Akt and Hypoxia-Inducible Factor-1 Independently Enhance Tumor Growth and Angiogenesis

Andrew M. Arsham,1,2 David R. Plas,2 Craig B. Thompson,2 and M. Celeste Simon2,3

1Committee on Genetics, University of Chicago, Chicago, Illinois, and 2Abramson Family Cancer Research Institute and 3Howard Hughes Medical Institute, University of Pennsylvania, Philadelphia, Pennsylvania

INTRODUCTION

Akt and hypoxia-inducible factor (HIF)-1 share the ability to induce angiogenesis, glucose transport, and glycolysis (reviewed in Refs. 1 and 2). Whether Akt is effecting these processes via HIF-1 has been the subject of much recent study. Many nonhypoxic stimuli, including inflammatory mediators and growth factors, can induce HIF-1 via phosphatididylinositol 3-kinase/Akt pathway (PI3K) and/or Akt. The tumor suppressor PTEN, which opposes PI3K/Akt signaling, can down-regulate HIF-1 (reviewed in Ref. 3). However, in a variety of other cell types and experimental systems, the PI3K/Akt pathway appears neither necessary nor sufficient for HIF-1 activity, suggesting that interactions between these two pathways are context dependent (4–6).

Akt is activated by growth factor signaling through PI3K (7, 8) and strongly enhances cell growth and proliferation by phosphorylating regulators of cell cycle, apoptosis, and metabolism (2). Akt also increases cellular glucose uptake and glycolysis (9–14), which may play a role in Akt-induced cell survival and growth. Consistent with its role as a mediator of cell growth and proliferation, Akt activity is oncogenic. Constitutive activation of Akt can convert anaplastic astrocytoma into high-grade glioblastoma (15, 16), and the activity of the tumor suppressor PTEN appears to depend on its ability to suppress Akt activity (17). Akt can also combine with c-ras (18, 19) to cause tumors in mice and enhance tumor growth in the skin (20), thymus (21, 22), and prostate tumor xenografts (23). Akt activation also appears to increase tumor cell invasiveness (24–26).

PI3K/Akt signaling can induce developmental and tumor angiogenesis by regulating the highly potent and endothelial cell-specific cytokine vascular endothelial growth factor (VEGF), which causes new blood vessel recruitment. This regulation occurs at both the mRNA (27–31) and protein (32–35) levels. PI3K/Akt regulation of VEGF mRNA appears to occur via both HIF-1-dependent (31, 36) and -independent (30, 37) mechanisms. Furthermore, in some contexts, this regulation may be dependent on differentiation (37, 38). Akt can also preferentially increase the rate of translation of VEGF mRNA (39–41). Consistent with these reports, Akt induces aggressive hemangioendotheliomas (22) and angiogenesis (28) in chickens.

HIF-1 is a heterodimer consisting of HIF-1α and HIF-1β/ARNT and is the major regulator of the transcriptional response to low O2 (hypoxia). HIF-1α (and all HIF-α subunits) is constitutively transcribed and translated. O2-dependent hydroxylation by a family of prolyl hydroxylases allows HIF-α to be targeted for degradation by the ubiquitin E3 ligase pVHL (42–47). HIF-α hydroxylation and ubiquitination are inhibited by hypoxia, allowing HIF dimers to transactivate a variety of genes containing hypoxia response elements, including glucose transporters, glycolytic enzymes, erythropoietin, and VEGF (reviewed in Ref. 1).

Like PI3K/Akt signaling, HIFs play a dual role in tumor growth by enabling both cell survival and angiogenesis. HIF-1 has been implicated in angiogenesis-independent tumor growth (48, 49) in part via effects on glucose metabolism (50, 51) and biosynthetic pathways (52). Although some reports suggest that HIF-1 retards tumor growth (53–55), overexpression of HIFs is generally associated with increased tumor growth and grade (56). HIF-1 also induces VEGF, and hypoxic induction of VEGF appears central to tumor angiogenesis in both embryonic stem (ES) cell– (57) and hepatocyte-derived (58) tumors. HIF-1 may also enhance tumor growth via secreted factors other than VEGF (59).

The ability of the PI3K/Akt pathway to regulate HIF-1 and fact that these two pathways each induce VEGF, angiogenesis, and tumor growth suggest that HIF-1 may be mediating Akt’s angiogenic activity. In fact, the existing literature strongly suggests a linear pathway from Akt to HIF-1 to VEGF. However, using an in vivo genetic approach, we show here that HIF-1 is dispensable for Akt’s oncogenic and angiogenic effects and that HIF-1 and Akt independently promote tumor growth and angiogenesis in vivo. Expression of constitutively active myristoylated Akt (myrAkt) in HIF-1-null tumors increases tumor growth, angiogenesis, and VEGF similarly to HIF-1 itself. Both myrAkt and HIF-1 also confer a cell-intrinsic competitive advantage in vivo. Thus, HIF-1 and Akt can independently effect both angiogenesis-dependent and -independent tumor growth and appear to perform similar functions in tumors via parallel independent pathways.

MATERIALS AND METHODS

Cell Culture and Generation of Stable Clones. All cells were propagated in D/10% medium: DMEM (Cellgro), 10% FBS (Gemini Bioproducts), 20 mM HEPES, 2 mM l-glutamine, 100 units/ml Penicillin, 100 mg/ml Streptomycin, and 100 mM nonessential amino acids (all from Invitrogen/Life Technologies, Inc.). Hypoxic conditions were 1.5% O2, 5% CO2, and 37°C and generated using an InVivo2 hypoxic workstation (Biotrace, Inc.) or IGT750 variable O2 incubator (Jouan).

The Station 1 vector used for ARNT expression has been described previ-
ously (60). pCDNA3/hemagglutinin (HA)-myrAkt was generously provided by A. Edinger and S. Talapatra. Simultaneous selection for both neomycin (0.4 mg/ml Geneticin) and hygromycin B (0.4 mg/ml) resistance was used to isolate transfected cells. pCDNA3/VEGF164 was constructed by excising the VEGF164 cDNA out of pEF2/VEGF164 (generously provided by W. Lee and N. Yielding) with EcoRI and cloning into pCDNA3 (Invirogen). Cells (2 × 10⁶ BpRc1) in 60-mm dishes were cotransfected with 1 μg each of the following combinations of linearized plasmids using Lipofectamine 2000 (Invitrogen): (a) pCDNA3 and Station I (Vector); (b) pCDNA3 and Station I/ARNT (HIF-1); (c) pCDNA3/HA-myrAkt and Station I (myrAkt); (d) pCDNA3/HA-myrAkt and Station I/ARNT (HIF/myr); or (e) pCDNA3/VEGF164 and Station I (VEGF). Expression of ARNT or HA-myrAkt in clones was confirmed by Western blot. Expression of VEGF was confirmed by Northern blot using VEGF cDNA as a probe and Quantikine M murine VEGF ELISA kit (R&D). Cell counts in Fig. 3 were obtained using a Coulter Counter ZZ (Beckman Coulter, Inc.). To generate green fluorescent protein (GFP)-tagged BpRc1 cells, a pHabeMN/IRE/GFP-based construct with LacZ as the first cistron and GFP as the second was transfected by CaPO4 into the Phoenix viral packaging line (61). Resultant viral supernatant was combined with 4 mg/ml hexadimethrine bromide (Sigma), transferred to 3.5-cm wells containing 5 × 10⁶ BpRc1 cells. After retroviral transduction, GFP-positive clones were selected, and expression was confirmed by fluorescence-activated cell sorting (FACS). Clones with high GFP expression and growth rates similar to vector control and parental cells were selected for experimental use.

**Western Blots and Histochemistry.** Cells (1 × 10⁶) were plated per 60-mm dish, starved for 18 h, and lysed in 150 mM NaCl, 50 mM Tris (pH 7.4), 5 mM EDTA, 0.1% SDS, 20 mM β-glycerophosphate, 10 mM NaF, 250 mM NaVO₃, 1 mM phenylmethylsulfonyl fluoride, and Complete protease inhibitor cocktail (Roche). Protein concentrations were measured using the protein assay reagent kit (Pierce), and 20 μg of protein were loaded per lane. Immunoblots were run according to standard protocols. Antibody against ARNT was obtained from Novus (NB 100–124); Akt and phospho-Akt Ser 73 were from Cell Signaling Technologies (9272 and 9271); and HA was from Roche (clone 3F10; 1 867 423). For histochemistry, tumors were fixed in 4% paraformaldehyde and paraffin embedded per standard protocols. Sections (6 μm) were treated with a citrate-based high temperature unmasking procedure (Vector Labs). Endothelial cells were stained with oCD34 polyclonal antibody (Abcam, clone MEC 14.7; 8158–100) according to standard protocols using horseradish peroxidase/3,3′-diaminobenzidine reaction and hematoxylin counterstain. All other reagents were from Vector Labs.

**Tumor Formation.** Cells (1 × 10⁶) in 100 μl of PBS were injected s.c. in nu/nu mice (Harlan). Tumors were measured twice weekly using calipers, and approximate tumor volume was calculated using the formula: (length × width)² × 0.5236 (mm³).

**VEGF ELISA.** VEGF protein levels were measured using a Quantikine M mouse VEGF ELISA kit (R&D) according to manufacturer’s instructions. For tumor ELISA, tumors were flash frozen in liquid N₂ and stored at −80°C before being diced and homogenized using an electric tissue homogenizer in 50 mM Tris (pH 7.4), 0.25% Triton X-100, 5 mM EDTA, 0.1% NP40, and Complete protease inhibitor (Roche). Homogenates were diluted 1:6 in 50 mM Tris (pH 7.5) and a further 1:5 in calibrator diluent (R&D) for ELISA measurements. All measurements were normalized to protein concentrations and performed in triplicate. For in vitro measurements, cells were plated in triplicate at 2.5 × 10⁵/well of a 12-well plate, washed in PBS, and serum starved for 5 h. Serum-free medium was replaced, and cells were incubated in normoxia or hypoxia for 20 h. Secreted VEGF was measured according to manufacturer’s instructions. All measurements were normalized to cell counts in each well and performed in duplicate. Intrawell averages were obtained and used to compute mean ± SE.

**Tumor Disaggregation and FACS Analysis.** Tumors were minced in 10 ml of PBS before 30–40-min digestion at 37°C in ∼200 units/ml Collagenase XI (Sigma), 0.3 mg/ml Proteinase (from Streptomyces Griseus; Sigma), and 0.03 units/ml DNase I (Roche) in PBS. Digestion was stopped with DMEM/10% FCS, and suspension was passed through a 100-μm cell strainer. Suspensions were spun at ∼300 g for 5 min, resuspended in ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM Na₂EDTA), and incubated on ice for 5 min to lyse RBCs. Suspensions were then diluted in 20 ml of PBS, passed through a 100-μm cell strainer, and spun at 300 g for 5 min. Cell pellets were resuspended in 500 μl of PBS, spun in strainer-capped FACS tubes (Falcon), and diluted to an appropriate density for antibody staining and FACS. The resulting single cell suspensions were incubated for 30 min on ice with an antibody, which recognizes tumor but not host cells (H-2D+, Pharmingen 60115A) to gate out any host contribution to the tumor cell suspensions. Cells were then briefly incubated in 4′,6-diamidino-2-phenylindole to stain for viability. For FACS analysis, cells were gated to include only live, H-2D⁺-positive cells in analysis of GFP expression. Some GFP expression was lost over the course of tumor growth, most likely attributable to silencing of the viral promoter-driving GFP expression. We therefore normalized the percentage of change in GFP expression in each chimeric tumor to that of control GFP-only tumors to account for this loss.

**Statistics.** All statistics were calculated using Instat 2.03 (GraphPad). All tests were one-way ANOVA comparing each column with vector control. Bonferroni corrections were performed to correct for multiple comparisons.

**RESULTS**

**HIF-1 and myrAkt Independently Promote Tumor Growth.** To investigate genetic interactions between Akt and HIF-1 in tumor growth, we used a murine hepatoma cell line, BpRc1 (62, 63), which lacks ARNT/HIF-1β and thus has no HIF-1 activity. This cell line enabled us to express myrAkt on a HIF-1-null genetic background and assess Akt function in the absence of HIF-1. We isolated stable transfected cells of this line, which expressed ARNT, thus restoring HIF-1 activity as measured by reporter assay (data not shown), myrAkt, or both ARNT and myrAkt. Cells stably transfected with both vectors were isolated as controls (Fig. 1A). Expression of ARNT and an HA-tagged myrAkt were assessed by Western blots for ARNT, total Akt, Akt phosphorylated at Ser473 (a marker of its activation), and the HA tag. Isolated clones had differing levels of ARNT and myrAkt, enabling us to assess any dose responsiveness of in vivo or in vitro effects (Fig. 1B). Analysis of in vitro growth rates (Fig. 1D) and cell cycle profiles (data not shown) of each clone revealed no consistent effect of HIF-1 or myrAkt, with the exception of one clone (HIF1.1) growing more slowly. In contrast, when s.c. tumors were generated in nude mice (Fig. 1, C and E), expression of either HIF-1 or myrAkt promoted dramatically faster growth rates and larger tumor volumes in two independent experiments using independent clones (represented by the two graphs). Importantly, clone HIF-1.1, which expresses high levels of ARNT, induced larger tumors (900 mm³) than clone HIF-1.2, which expresses low levels of ARNT, at 30 days postinjection (580 mm³). These results indicate that HIF-1 can induce tumorigenesis in a dose-dependent fashion. The same is true for myrAkt (Fig. 1E). Coexpression of HIF-1 and myrAkt did not have additive or synergistic effects on tumor growth, suggesting that myrAkt-induced tumor growth was not impeded by the absence of HIF-1. These data demonstrate that HIF-1 and myrAkt independently promote tumor growth and suggest that myrAkt can overcome the deleterious effects of the absence of HIF-1.

**HIF-1 and myrAkt Independently Enhance Tumor Vasculature and VEGF Production.** Visual analysis of the tumors indicated that HIF-1, myrAkt, and HIF-1/myrAkt tumors contained markedly more blood than vector controls, and this was confirmed by H&E staining of tumor sections (Fig. 2, A–D). In addition, HIF-1 tumors appeared to be more hemorrhagic than the other tumor groups and, as a result, have less structural integrity. To specifically assess vascular parameters within the tumors, immunohistochemical staining for vascular endothelial cells was performed with an antibody to CD34. Although vector control tumors had many small blood vessels, HIF-1, myrAkt, and HIF-1/myrAkt tumors were characterized by a dramatic increase in vessel size (Fig. 2E–H), indicating that HIF-1 and myrAkt can independently affect tumor vascularization. Furthermore, myrAkt-induced vascularization can occur in the absence of HIF-1.
To determine whether vascular differences between tumors were associated with differences in VEGF expression, we measured VEGF protein levels in tumor homogenates. Both HIF-1 and myrAkt tumors had a 3-fold increase in VEGF protein levels over vector controls (Fig. 3A; P < 0.01 for both), and VEGF levels were strongly correlated with individual tumor volume (Fig. 3B), implicating tumor-derived VEGF in the increased growth of HIF-1 and myrAkt tumors.

HIF-1 is a known activator of VEGF transcription, but less is known about how Akt regulates VEGF. It was also unclear whether the increased VEGF we observed in myrAkt tumors was a direct effect of myrAkt itself or was secondary to changes in tumor physiology. We thus analyzed VEGF protein secretion (Fig. 3C) and mRNA levels (Fig. 3D) in vitro. Restoration of HIF-1 activity in two independent clones increased basal VEGF levels and restored hypoxic induction of VEGF secretion compared with vector controls. VEGF secretion corresponded to levels of ARNT protein, as higher levels of VEGF were detected in clone HIF-1.1 (Fig. 1B). In myrAkt cells, basal levels of VEGF were elevated ~2-fold in two independent clones and not further inducible by hypoxia. Double transfected clones had restored hypoxic induction of VEGF secretion but showed no consistent increase in basal levels, suggesting a complex regulatory interaction. Consistent with VEGF secretion data, restoration of HIF-1 increased basal levels and restored hypoxic induction of VEGF mRNA (Fig. 3D). Steady-state levels of VEGF mRNA were also increased in myrAkt-expressing cells lacking HIF-1 and cells expressing both. Hypoxic induction of VEGF mRNA in myrAkt-expressing, HIF-1-null cells is likely to be caused by HIF-independent hypoxic regulation of VEGF mRNA stability. Although VEGF secretion measured by ELISA did not always directly correlate with mRNA measured by Northern blot, this may be caused by HIF-independent effects on secretion or myrAkt regulation of secretion. VEGF production is complex and regulated at many levels by kinase signaling cascades, such as mitogen-activated protein kinase, stress-activated protein kinase, and PI3K, and transcription factors, including SP1/AP2, HIFs, and nuclear factor-κB (64–66). These data clearly show Akt induction of VEGF mRNA and protein, despite the absence of HIF-1.
**HIF-1 and myrAkt Independently Confer a Cell-Autonomous Competitive Advantage in Vivo.** Although intratumor VEGF levels correlated well with tumor volume, both HIF-1 and Akt may also increase tumor growth independently of angiogenesis (48–50, 67). To examine the cell-intrinsic effects of HIF-1 and myrAkt on tumor growth, we performed in vivo competition experiments using the clones described in Fig. 1 and a GFP-tagged clone of the BpRc1 cell line (Fig. 4A). To compare the effects of HIF-1 and myrAkt with the effects of increased VEGF alone, we generated BpRc1 cells which overexpressed VEGF cDNA to approximately the same degree as the myrAkt-expressing cells (Fig. 4B). If HIF-1 or myrAkt enhances tumor growth exclusively via angiogenesis, all cells within the tumor should benefit equally, irrespective of their genotype, and GFP cells will be maintained. In contrast, if HIF-1 or myrAkt enhances cell-autonomous survival or proliferation in addition to angiogenesis, these cells should have a competitive advantage in vivo and thus end up over-represented relative to the GFP-tagged index population.

Competition experiments were first carried out in vitro by mixing GFP cells with vector, HIF-1, myrAkt, or VEGF clones. For each in vitro competition, we measured the percentage of GFP-positive cells by FACS immediately after mixing and after one and two passages. As Fig. 4C shows, GFP cells did not proliferate significantly differently from vector controls. Furthermore, in agreement with Fig. 1C, expression of HIF-1, myrAkt, or VEGF conferred no competitive advantage over the GFP population in vitro, although HIF-1 and myrAkt clones appeared to display a slight proliferative disadvantage.

Chimeric tumors were then generated by mixing cells (66% GFP to 33% competitor) and injecting these mixed populations s.c. into nude mice. Tumors consisting of only GFP-tagged cells or GFP/vector mixtures grew at the same rate, demonstrating that the GFP and vector control cells were similarly tumorigenic. Furthermore, expression of HIF-1, myrAkt, or VEGF within a subpopulation of tumor cells was sufficient to increase mean tumor volume 4.9-, 2.5-, and 3.3-fold, respectively (Fig. 4D).
After 21 days, chimeric tumors were disaggregated to single cell suspensions and analyzed for GFP content (Fig. 4E). Of note, any contribution of host cells to tumor cell suspensions was eliminated using histocompatibility antigens, i.e., H-2D<sup>b</sup> (see “Materials and Methods”). Tumors comprised of GFP-positive cells alone exhibited a decline in GFP expression, possibly because of silencing of the viral promoter driving GFP mRNA. Correcting for this decline, the percentage of change in GFP-positive cells was indistinguishable between GFP only and GFP/vector chimeric tumors. Similarly, the percentage of change of GFP expression in GFP/VEGF chimeric tumors was not significantly different from GFP or GFP/vector tumors, although GFP/VEGF chimeric tumors were, on average, 3-fold larger. Thus, VEGF expression in a subpopulation of tumor cells is able to promote the growth of all cells and confers no cell-autonomous advantage. In direct contrast, tumors containing either HIF-1- or myrAkt-expressing cells displayed a significant decrease in GFP-positive cells relative to controls (59 and 63%; P < 0.01 for both). This increase in HIF-1- or myrAkt-expressing cells at the expense of GFP-expressing cells demonstrates that expression of either HIF-1 or myrAkt increases a cell’s fitness in vivo, giving such cells a competitive advantage that cannot be explained by their increased VEGF expression alone. Therefore, both HIF-1 and myrAkt confer a cell-autonomous competitive advantage in vivo.

**DISCUSSION**

Using both in vitro and in vivo approaches, we have shown that HIF-1 is not necessary for myrAkt’s effects on tumor growth and angiogenesis and Akt can induce VEGF independently of HIF-1. However, these results do not exclude interaction between these two pathways, nor do they invalidate the emerging model of nonhypoxic activation of HIF-1 by the PI3K/Akt/mammalian target of rapamycin (mTOR) signaling cascade. Rather, these findings suggest that HIF-1 and Akt can carry out similar but independent functions with respect to tumor growth and angiogenesis separate from Akt’s ability to induce HIF-1.

Existing literature strongly, if circumstantially, suggests that HIF-1 mediates Akt’s effects on angiogenesis; in fact, several groups have demonstrated that HIF-1 is sufficient for VEGF induction downstream of Akt. We have tested for the first time using a genetic strategy...
whether HIF-1 is necessary for Akt regulation of VEGF. Although activation of the PI3K/Akt pathway can lead to increased HIF-1 (3), this induction is generally of a lower magnitude than hypoxia itself and appears to be through a distinct, hydroxylation- and pVHL-independent mechanism (68) involving increased translation (69). Given this difference in magnitude and mechanism, the precise role these modes of activation play in normal hypoxic signal transduction is unclear.

Furthermore, Akt activation of HIF-1 appears to depend on mTOR, a central regulator of protein synthesis in response to growth factor signaling, nutritional cues, and stress. However, we have observed that hypoxia inhibits mTOR pathway signaling (70), suggesting that hypoxia may inhibit or exclude Akt/mTOR of HIF-1α (71). In addition, although some reports have proposed that the PI3K pathway is necessary for hypoxic induction of HIF-1, we and others (5, 6) have shown that this requirement is not universal using a variety of experimental systems.

From a functional standpoint, myrAkt seems able to compensate for whatever in vivo growth defects are caused by a deficiency in HIF-1. To what degree this depends on regulation of VEGF, glucose metabolism, or other shared targets of Akt and HIF-1 remains to be elucidated. Nevertheless, the fact that HIF-1 and Akt can operate via parallel independent mechanisms has implications for cancer therapy. HIF-1 has become a strong candidate target for anticancer therapies (56), based on the model that by blocking HIF-1, one can block both hypoxic survival of cancer cells and tumor angiogenesis without excessive harm to normal tissues. Our data indicate that activation of either HIF-1 or Akt can be sufficient to promote tumor vascularization and growth and that HIF-1 activity is not required for the oncogenic activity of Akt. This suggests that antiangiogenic tumor therapies may require independent strategies to simultaneously inactivate both HIF-1 and Akt to successfully inhibit tumor vascularization.

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