotion of cell survival was dominant over any other soluble growth factor signal. In addition, Flt1 expression correlated with

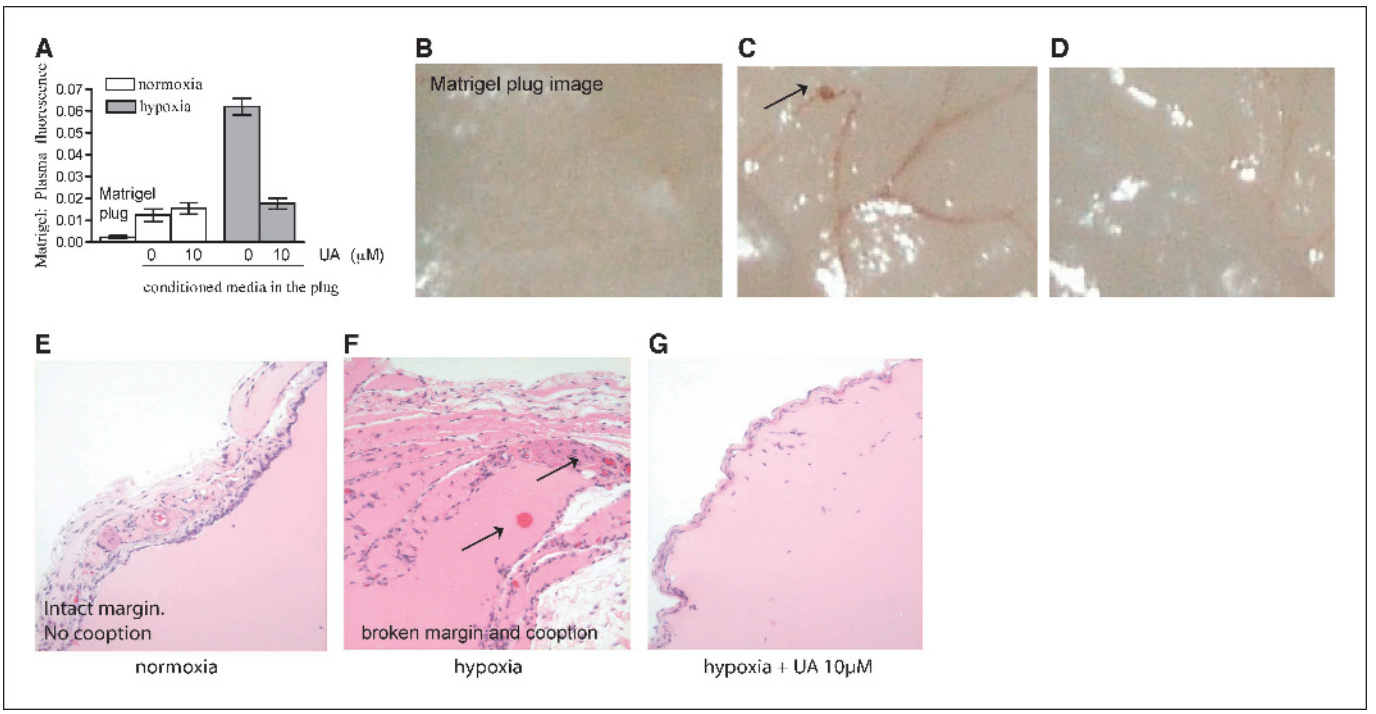

Figure 7. Effect of VEGF/Flt-1 and HIF-1α autocrine activity on *in vivo* angiogenesis. SK-N-BE(2) cells were incubated in 24 hours of hypoxia in the presence or absence of ursolic acid 10 μmol/L, and the conditioned media used to quantify induction of angiogenesis using a Matrigel plug assay as described in Materials and Methods. Whereas the hypoxic conditioned media markedly enhanced Matrigel perfusion, the ursolic acid treatment reduced the perfusion significantly. *B-D,* digital microscopic images of the Matrigel plugs recovered from the experiment. *A,* hypoxia groups shows the invasion of a capillary-like structure into the plug (*arrow*, *C*), whereas normoxia group did not show any capillaries (*E-G*) The plugs from experiment (*A*) were fixed in 10% formalin, paraffin-embedded, and H&E staining done to reveal the morphology of the plug. The plug with hypoxia-conditioned media shows marked invasion of host vessels (*arrows*) resembling vessel cooption.

malignant transformation and subsequent progression of human colon carcinoma (12). In the prostate carcinoma cell line FB2, which is the malignant counterpart of the rat prostate epithelial cell line NbE-1, Flt1 expression was correlated with malignant transformation and lung metastasis (13). In stage I and II neuroblastoma, Flt1 mRNA was detected in two of nine tumors, whereas in stage IV tumors, Flt1 mRNA was detected in 7 of 10 samples suggesting the role of Flt1 in neuroblastoma progression (15). We found that Flt1 mRNA is present in 8 of 15 cell lines and 5 of 10 primary neuroblastoma tumors. We found that VEGF/Flt1 signaling plays a significant role in neuroblastoma cell survival during hypoxia. The inhibition of Flt1 activity by siRNA knockdown significantly reduced SK-N-BE(2) cell survival during hypoxia, whereas overexpression of Flt1 in the IMR-5 cell line significantly increased survival during hypoxia. The VEGF/Flt1 activity was associated with up-regulation of bcl-2, an important antiapoptotic protein, and bFGF, a potent angiogenic factor. Most importantly, we found that inhibition of VEGF/Flt1 system led to the reduction of HIF-1α phosphorylation. This suggests a strong interaction between the VEGF/Flt1 autocrine loop and HIF-1α activation during initial hours of hypoxia. Based on our findings, here we propose an autocrine loop between VEGF/Flt1 signaling and HIF-1α. In this loop. VEGF/Flt1 first activates ERK1/2 during the initial period of hypoxia. ERK1/2 in turn activates HIF-1α leading to the up-regulation of VEGF, and Flt1, which further stimulates the ERK1/2 system (Fig. 6). Flt1 is the only receptor associated with VEGF that contains a binding site for HIF-1α (2), which further supports the recruitment of VEGF/Flt1 system in HIF-1α-driven tumor progression. Recently, Tang et al. showed the interaction between a hypoxia-driven VEGF/VEGFR2 autocrine loop and HIF-1α in endothelial cells to regulate early angiogenic process including cooption-like events (46). Our results suggest that the VEGF/Flt1 and HIF-1α loop may be involved in cooption in neuroblastoma (Fig. 7*F*). In addition, Flt1, being involved in endothelial cell migration (47), may also help to stimulate migration of tumor cells towards the nearby capillaries. Resistance to apoptosis and increased angiogenesis are two important hallmarks of tumor progression (48), and tumor hypoxia has been known to play an important role in both angiogenesis and the selection of apoptosis resistant cells (2). In this context, our findings of a hypoxia-driven VEGF/Flt1 autocrine survival pathway and its close cooperation with HIF-1α is significant in terms of tumor angiogenesis and drug resistance.

Studies have shown that *in vitro* exposure of cancer cell lines to hypoxia (24-72 hours) may lead to resistance against multiple chemotherapeutic agents (49). Earlier studies focused on hypoxia-induced cell cycle arrest and subsequent decreased sensitivity to chemotherapeutic agents as the mechanism of hypoxia-mediated drug resistance (50). However, recent investigations suggest that hypoxia-driven resistance may involve specific cellular survival pathways independent of cell cycle arrest. Erler et al. found that colon carcinoma cells develop resistance against etoposide-induced apoptosis via up-regulation of HIF-1α and down-regulation of Bid and Bax (37). We found that hypoxia decreases toxicity of several chemotherapeutic agents (etoposide, melphalan, cyclophosphamide, and doxorubicin) to neuroblastoma (Supplementary Table 1). We also found that HIF-1α and VEGF/Flt1 autocrine loop may play an important role in the hypoxia-mediated drug resistance. Neuroblastoma is the most common solid tumor of childhood, and a subset of older patients frequently succumb to metastatic disease despite even the most intensive chemotherapy (51). Metastatic and drug resistance neuroblastoma secretes high level of VEGF and other angiogenic factors (52). Our findings suggest that targeting VEGF-mediated autocrine activity may reduce drug resistance and thus increase the chemotherapeutic efficacy in metastatic neuroblastoma.

We previously showed that the combination of low-dose vinblastine and VEGFR2 antibody led to significant antiangiogenic response and an increase in neuroblastoma cell apoptosis (31). Effective blockade of the VEGF pathway by the neutralizing antibody, bevacizumab (avastin) has been shown to reduce tumor progression in colorectal cancer (53) and leukemia (6). Findings of the present work suggest that apart from its antiangiogenic activity, the anti-VEGF treatment strategy may also target hypoxia-mediated survival and drug resistance thus increasing the efficacy of the chemotherapeutic agents and reducing tumor burden. Anti-VEGF therapy may be combined with novel agents that target the hypoxia pathway to achieve greater tumor regression. Interestingly, ursolic acid has been shown to exert inhibitory activity against multiple kinase pathways including nuclear factor-κB, activator protein, protein kinase C, and ERK1/2 (41, 54). We found that ursolic acid reduces cellular survival during hypoxia by targeting VEGF/Flt1 related ERK1/2 activation. Ursolic acid, being a dietary compound, may be further explored for such uses.

In summary, we report a novel autocrine loop between the hypoxia-driven VEGF/Flt1 signaling and HIF-1α activity. We suggest that the loop serves the tumor cells to survive hypoxia, cytotoxic drugs such as etoposide, and recruits surrounding vessels (cooption) for the early phase of angiogenesis. We have shown that a naturally occurring molecule, ursolic acid, can inhibit this autocrine loop. Further understanding of the loop may help to decipher the early stages of tumor progression/relapse in neuroblastoma and could lead to more effective treatment strategies.

Acknowledgments

Received 12/22/2004; revised 4/30/2005; accepted 5/27/2005.

Grant support: James Birrell Neuroblastoma Research Fund; B.R.A.I.N Child (Meagan Bebenek) fund; Toronto and the Andrew Mizzoni Cancer Research Fund, The Hospital for Sick Children, Toronto, Canada; Hospital for Sick Children's Research Training Centre, Canada (B. Das, R. Tsuchida, and M.F.W. Gee); and National Cancer Institute of Canada Fellowship with funds from the Terry Fox Foundation (R. Tsuchida).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Drs. Robert Kerbel, David Kaplan, Meredith Irwin, Maria Zielenska, and Diana Stempak for critical review of the article; Michael Ho and Jean-François Lavoie for technical assistance; and the staff of the animal facility of Hospital for Sick Children for their technical support.

References

1. Yokoi K, Fidler I. Hypoxia increases resistance of human pancreatic cancer cells to apoptosis induced by gemcitabine. Clin Cancer Res 2004;10:2299–306.
2. Harris A. Hypoxia: a key regulatory factor in tumor growth. Nat Rev Cancer 2002;2:38.
3. Semenza G. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003;3:721–32.
4. Wang ES, Teruya-Feldstein J, Wu Y, Zhu Z, Hicklin D, Moore MA. Targeting autocrine and paracrine VEGF receptor pathways inhibits human lymphoma xenografts *in vivo*. Blood 2004;104:2893–902.
5. Steiner HH, Karcher S, Mueller MM, Nalbantis E, Kunze S, Herold-Mende C. Autocrine pathways of the vascular endothelial growth factor (VEGF) in glioblastoma multiforme: clinical relevance of radiation-induced increase of VEGF levels. J Neurooncol 2004;66:129–38.
6. Karp JE, Gojo I, Pili R, et al. Targeting vascular endothelial growth factor for relapsed and refractory adult acute myelogenous leukemias: therapy with

sequential 1-β-D-arabinofuranosylcytosine, mitoxantrone, and bevacizumab. Clin Cancer Res 2004;10:3577–85.
7. Santos SC, Dias S. Internal and external autocrine VEGF/KDR loops regulate survival of subsets of acute leukemia through distinct signaling pathways. Blood 2004;103:3883–9.
8. Lee Y, Bone N, Strege A, Shanafelt T, Jelinek D, Kay N. VEGF receptor phosphorylation status and apoptosis is modulated by a green tea component, epigallocatechin-3-gallate (EGCG), in B-cell chronic lymphocytic leukemia. Blood 2004;104:788–94.
9. Qi L, Robinson WA, Brady BM, Glode LM. Migration and invasion of human prostate cancer cells is related to expression of VEGF and its receptors. Anticancer Res 2003;23:3917–22.
10. Strizzi L, Catalano A, Vianale G, et al. Vascular endothelial growth factor is an autocrine growth factor in human malignant mesothelioma. J Pathol 2001;193:468–75.
11. Bellamy WT, Richter L, Sirjani D, et al. Vascular endothelial cell growth factor is an autocrine promoter of abnormal localized immature myeloid precursors and leukemia progenitor formation in myelodysplastic syndromes. Blood 2001;97:1427–34.
12. Bates RC, Goldsmith JD, Bachelder RE, et al. Flt-1-dependent survival characterizes the epithelial-mesenchymal transition of colonic organoids. Curr Biol 2003;13:1721–7.
13. Soker S, Kaefer M, Johnson M, Klagsbrun M, Atala A, Freeman MR. Vascular endothelial growth factor-mediated autocrine stimulation of prostate tumor cells coincides with progression to a malignant phenotype. Am J Pathol 2001;159:651–9.
14. Ferrara N. Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. Semin Oncol 2002;29:10–4.
15. Langer I, Vertongen P, Perret J, Fontaine J, Atassi G, Robberecht P. Expression of vascular endothelial growth factor (VEGF) and VEGF receptors in human neuroblastomas. Med Pediatr Oncol 2000;34:386–93.
16. Das B, Yeger H, Baruchel H, Freedman M, Koren G, Baruchel S. *In vitro* cytoprotective activity of squalene on a bone marrow versus neuroblastoma model of cisplatin-induced toxicity. Implications in cancer chemotherapy. Eur J Cancer 2003;39:2556–65.
17. Jogi A, Ora I, Nilsson H, et al. Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype. Proc Natl Acad Sci U S A 2002;99:7021–6.
18. Dimitroulakos J, Squire J, Pawlin G, Yeger H. NUB-7: a stable I-type human neuroblastoma cell line inducible along N- and S-type cell lineages. Cell Growth Differ 1994;5:373–84.
19. Feldkamp M, Lau N, Rak J, Kerbel R, Guha A. Normoxic and hypoxic regulation of vascular endothelial growth factor (VEGF) by astrocytoma cells is mediated by Ras. Int J Cancer 1999;81:118–24.
20. Fotsis T, Breit S, Lutz W, et al. Down-regulation of endothelial cell growth inhibitors by enhanced MYCN oncogene expression in human neuroblastoma cells. Eur J Biochem 1999;263:757–64.
21. Gee MFW, Tsuchida R, Malkin D. Expression of vascular endothelial growth factor (VEGF) and VEGF tyrosine-kinase receptor expression in rhabdomyosarcoma [abstract]. Proc Am Assoc Cancer Res 2003;44:842.
22. Beierle EA, Dai W, Langham MR Jr, Copeland EM III, Chen MK. Expression of VEGF receptors in cocultured neuroblastoma cells. J Surg Res 2004;119:56–65.
23. Fields R, Lancaster M. Dual-attribute continuous monitoring of cell proliferation/cytotoxicity. Am Biotechnol Lab 1993;11:48–50.
24. Carrasco R, Stamm N, Patel B. One-step cellular caspase-3/7 assay. Biotechniques 2003;34:1064–7.
25. Torkin R, Lavoie JF, Kaplan DR, Yeger H. Induction of caspase-dependent, p53-mediated apoptosis by apigenin in human neuroblastoma. Mol Cancer Ther 2005;4:1–11.
26. Minet E, Arnould T, Michel G, et al. ERK activation upon hypoxia: involvement in HIF-1 activation. FEBS Lett 2000;468:53–8.
27. Nagamatsu S, Kornhauser J, Burant C, Seino S, Mayo K, Bell G. Glucose transporter expression in brain. cDNA sequence of mouse GLUT3, the brain facilitative glucose transporter isoform, and identification of sites of expression by *in situ* hybridization. J Biol Chem 1992;267:467–72.
28. Chevalier S, Defoy I, Lacoste J, et al. Vascular endothelial growth factor and signaling in the prostate: more than angiogenesis. Mol Cell Endocrinol 2002;189:169–79.
29. Tahir S, Gu W, Zhang H, et al. Inhibition of farnesyltransferase with A-176120, a novel and potent farnesyl pyrophosphate analogue. Eur J Cancer 2000;36:1161–70.
30. Sawano A, Takahashi T, Yamaguchi S, Aonuma M, Shibuya M. Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular endothelial growth factor. Cell Growth Differ 1996;7:213–21.
31. Klement G, Baruchel S, Rak J, et al. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J Clin Oncol 2000;105:15–24.
32. Takahashi M, Matsui A, Inao M, Mochida S, Fujiwara K. ERK/MAPK-dependent PI3K/Akt phosphorylation through VEGFR-1 after VEGF stimulation in activated hepatic stellate cells. Hepatol Res 2003;26:232–6.
33. Kazi AS, Lotfi S, Goncharova EA, et al. Vascular endothelial growth factor-induced secretion of fibronectin is ERK dependent. Am J Physiol Lung Cell Mol Physiol 2004;286:L539–45.
34. Deveraux QL, Roy N, Stennicke HR, et al. IAPs block apoptotic events induced by caspase-8 and cytochrome *c* by direct inhibition of distinct caspases. EMBO J 1998;17:2215–23.
35. Tamm I, Wang Y, Sausville E, et al. IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. Cancer Res 1998;58:5315–20.
36. Cervinka M, Cerman J, Rudolf E. Apoptosis in Hep2 cells treated with etoposide and colchicine. Cancer Detect Prev 2004;28:214–26.
37. Erler T, Cawthorne C, Williams K, et al. Hypoxia-mediated down-regulation of Bid and Bax in tumors occurs via hypoxia-inducible factor 1-dependent and -independent mechanisms and contributes to drug resistance. Mol Cell Biology 2004;24:2875–89.
38. Beierle EA, Strande LF, Chen MK. VEGF upregulates Bcl-2 expression and is associated with decreased apoptosis in neuroblastoma cells. J Pediatr Surg 2002;37:467–71.
39. Baek J, Jang J, Kang C, Chung H, Kim N, Kim K. Hypoxia-induced VEGF enhances tumor survivability via suppression of serum deprivation-induced apoptosis. Oncogene 2000;19:4621–31.
40. Ballif B, Blenis J. Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. Cell Growth Differ 2001;12:397–408.
41. Subbaramaiah K, Michaluart P, Sporn MB, Dannenberg AJ. Ursolic acid inhibits cyclooxygenase-2 transcription in human mammary epithelial cells. Cancer Res 2000;60:2399–404.
42. Moeller B, Cao Y, Li C, Dewhirst M. Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. Cancer Cell 2004;5:429–41.
43. Jonca F, Ortega N, Gleizes P-E, Bertrand N, Plouet J. Cell release of bioactive fibroblast growth factor 2 by exon 6-encoded sequence of vascular endothelial growth factor. J Biol Chem 1997;272:24203–9.
44. Holash J, Maisonpierre PC, Compton D, et al. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science 1999;284:1994–8.
45. Graeber G, Osmanian C, Jacks T, et al. Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumors. Nature 1996;379:88–90.
46. Tang N, Wang L, Esko J, et al. Loss of HIF-1α in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. Cancer Cell 2004;6:485–95.
47. Orecchia A, Lacal PM, Schietroma C, Morea V, Zambruno G, Failla CM. Vascular endothelial growth factor receptor-1 is deposited in the extracellular matrix by endothelial cells and is a ligand for the α 5 β 1 integrin. J Cell Sci 2003;116:3479–89.
48. Hanahan D, Weinberg R. The hallmarks of cancer. Cell 2000;100:57–70.
49. Koch S, Mayer F, Honecker F, Schitternhelm M, Bokemeyer C. Efficacy of cytotoxic agents used in the treatment of testicular germ cell tumours under normoxic and hypoxic conditions *in vitro*. Br J Cancer 2003;89:2133–9.
50. Teicher B. Hypoxia and drug resistance. Cancer Metastasis Rev 1994;13:139–68.
51. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 2003;3:203–16.
52. Eggert A, Ikegaki N, Kwiatkowski J, Zhao H, Brodeur G, Himelstein B. High-level expression of angiogenic factors is associated with advanced tumor stage in human neuroblastomas. Clin Cancer Res 2000;6:1900–8.
53. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004;25:581–611.
54. Shishodia S, Majumdar S, Banerjee S, Aggarwal BB. Ursolic acid inhibits nuclear factor-κB activation induced by carcinogenic agents through suppression of IκBα kinase and p65 phosphorylation: correlation with down-regulation of cyclooxygenase 2, matrix metalloproteinase 9, and cyclin D1. Cancer Res 2003;63:4375–83.

A Hypoxia-Driven Vascular Endothelial Growth Factor/Flt1 Autocrine Loop Interacts with Hypoxia-Inducible Factor-1 α through Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase 1/2 Pathway in Neuroblastoma

Bikul Das, Herman Yeger, Rika Tsuchida, et al.

Cancer Res 2005;65:7267-7275.