Involvement of PI3K and MAPK Signaling in bcl-2-induced Vascular Endothelial Growth Factor Expression in Melanoma Cells

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Submitted December 17, 2004; Accepted June 16, 2005
Monitoring Editor: Gerard Evan

We have previously demonstrated that bcl-2 overexpression in tumor cells exposed to hypoxia increases the expression of vascular endothelial growth factor (VEGF) gene through the hypoxia-inducible factor-1 (HIF-1). In this article, we demonstrate that exposure of bcl-2 overexpressing melanoma cells to hypoxia induced phosphorylation of AKT and extracellular signal-regulated kinase (ERK)1/2 proteins. On the contrary, no modulation of these pathways by bcl-2 was observed under normoxic conditions. When HIF-1 α expression was reduced by RNA interference, AKT and ERK1/2 phosphorylation were still induced by bcl-2. Pharmacological inhibition of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways reduced the induction of VEGF and HIF-1 in response to bcl-2 overexpression in hypoxia. No differences were observed between control and bcl-2-overexpressing cells in normoxia, in terms of VEGF protein secretion and in response to PI3K and MAPK inhibitors. We also demonstrated that RNA interference-mediated down-regulation of bcl-2 expression resulted in a decrease in the ERK1/2 phosphorylation and VEGF secretion only in bcl-2-overexpressing cell exposed to hypoxia but not in control cells. In conclusion, our results indicate, for the first time, that bcl-2 synergizes with hypoxia to promote expression of angiogenesis factors in melanoma cells through both PI3K- and MAPK-dependent pathways.

INTRODUCTION

Angiogenesis is a complex and multilevel process of new capillary formation on the basis of already existing blood vessels. Physiologically, it is a very strictly regulated process, which results in a balance between stimulatory (angiogenic) and inhibitory (angiostatic) factors to control the correct development of blood vessels. Angiogenesis is a rate-limiting step in tumor growth and progression, and many tumors develop a severely hypoxic microenvironment and secrete angiogenesis-stimulating factors (Folkman *et al.*, 1971; Talks *et al.*, 2000). Vascular endothelial growth factor (VEGF), a potent and specific mitogen for vascular endothelial cells, is a critical mediator of angiogenesis. Its expression is induced in cancer cells as a result of hypoxia and multiple genetic alterations, including p53 and PTEN loss-of-function, RAS and SRC gain-of-function, and autocrine tyrosine kinase signaling pathways (Mukhopadhyay *et al.*, 1995; Arbiser *et al.*, 1997; Petit *et al.*, 1997; Akagi *et al.*, 1998; Ellis *et al.*, 1998; Yen *et al.*, 2000). Based on the analysis of melanoma

and breast cancer cells recently reported by our group (Biroccio *et al.*, 2000; Iervolino *et al.*, 2002; Del Bufalo *et al.*, 2003; Trisciuglio *et al.*, 2004), this list can now be extended to include the increased VEGF expression resulting from bcl-2 overexpression. Hypoxia- and growth factor-induced VEGF expression in tumor cells is regulated by the hypoxia inducible factor 1 (HIF-1) (Jiang *et al.*, 1997; Mazure *et al.*, 1997; Ravi *et al.*, 2000; Zhong *et al.*, 2000; Zundel *et al.*, 2000; Laughner *et al.*, 2001; Fukuda *et al.*, 2002), a transcription factor that is composed of an hypoxia-inducible-HIF-1 α subunit and a constitutive expressed HIF-1 β subunit (Wang and Semenza, 1995; Semenza, 2002a). The unique feature of HIF-1 is the regulation of HIF-1 α expression and activity based upon the cellular O₂ concentration. HIF-1 α expression and activity also are regulated by major signal transduction pathways, including those involving phosphatidylinositol 3-kinase (PI3K) and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) (Berra *et al.*, 2000; Semenza, 2002b). Stimulation of cells with a variety of growth factors and cytokines induces HIF-1 target gene expression under nonhypoxic conditions through activation of a variety of signal transduction pathways, including PI3K and the downstream serine-threonine protein kinase AKT (protein kinase B) and FKBP/rapamycin-associated protein (mTOR). Receptor tyrosine kinase activity also leads to signaling via the ERK and p38 MAP kinase pathways. PI3K and AKT also have been implicated in the induction of HIF-1 expression by hypoxia (Berra *et al.*, 2000; Semenza, 2002b).

We previously demonstrated that bcl-2 overexpression in human melanoma cells exposed to hypoxic conditions increases VEGF promoter activity and mRNA stability and enhances HIF-1 DNA binding activity (Iervolino *et al.*, 2002).

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E04-12-1087>) on June 29, 2005.

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Abbreviations used: ERK1/2, extracellular signal-regulated kinase 1/2; HIF-1, hypoxia-inducible factor-1; HRE, hypoxic responsive element; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; VEGF, vascular endothelial growth factor; Wort, wortmannin.

However, the steps that participate in VEGF expression mediated by HIF-1 activity in response to bcl-2 overexpression in human melanoma cells exposed to hypoxia are still unknown. We have demonstrated that the MAPK pathway is implicated in signal transduction by bcl-2 (Trisciuglio *et al.*, 2004). In particular, our studies showed that ERK1/2 is activated in bcl-2-overexpressing cancer cells under hypoxic conditions (Trisciuglio *et al.*, 2004). However, to which extent this route may participate in VEGF accumulation by bcl-2 in human melanoma cells is still unsolved. Because VEGF up-regulation via both PI3K and MAPK pathways has been demonstrated (Berra *et al.*, 2000; Semenza, 2002b) and the ability of MAPK to regulate HIF-1 α may be stimulus- and/or cell type specific (Rak *et al.*, 2000), we investigated the role of PI3K and MAPK pathways on bcl-2 induced VEGF/HIF-1 up-regulation in melanoma cells exposed to hypoxia. In this article, we provide evidence that the activity of both signal transduction pathways is required for the HIF-1-mediated induction of VEGF expression by bcl-2.

MATERIALS AND METHODS

Cell Cultures, Hypoxic Exposure, and Inhibitor Treatments

M14 human melanoma parental cells, MN8 control and bcl-2-overexpressing (MB5 and MB6) clones, previously obtained after transfection (Iervolino *et al.*, 2002), were used for all the experiments. The cells were cultured in a humidified atmosphere with 95% air/5% CO₂ (normoxia) or in specially designed aluminium chambers flushed with a gas mixture containing 5% CO₂ and 95% N₂ (hypoxia). Under hypoxic conditions (24 h), oxygen concentration remains at ~1% throughout the incubation period. In some experiments, UO126 (5 and 10 μ M; Promega, Madison, WI) and PD98059 (25 and 50 μ M; Promega), which are selective pharmacological inhibitors of ERK, and wortmannin (Wort) (35 and 50 nM; Calbiochem, San Diego, CA) and LY29402 (20 and 40 μ M; Calbiochem), which are selective pharmacological inhibitors of PI3K, were added before exposure to normoxia or hypoxia. At the end of treatment, the cells were harvested, counted, and used for protein preparation and/or total RNA extraction.

RNA Interference (RNAi) Experiments

For silencing of bcl-2, cells were transfected with different amount (2–6 μ g) of the RNAi expression vector against bcl-2 (IMG-819; Imgenex, San Diego, CA) or with negative control vectors (IMG-700; Imgenex) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were exposed in serum-free medium under hypoxic conditions for 24 h.

For silencing of HIF-1 α , two oligonucleotides consisting of ribonucleosides except for the presence of 2'-deoxyribonucleosides (dTdT) at the 3' end, 5'-AGAGGUGGAUAGUGUGGGdTdT-3' and 5'-CCCACAUUAUCCAC-CUCUdTdT-3', were synthesized and annealed (Dharmacon Research, Lafayette, CO). The cells were exposed to 100 nM small interference RNA (siRNA) in the presence of Lipofectamine 2000 (Invitrogen) for 48 h and then exposed in serum-free medium under hypoxic conditions for 24 h. Control experiments were performed using siRNA directed against unrelated mRNA.

Analysis of Apoptosis

To measure the percentage of apoptotic cells, 1×10^6 adherent cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V using the Vybrant apoptosis kit (Molecular Probes, Eugene, OR) according to manufacturer's instructions and analyzed by flow cytometry, while simultaneously assessing membrane integrity by propidium iodide exclusion.

VEGF and Interleukin (IL)-8 Analysis

Enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) was used to determine the amount of VEGF and IL-8 proteins in the conditioned medium. Northern blot analysis was performed using a 600-base pairs fragment of the plasmid specific for VEGF and the probe for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as described previously (Biroccio *et al.*, 2000). Densitometric analysis was performed after Northern blot analysis.

Protein Extracts

Total protein extracts of cell cultures were prepared as described previously (Iervolino *et al.*, 2002). Cytosolic extracts were harvested by lysing cells with buffer containing 10 mM HEPES, pH 7.5, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.7% Nonidet P-40, and inhibitors proteases and phosphatases for 10

min at 4°C and centrifuged at 10,000 rpm for 15 min, and then supernatants (cytosolic extract) were harvested. Pellets were further lysed with buffer containing 20 mM HEPES, pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, and supernatants (nuclear extract) were harvested after centrifugation at 12,000 rpm at 4°C for 10 min.

Western Blot Analysis

Nuclear, cytosolic, and total extracts (60 μ g) were fractionated by SDS-PAGE, transferred to a nitrocellulose filter, and subjected to immunoblot assays. For analysis of HIF-1 α and HIF-1 β , nuclear proteins were analyzed using monoclonal antibodies against HIF-1 α (H1 α 67; Novus Biologicals, Littleton, CO) or HIF-1 β (Transduction Laboratories, San Diego, CA) at 1:500 dilution. Antibodies (Cell Signaling Technology, Beverly, MA) specific for phosphorylated (Thr202/Tyr204) or total ERK1/2, phosphorylated (Ser473) or total AKT were used at 1:1000 dilution. Immunoreactive bands were visualized using horseradish peroxidase-coupled goat anti-rabbit immunoglobulin and the ECL detection system (Amersham Biosciences, Piscataway, NJ). To check the amount of proteins transferred to the nitrocellulose membranes, β -actin was used as control and detected by anti- β -actin polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution. Densitometric analysis was performed after Western blot analysis.

Promoter Activity

Promoter activity was evaluated using the VEGF 1511 fragment of the VEGF promoter construct, and the HIF-1-like wild-type (wt; 808-base pair) binding site polymers as reported previously (Iervolino *et al.*, 2002). Twenty-four hours after transfection with plasmids legated to luciferase, half of the cells were subjected to normoxia and the other half was kept under hypoxia in the presence or absence of UO126, PD98059, Wort, or LY29402. Samples were collected 24 h after the induction of hypoxia and analyzed for luciferase and β -galactosidase activity. Relative luciferase expression was determined as ratio of β -galactosidase activity. The mean of five independent experiments was calculated for each condition. To normalize for transfection efficiency, PEQ-176 plasmid (0.2 μ g) was included in the transfections.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed, as described previously (Iervolino *et al.*, 2002), after exposure to hypoxia for 24 h in the presence or absence of UO126, PD98059, Wort, or LY29402. The following double-strand oligomer as radiolabeled probes or cold competitor: HIF-1 (–985 to –951 of human VEGF 5' gene promoter), 5'TCGACCACAGTGCATACGTGGGCTCCACAGGTCTCTTC-3'.

Statistical Analysis

The statistical significance of the differences between M14 cells, MN8 control clone, and bcl-2 transfectants was determined by the Student's *t* test for unpaired data (two sided).

RESULTS

Effect of bcl-2 Overexpression on AKT and ERK1/2 Phosphorylation and Cellular Distribution

We previously demonstrated that ERK kinase phosphorylation is induced in bcl-2-overexpressing melanoma clones exposed to hypoxic conditions for 24 h (Trisciuglio *et al.*, 2004). Because phosphorylation of cytosolic ERK1/2 leads to their activation and translocation within the nucleus (Brunet *et al.*, 1999; Volmat *et al.*, 2001), we first confirmed ERK activation by Western blot. Using an anti-phospho-ERK antibody that recognizes the active forms of ERK1/2, an increase in both ERK1 and ERK2 phosphorylation, of about two- to threefold, was observed in total extracts from MB5- and MB6 bcl-2-overexpressing cells exposed to hypoxia for 24 h compared with the M14 parental line and MN8 control clone exposed at the same experimental conditions (Figure 1A). As evidenced in Figure 1B, bcl-2 transfectants showed an increased amount (about fivefold) of nuclear phosphorylated ERK1/2 compared with control cells: whereas ERK1/2 were slightly detectable in nuclei of control cells, they were very evident in nuclei of bcl-2-overexpressing clones. The difference between control and bcl-2-overexpressing cells, even though to a lesser extent (about a threefold increase), was also evident when comparing the amount of cytoplasmatic phosphorylated ERK1/2. These results

Figure 1. Effect of bcl-2 overexpression on AKT and ERK1/2 phosphorylation and cellular distribution under normoxic and hypoxic conditions. Western Blot analysis of bcl-2 and ERK1/2 expression and phosphorylation in total extracts (A), ERK1/2 expression and phosphorylation in nuclear and cytoplasmatic protein extracts (B), and AKT expression and phosphorylation in total extracts (C). Western Blot analyses were performed 24 h after exposure to hypoxia. (D) ERK1/2 and AKT expression and phosphorylation in total extracts of cells exposed to normoxia for 24 h. β -actin expression served as controls for uniformity of protein gel loading and blotting. Representative experiment out of two is shown.

were confirmed by immunofluorescence experiments that showed an elevated phospho-ERK nuclear translocation in bcl-2 transfectants compared with the control cells (our unpublished data).

Because PI3K and AKT have also been implicated in the induction of angiogenesis in response to hypoxia (Richard *et al.*, 1999; Berra *et al.*, 2000; Semenza, 2002b; Arsham *et al.*, 2004), we evaluated AKT phosphorylation in parental cells and bcl-2 transfectants exposed to hypoxia for 24 h. Using an anti-phospho-AKT (Ser-473) antibody that recognizes the active form of AKT (Figure 1C), an increase in AKT phosphorylation of about five- to sixfold was observed in bcl-2 transfectants compared with the parental line or control clone. Using a polyclonal antibody raised against total AKT, no modification in the level of AKT kinase expression was detected, indicating that the increase in AKT phosphorylation was not due to an increase in total AKT protein. ERK1/2 and AKT phosphorylation under hypoxic conditions also were observed after transient transfection of M14 cells with a bcl-2 expression vector (our unpublished data). Thus, indicating that the effects seen were a direct consequence of bcl-2 overexpression and did not represent a chronic adaptation to higher levels of bcl-2.

Experiments also were performed to evaluate the effect of bcl-2 overexpression on ERK1/2 and AKT phosphorylation

under normoxic conditions. As reported in Figure 1D, a similar ERK1/2 and AKT phosphorylation was observed between the parental, control, and bcl-2-overexpressing cells.

Effect of PI3K and MAPK Inhibitors on the VEGF Protein, mRNA, and Promoter Activity

To determine the signal transduction pathways mediating the effects of bcl-2 on VEGF protein expression, M14 parental cells, MN8 control clone and two bcl-2 transfectants (MB5 and MB6) were pretreated with PI3K (Wort and LY29402) and ERK (UO126 and PD98059) inhibitors, and the level of VEGF protein secretion was determined by ELISA assay after exposure to normoxic or hypoxic conditions for 24 h.

As demonstrated previously (Iervolino *et al.*, 2002) and reported in Figure 2A, no differences were observed between control cells and bcl-2 transfectants, exposed to normoxic conditions, in terms of VEGF protein secretion: the VEGF protein ranged from 1656 ± 284 to 1880 ± 335 pg/10⁶ cells/24 h for the different lines ($p > 0.05$). VEGF secretion was decreased by PI3K (~50%) and ERK1/2 (~70%) inhibitors to the same extent in both parental and bcl-2-overexpressing cells when exposed to normoxia.

As expected (Iervolino *et al.*, 2002) bcl-2-overexpressing cells exposed to hypoxia for 24 h secrete higher level of

Figure 2. Effect of PI3K and ERK inhibitors on the VEGF protein expression. Expression of VEGF was evaluated by ELISA in conditioned medium of M14 parental line (white columns), MN8 control clone (black columns), and bcl-2 transfectants (dark and light gray columns) grown under normoxic (A) or hypoxic (B) conditions for 24 h in absence or in presence of wortmannin (50 nM) or UO126 (10 μM). Two representative experiments out of six with standard deviations are reported. * Indicates that values are significantly different from the corresponding value of control (* $p < 0.05$, ** $p < 0.001$).

VEGF protein than the parental cells (Figure 2B). Although a decrease of ~20 and 40% of VEGF secretion was observed after treatment of the parental cells with Wort (50 nM; $p < 0.05$) and UO126 (10 μM; $p < 0.05$) inhibitors, respectively, VEGF secretion in bcl-2 transfectants was reduced by 40% ($p < 0.001$) and 60% ($p < 0.001$) after treatment with Wort (50 nM) and UO126 (10 μM) inhibitors, respectively. Similar results were obtained using LY29402 and PD98059 that inhibit PI3K and MAPK, respectively. After treatment with the 35 nM dose of Wort, a significant VEGF reduction was only observed in bcl-2 transfectants ($p < 0.001$) but not in the parental cells ($p > 0.05$) (our unpublished data).

We further investigated the effect of PI3K and MAPK inhibitors on VEGF mRNA-induction by bcl-2/hypoxia in MB5 and MB6 bcl-2-overexpressing clones (Figure 3A). As expected (Iervolino *et al.*, 2002), 24-h hypoxia induced about a three- to fourfold increase in mRNA expression in MB5 and MB6 clones compared with normoxic conditions. The two inhibitors reduced the induction of VEGF mRNA expression by bcl-2 in both bcl-2 transfectants. In particular, under these conditions the accumulation of VEGF mRNA,

after GAPDH normalization, was reduced by 30–40% in Wort-treated cells and by 50–70% in UO126-treated cells (Figure 3A).

Because we previously demonstrated the ability of bcl-2 to increase VEGF promoter activity in hypoxia (Iervolino *et al.*, 2002), we also evaluated whether PI3K and MAPK inhibitors were able to affect the increase of VEGF promoter expression induced by bcl-2/hypoxia (Figure 3B). Transient transfections were performed using the VEGF promoter fused to the luciferase-reporter gene (Iervolino *et al.*, 2002), and the M14 parental, MN8 control clone, and bcl-2-overexpressing cells were exposed to hypoxia for 24 h with or without UO126 or Wort pretreatment. As expected (Iervolino *et al.*, 2002), under 24 h of hypoxic conditions, an increase in promoter activity of about twofold was observed in both bcl-2-overexpressing clones compared with control cells (Figure 3B). Although a significant reduction of the VEGF promoter activity was observed in the parental cells treated with the highest dose of Wort (50 nM), preexposure to UO126 at both the doses (5 and 10 μM) used, significantly ($p < 0.001$) reduced the promoter activity. Pretreatment of bcl-2 transfectants with Wort or UO126 significantly ($p < 0.001$) inhibited bcl-2/hypoxia induced VEGF promoter expression in a dose-dependent manner, but the inhibitory effect was more evident when using UO126 (~80%) than Wort (~50%). Results obtained with PD98058 or LY29402 were similar to those obtained when using UO126 and Wort, respectively (our unpublished data).

Effect of bcl-2 RNAi on bcl-2 Expression, Apoptosis, and ERK1/2 Phosphorylation

To confirm the results obtained through up-regulation of bcl-2, we specifically silenced the bcl-2 gene. For this purpose, the MN8 control clone and bcl-2-overexpressing cells were transfected with RNAi-targeting bcl-2 mRNA (bcl-2 RNAi) and then exposed to hypoxia for 24 h. As a control for specificity of RNAi, cells were transfected with a scrambled RNAi vector. Western blot analysis demonstrated that bcl-2 RNAi severely suppressed expression of bcl-2 in a dose-dependent manner compared with untransfected or control transfected cells (Figure 4A). A decrease in bcl-2 expression had already been observed after transfection with the lowest concentration (2 μg) of bcl-2 RNAi both in parental (about a twofold decrease) and bcl-2-overexpressing (about a sixfold decrease) cells, whereas the bcl-2 protein was not detectable after transfection with the highest concentration (6 μg). No effects of RNAi were observed on the expression of β-actin, which was used as an internal control for specificity and loading. Cytofluorimetric analysis for detection of apoptosis revealed the presence of apoptotic cells in the control cells (~30%) and bcl-2 overexpressing clones (~25%) only when transfected with the highest concentration (6 μg) of bcl-2 RNAi (Figure 4B). Then, we evaluated the impact of reduced bcl-2 expression on ERK1/2 phosphorylation (Figure 4C). Bcl-2 RNAi induced a dose-dependent decrease in ERK1/2 phosphorylation in bcl-2 overexpressing cells compared with untreated cells: about a 10-fold decrease in ERK1/2 phosphorylation was already evident after transfection with the lowest concentration (2 μg) of bcl-2 RNAi. On the contrary, no modulation of ERK1/2 phosphorylation was evidenced in the MN8 control clone after down-regulation of the bcl-2 protein (Figure 4C).

As mentioned above and previously described, bcl-2 is able to increase VEGF expression and transcriptional activity (Iervolino *et al.*, 2002). To exclude the possibility that the decrease in cell survival induced by bcl-2 RNAi (Figure 4) affects VEGF expression, conditions were chosen under

Figure 3. Effect of PI3K and ERK inhibitors on VEGF mRNA expression and promoter activity. VEGF mRNA expression by Northern blot analysis (A) and VEGF transcriptional activity (B) were performed in M14 parental line (white columns), MN8 control clone (black columns), and two bcl-2 transfectants (dark and light gray columns) grown under normoxic or hypoxic conditions for 24 h in the absence or presence of Wort or UO126. (A) Fold increase relative to normoxia was calculated by densitometric analysis. A representative experiment out of two is shown. (B) Cells were transfected with a reporter plasmid (VEGF 1511 base pairs fragment of the VEGF promoter) and exposed to hypoxia before assaying for luciferase activity. Luciferase values were normalized for transfection efficiency (luciferase/ β galactosidase ratios). A representative experiment out of six with standard deviations is reported. * Indicates that values are significantly different from the corresponding value of control (* p < 0.05, ** p < 0.001).

which bcl-2 RNAi did not activate the apoptotic program in the cells. Thus, after transfection with bcl-2 RNAi, the cells were exposed to hypoxia for 24 h, and the VEGF secretion was evaluated. No modulation of VEGF expression was evidenced in the M14 parental cells or MN8 control clone after down-regulation of bcl-2: similar VEGF protein levels ($\sim 3125 \pm 180$ pg/10⁶ cells/24 h) were produced by control and parental cells treated or untreated with bcl-2 RNAi. On the contrary, about a 50 and 70% decrease in VEGF secretion was observed in bcl-2-overexpressing clones after transfection with bcl-2 RNAi at 2 and 4 μ g concentrations, respectively (Figure 4D). No effects of bcl-2 RNAi were observed on the secretion of IL-8, which was never found to be modulated by bcl-2 overexpression in our experimental model (our unpublished data).

Effect of PI3K and MAPK Inhibitors on HIF-1 α Protein Expression and DNA Binding Activity

We previously demonstrated that Bcl-2 overexpression in human melanoma cells increases HIF-1 α expression and DNA binding activity (Iervolino *et al.*, 2002). To examine the contribution of the MAPK and PI3K signaling pathways to bcl-2/hypoxia-induced HIF-1 α protein expression, we exposed the cells to 24 h hypoxia after treatment with UO126 and Wort. As shown in Figure 5A, the levels of HIF-1 α protein was increased by about threefold in bcl-2-overexpressing clones exposed to hypoxia compared with parental cells at the same experimental conditions. The expression of HIF-1 α protein was not detected in bcl-2 transfectants pre-

treated with UO126 and was almost absent in cells pre-treated with Wort (Figure 5A). No changes in HIF-1 β were observed after bcl-2 overexpression and treatment with inhibitors.

To analyze the contribution of the MAPK and PI3K pathways in HIF-1 DNA binding activity, we performed EMSA assays in the absence or presence of PI3K and MAPK inhibitors (Figure 5B). Treatment with either UO126 or Wort strongly reduced the DNA binding activity of bcl-2 transfectants, clearly suggesting that HIF-1 DNA binding activity was MAPK and PI3K dependent. On the contrary, no significant reduction of DNA binding activity was observed in the parental cells exposed to either inhibitor.

Because we previously found the ability of bcl-2 to increase hypoxic responsive element (HRE) promoter activity in hypoxia (Iervolino *et al.*, 2002), we evaluated the role of the PI3K and MAPK signaling pathways in the transcriptional activation of HIF-1 by bcl-2/hypoxia. Parental and bcl-2-overexpressing cells were transiently transfected with a pHRE-Luc reporter plasmid, which contains four concatamerized HIF-1 binding sites, and then exposed to normoxia or hypoxia for 24 h in the presence or absence of PI3K and MAPK inhibitors. As shown in Figure 5C, HRE promoter activity in bcl-2 transfectants under hypoxic conditions was about twofold higher than in M14 parental cells exposed to the same experimental conditions. Although a decrease of $\sim 25\%$ in HRE promoter activity was observed in parental cells treated with UO126, no inhibition was revealed after treatment with Wort. On the contrary, the in-

Figure 4. Effect of bcl-2 RNAi on the bcl-2 expression, induction of apoptosis, ERK1/2 phosphorylation, and VEGF protein levels. (A) Western blot analysis of bcl-2 protein in MN8 control clone and MB5 bcl-2-overexpressing clone transfected with different amount (2–6 μ g) of the RNAi expression vector against bcl-2 (bcl-2 RNAi) or with negative control vector (6 μ g) and then exposed for 24 h to hypoxia. β -actin was used as a control for equal protein loading. A representative experiment out of two is shown. (B) Detection of apoptosis by annexin V/propidium iodide (PI) staining in MN8 control clone and bcl-2-overexpressing clones (MB5 and MB6) transfected with bcl-2 RNAi or with negative control vector and then exposed for 24 h to hypoxia. The percentage of apoptotic cells (annexin V+/PI-) is reported. (C) Western Blot analysis of ERK1/2 expression and phosphorylation in total extracts of MN8 control cells and MB5 bcl-2-overexpressing clone transfected with the bcl-2 RNAi expression vector or with negative control vector and then exposed for 24 h to hypoxia. (D) Expression of VEGF protein evaluated by ELISA in conditioned medium of M14 parental line (white columns), MN8 control clone (black columns), and bcl-2-overexpressing cells (dark and light gray columns) transfected with the bcl-2 RNAi or with negative control vector and then grown under hypoxic conditions for 24 h. * Indicates that values are significantly different from the corresponding value of control (* $p < 0.01$).

crease in HIF-1-dependent reporter gene transcription observed in bcl-2-overexpressing clones exposed to hypoxia was significantly reduced by cellular pretreatment with either Wort (~30% reduction) or UO126 (~70% reduction). Results obtained with PD98059 or LY29402 were similar to those obtained when using UO126 and Wort, respectively (our unpublished data). Results obtained with the MN8 control clone were superimposable to those obtained when using M14 (our unpublished data).

To evaluate whether MAPK and PI3K pathways were still induced in hypoxia by bcl-2 in the absence of HIF-1 α activity, siRNA was used to reduce the expression of HIF-1 α transcription factor. MN8 control and bcl-2-overexpressing cells were transfected with siRNA-targeting HIF-1 α mRNA (HIF-1 α siRNA) and then exposed to hypoxia for 24 h. As a control for specificity of siRNA, cells were transfected with siRNA directed against unrelated control mRNA. Western blot analysis demonstrated that HIF-1 α siRNA severely suppressed expression of HIF-1 α in both the MN8 control cells

and bcl-2 transfectants compared with control transfected cells (Figure 5D). As expected, no effects of HIF-1 α siRNA were observed on the expression of HIF-1 β , and VEGF expression was reduced both in parental and bcl-2 overexpressing cells (our unpublished data). Thus, we also evaluated the impact of reduced HIF-1 α expression on ERK1/2 and AKT phosphorylation. No modulation of ERK1/2 and AKT phosphorylation was observed in parental or bcl-2-overexpressing cells after HIF-1 α silencing.

DISCUSSION

Several studies have demonstrated that in addition to mediating antiapoptotic (Cory *et al.*, 2003) and proliferative signals (Quinn and Richardson, 2004), bcl-2 also promotes tumor angiogenesis (Iervolino *et al.*, 2002; Trisciuglio *et al.*, 2004). Moreover, Bcl-2 regulates gene expression and modulates the transactivity of several transcription factors (Feng

Figure 5. Effect of bcl-2 overexpression on HIF-1 α protein expression, DNA binding activity and transcriptional activity under hypoxic conditions. Western blot analysis of HIF-1 α and HIF-1 β protein expressions (A), EMSA (B) and HRE transcriptional activity (C) were performed in the nuclear extracts of M14 parental line and two bcl-2 transfectants (MB5, MB6) grown under hypoxic conditions for 24 h in the presence or absence of UO126 (5 μ M) or Wort (35 nM). (B) EMSA was carried out using the double-strand oligomer as radiolabeled probes or cold competitor (cold). Supershift assay (SS) of HIF-1 DNA-binding complexes in MB6 cells was performed by addition of anti-HIF-1 antibody (antibody) to the reaction mixture. Representative experiment out of two is shown. (C) M14 parental line (white columns), MN8 control clone (black column), and two bcl-2 transfectants (dark and light gray columns) were transfected with a pHRE-Luc reporter plasmid. After 24 h, the cells were exposed to normoxia, hypoxia, or hypoxia in presence of UO126 or wortmannin. Luciferase values were normalized for transfection efficiency (luciferase/ β galactosidase ratios). Results are means of five independent experiments. * Indicates that values are significantly different from the corresponding value of control (* p < 0.05, ** p < 0.01). (D) Analysis of HIF-1 α , HIF-1 β protein expression and ERK1/2 and AKT expression and phosphorylation in total extracts obtained from MN8 control line and bcl-2 transfectants (MB5, MB6), after transfection with small interference RNA directed against HIF-1 α (siRNA) or with small interference RNA directed against unrelated control mRNA. β -actin expression served as controls for uniformity of protein gel loading and blotting. Representative experiments out of two is shown.

et al., 1999; Ricca *et al.*, 2000; Schwarz *et al.*, 2002; Cory *et al.*, 2003; Quinn and Richardson, 2004).

Our previous results, demonstrating that forced overexpression of bcl-2 in melanoma cells exposed to hypoxia enhances HIF-1-mediated-VEGF expression (Iervolino *et al.*, 2002) and induces ERK1/2 activation (Trisciunglio *et al.*, 2004), persuaded our group to investigate the participation of MAPK and PI3K signaling pathways in bcl-2-mediated

VEGF/HIF-1 expression and activity. Even though the role of MAPK and PI3K pathways in the process of HIF-1 α /VEGF activation is still controversial (Alvarez-Tejado *et al.*, 2002; Stiehl *et al.*, 2002), several studies evidenced that these two specific intracellular signaling pathways are actively involved in angiogenesis mediated by VEGF (Berra *et al.*, 2000; Zhong *et al.*, 2000; Semenza, 2002; Sang *et al.*, 2003). Recognizing which signal transduction pathway is active in

a tumor-overexpressing bcl-2 may have prognostic information and also may identify a potential target for antiangiogenic therapy.

Our results demonstrated that the effect of bcl-2 overexpression on VEGF expression, ERK and AKT phosphorylation, and on the response to PI3K and MAPK inhibitors was strictly dependent on the oxygen concentration. In fact, all of these parameters were similar in the control and bcl-2-overexpressing cells exposed to normoxic conditions, and as reported for other tumor histotypes (Chelouche-Lev *et al.*, 2004), both PI3K and MAPK pathways were essential for the constitutive expression of VEGF in normoxia. On the contrary, under hypoxic conditions increased VEGF secretion, AKT and ERK phosphorylation were evidenced in both transiently and stably bcl-2-overexpressing cells. A strong involvement of PI3K and MAPK in the induction of HIF-1-mediated VEGF expression by bcl-2 also was evidenced. Moreover, when HIF-1 α expression was reduced by siRNA, AKT and ERK1/2 phosphorylation were still induced by bcl-2, indicating that the role of bcl-2 as an enhancer in PI3K and MAPK pathways under hypoxic conditions is not related to HIF-1 induction.

The cooperation of bcl-2 overexpression with hypoxia on VEGF and HIF-1 expression confirmed in the present study, corroborates the importance of two parallel pathways for induction of HIF-1 in human cancer, one based on physiological stimulation and the other on genetic alterations.

Confirming the involvement of the MAPK pathway in bcl-2 overexpression in hypoxia (Trisciuglio *et al.*, 2004), the present study shows that bcl-2 and hypoxia cooperate to promote ERK phosphorylation and translocation to the nucleus. The evidence that the phosphorylation of ERK1/2 regulatory sites can drive their translocation to the nucleus where ERK exerts part of its biological activity (Brunet *et al.*, 1999) and that ERK1/2 phosphorylate HIF-1 α and enhance HIF-1 transcriptional activity (Feng *et al.*, 2004) indicates a possible mechanism by which bcl-2 increases HIF-1-mediated VEGF induction in our experimental models. Furthermore, the previously demonstrated effect of MAPK signaling on HIF activation through p300/CBP can be postulated as a possible mechanism by which bcl-2/hypoxia induces VEGF expression (Sang *et al.*, 2003). Moreover, the fact that RNAi-mediated down-regulation of bcl-2 expression resulted in a decrease in the ERK1/2 phosphorylation and VEGF secretion in bcl-2 transfectants exposed to hypoxia, but not in the control cells under the same experimental conditions, confirms the importance of bcl-2 on angiogenesis in conditions of low oxygen concentration. From a clinical perspective, these findings are relevant only for melanoma expressing high bcl-2 protein levels.

As an additional parameter to confirm the involvement of PI3K and MAPK on VEGF/HIF-1 expression, we have shown that the inhibition of MAPK and PI3K pathways successfully reduced bcl-2 induction of these molecules under hypoxic conditions. In particular, using selective pharmacologic inhibitors of PI3K (Wort or LY29402) and MAPK (UO126 or PD98059), we have found that the induction of VEGF and HIF-1 α in bcl-2-overexpressing cells is dependent upon activity of both the MAPK and PI3K pathways. In particular, VEGF protein and mRNA expression and VEGF promoter activity induced after bcl-2 transfection in hypoxia were reduced in a dose-dependent manner by inhibition of either MAPK or PI3K pathways. The induction of the HIF-1 α protein, DNA binding, and transcriptional activity also were partially or completely blocked by PI3K and MAPK pathways. These data are in agreement with results demonstrating PI3K and MAPK signaling-mediated HIF-1 α and VEGF

induction in several experimental models and in response to different stimuli (Fukuda *et al.*, 2002; Semenza, 2002b).

Together, our results point to the important role of the MAPK and PI3K signaling pathways in the modulation of the bcl-2 effect on VEGF/HIF-1 expression and indicate that at least two different signal transduction pathways are activated by bcl-2 overexpression in melanoma cells exposed to hypoxic conditions. The inhibitory effect of UO126 was more evident than the inhibitory effect of Wort for all the experiments performed and at the concentrations used. Although the inhibition of MAPK pathway seems to affect VEGF/HIF-1 expression more than the inhibition of PI3K, it should be considered that the effect of PI3K inhibition is more specific for the bcl-2-overexpressing clones than for parental cells. Even though it is difficult to ascertain which pathway plays a more important role in our model system, the involvement of PI3K and MAPK in the induction of HIF-1-mediated VEGF expression by bcl-2 in hypoxia, represent two important signaling pathways. These data are in agreement with those demonstrating the importance of both the PI3K and MAPK pathways on HIF-1 α and VEGF expression (Jiang *et al.*, 2000; Sang *et al.*, 2003).

The signal transduction pathway involving PI3K, AKT, and FRAP has been shown to regulate the translation of several proteins, including VEGF (Graff and Zimmer, 2003), via phosphorylation of eukaryotic initiation factor 4E-binding protein 1 and p70 ribosomal protein S6 kinase (Gingras *et al.*, 1999; Peterson *et al.*, 1999; Fukuda *et al.*, 2002), and ERK also has been shown to phosphorylate the eukaryotic initiation factor 4E-binding protein 1 (Haystead *et al.*, 1994; Fukuda *et al.*, 2002). Thus, it is possible that PI3K and MAPK pathways are responsible for the increased VEGF and HIF-1 protein expression induced by bcl-2/hypoxia in our experimental model through this mechanism.

Regarding the possible mechanism by which bcl-2 modulates transcription factors and gene expression in our experimental model, it is possible that bcl-2 activates ERK signaling through the RAS/RAF/MEK pathway. Recently, upstream activators of ERK1/2, such as RAS and RAF, have been demonstrated to play a role in bcl-2-induced gene expression in nontumoral cells such as rabbit lens epithelial cells (Feng *et al.*, 2004), and bcl-2 overexpression in PC12 pheochromocytoma cells has been found to affect cellular signaling pathway via an up-regulation of Ras (Schwarz *et al.*, 2002).

Because we previously demonstrated the ability of bcl-2 to modulate Sp1 DNA binding activity through the ERK signaling pathway, we do not exclude a possible involvement of Sp1 in VEGF modulation by bcl-2. In fact, data from other groups evidenced that Sp1 is required for PI3K-mediated induction of VEGF (Pore *et al.*, 2004) and that phosphorylation of Sp1 by ERK1/2 MAPK is a crucial event for the regulation of VEGF (Berra *et al.*, 2000; Milanini-Mongiati *et al.*, 2002). We are currently investigating the involvement of Sp1 and the RAS/RAF pathway on bcl-2-induced HIF-1/VEGF expression.

ACKNOWLEDGMENTS

D. T. and A. I. are the recipient of a fellowship from the Italian Foundation for Cancer Research. We are grateful to Dr. Adele Petricca for secretarial assistance in preparation of the manuscript, to Paula Franke for revising the English language, and to Drs. Maurizio Fanciulli and Oreste Segatto for critical reading of the manuscript. This study was supported by Ministero della Sanità (D.D.B.) and Italian Association for Cancer Research (D.D.B.).

REFERENCES

- Akagi, Y., Liu, W., Zebrowski, B., Xie, K., and Ellis, L. M. (1998). Regulation of vascular endothelial growth factor expression in human colon cancer by insulin-like growth factor-I. *Cancer Res.* 58, 4008–4014.
- Alvarez-Tejado, M., Alfranca, A., Aragones, J., Vara, A., Landazuri, M. O., and del Peso, L. (2002). Lack of evidence for the involvement of the phosphoinositide 3-kinase/Akt pathway in the activation of hypoxia-inducible factors by low oxygen tension. *J. Biol. Chem.* 277, 13508–13517.
- Arbiser, J. L., et al. (1997). Oncogenic H-ras stimulates tumor angiogenesis by two distinct pathways. *Proc. Natl. Acad. Sci. USA* 94, 861–866.
- Arsham, A. M., Plas, D. R., Thompson, C. B., and Simon, M. C. (2004). Akt and hypoxia-inducible factor-1 independently enhance tumor growth and angiogenesis. *Cancer Res.* 64, 3500–3507.
- Berra, E., Milanini, J., Richard, D. E., Le Gall, M., Vinals, F., Gothie, E., Roux, D., Pages, G., and Pouyssegur, J. (2000). Signaling angiogenesis via p42/p44 MAP kinase and hypoxia. *Biochem. Pharmacol.* 60, 1171–1178.
- Biroccio, A., Candiloro, A., Mottolise, M., Sapor, O., Albini, A., Zupi, G., and Del Bufalo, D. (2000). Bcl-2 overexpression and hypoxia synergistically act to modulate vascular endothelial growth factor expression and in vivo angiogenesis in a breast carcinoma line. *FASEB J.* 14, 652–660.
- Brunet, A., Roux, D., Lenormand, P., Dowd, S., Keyse, S., and Pouyssegur, J. (1999). Nuclear translocation of p42/p44 mitogen-activated protein kinase is required for growth factor-induced gene expression and cell cycle entry. *EMBO J.* 18, 664–674.
- Chelouche-Lev, D., Miller, C. P., Tellez, C., Ruiz, M., Bar-Eli, M., and Price, J. E. (2004). Different signalling pathways regulate VEGF and IL-8 expression in breast cancer: implications for therapy. *Eur. J. Cancer* 40, 2509–2518.
- Cory, S., Huang, D. C., and Adams, J. M. (2003). The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene* 22, 8590–8607.
- Del Bufalo, D., Trisciuglio, D., Scarsella, M., Zangemeister-Wittke, U., and Zupi, G. (2003). Treatment of melanoma cells with a bcl-2/bcl-xL antisense oligonucleotide induces antiangiogenic activity. *Oncogene* 22, 8441–8447.
- Ellis, L. M., Staley, C. A., Liu, W., Fleming, R. Y., Parikh, N. U., Bucana, C. D., and Gallick, G. E. (1998). Down-regulation of vascular endothelial growth factor in a human colon carcinoma cell line transfected with an antisense expression vector specific for c-src. *J. Biol. Chem.* 273, 1052–1057.
- Feng, H., et al. (2004). Human Bcl-2 activates ERK signaling pathway to regulate activating protein-1, lens epithelium-derived growth factor and downstream genes. *Oncogene* 23, 7310–7321.
- Feng, L., Balakir, R., Precht, P., and Horton, W. E., Jr. (1999). Bcl-2 regulates chondrocyte morphology and aggrecan gene expression independent of caspase activation and full apoptosis. *J. Cell. Biochem.* 74, 576–586.
- Folkman, J., Merler, E., Abernathy, C., and Williams, G. (1971). Isolation of a tumor factor responsible for angiogenesis. *J. Exp. Med.* 133, 275–288.
- Fukuda, R., Hirota, K., Fan, F., Jung, Y. D., Ellis, L. M., and Semenza, G. L. (2002). Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. *J. Biol. Chem.* 277, 38205–38211.
- Gingras, A. C., Gygi, S. P., Raught, B., Polakiewicz, R. D., Abraham, R. T., Hoekstra, M. F., Aebersold, R., and Sonenberg, N. (1999). Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev.* 13, 1422–1437.
- Graff, J. R., and Zimmer, S. G. (2003). Translational control and metastatic progression: enhanced activity of the mRNA cap-binding protein eIF-4E selectively enhances translation of metastasis-related mRNAs. *Clin. Exp. Metastasis* 20, 265–273.
- Haystead, T. A., Haystead, C. M., Hu, C., Lin, T. A., and Lawrence, J. C., Jr. (1994). Phosphorylation of PHAS-I by mitogen-activated protein (MAP) kinase. Identification of a site phosphorylated by MAP kinase in vitro and in response to insulin in rat adipocytes. *J. Biol. Chem.* 269, 23185–23191.
- Iervolino, A., Trisciuglio, D., Ribatti, D., Candiloro, A., Biroccio, A., Zupi, G., and Del Bufalo, D. (2002). Bcl-2 overexpression in human melanoma cells increases angiogenesis through VEGF mRNA stabilization and HIF-1-mediated transcriptional activity. *FASEB J.* 16, 1453–1455.
- Jiang, B. H., Agani, F., Passaniti, A., and Semenza, G. L. (1997). V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1, involvement of HIF-1 in tumor progression. *Cancer Res.* 57, 5328–5335.
- Jiang, B. H., Zheng, J. Z., Aoki, M., and Vogt, P. K. (2000). Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc. Natl. Acad. Sci. USA* 97, 1749–1753.
- Laughner, E., Taghavi, P., Chiles, K., Mahon, P. C., and Semenza, G. L. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1 α (HIF-1 α) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol. Cell. Biol.* 21, 3995–4004.
- Mazure, N. M., Chen, E. Y., Laderoute, K. R., and Giaccia, A. J. (1997). Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. *Blood* 90, 3322–3331.
- Milanini-Mongiat, J., Pouyssegur, J., and Pages, G. (2002). Identification of two Sp1 phosphorylation sites for p42/p44 mitogen-activated protein kinases: their implication in vascular endothelial growth factor gene transcription. *J. Biol. Chem.* 277, 20631–20639.
- Mukhopadhyay, D., Tsiokas, L., and Sukhatme, V. P. (1995). Wild-type p53 and v-Src exert opposing influences on human vascular endothelial growth factor gene expression. *Cancer Res.* 55, 6161–6165.
- Peterson, R. T., Desai, B. N., Hardwick, J. S., and Schreiber, S. L. (1999). Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycin-associated protein. *Proc. Natl. Acad. Sci. USA* 96, 4438–4442.
- Petit, A. M., Rak, J., Hung, M. C., Rockwell, P., Goldstein, N., Fendly, B., and Kerbel, R. S. (1997). Neutralizing antibodies against epidermal growth factor (EGF) and ErbB-2/neu receptor tyrosine kinases down-regulate vascular endothelial growth factor production by tumor cells in vitro and in vivo: angiogenic implications for signal transduction therapy of solid tumors. *Am. J. Pathol.* 151, 1523–1530.
- Pore, N., et al. (2004). Sp1 is involved in Akt-mediated induction of VEGF expression through an HIF-1-independent mechanism. *Mol. Biol. Cell* 15, 4841–4853.
- Quinn, L. M., and Richardson, H. (2004). Bcl-2 in cell cycle regulation. *Cell Cycle* 3, 7–9.
- Rak, J., Mitsushashi, Y., Sheehan, C., Tamir, A., Vilorio-Petit, A., Filmus, J., Mansour, S. J., Ahn, N. G., and Kerbel, R. S. (2000). Oncogenes and tumor angiogenesis: differential modes of vascular endothelial growth factor up-regulation in ras-transformed epithelial cells and fibroblasts. *Cancer Res.* 60, 490–498.
- Ravi, R., Moorerjee, B., Bhujwalla, Z. M., Sutter, C. H., Artemov, D., Zeng, Q., Dillehay, L. E., Madan, A., Semenza, G. L., and Bedi, A. (2000). Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1 α . *Genes Dev.* 14, 34–44.
- Ricca, A., Biroccio, A., Del Bufalo, D., Mackay, A. R., Santoni, A., and Cippitelli, M. (2000). bcl-2 Over-expression enhances NF-kappaB activity and induces mmp-9 transcription in human MCF7(ADR) breast-cancer cells. *Int. J. Cancer* 86, 188–196.
- Richard, D. E., Berra, E., Gothie, E., Roux, D., and Pouyssegur, J. (1999). p42/p44 mitogen-activated protein kinases phosphorylate hypoxia-inducible factor 1 α (HIF-1 α) and enhance the transcriptional activity of HIF-1. *J. Biol. Chem.* 274, 32631–32637.
- Sang, N., Stiehl, D. P., Bohensky, J., Leshchinsky, I., Srinivas, V., and Caro, J. (2003). MAPK signaling up-regulates the activity of hypoxia-inducible factors by its effects on p300. *J. Biol. Chem.* 278, 14013–14019.
- Schwarz, C. S., Seyfried, J., Evert, B. O., Klockgether, T., and Wullner, U. (2002). Bcl-2 up-regulates ha-ras mRNA expression and induces c-Jun phosphorylation at Ser73 via an ERK-dependent pathway in PC 12 cells. *Neuroreport* 13, 2439–2442.
- Semenza, G. L. (2002a). HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol. Med.* 8, S62–S67.
- Semenza, G. L. (2002b). Signal transduction to hypoxia-inducible factor 1. *Biochem. Pharmacol.* 64, 993–998.
- Stiehl, D. P., Jelkmann, W., Wenger, R. H., and Hellwig-Burgel, T. (2002). Normoxic induction of the hypoxia-inducible factor 1 α by insulin and interleukin-1 β involves the phosphatidylinositol 3-kinase pathway. *FEBS Lett.* 512, 157–162.
- Talks, K. L., Turley, H., Gatter, K. C., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J., and Harris, A. L. (2000). The expression and distribution of the hypoxia-inducible factors HIF-1 α and HIF-2 α in normal human tissues, cancers, and tumor-associated macrophages. *Am. J. Pathol.* 157, 411–421.
- Trisciuglio, D., Iervolino, A., Candiloro, A., Fibbi, G., Fanciulli, M., Zangemeister-Wittke, U., Zupi, G., and Del Bufalo, D. (2004). Bcl-2 overexpression in human melanoma cells increases angiogenesis through VEGF mRNA sta-

- bilization and HIF-1-mediated transcriptional activity. *J. Biol. Chem.* 279, 6737–6745.
- Volmat, V., Camps, M., Arkinstall, S., Pouyssegur, J., and Lenormand, P. (2001). The nucleus, a site for signal termination by sequestration and inactivation of p42/p44 MAP kinases. *J. Cell Sci.* 114, 3433–3443.
- Wang, G. L., and Semenza, G. L. (1995). Purification and characterization of hypoxia-inducible factor 1. *J. Biol. Chem.* 270, 1230–1237.
- Yen, L., You, X. L., Al Moustafa, A. E., Batist, G., Hynes, N. E., Mader, S., Meloche, S., and Alaoui-Jamali, M. A. (2000). Heregulin selectively upregulates vascular endothelial growth factor secretion in cancer cells and stimulates angiogenesis. *Oncogene* 19, 3460–3469.
- Zhong, H., Chiles, K., Feldser, D., Laughner, E., Hanrahan, C., Georgescu, M. M., Simons, J. W., and Semenza, G. L. (2000). Modulation of hypoxia-inducible factor 1 α expression by the EGF/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res.* 60, 1541–1545.
- Zundel, W., *et al.* (2000). Loss of PTEN facilitates HIF-1-mediated gene expression. *Genes Dev.* 14, 391–396.