Phosphatidylinositol 3′-kinase and MAPK/ERK kinase 1/2 differentially regulate expression of vascular endothelial growth factor in human malignant astrocytoma cells[1]

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Malignant astrocytomas are characterized by extensive vascularization attributed to increased expression of the angiogenic cytokine vascular endothelial growth factor (VEGF). VEGF is elevated in astrocytomas under normal oxygen conditions and undergoes induction in hypoxic stress. Prior studies have shown that both the phosphatidylinositol 3′-kinase (PI3-kinase) and MEK1/2 (MAPK/ERK kinase 1/2) pathways promote proliferation of astrocytoma cells and growth of astrocytic tumors. Whether these pathways regulate growth by modulating angiogenesis as well as proliferation is not clear. In this study, pharmacologic inhibitors were used to specifically inhibit PI3-kinase and MEK1/2 activity in human malignant astrocytoma cell lines, and their effects on VEGF expression were determined. Northern blot analysis of VEGF messenger RNA (mRNA) from cells treated with inhibitors demonstrated cell line–specific responses. The PI3-kinase pathway regulated both the normoxic expression and hypoxic induction of VEGF in 2 cell lines, whereas MEK1/2 regulated only the normoxic expression in the same 2 lines. The third cell line showed no change in VEGF mRNA with inhibition of either of these 2 pathways. This study suggests that modulation of signaling pathways implicated in proliferation of astrocytoma cell lines may have varying effects in vivo depending on the role these pathways play in regulating tumor angiogenesis.
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[3]Abbreviations used are as follows: AKT, v-akt murine thymoma viral oncogene homolog; ARNT, aryl hydrocarbon nuclear translocator; DMEM, Dulbecco's modified Eagle's medium; ERK1/2, extracellular signal–regulated kinase 1/2; FCS, fetal calf serum; HIF-1, hypoxia inducible factor-1; MAPK, mitogen-activated protein kinase; MEK1/2, MAPK/ERK kinase 1/2; mRNA, messenger RNA; PI3-kinase, phosphatidylinositol 3′-kinase; PKB, protein kinase B; SDS, sodium dodecyl sulfate; VEGF, vascular endothelial growth factor.

Human malignant astrocytomas are the most common tumor of the CNS, being responsible for approximately 5% of all cancer-related deaths every year (Mahaley et al., 1989). A trademark characteristic of these tumors is their high degree of vascularity, the result of tumor angiogenesis. One of the most potent angiogenic factors implicated in solid tumor angiogenesis is the homodimeric cytokine VEGF.[3] This protein is an endothelial cell–specific mitogen, expressed in response to a number of stimuli including growth factors, hormones, oncogenes, and hypoxia (Grugel et al., 1995; Hashimoto et al., 1994; Horiuchi and Weller, 1997; Jiang et al., 2000; Ladoux and Frelin, 1993; Ryuto et al., 1996; Tsai et al., 1995; Warren et al., 1996). Because of its potent angiogenic effect, VEGF

expression is highly regulated, and few tissues express VEGF after embryonic development.

Regulation of VEGF is complex and occurs at many different levels, including induction of VEGF transcription, stabilization of VEGF mRNA, and secretion and diffusion of mature protein. Hypoxia is the major physiologic stimulus of VEGF expression. It induces transcriptional activity of the *VEGF* gene (Finkenzeller et al., 1995) and significantly increases the stability of the normally labile VEGF mRNA (Levy et al., 1996). Induction of VEGF transcription is largely mediated through the HIF1 transcription factor, a heterodimeric complex consisting of HIF1α and HIF1β/ARNT (Forsythe et al., 1996; Wood et al., 1996). HIF1 specifically binds hypoxia response elements, DNA sequences found in the promoter of a number of hypoxia-regulated genes, including VEGF (Wang and Semenza, 1993). The HIF1 transcription factor becomes active in hypoxic conditions through stabilization of the HIF1α subunit (Wang and Semenza, 1993; Wang et al., 1995).

Malignant astrocytomas and cell lines derived from these tumors frequently show aberrant expression of VEGF under normal oxygen conditions while still undergoing significant hypoxic induction. Recent evidence has demonstrated an important role for the small G-protein p21-RAS in regulating astrocytoma cell proliferation (Bouterfa et al., 2000; Feldkamp et al., 1999a; Guha et al., 1997) and VEGF expression under normal and hypoxic conditions (Feldkamp et al., 1999b, 1999c). Oncogenic mutations of p21-RAS have been described in more than 40% of human cancers, though astrocytomas do not harbor such mutations. However, an increase in mitogenic signals from overexpressed and constitutively active receptor tyrosine kinases results in elevated levels of activated p21-RAS (GTP-bound p21-RAS) in both astrocytoma tumor samples and established astrocytoma cell lines (Guha et al., 1997). Activation of p21-RAS is known to be functionally important in the proliferation and growth of astrocytomas both in vitro and in vivo, which has led to the targeting of these tumors by agents that inhibit p21-RAS (Feldkamp et al., 1999a). In addition to the antiproliferative effects observed in astrocytomas, use of p21-RAS inhibitors also results in both a significant decrease in VEGF expression under normal oxygen conditions and a significant blunting of the hypoxic induction of VEGF (Feldkamp et al., 1999c).

Although it is clear that p21-RAS is involved in regulating VEGF in astrocytomas, the downstream effectors that are responsible for transmitting this angiogenic signal have not yet been clearly elucidated. The 2 most commonly studied p21-RAS effector pathways are the PI3-kinase–AKT/PKB pathway and the MEK1/2-ERK1/2 MAPK pathway, both of which have been strongly implicated as major regulators of astrocytoma proliferation. Multiple studies have shown that inhibition of PI3-kinase reduces VEGF expression (Jiang et al., 2000; Mazure et al., 1997; Rak et al., 2000; Wang et al., 1999), whereas others report a role for ERK1/2 in regulating VEGF (Jung et al., 1999; Milanini et al., 1998; Rak et al., 2000). However, most of the studies to date have been undertaken in cell lines that are not tumorigenic or astrocytic in origin, such as fibroblasts and endothelial cells, and therefore may not be reflective of the method through which astrocytomas regulate VEGF expression. In this study we use established astrocytoma cell lines as models for studying VEGF regulation in astrocytic tumors. Using pharmacological inhibitors, we have blocked signaling via PI3-kinase and MEK1/2 to determine the contribution of these 2 major p21-RAS effector pathways in regulating aberrant expression of VEGF in normal oxygen conditions, as well as the hypoxic induction of VEGF expression in human malignant astrocytomas.

Materials and Methods

Cell Lines and Culture Conditions

Three established human malignant astrocytoma cell lines were used in these experiments: U-373 MG and U-343 MG cells (a gift from B. Westermark, Uppsala, Sweden) and U-87 MG cells (obtained from the American Type Culture Collection, Rockville, Md.). All cells were grown at 37°C/5% CO_2 in DMEM supplemented with 10% FCS. Hypoxia was induced using BBL GasPak Plus anaerobic system envelopes with palladium catalyst (Becton Dickinson, Cockeysville, Md.), in an air-tight hypoxia chamber. Hypoxic conditions were confirmed using BBL Dry Anaerobic Indicator Strips (BD Biosciences, San Jose, Calif.).

Use of Pharmacologic Inhibitors

The PI3-kinase–specific inhibitor LY294002 (Calbiochem, La Jolla, Calif.) was used at concentrations ranging from 1 to 25 μM, diluted in culture medium (DMEM + 10% FCS) from concentrated stocks (100 mM) using MeOH as the solvent. U0126, a specific inhibitor of MEK1/2 (Promega, Madison, Wis.), was dissolved in MeOH and used at concentrations ranging from 1 to 20 μM, diluted in culture medium (DMEM + 10% FCS). For transcriptional studies, cells were treated with 1 μg/ml actinomycin-D (Calbiochem), diluted in culture medium (DMEM + 10% FCS) for the times indicated in different experiments.

Western Blot Analysis and Antibodies

Antibodies against AKT/PKB, phospho-AKT/PKB(Ser473), p44/42 MAPK (ERK1/2), phospho-p44/42 MAPK, MEK1/2 (New England Biolabs Inc., Beverly, Mass.), and HIF1β (Transduction Laboratories, Lexington, Ky.) were all used at a dilution of 1:1000 for Western blotting. The antibody against p85 PI3-kinase (Upstate Biotechnology Inc., Lake Placid, N.Y.) was used at a dilution of 1:2000. The antibody against HIF1α (Transduction Laboratories) was used at a dilution of 1:250 for Western blotting. Horseradish peroxidase–conjugated goat anti-rabbit and goat anti-mouse antibodies (Bio-Rad, Hercules, Calif.) were both used at a dilution of 1:3000.

For analysis of HIF1α and HIF1β, monolayered cells were lysed in RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and leupeptin, and 1 mM sodium orthovanadate). For analysis of all other proteins, monolayer cells were lysed in PLC buffer

(50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 10 mM NaPP, 100 mM NaF, 10 μg/ml aprotinin and leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 0.2 mM sodium orthovanadate). Cell debris was removed from all cell lysates by centrifugation, and protein lysates were flash-frozen and stored at –80°C.

For Western blot detection of HIF1α, 500 μg cell lysate was immunoprecipitated with 1 μg anti-HIF1α antibody (Transduction Laboratories), 1 μg rabbit anti-mouse polyclonal antibody (Upstate Biotechnology Inc.), and 50 μl of 50% protein-A Sepharose in phosphate-buffered saline. The immunoprecipitants were washed in RIPA buffer, and proteins were eluted off the Sepharose beads with 2× SDS sample buffer (125 mM Tris-Cl, pH 6.8, 4% wt/vol SDS, 20% glycerol, 100 mM dithiothreitol, and 0.1% wt/vol bromophenol blue). The immunoprecipitants were then separated by SDS–polyacrylamide gel electrophoresis. For analysis of all other proteins, 15 μg protein lysate was boiled with an equivalent volume of 2× SDS sample buffer and separated by SDS–polyacrylamide gel electrophoresis. Proteins were transferred to PolyScreen polyvinylidene fluoride membranes (NEN, Boston, Mass.) using a semi-dry transfer apparatus (Bio-Rad) and were then probed overnight at 4°C using the appropriate antibody. Proteins were detected by enhanced chemiluminescence (Amersham Pharmacia, Baie d'Urfé, Quebec, Canada) and exposing the membranes to autoradiography film (Eastman Kodak, Rochester, N.Y.).

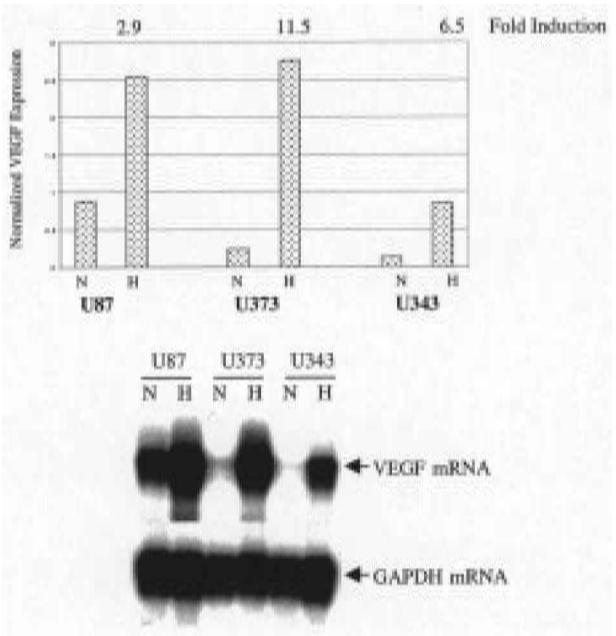

Fig. 1. Increased VEGF mRNA under hypoxic conditions in established astrocytoma cell lines. VEGF and GAPDH mRNA levels in U-87 MG, U-373 MG, and U-343 MG cell lines after incubation in normal oxygen (N) or hypoxic (H) conditions for 6 h were determined via Northern blot analysis. VEGF mRNA was normalized to GAPDH in each lane and is represented graphically. Fold induction of VEGF by hypoxia is indicated on the top of the graph.

Northern Blot Analysis of mRNA

Total RNA was extracted from cells using RNeasy columns (Qiagen, Mississauga, Ontario, Canada); 5 to 10 μg of total RNA was separated in an agarose-formaldehyde gel and transferred to Hybond neutral nylon (Amersham Pharmacia) by capillary action. Membranes were prehybridized for 30 min at 68°C using ExpressHyb hybridization solution (Clontech, Palo Alto, Calif.) containing 0.1 mg/ml single-stranded salmon sperm DNA. A VEGF cDNA probe that recognizes all 4 VEGF mRNA isoforms (gift from Brygida Berse, Beth Israel Hospital, Boston, Mass.) was radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol) using the T7 QuickPrime Kit (Amersham Pharmacia). The probe was purified through a Sephadex column (Amersham Pharmacia) and incubated with the Hybond membrane in ExpressHyb solution at 68°C. The membranes were washed with 2× saline-sodium citrate/0.05% SDS and 0.1× saline-sodium citrate/0.1% SDS and exposed to a PhosphorImager cassette, which was then analyzed with a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). The membranes were then reprobed with a GAPDH cDNA probe using the same conditions as above.

Proliferation Analysis of Astrocytoma Cells

U-87 MG, U-373 MG, and U-343 MG cells were plated on five 24-well tissue culture plates at a density of 5000 cells per well. Sixteen hours after plating, cells were treated with MeOH, 25 μM LY294002, and 20 μM U0126 or were left untreated. MeOH and U0126 were replaced every 24 h, and LY294002 was replaced every 8 h for the duration of the study. Four hours after the initial treatment, one plate of cells was stained with trypan blue, and viable cells were counted using a hemocytometer. A new plate of cells was then counted every 24 h after this initial count. Cell numbers from 4 independent experiments were averaged and plotted on a graph.

Statistical Analysis

All error terms are expressed as SD. For Figs. 1, 4, and 5, graphs represent the amount of VEGF mRNA as detected by the STORM PhosphorImager normalized to the amount of GAPDH in each lane and expressed relative to the amount of VEGF mRNA in the control lanes. A significant effect of the drug was determined by performing Student's *t* test between cells treated with the solvent alone (MeOH) versus cells treated with the highest dose of the given drug. These analyses were completed using Microsoft Excel for Macintosh (Redmond, Wash.).

Results

VEGF Expression Is Induced by Hypoxia

Northern blot analysis was used to assess levels of VEGF mRNA in each of 3 established malignant astrocytoma cell lines after incubation in normal oxygen or hypoxia for 6 h (Fig. 1). The U-87 MG cell line expressed the most VEGF

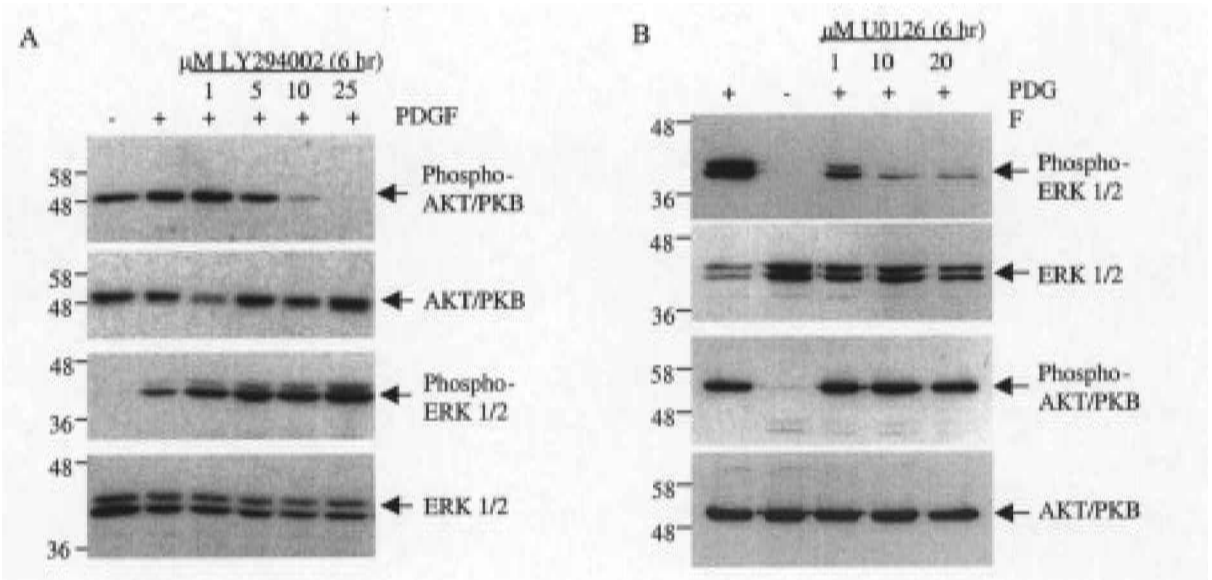

Fig. 2. Inhibited phosphorylation of AKT/PKB by LY294002 and of ERK1/2 by U0126 in U-87 MG astrocytoma cell lines. Confluent dishes of U-87 MG cells were serum starved for 24 h. The cells were then treated with increasing doses of LY294002 or U0126 or were left untreated for 6 h. Cells were then stimulated with platelet-derived growth factor (50 ng/ml) for 15 min at 37°C before lysis. A. Western blot analysis of cell lysates showing that 10 to 25 μM LY294002 inhibits phosphorylation of the PI3-kinase effector AKT/PKB. LY294002 does not affect steady-state levels of AKT/PKB, nor does it inhibit phosphorylation of ERK1/2. B. Western blot analysis also showing that 20 μM U0126 specifically inhibits phosphorylation of ERK1/2 without reducing levels of ERK1/2 or phosphorylated AKT/PKB.

mRNA under normal oxygen conditions; the U-373 MG cells expressed very little, with undetectable levels in U-343 MG cells. All 3 cell lines showed an induction in VEGF mRNA levels under hypoxic stress, with the U-373 MG cells undergoing the largest induction (11.5-fold increase), U-343 MG showing the second largest induction (6.5-fold), and the U-87 MG cells undergoing the least induction (2.9-fold).

LY294002 and U0126 Specifically Inhibit Proliferative Signals from PI3-kinase and MEK1/2

Phosphorylation of the PI3-kinase effector AKT/PKB and phosphorylation of the MAPK family member ERK1/2 (downstream effector of MEK1/2) were used as measures of PI3-kinase and MEK1/2 activity, respectively. Serum-starved U-87 MG cells were treated with increasing concentrations of LY294002 or U0126 for a period of 6 h, at which time the cells were stimulated with platelet-derived growth factor (50 ng/ml for 15 min). Western blot analysis showed that 25 μM of LY294002 inhibited the phosphorylation of AKT/PKB, whereas there was no significant change in the levels of whole-cell AKT/PKB or the phosphorylation of ERK1/2 (Fig. 2A). Activity of U0126 was also assessed, and Western blot analysis showed that phosphorylation of ERK1/2 was inhibited by a 20-μM dose of U0126 (Fig. 2B). Phosphorylation of the PI3-kinase effector AKT/PKB was not affected by U0126, demonstrating that the compound specifically inhibits the MEK1/2 pathway under these conditions.

The effect of LY294002 and U0126 on proliferation of U-87 MG, U-373 MG, and U-343 MG cells was also determined. Inhibition of PI3-kinase activity by LY294002 significantly reduced proliferation of all 3 cell lines, whereas inhibition of MEK1/2 activity by U1026 reduced proliferation in the U-87 MG and U-373 MG cells but had little effect on proliferation of U-343 MG cells (Fig. 3).

Differential Regulation of VEGF Expression in Normal Oxygen Conditions in Astrocytoma Cell Lines

To determine the contribution of PI3-kinase activity to the levels of VEGF mRNA in astrocytoma cell lines under normal oxygen conditions, U-87 MG, U-373 MG, and U-343 MG cells were treated with increasing doses of LY294002 for 6 h (Fig. 4A). Northern blot analysis was used to assess levels of VEGF mRNA in untreated control cells, in cells treated with the drug solvent MeOH (0.025%), or with doses of LY294002 as indicated. VEGF mRNA levels for each cell line were normalized to GAPDH in each lane and were expressed relative to the amount of VEGF mRNA in the control lane of that cell line. Experiments were repeated in triplicate, and results are expressed graphically, with one representative Northern blot shown (Fig. 4A). For each cell line, the amount of VEGF mRNA in the solvent lane was compared to the VEGF mRNA level in the 25-μM LY294002 lane using the 2-tailed Student's *t* test. In normal oxygen, U-87 MG cells showed a significant decrease in the level of VEGF mRNA present with inhibition of PI3-kinase ($P = 0.03$). Both U-373 MG and U-343 MG cells also showed a downward trend; however, this decrease was not significant.

The above studies were repeated using the MEK1/2 inhibitor U0126 (Fig. 4B). Treatment of U-87 MG, U-373 MG, and U-343 MG cells with increasing doses of U0126 for 6 h in normal oxygen significantly decreased the level of VEGF mRNA in both the U-87 MG and

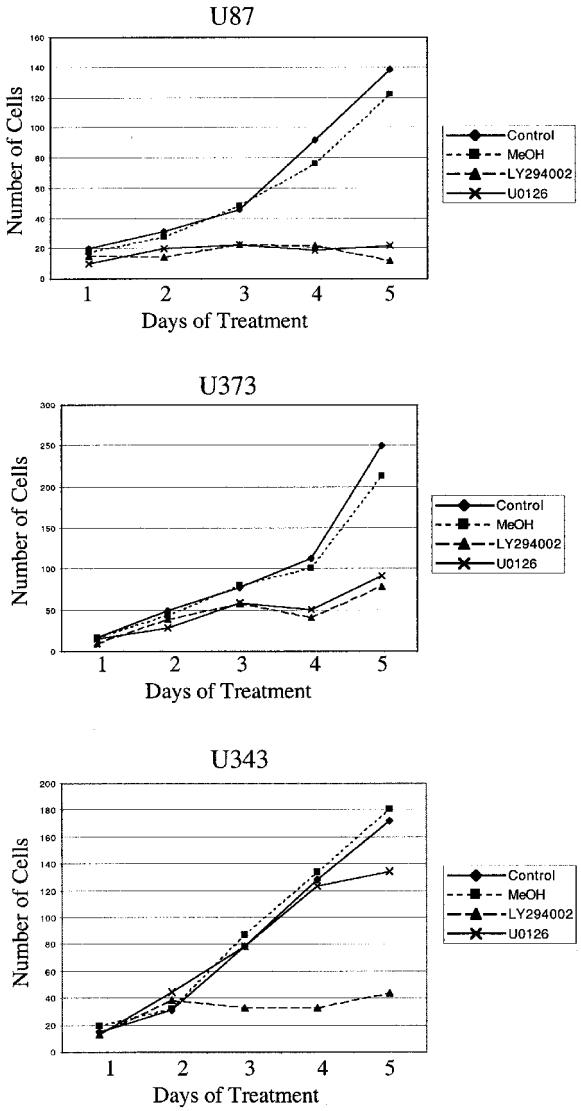

Fig. 3. Proliferation of astrocytoma cells in the presence of the PI3-kinase inhibitor LY294002 and the MEK1/2 inhibitor U0126. U-87 MG (A), U-373 MG (B), or U-343 MG (C) cells were plated at an initial density of 5000 cells per well. Sixteen hours after plating, cells were treated with MeOH, 25 μM LY294002, or 20 μM U0126 or were left untreated (control). Four hours after beginning treatment, cells were stained with trypan blue and counted with a hemocytometer. Cells were then counted every 24 h for 5 days. Cell counts for 3 independent experiments were averaged and are displayed graphically.

U-343 MG cells ($P = 0.004$ and $P = 0.042$, respectively). The U-373 MG cells showed no significant change in VEGF mRNA level, even at the highest dose of U0126.

Differential Regulation of the Hypoxic Induction of VEGF in Astrocytoma Cell Lines

The role of signaling via PI3-kinase and MEK1/2 pathways in the hypoxic induction of VEGF in astrocytoma cell lines was examined. U-87 MG, U-373 MG, and U-343 MG cells were treated with LY294002 or MeOH (0.025%) or were untreated, as in the previous experiment. The cells were then incubated for 6 h in a hypoxic chamber at 37°C. Northern blot analysis of VEGF mRNA and GAPDH mRNA was used to assess the effect of PI3-kinase inhibition on VEGF expression (Fig. 5A). Under hypoxic stress, VEGF induction was sensitive to PI3-kinase inhibition in both U-87 MG and U-343 MG cells ($P = 0.04$ and $P = 0.03$, respectively); however, VEGF mRNA levels in U-373 MG cells did not significantly change. The same studies were completed with the MEK1/2-specific inhibitor U0126 under hypoxic stress for 6 hours (Fig. 5B). In this case, all 3 cell lines showed a downward trend in the levels of VEGF mRNA with increasing doses of U0126; however, none of these decreases were statistically significant.

LY294002 and U0126 Do Not Affect VEGF mRNA Stability in Astrocytoma Cells in Normal Oxygen

To determine if the significant decrease in VEGF mRNA levels seen in U-87 cells treated with LY294002 under normal oxygen conditions was due to a decrease in the VEGF mRNA stability or a decrease in VEGF transcription, cells were preincubated with the transcriptional inhibitor actinomycin D for 20 min. Cells were then left untreated, treated with MeOH, or treated with 25 μM LY294002. RNA was collected from the cells at various time points, and the level of VEGF mRNA was assessed via Northern blot analysis. The decrease in VEGF mRNA was plotted for each treatment, and the half-life was extrapolated from the logarithmic plot of each line. As can be seen in Fig. 6, there was no significant difference in the rate of VEGF mRNA degradation between each treatment, and therefore no difference in the VEGF mRNA half-life ($t_{1/2}$). This experiment was repeated with 20 μM U0126 in place of LY294002, and no significant difference in the VEGF mRNA half-life from cells treated with U0126 or control MeOH was observed (data not shown).

Expression and Activity of PI3-kinase and MEK1/2 Signaling Components in Astrocytoma Cell Lines

The cell line–specific responses to inhibition of PI3-kinase and MEK1/2 suggested that differences in the genotypic background of each line may affect the manner in which VEGF is regulated. Western blot analysis was used to determine if there were any differences among the astrocytoma cell lines in protein levels or activation status of the proteins closely involved in the PI3-kinase and MEK1/2-ERK1/2 pathways. Western blot analysis showed no significant difference in the levels of AKT/PKB or phosphorylated AKT/PKB in either normal oxygen or hypoxia (Fig. 7A). Levels of proteins involved in the MAPK pathway were also examined (Fig. 7B). No significant difference in the levels of MEK1/2 or the downstream effector ERK1/2 was observed between the 3 cell lines. However, it does appear that phosphorylation of ERK1/2 is highest in the U-87 MG cells, in both normal oxygen conditions and under hypoxic stress, whereas very little phosphorylated ERK1/2 is present in U-373 and U-343 cells.

Finally, we examined expression of the hypoxia-inducible transcription factor HIF1. Stabilization and

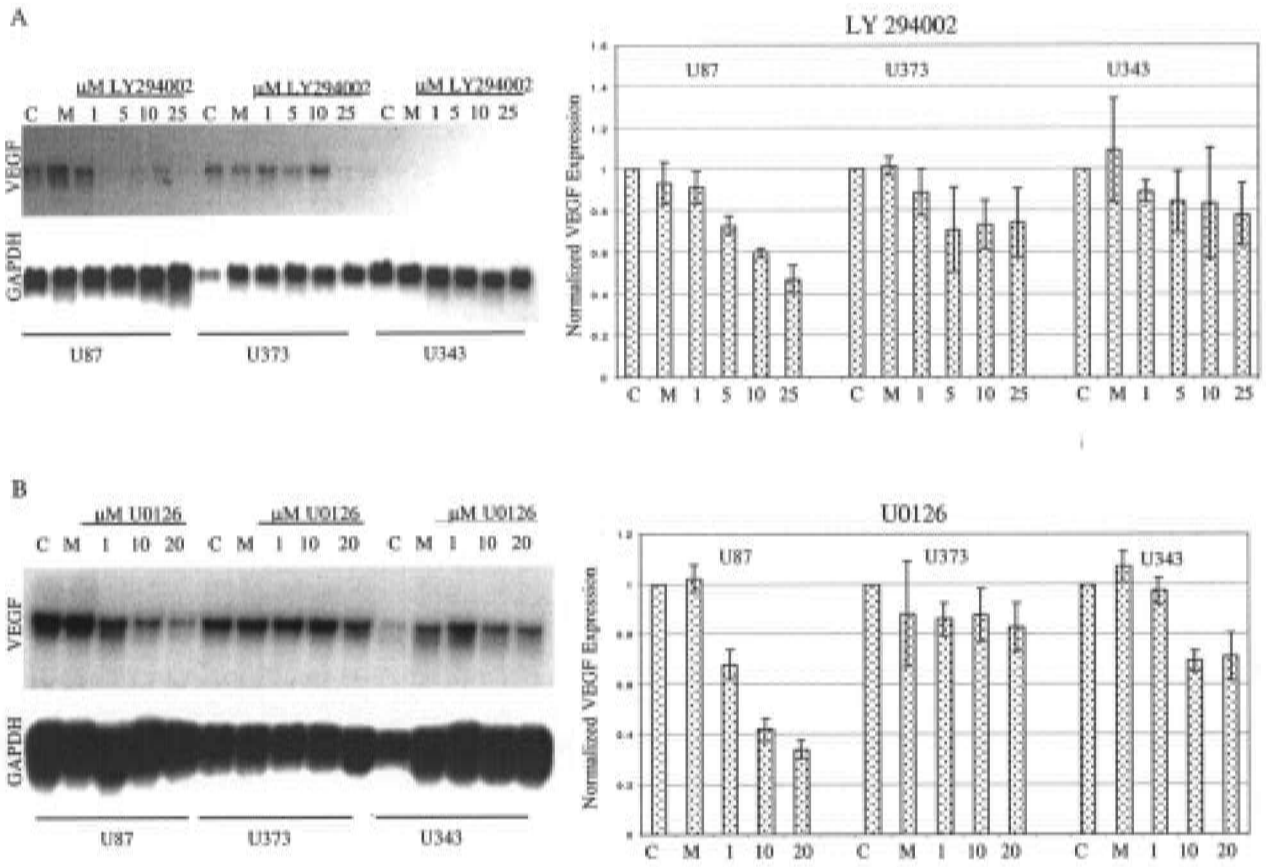

Fig. 4. Effect of LY294002 or U0126 treatment on VEGF expression in normoxia. Confluent astrocytoma cells were treated with increasing doses of LY294002, U0126, or MeOH (M) or were left untreated (C) in normal oxygen conditions for 6 h. Total RNA was extracted, and VEGF and GAPDH mRNA levels were assessed via Northern blot analysis. VEGF mRNA levels expressed graphically were normalized to GAPDH, and the mean values of triplicate experiments are represented along with error bars (SD). A. Inhibition of PI3 kinase. Normoxic VEGF mRNA levels were significantly reduced in U-87 MG cells, but not in U-373 MG or U-343 MG cells. B. Inhibition of MEK1/2. Normoxic VEGF mRNA levels were significantly reduced in U-87 MG and U-343 MG cells, but not in U-373 MG cells.

activation of HIF1 in hypoxic conditions have been attributed to both the PI3-kinase pathway and the MEK1/2-ERK1/2 MAPK pathway (Minet et al., 2000; Richard et al., 1999; Zhong et al., 2000; Zundel et al., 2000). Western blot analysis of HIF1α in the astrocytoma cell lines showed an increase in the steady-state levels of HIF1α under hypoxic conditions as well as an apparent increase in its molecular size, most likely due to phosphorylation of the protein (Fig. 8). In addition, HIF1α was present in normal oxygen conditions in both U-87 MG cells and U-373 MG cells, suggesting aberrant stabilization of this protein. As expected, Western blot analysis of HIF1β/ARNT shows that it is highly expressed by all 3 cell lines, and not induced by hypoxic conditions.

Discussion

Genetic mutations that result in the gain of oncogenes and loss of tumor suppressors are usually thought to promote tumorigenesis by inducing signaling cascades that lead to aberrant cell proliferation and survival. More recently, the role of such mutations in regulating tumor angiogenesis has been appreciated, with the realization that induction of angiogenesis is a vital step in the successful growth, progression, and metastasis of a solid tumor. As these signaling pathways are being targeted in anticancer therapy, a better understanding of their role in regulating the crucial angiogenic process of solid tumors is required. This may lead to the development of compounds targeting proteins that play critical roles in regulating both proliferation and angiogenesis. Such compounds may have a more profound effect in vivo than those that inhibit proliferation alone.

One of the major angiogenic factors regulated by oncogenes and tumor suppressor genes is VEGF, an endothelial cell–specific cytokine that promotes destabilization of mature blood vessels and growth of new vessels. Our interest in VEGF regulation by activated p21-RAS in malignant astrocytomas stems from our prior observation that this signaling pathway is activated and functionally relevant in the proliferation and growth of these human tumors in vitro and in vivo (Feldkamp et al., 1999a, 1999b; Guha et al., 1997). Furthermore, we and others have demonstrated that activated p21-RAS positively regulates VEGF expression in several transformed cell lines including established astrocytoma cell lines (Feldkamp et al., 1999b, 1999c; Grugel et al., 1995;

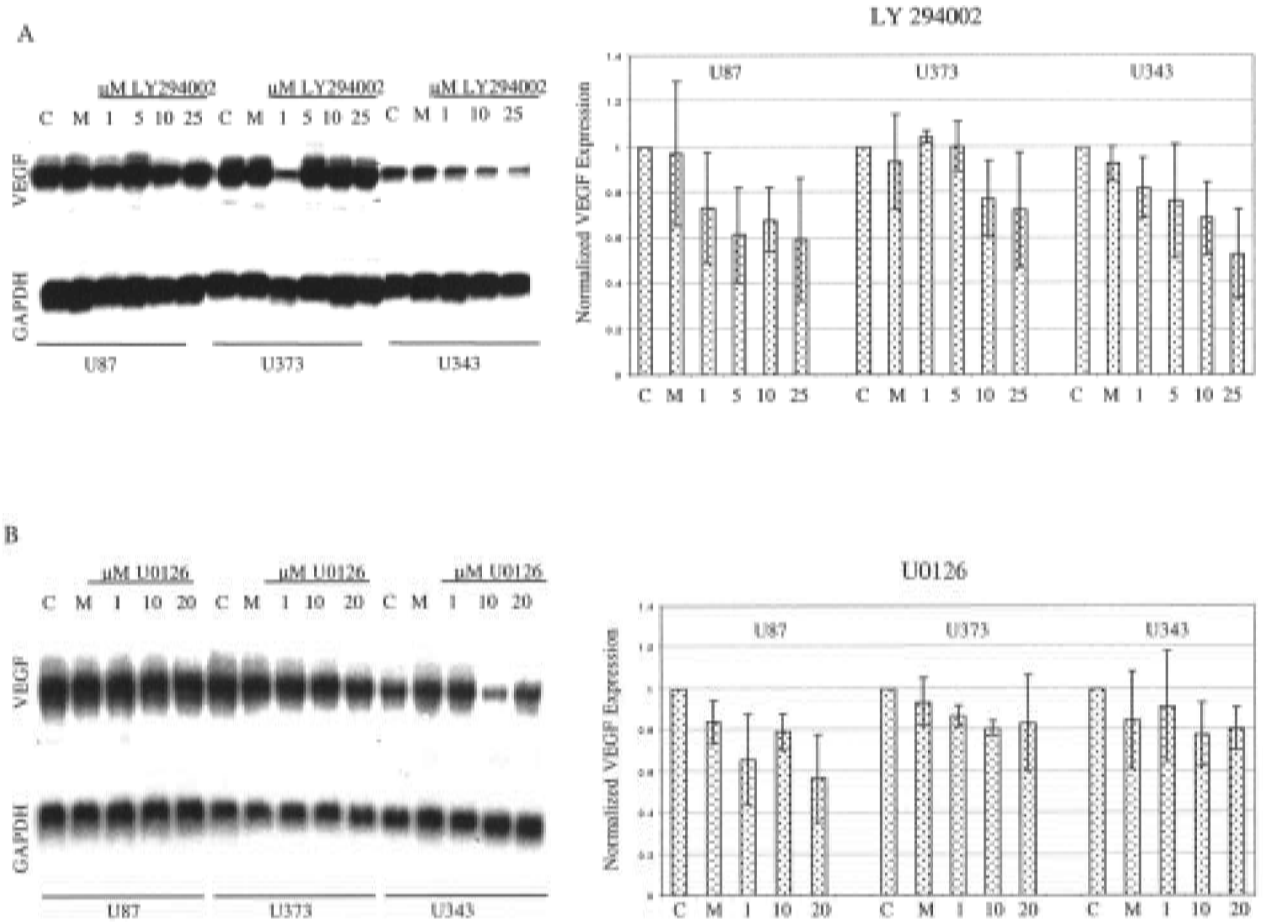

Fig. 5. Effect of LY294002 or U0126 treatment on VEGF expression in hypoxia. Confluent astrocytoma cells were treated with increasing doses of LY294002, U0126, or MeOH (M) or were left untreated (C) in hypoxic conditions for 6 h. RNA was extracted, and VEGF and GAPDH mRNA levels were assessed via Northern blot analysis. VEGF mRNA levels expressed graphically were normalized to GAPDH, and results for the mean values of triplicate experiments are represented along with error bars (SD). A. Inhibition of PI3-kinase activity in hypoxic conditions. VEGF mRNA levels were significantly reduced in both U-87 MG and U-343 MG cells, but not in U-373 MG cells. B. Inhibition of MEK1/2 activity in hypoxic conditions. VEGF mRNA levels in none of 3 astrocytoma cell lines were significantly affected.

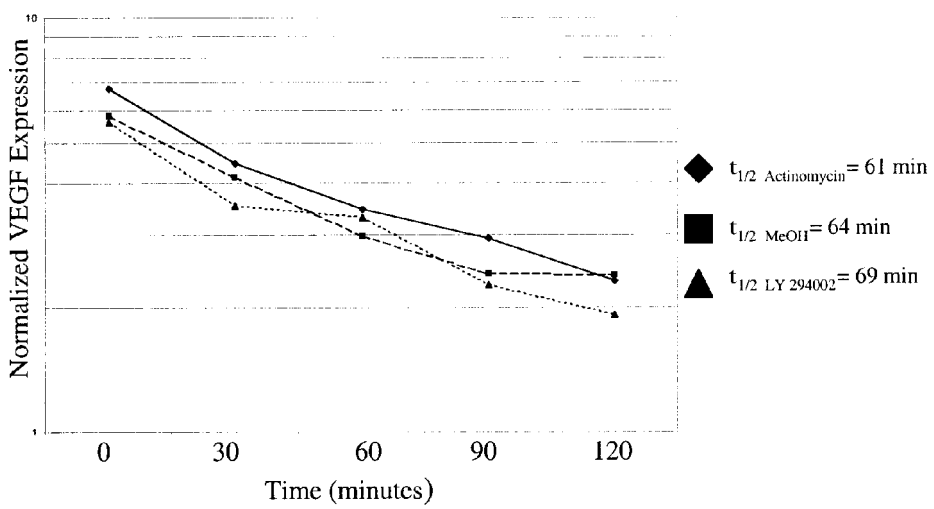

Fig. 6. VEGF mRNA rate of degradation not significantly changed by LY294002. Confluent U-87 MG cells were pretreated with transcriptional inhibitor actinomycin-D (1 μg/ml) for 20 min. Cells were then left untreated (◆), treated with MeOH (■), or treated with 25 μM LY294002 (▲) for 0, 30, 60, 90, or 120 min. Total RNA was collected at each time point, and Northern blot analysis was used to determine VEGF and GAPDH mRNA levels. VEGF mRNA was normalized to GAPDH mRNA and graphed. The VEGF mRNA half-life ($t_{1/2}$) for each treatment was extrapolated from the logarithmic graph. There was no significant difference in rate of degradation between any of the 3 treatments.

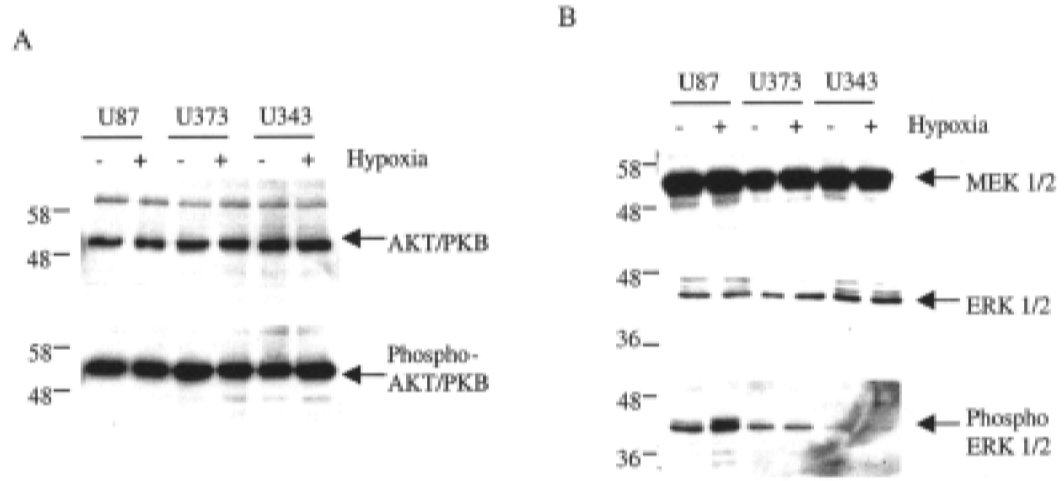

Fig. 7. Analysis of PI3-kinase and MEK1/2 signaling pathway components. U-87 MG, U-373 MG, and U-343 MG cells were incubated in normal oxygen or hypoxia for 6 h. A. Examination of cell lysates from all 3 astrocytoma cell lines using anti-AKT/PKB, anti–phospho-AKT/PKB, and anti-PTEN. The cell lines were found to express similar levels of the PI3-kinase downstream effector AKT/PKB, as well as the active (phosphorylated) form of AKT/PKB. Note that although the U-343 MG cells were the only line to express PTEN protein, this protein has been shown to be inactive because of a point mutation. B. MEK1/2, ERK1/2, and phospho-ERK1/2 levels examined via Western blot. The cell lines again expressed similar levels of MEK1/2 and ERK1/2; however, the U-87 MG cells were found to express higher levels of activated (phosphorylated) ERK1/2 than either the U-373 MG or U-343 MG cells.

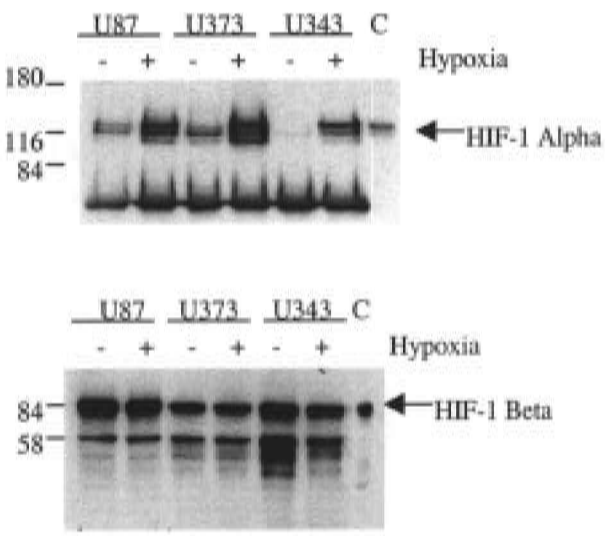

Fig. 8. Normoxic expression of HIF1α in astrocytoma cell lines. U-87 MG, U-373 MG, and U-343 MG astrocytoma cell lines incubated in normal oxygen or hypoxia for 6 h were lysed and assessed for levels of HIF1α (upper panel) and HIF1β (lower panel). Expression of HIF1α under normal oxygen conditions was observed in U-87 MG and U-373 MG cells, and all 3 cell lines show increased levels of HIF1α in hypoxic conditions. All 3 cell lines expressed similar levels of HIF1β in both normal oxygen and hypoxia (lower panel). Lane C in each blot contains control lysate as a marker for HIF1α or HIF1β migration.

Larcher et al., 1996; White et al., 1997). In addition, pharmacologic inhibition of p21-RAS and associated pathways with farnesyl transferase inhibitors resulted in decreased astrocytoma xenograft growth, reduced tumor vasculature and VEGF secretion, and increased apoptosis of endothelial cells (Feldkamp et al., 2001). This suggests that targeting of p21-RAS–regulated pathways may be of benefit in these highly malignant and vascularized human tumors. Multiple signal transduction pathways downstream of p21-RAS could be responsible for transmitting the VEGF regulatory signals, knowledge of which may lead to more effective targets in astrocytomas. In this study we examined the role of 2 main p21-RAS effectors, PI3-kinase and MEK1/2, both of which have been implicated in astrocytoma growth and in regulation of VEGF expression in both normoxic and hypoxic conditions.

We found cell line–specific differences in regulation of VEGF mRNA and regulation of proliferative signals by the PI3-kinase and MEK1/2 pathways in human malignant astrocytomas. These results are very intriguing, since our previous studies showed that inhibition of p21-RAS activity reduced both cell proliferation and production of VEGF in all 3 astrocytoma cell lines (Feldkamp et al., 1999b). This present study suggests that each of the 3 cell lines transmits the angiogenic and proliferative signals downstream of p21-RAS in a unique manner. In normal oxygen conditions, inhibition of PI3-kinase activity significantly inhibited proliferation of all 3 astrocytoma lines, but reduced the amount of VEGF mRNA only in the U-87 MG cells, not the U-343 MG or U-373 MG cells. The U-87 MG cells expressed much higher levels of VEGF in normal oxygen conditions than any of the other astrocytoma cell lines and, interestingly, U-87 MG cells grew the best in xenograft experiments. These data suggest that activity of the PI3-kinase pathway may be critical in mediating both the proliferative and angiogenic signals in the growth of U-87 MG cells in vivo. In the U-343 MG and U-373 MG cells, the PI3-kinase pathway is important in regulating proliferation in vitro, but may not have a major role in in vivo growth. The observed differences in VEGF regulation between the 3 cell lines in normal oxygen may indicate the presence of an addi-

tional mutation in the U-87 MG cells that deregulates the PI3-kinase pathway. All 3 lines were null for phosphatase and tensin homolog (data not shown) and expressed similar levels of the p85 and p110 subunits of PI3-kinase (Fig. 7). Therefore, the mutation may be in a different gene, the protein for which is a component regulating the PI3-kinase signaling pathway.

Regulation of VEGF under hypoxia is of importance because it is the main physiologic and pathologic regulator of VEGF expression. All 3 astrocytoma lines underwent a significant increase in VEGF mRNA levels under hypoxic conditions. When treated with the PI3-kinase inhibitor LY294002, the VEGF hypoxic induction was significantly blunted in the U-87 MG and U-343 MG cells, whereas there was only a minimal decrease in U-373 MG VEGF mRNA levels. This suggests that U-373 MG cells either induce VEGF expression through a different signaling pathway or perhaps have aberrant hypoxic regulation of activity downstream of PI3-kinase that promotes VEGF expression. It has been noted by one group that oncogenic transformation alone was not enough to induce VEGF expression, but that certain oncogenes amplify the hypoxic induction, promoting very strong angiogenesis (Mazure et al., 1996). The U-373 MG cells undergo the largest hypoxia-induced increase in VEGF mRNA, which may be due to synergistic activity between hypoxia and an activated oncoprotein downstream of PI3-kinase.

The observed decrease in the level of VEGF mRNA could have been the result of changes in VEGF mRNA stability or changes in transcription of the VEGF gene. In the presence of the transcriptional inhibitor actinomycin-D, the degradation rate of VEGF mRNA was not altered by inhibition of the PI3-kinase pathway. These findings suggest that the major method through which inhibition of PI3-kinase activity reduces VEGF mRNA levels is through changes in transcription of the VEGF gene rather than by decreasing VEGF mRNA stability. These data are in agreement with the results of other studies in which signaling through PI3-kinase altered VEGF gene transcription, not mRNA stability (Jiang et al., 2000; Mazure et al., 1997).

Inhibition of the MEK1/2-ERK1/2 MAPK pathway by U0126 in normal oxygen significantly reduced the levels of VEGF mRNA in both the U-87 MG and U-343 MG cell lines, yet had little effect on U-373 MG cells. In contrast, U0126 inhibited proliferation of both the U-87 MG and U-373 MG cell lines, while having little to no effect on U-343 MG cell proliferation. This suggests that in both U-87 MG and U-343 MG cells, MEK1/2 activity contributes to angiogenesis, whereas only in U-87 MG cells does MEK1/2 activity contribute to both angiogenesis and cell proliferation. Of interest, U-87 MG cells have the highest level of MEK1/2 activity among the 3 cell lines examined, which may account for the increased sensitivity of U-87 MG cells to U0126 with respect to both antiproliferative effects and decreased VEGF expression. These results are in contrast to a recent report that demonstrated no decrease in VEGF mRNA levels with inhibition of MEK1/2 in U-87 MG astrocytoma cells; however, a different pharmacologic inhibitor was used, which may account for the observed differences (Maity et al., 2000). Normoxic levels of VEGF mRNA in the U-373 MG cells were not regulated by MEK1/2, but this pathway did alter proliferation in these cells, suggesting that separate p21-RAS effector pathways are responsible for mediating these 2 different responses in U-373 MG cells.

The hypoxic induction of VEGF was not altered by U0126 in any of the 3 cell lines. This is in agreement with studies showing that MEK1/2 activity is not necessary for the hypoxic induction of VEGF in fibroblasts (Mazure et al., 1997; Milanini et al., 1998). Recent studies have identified ERK1/2 (MEK1/2 downstream effector) as a regulator of the hypoxia-inducible transcription factor HIF1, which is known to up regulate VEGF expression through HIF1 binding sites in the VEGF promoter (Pages et al., 2000; Richard et al., 1999). These data would implicate ERK1/2 activity in the hypoxic induction of VEGF. However, our data suggest that VEGF induction in hypoxia is regulated in an MEK1/2-independent manner; therefore hypoxic induction of ERK1/2 may also be MEK1/2 independent.

The HIF1 transcription factor is a major regulator of VEGF transcription, and recent reports have shown that HIF1α stability and subsequent HIF1 activity are dependent on signals from both PI3-kinase-AKT/PKB and ERK1/2. This would suggest that down regulation of VEGF mRNA by either LY294002 or U0126 could be the result of a decrease in HIF1α stability and HIF1 activity. HIF1α is the oxygen-sensitive component of the HIF1 transcription factor and is not detected in normoxia in normal cells, though some transformed cells have been found to have aberrant stabilization and expression of HIF1α in normal oxygen conditions. Of interest, both U-87 MG and U-373 MG cells have deregulated stabilization of HIF1α in normal oxygen, correlating with aberrant VEGF expression in normoxia by these 2 lines. However, our preliminary studies showed that neither LY294002 nor U0126 affects the steady-state levels of HIF1α or the phosphorylation of HIF1α in these astrocytoma lines (data not shown). The activity of the HIF1 transcription factor in the absence or presence of these drugs has yet to be determined. In addition to HIF1, other VEGF transcriptional regulators are present (Damert et al., 1997; Finkenzeller et al., 1997; Sheta et al., 2000) and may also be the target of the PI3-kinase and MEK1/2 pathways.

In conclusion, this work indicates that neither the PI3-kinase nor the MEK1/2 pathway is solely responsible for mediating angiogenic and proliferative signals from p21-RAS in astrocytomas. This is not surprising, given that astrocytomas are well known for their pathologic, molecular, and therapeutic response heterogeneity. These results do have potential important implications in the development of novel therapeutics that target one particular signaling pathway. Our data suggest that treatment of astrocytomas, and perhaps other solid cancers, with compounds that inhibit multiple signaling pathways may be necessary to promote both antiproliferative and anti-angiogenic beneficial effects. Furthermore, the effects of these inhibitors may not be predictable because of tumor heterogeneity, and assays designed to determine the activity of signaling pathways in a particular tumor may be required to optimize therapy.

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