Critical Reviews in Biochemistry and Molecular Biology, 35(2):71–103 (2000)

Hypoxia, Clonal Selection, and the Role of HIF-1 in Tumor Progression

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Abstract: Tumor progression occurs as a result of the clonal selection of cells in which somatic mutations have activated oncogenes or inactivated tumor suppressor genes leading to increased proliferation and/or survival within the hypoxic tumor microenvironment. Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that mediates adaptive responses to reduced O_2 availability, including angiogenesis and glycolysis. Expression of the O_2-regulated HIF-1α subunit and HIF-1 transcriptional activity are increased dramatically in hypoxic cells. Recent studies indicate that many common tumor-specific genetic alterations also lead to increased HIF-1α expression and/or activity. Thus, genetic and physiologic alterations within tumors act synergistically to increase HIF-1 transcriptional activity, which appears to play a critical role in the development of invasive and metastatic properties that define the lethal cancer phenotype.

Key Words: angiogenesis, cancer, glycolysis p53; Ras, vascular endothelial growth factor.

I. INTRODUCTION: GENETIC ALTERATIONS IN TUMOR CELLS

A major focus of oncology research over the last quarter-century spanning the era of molecular biology has been the identification of genetic alterations arising as a result of somatic mutation within tumor cells. A large body of research data established the existence of several groups of genes that, when mutated, contribute to the process of tumor progression (for review see Vogelstein and Kinzler, 1998). The first group that was identified consists of oncogenes, in which gain-of-function mutations result in increased expression of a gene product and/or expression of a mutant product with constitutive activity that is not responsive to normal molecular mechanisms of downregulation. The second group consists of tumor suppressor genes, in which loss-of-function mutations eliminate the expression or activity of a gene product. A third group consists of mutator genes involved in DNA repair, in which loss-of-function mu-

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tations increase the rate at which mutations occur within oncogenes and tumor suppressor genes.

The high frequency of mutations involving specific genes within specific tumor cell types implies that these genetic alterations play a key role in the oncogenic transformation of that cell type. Tumor-specific mutations do not result from targeted mutagenesis, but instead represent the effect of selection: cells in which a mutation occurs by chance within a specific gene that results in an increased rate of survival and/or division (that together determine the rate of cell proliferation) become overrepresented in the tumor relative to cells lacking the mutation. Along with the discovery of oncogenes, tumor suppressor genes, and mutator genes, the concept of clonal selection (Nowell, 1976) represented a critical advance in our understanding of tumor biology.

Because it is much easier to manipulate tumor cells in plastic dishes than in living animals, a great deal of research investigating the biological function of oncogenes and tumor suppressor genes has been performed in cultured cells. In this context, studying the rate at which cells divide is accomplished easily. For this reason, the functions attributed to the protein products of oncogenes and tumor suppressor genes have primarily related to the control of cellular proliferation in tissue culture: oncogenes promote cell proliferation whereas tumor suppressor genes counteract this effect. Likewise, the influence of anti- and proapoptotic proteins, such as members of the BCL2 family, on tumor cell survival has been analyzed primarily in the tissue culture milieu.

The goal of this review is to focus on adaptive mechanisms by which selected tumor cells are able to survive the harsh microenvironmental conditions that they impose on themselves *in vivo.* The adaptations that are described have in common the fact that they are mediated, at least in part, by the transcriptional activator hypoxia-inducible factor 1 (HIF-1). Important properties of HIF-1 and common characteristics of solid tumors are reviewed first, followed by a summary of recent data that have delineated specific functional relationships between genetic alterations, HIF-1, and tumor progression. Finally, the clinical implications of these results are considered.

II. HIF-1 STRUCTURE AND FUNCTION

HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits (Wang and Semenza, 1995). Both subunits contain basic-helix-loop-helix (bHLH)-PAS domains (Wang et al., 1995a). Whereas the bHLH domain defines a superfamily of eukaryotic transcription factors (reviewed by Semenza, 1998), the PAS domain, which was first identified in the PER, ARNT, and SIM proteins, defines a subfamily of bHLH proteins that is unique to metazoans (reviewed by Crews and Fan, 1999). The HIF-1β subunit is also known as the aryl hydrocarbon nuclear translocator (ARNT), as it was first shown to dimerize with the aryl hydrocarbon receptor (Hoffman et al., 1991). Recently, proteins with sequence similarity to HIF-1α (HIF-2α and HIF-3α) and HIF-1β (ARNT2 and ARNT3) have been identified, and all HIF-1α-like polypeptides appear able to dimerize with all HIF-1β-like polypeptides, at least *in vitro* (reviewed by Semenza, 2000). In knockout mice, homozygosity for a targeted loss-of-function mutation in the *Hif1a*, *Epas1,* or *Arnt* gene encoding HIF-1α, HIF-2α, or HIF-1β, respectively, results in embryonic lethality (Iyer et al., 1998; Kozak et al., 1998; Maltepe et al., 1997; Tian et al., 1998). The developmental and physiological roles of these fac-

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tors have been reviewed elsewhere recently (Crews and Fan, 1999; Semenza, 1999, 2000).

The HLH and PAS domains of HIF-1α and HIF-1β are required for the formation of a dimer in which conformation of the basic domains allows their recognition of specific DNA binding sites containing the core sequence 5'-RCGTG-3' (Jiang et al., 1996a; Semenza et al., 1996; Wood et al., 1996). HIF-1 functions as a sequence-specific transcriptional activator (Forsythe et al., 1996; Jiang et al., 1996a; Semenza et al. 1996). Both the expression of HIF-1α and its ability to activate transcription are regulated by the cellular O_2 concentration (Huang et al., 1996; Jiang et al., 1996b, 1997b; Pugh et al., 1997; Wang et al., 1995a). HIF-1α has a half-life of < 5 min under posthypoxic conditions, both in cultured cells and *in vivo* (Wang et al., 1995a; Yu et al., 1998). Under nonhypoxic conditions, HIF-1α is subject to ubiquitination and proteasomal degradation (Huang et al., 1998; Kallio et al., 1999; Salceda and Caro, 1997). Under hypoxic conditions, ubiquitination of HIF-1α is dramatically reduced (Sutter et al., 2000). The introduction of specific missense mutations and deletions into the HIF-1a sequence results in decreased ubiquitination and increased expression of HIF-1a protein under nonhypoxic conditions (Sutter et al., 2000). Transactivation domain function is also under negative regulation in nonhypoxic cells and deletions within these domains also increase their activity in nonhypoxic cells (Jiang et al., 1997b; Pugh et al., 1997). Thus, under hypoxic conditions, HIF-1 transcriptional activity increases rapidly due to synergistic effects on HIF-1a protein expression and transactivation domain function. The HIF-1 transactivation domains have been shown to interact with the coactivator proteins CBP, p300, SRC-1, and TIF2, and these interactions are promoted by the redox regulatory proteins thioredoxin and Ref-1 (Arany et al., 1997; Carrero et al., 2000; Ema et al., 1999).

To date, approximately 30 target genes that are transactivated by HIF-1 have been identified (Table 1 and data not shown). These genes encode proteins that are required for angiogenesis, regulation of blood vessel tone, and vascular remodeling; cell proliferation and viability; erythropoiesis and iron metabolism; glucose transport and glycolysis. Remarkably, the vast majority of these gene products have been shown to be overexpressed in human tumor cells. Each HIF-1 target gene contains a hypoxia response element, a *cis*-acting transcriptional regulatory sequence. The presence of a HIF-1 binding site is necessary but not sufficient to constitute a functional hypoxia response element (Forsythe et al., 1996; Semenza et al., 1996; Semenza and Wang, 1992).

III. COMMON BIOCHEMICAL AND PHYSIOLOGICAL CHARACTERISTICS OF SOLID TUMORS

A. The Warburg Effect

Seventy years ago, Warburg demonstrated that compared with normal cells, tumor cells are characterized by a marked increase in glycolytic metabolism, even when cultured in the presence of high O_2 concentrations (Warburg, 1930). Glucose transport into tumor cells is also markedly increased in order to provide increased amounts of the substrate that is ultimately converted to lactate by the glycolytic enzymes. Indeed, increased uptake of labeled glucose derivatives is utilized in clinical diagnostic tests to identify occult tumors in patients. Furthermore, glycolytic metabolism is correlated with disease progression,

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Table 1.

Known HIF-1 Target Genes

Gene product	Ref.
Adenylate kinase 3	Wood et al., 1998
α_{1B}-Adrenergic receptor	Eckhart et al., 1997
Adrenomedullin	Cormier-Regard et al., 1998
Aldolase A	Iyer et al., 1998; Ryan et al., 1998; Semenza et al.,1996
Aldolase C	Iyer et al., 1998
Endothelin-1 (ET-1)	Hu et al., 1998
Enolase 1 (ENO1)	Semenza et al., 1996; Iyer et al., 1998
Erythropoietin (EPO)	Semenza and Wang, 1992; Jiang et al.,1996a
Glucose transporter 1	Iyer et al., 1998; Ryan et al., 1998; Wood et al., 1998
Glucose transporter 3	Iyer et al., 1998
Glyceraldehyde phosphate dehydrogenase	Iyer et al., 1998; Ryan et al., 1998
Heme oxygenase 1	Lee et al., 1997
Hexokinase 1	Iyer et al., 1998
Hexokinase 2	Iyer et al., 1998
Insulin-like growth factor 2 (IGF-2)	Feldser et al., 1999
IGF binding protein 1	Tazuke et al., 1998
IGF factor binding protein 3	Feldser et al., 1999
Lactate dehydrogenase A	Iyer et al., 1998; Ryan et al., 1998; Semenza et al., 1996
Nitric oxide synthase 2 (NOS2)	Melillo et al., 1995; Palmer et al., 1998
p21	Carmeliet et al., 1998
p35srj	Bhattacharya et al., 1999
Phosphofructokinase L	Iyer et al., 1998
Phosphoglycerate kinase 1	Carmeliet et al., 1998; Iyer et al., 1998; Ryan et al., 1998
Plasminogen activator inhibitor-1	Kietzmann et al., 1999
Pyruvate kinase M	Iyer et al., 1998
Transferrin	Rolfs et al., 1997
Transferrin receptor	Lok and Ponka, 1999; Tacchini et al., 1999
Vascular endothelial growth factor (VEGF)	Carmeliet et al., 1998; Forsythe et al., 1996; Iyer et al., 1998; Ryan et al., 1998
VEGF receptor FLT-1	Gerber et al., 1997

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because lactate concentrations of > 20 μmol/g are 6.5-fold more common in primary cervical cancers with metastatic spread compared with non-metastasizing primary tumors (Schwickert et al., 1995). A correlation between lactate production and metastasis was also reported for head and neck tumors (Walenta et al., 1997). In addition to aerobic glycolysis (the Warburg effect), the existence of intratumoral hypoxia provides an additional stimulus for glycolysis, as described below.

Even though the Warburg effect is one of the most universal characteristics of solid tumors, only recently has insight been gained into the molecular mechanisms by which energy metabolism is shifted from oxidative to glycolytic pathways. Quiescent thymocytes cultured in the absence of mitogens have been shown to derive > 80% of their ATP from oxidative phosphorylation, whereas mitogen-stimulated thymocytes derive > 80% of their ATP from glycolysis (Brand and Hermfisse, 1997). It has been proposed that the switch from oxidative to glycolytic metabolism occurs in order to reduce the generation of reactive oxygen species that might otherwise damage replicating DNA. If this switch is hard-wired into the program of cellular proliferation, then it is not surprising that virtually all tumor cells would manifest the Warburg effect despite their thorough disregard for genomic integrity.

Hypoxia response elements have been identified in the promoters of genes encoding aldolase A (ALDA), enolase 1 (ENO1), glucose transporter 3 (GLUT3), lactate dehydrogenase A (LDHA), phosphoglycerate kinase 1, and phosphofructokinase L (Ebert et al., 1995; Firth et al., 1994, 1995; Semenza et al., 1994, 1996) and HIF-1-dependent transactivation of the *ALDA* and *ENO1* promoters has been demonstrated (Semenza et al., 1996). A 68-bp fragment of the *ENO1* promoter, which is the most powerful hypoxia response element identified to date, contains three HIF-1 binding sites (Semenza et al., 1996). Analysis of mouse embryonic stem cells that are homozygous for a targeted loss-of-function mutation in the *Hif1a* gene encoding HIF-1α revealed decreased expression of 13 different genes encoding glucose transporters and glycolytic enzymes (Iyer et al., 1998; Ryan et al., 1998). Thus, HIF-1 mediates coordinate transcriptional activation of the entire pathway from glucose uptake to lactate production. Furthermore, expression of HIF-1α is induced when tumor cells are cultured under conditions associated with cellular proliferation such as low cell density and growth factor stimulation (Feldser et al., 1999; Zhong et al., 1998; Zhong et al., 2000). C-MYC, a transcription factor whose primary role is to stimulate cellular proliferation, also transactivates several genes encoding glycolytic enzymes, including *LDHA* (Dang and Semenza, 1999; Shim et al., 1997). HIF-1 (consensus binding site sequence, 5'-RCGTG-3') and C-MYC (consensus binding site sequence, 5'-CACGTG-3') recognize overlapping binding sites in the *LDHA* gene (Dang and Semenza, 1999; Semenza et al., 1996; Shim et al., 1997).

B. Angiogenesis

A large body of experimental evidence indicates that primary tumors and metastases will not grow beyond a volume of several mm^3 without establishing a blood supply (reviewed in Fidler and Ellis, 1994; Folkman, 1971, 1990, 1992; Hanahan and Folkman, 1996; Zetter, 1998). In the absence of vascularization, cell viability is limited by the diffusion of O_2 and glucose from host vessels. Under these circumstances, cell division and cell death occur at equal rates and no net tumor growth occurs. In

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RIP1Tag2 transgenic mice, every pancreatic-islet β cell expresses SV40 T antigen, approximately 50% of the islets demonstrate β cell hyperplasia, but only a subset of hyperplastic islets demonstrate neovascularization; an even smaller subset of islets progress to carcinoma, but, notably, all of these manifest neovascularization (Folkman et al., 1989). The onset of angiogenesis permits rapid tumor growth, thus setting the stage for subsequent development of the invasive and metastatic properties that define the lethal cancer phenotype. Indeed, for many tumor types there is a statistically significant correlation between tumor angiogenesis (as measured by blood vessel density) and patient survival (reviewed in Fox, 1997; Jekunen and Kairemo, 1997; Zetter, 1998).

Tumor angiogenesis occurs as a result of increased expression of angiogenic factors and decreased expression of antiangiogenic factors. The angiogenic factor that has been shown to have the most important role in mediating blood vessel formation in response to developmental, physiological, or oncogenic stimuli is vascular endothelial growth factor (VEGF) (reviewed in Ferrara and Davis-Smyth, 1997). There is a strong correlation between VEGF expression and blood vessel density and clinical outcome in many tumor types (Ferrara and Davis-Smyth, 1997). Inhibition of VEGF expression or binding to its receptor on endothelial cells has a dramatic effect on tumor growth, invasion, and metastasis in animal models (Benjamin et al., 1999; Benjamin and Keshet, 1997; Borgstrom et al., 1996; Cheng et al., 1996; Grunstein et al., 1999; Im et al., 1999; Jain et al., 1998; Kim et al., 1993; Millauer et al., 1994, 1996; Saleh et al., 1996; Shaheen et al., 1999; Skobe et al., 1997; Yuan et al., 1996). Somatic mutations resulting in oncogene activation (e.g., *H-Ras* and *v-Src*) and tumor suppressor gene inactivation (e.g., *p53* and *VHL*) are associated with increased *VEGF* gene expression (reviewed in Ferrara and Davis-Smyth, 1997). In addition, in several tumor types, most notably glioblastoma multiforme (Shweiki et al., 1992), *VEGF* expression is correlated with intratumoral hypoxia (Ferrara and Davis-Smyth, 1997).

The role of HIF-1 in activating hypoxia-induced transcription of the *VEGF* gene is well established. A hypoxia response element is located approximately 1 kb 5' to the transcription start site of the human *VEGF* gene (Forsythe et al., 1996; Liu et al., 1995) and is also present in the mouse and rat *Vegf* genes (Levy et al., 1995; Shima et al., 1996). A reporter gene containing a 48-bp *VEGF* hypoxia response element upstream of an SV40 promoter and luciferase coding sequences is expressed in a hypoxia-inducible manner. Forced overexpression of HIF-1α and HIF-1β results in reporter gene expression under nonhypoxic conditions and a superinduction in response to hypoxia (Forsythe et al., 1996). A 3-bp mutation that disrupts the HIF-1 binding site in the hypoxia response element resulted in a loss of reporter gene expression in response to hypoxia or coexpressed HIF-1α and HIF-1β (Forsythe et al., 1996). In mouse embryonic stem cells homozygous for a targeted mutation in the *Hif1a* gene encoding HIF-1α there is no induction of VEGF mRNA expression in response to hypoxia (Carmeliet et al., 1998; Iyer et al., 1998; Ryan et al., 1998), although VEGF expression is still induced by glucose deprivation (Iyer et al., 1998).

In a series of mouse hepatoma subclones, there is a strong correlation between the level of hypoxia-induced HIF-1 DNA-binding activity and VEGF mRNA expression (Forsythe et al., 1996; Salceda et al., 1996; Wood et al., 1996). Furthermore, when these subclones are injected into nude mice, there is a strong correlation between the levels of hypoxia-induced HIF-1 and VEGF expression *ex vivo* and tu-

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mor xenograft growth and angiogenesis *in vivo* (Jiang et al., 1997a; Maxwell et al., 1997). *In situ* hybridization analysis of tumors derived from HIF-1expressing subclones has demonstrated that viable cells surrounding areas of necrosis express high levels of VEGF and GLUT3 mRNA, whereas such expression is not seen at similar locations in tumors derived from HIF-1 nonexpressing subclones (Maxwell et al., 1997), suggesting that physiologic induction of hypoxia-inducible genes mediated by HIF-1 is important for tumor growth and vascularization. Vascularization of teratomas derived from mouse embryonic stem cells is also dramatically affected by loss of HIF-1α expression (Carmeliet et al., 1998; Ryan et al. 1998).

In addition to hypoxia, VEGF expression is also induced by exposure of both transformed and nontransformed cells to a variety of growth factors, including epidermal growth factor, basic fibroblast growth factor (FGF-2), insulin-like growth factor 1 (IGF-1), interleukin 1β, platelet-derived growth factor, and tumor necrosis factor α (TNF-α) (Jackson et al., 1997; Ryuto et al., 1996; Stavri et al., 1995a, 1995b; Warren et al., 1996). Remarkably, all of these cytokines/growth factors also induce HIF-1α protein expression and/or HIF-1 DNA-binding activity (Feldser et al., 1999; Hellwig-Burgel et al., 1999; Zelzer et al., 1998; Zhong et al., 2000).

C. Hypoxia

Given the strong correlation between angiogenesis and tumor progression described above, one might predict that that there is a positive correlation between tumor oxygenation and tumor progression. Remarkably, the opposite is true: first, in the majority of human cancers that have been analyzed, the mean pO_2 is markedly lower than that of normal tissue in the same organ (reviewed in Brown and Giaccia, 1998; Vaupel, 1996; Vaupel et al., 1989). Second, clinical studies in which oxygenation of cervical or head and neck tumors has been measured *in situ* have demonstrated that an intratumoral pO_2 of < 10 mmHg is associated with a significantly increased frequency of tumor invasion and metastasis and of patient death (Brizel et al., 1996; Hockel et al., 1996). In both cervical and head and neck cancers, a correlation between elevated lactate levels and metastasis has also been reported (Schwickert et al., 1995; Walenta et al., 1997).

What is the basis for the apparent paradox regarding the effects of tumor hypoxia and angiogenesis on clinical outcome? The blood vessels induced by tumors are structurally and functionally abnormal, resulting in marked regional heterogeneity in tumor perfusion (reviewed in Brown and Giaccia, 1998; Gillies et al., 1999; Vaupel, 1996; Vaupel et al., 1989). Analysis of tumor xenografts has revealed that although there is an inverse correlation between mean pO_2 and distance of a tumor cell from the nearest capillary (reaching a mean pO_2 of 0 at > 200 μm), individual tumor cells with a pO_2 of 0 can be identified immediately adjacent to a blood vessel, indicating an absence of blood flow (Helmlinger et al., 1997). These data suggest that the induction of angiogenesis is necessary but not sufficient to ensure tumor progression.

Intratumoral hypoxia is also associated with genetic instability and resistance to chemotherapy and radiation, thus providing a basis for tumor recurrence (reviewed by Brown and Giaccia, 1994, 1998; Yuan and Glazer, 1998). For chemotherapy, it is has been hypothesized that hypoperfused tumor tissue would be deprived both of O_2 and any chemotherapeutic agent administered parenterally; alternatively, cell cycle arrest under hypoxic conditions may provide pro-

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tection against agents that induce apoptosis in proliferating cells. For radiation, it has been hypothesized that decreased O_2 concentrations would result in decreased formation of reactive oxygen species, which are believed to trigger radiation-induced cell death. Whereas these proposed mechanisms may indeed contribute to the survival of hypoxic tumor cells following cancer therapy, it is also possible that hypoxic tumor cells express survival factors that mediate protection against any apoptosis-inducing stimulus. Exposure of cultured glioma cells to hypoxia results in increased survival when the cells are subsequently exposed to chemotherapeutic agents (Liang, 1996), an effect that is obviously independent of vascularization/perfusion. Insulin-like growth factor 2 (IGF-2) represents an example of a hypoxia-induced survival factor (Kim et al., 1998), and its expression appears to be regulated by HIF-1 (Feldser et al., 1999).

IV. IMMUNOHISTOCHEMICAL ANALYSIS OF HIF-1α EXPRESSION IN TUMORS

A. Increased Expression of HIF-1α in Common Human Cancers and Their Metastases

The analysis of tumor-derived cell lines either in tissue culture or xenograft assays provides a means to investigate molecular mechanisms underlying tumor biology. Yet, such investigations must rest on a firm foundation of histopathological studies utilizing clinical material that provide the basis for generating hypotheses to be tested in model systems. Although clinical investigation is often limited by the lack of optimal controls, in the case of cancer, the patient's normal tissue often can be utilized effectively for this purpose. With these principles in mind, a mouse monoclonal antibody specific for HIF-1α was generated, and a sensitive immunohistochemical assay was developed to detect HIF-1α expression in formalin-fixed and paraffin-imbedded human tumor biopsy specimens (Zhong et al., 1999). Whereas HIF-1α expression was not detected in the corresponding normal tissue (either adjacent to the tumor in the biopsy or in separate autopsy specimens), HIF-1α expression was detected in two-thirds of the primary tumors of the brain, breast, colon, lung, ovary, and prostate that were analyzed (Table 2). Although HIF-1α expression was frequently detected in many, but not all, types of malignant tumors, it was not detected in benign tumors such as breast fibroadenoma and uterine leiomyoma (Zhong et al., 1999). In contrast, HIF-1α expression was detected in early neoplastic lesions (breast comedo-type ductal carcinoma *in situ*, colon adenoma, and prostatic intraepithelial neoplasia) detected incidentally in tumor specimens, suggesting that increased HIF-1α expression can occur early in tumor progression (Zhong et al., 1999). In animal models, induction of angiogenesis has been demonstrated during the transition from hyperplasia to neoplasia (Folkman et al., 1989). HIF-1α expression in early neoplastic lesions may promote vascularization mediated by VEGF and possibly other HIF-1-regulated angiogenic factors.

B. HIF-1α Expression in Brain Tumors

Immunohistochemical analysis of brain tumors was particularly instructive (Zagzag et al., 2000). Several studies have shown that compared with low-grade gliomas, the

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Table 2
Expression of HIF-1a in Human Cancers Compared with Corresponding Normal Tissue[a]

Organ	Normal Tissue	Benign Tumors	Primary Cancers	Metastases
Brain	**0/10**	**—**	**5/9**	**—**
Breast	**0/18**	**0/10[b]**	**25/52**	**9/13[c]**
Colon	**0/24**	**—**	**22/22**	**9/10[c]**
Lung	**0/10**	**—**	**3/3**	**—**
Ovary	**0/10**	**—**	**2/2**	**—**
Prostate	**0/12**	**—**	**9/11**	**5/10[c,d]**
Uterus	**0/3**	**0/2[e]**	**—**	**—**

[a]Data from Zhong et al., 1999.
[b]Fibroadenomas
[c]Lymph node metastases
[d]Bone metastases
[e]Leiomyomas

high-grade glioblastoma multiforme (GBM) has significantly higher levels of VEGF expression and neovascularization (Leung et al., 1997; Pietsch et al., 1997; Plate et al., 1992; Plate and Mennel, 1995; Plate and Warnke, 1997; Takano et al., 1996; Takekawa and Sawada, 1998). Even in low-grade astrocytomas, patient survival is inversely correlated with VEGF expression and vascular density (Abdulrauf et al., 1998). GBM is also characterized by extensive areas of necrosis, representing tumor cells located beyond the effective diffusion distance of O_2 from the nearest blood vessel. VEGF mRNA is highly expressed in the pseudopalisading cells surrounding necrotic areas, suggesting that hypoxia is a stimulus for VEGF expression by these cells (Plate et al., 1992; Shweiki et al., 1992). Remarkably, the expression of HIF-1α is correlated with tumor grade and, in particular, with vascularity (Table 3 and Figure 1). Expression of HIF-1β was also analyzed by immunohistochemistry, and its expression is also correlated with tumor grade, although it is more widely expressed than HIF-1α and is not as strongly correlated with tumor vascularity. Most striking is the pattern of HIF-1α protein expression in GBM which are identical to that described

Table 3
Vascularity and Expression of HIF-1α and HIF-1β in Human Brain Tumors[a]

Tumor	*n*	Vascularity[b]	HIF-1α[c]	HIF-1β[c]
Low grade astrocytoma	2	1.0	1.5	1.0
Low grade mixed glioma	2	2.5	2.5	0.5
Anaplastic astrocytoma	2	3.5	3.5	2.5
Anaplastic oligodendroglioma	3	3.7	3.0	2.3
Anaplastic mixed glioma[d]	9	2.4	2.8	2.4
Glioblastoma multiforme	14	3.9	4.0	3.2
Hemangioblastoma	10	3.7	3.2	3.2

[a]Data from Zagzag et al., 2000.
[b]Vascularity: 0, vascular hyperplasia not detected; 1, minimal; 2, mild; 3, moderate; 4, marked increase in tumor vascularity compared with surrounding normal brain tissue
[c]HIF-1α or HIF-1β: 0, nuclear staining not detected by immunohistochemistry; 1, staining in less than 1% of nuclei; 2, 1–10% of nuclei; 3, 10–50%; 4, greater than 50%.
[d]astrocytoma/oligodendroglioma

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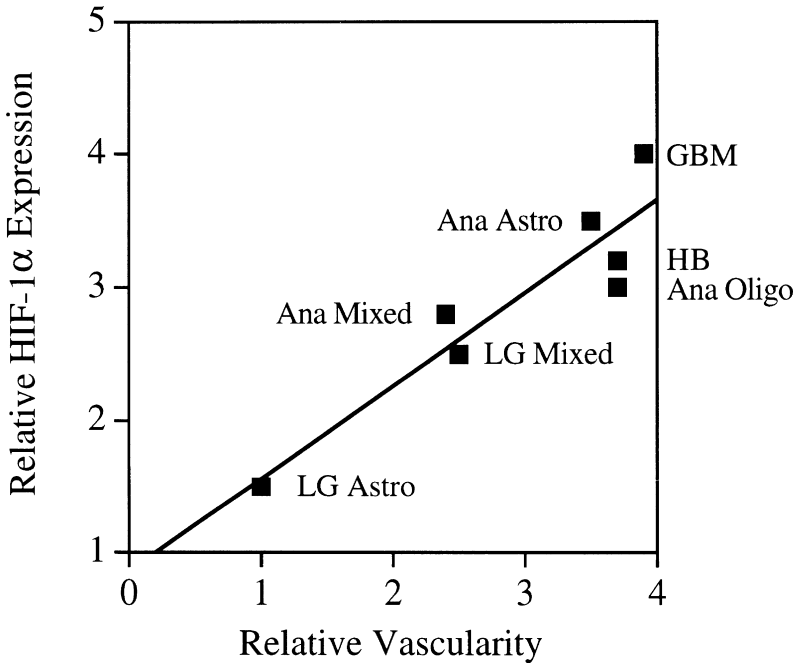

Figure 1. Correlation between brain tumor grade, vascularity, and HIF-1a expression. Based on an analysis of 42 human brain tumors (Zagzag et al., 2000). See Table 3 for definitions.

for VEGF mRNA, with high levels of HIF-1α detected in pseudopalisading cells surrounding areas of necrosis (Zagzag et al., 2000). Taken together, these data are consistent with the hypothesis that HIF-1 mediates hypoxia-induced VEGF expression in GBM.

Hemangioblastoma is another extremely vascular brain tumor. The vascularity of hemangioblastomas is so great that, as the name implies, they were originally believed to be tumors of hemangioblasts, the stem cells from which hematopoietic and endothelial cells are derived. However, it is now apparent that the "stromal" cells (as opposed to the blood vessels) are in fact tumor cells that produce extremely high levels of VEGF (Flamme et al., 1998; Krieg et al., 1998; Stratmann et al., 1997; Wizigman-Voos et al., 1995). Unlike GBM, hemangioblastomas are so richly vascularized that areas of necrosis are unusual. Yet, immunohistochemistry revealed that in those hemangioblastomas with the greatest degree of vascularization, HIF-1α was expressed in the majority of tumor cells, whereas expression was not detected in vascular cells (Zagzag et al., 2000; Zhong et al., 1999). The detection of high levels of HIF-1α in tumor cells immediately adjacent to most blood vessels suggests that such expression is not hypoxia induced.

A similar pattern of expression was detected in another extremely vascularized tumor, clear cell renal carcinoma (Zhong et al., 1999). Hemangioblastoma and clear cell renal carcinoma share in common inactivation of the *VHL* gene encoding the von Hippel-Lindau tumor suppressor protein (Gnarra et al., 1994; Herman et al., 1994; Kanno et al., 1994; Shuin et al., 1994). In renal carcinoma cell lines lacking VHL expression, the normal O_2-regulated expression of HIF-1α and HIF-2α is lost; these proteins are constitutively expressed at high levels under nonhypoxic conditions and activate transcription of downstream target genes, including *VEGF* (Maxwell et al.,

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1999). Analysis of multiple renal carcinoma cell lines with VHL loss-of-function revealed that HIF-2α was overexpressed in all, whereas in several lines HIF-1α expression was completely absent. In contrast, HIF-1α was highly expressed in all of the hemangioblastoma (n = 10) and clear cell renal carcinoma (n = 1) biopsies that were analyzed by immunohistochemistry (Zagzag et al., 2000; Zhong et al., 1999), suggesting that there may be a selection against HIF-1α overexpression in cultured renal carcinoma cells that is not relevant to their biological behavior *in vivo*. However, primary clear cell renal carcinomas express an antisense HIF-1α RNA species (Thrash-Bingham and Tartof, 1999). Thus, the role of HIF-1α expression in these tumors requires further analysis.

C. HIF-1α and Tumor Invasion

GBM is one of the most highly invasive forms of human cancer and patients with this tumor have a life expectancy of less than 1 year regardless of whether they are treated by chemotherapy, radiation, or surgery. In addition to expression in pseudopalisading cells surrounding necrotic areas, HIF-1α was also detected within GBM cells infiltrating the brain at tumor margins (Zagzag et al., 2000). Similar results were observed in biopsies of colon cancer (Zhong et al., 1999). In addition, when GL261 glioma cells were implanted into mouse brains, HIF-1α expression was detected within pseudopalisading and infiltrating tumor cells as observed in the human GBM biopsies (Zagzag et al., 2000). Among normal developmental and physiological examples of invasion, several are clearly related to O_2 homeostasis, such as the invasion of the uterine wall by placental trophoblasts and the invasion of hypoperfused tissue by vascular endothelial cells. The expression of cytoskeletal and cell surface proteins that are required for motility and invasion therefore may be regulated by HIF-1. In this regard, the hypoxia-inducible expression of the urokinase-type plasminogen-activated receptor (Graham et al., 1998) is notable, because this protein has been implicated in tumor vascularization, invasion, and metastasis (Crowley et al., 1993; Evans et al., 1997; Graham et al., 1999; Min et al., 1996; Ossowski et al., 1991; reviewed by Andreasen et al., 1997).

D. HIF-1α and Tumor Cell Proliferation

Further evidence that HIF-1α expression is induced in tumor cells by stimuli other than hypoxia was obtained by an immunohistochemical comparison of HIF-1α and Ki67 staining in human cancer biopsies (Zhong et al., 1999). Ki67 staining, which identifies proliferating cells, was highly correlated with HIF-1α expression (Table 4). Because hypoxia is believed to inhibit tumor cell proliferation (Brown and Giaccia, 1998), this result is the opposite of what would be expected if HIF-1α expression in these tumors was induced solely by hypoxia. However, several recent studies have revealed increased HIF-1α expression in proliferating and/or growth factor-stimulated cells. Human prostate cancer cells express higher levels of HIF-1α when assayed under low-density, as opposed to high-density, culture conditions (Zhong et al., 1998). HIF-1α expression is also induced when tumor cell lines are exposed to any one of a variety of growth factors, including epidermal growth factor, fibroblast growth factor 2, insulin, IGF-1, or IGF-2 (Feldser et al., 1999; Zelzer et al., 1998; Zhong et al., 2000). HIF-1α expression is induced by insulin in cells under low-density, but not under high-

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Table 4
p53 Mutation, Cell Proliferation, and HIF-1α Overexpression[a]

	p53 [b]		Ki67 Labeling Index (%)[c]			
HIF-1α[d]	**-**	**+**	**<5**	**5-15**	**15-50**	**>50**
0	38	11	38	10	2	6
1–2	3	9	9	8	7	3
3–4	7	7	0	2	8	33
	P < 0.01[e]		P < 0.001[f]			

[a]Data from Zhong et al., 1999. Number of tumor sections in each category is indicated.
[b]p53 staining in > 10% of tumor cell nuclei (+) indicates overexpression of mutant p53 protein
[c]Percentage of tumor cells expressing Ki67 antigen.
[d]HIF-1a immunohistochemistry: 0, nuclear staining not detected; 1, staining in < 1% of nuclei; 2, 1-10% of nuclei; 3, 10-50%; 4, > 50% of nuclei.
[e]Kruskal-Wallis test
[f]Nonparametric Jonckhere-Terpstra test

density, culture conditions (Feldser et al., 1999), again suggesting a correlation with cell proliferation. The mitogen PMA also induces HIF-1α expression in human prostate cancer cell lines (Zhong et al., 2000). As described above, the preferential utilization of glycolytic metabolism provides a rationale for the induction of HIF-1α in proliferating cells. In addition, because cell proliferation inevitably results in increased O_2 consumption, it is possible that linking VEGF production to cell proliferation via induction of HIF-1α may provide a means to maintain adequate perfusion during development.

V. ONCOGENE AND TUMOR SUPPRESSOR GENE MUTATIONS THAT INCREASE HIF-1α EXPRESSION OR ACTIVITY

Studies described above provide evidence that HIF-1α expression can be induced by signals other than hypoxia. Cancer cells are distinguished from normal cells by the presence of genetic alterations that cause major derangements in cellular physiology. Recent studies have revealed that mutations that activate oncogenes and those that inactivate tumor suppressor genes result in increased HIF-1α expression or activity in tumor cells, which may contribute to the selective advantage provided by these mutations *in vivo*.

A. V-SRC

Oncogenes were first identified in tumorigenic avian retroviruses. The prototype oncogene is *v-src*, which encodes the transforming protein of Rous sarcoma virus (RSV) and which was derived from the cellular protooncogene *c-src* (Czernilofsky et al., 1980; Stehelin et al., 1976; Swanstrom et al., 1983). RSV infection is associated with increased rates of glucose transport and glycolysis (Singh et al., 1974; Steck et al., 1968; Venuta and Rubin, 1973; Weber, 1973). Rat kidney cells infected with RSV containing a temperature-sensitive mutation in the *v-src* gene exhibit a transformed phenotype and increased rates of aerobic glyco-

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lysis at the permissive, but not at the nonpermissive, temperature (Carroll et al., 1978). Thus, V-SRC expression mediates both cellular transformation and the Warburg effect (see above) in RSV-infected cells. In humans, activating mutations of the *C-SRC* gene have been identified in advanced human colon cancers (Irby et al., 1999). Expression of C-SRC antisense RNA in HT29 colon cancer cells is associated with decreased VEGF mRNA expression both in culture and in tumor xenografts (Ellis et al., 1998). In transgenic mice with V-SRC expression targeted to astrocytes, VEGF expression occurs at an early step in glioma formation (Theurillat et al., 1999).

Compared with parental cells, *v-src*-transformed Rat1 fibroblasts manifest a striking increase in the expression of HIF-1α protein and HIF-1 DNA-binding activity under nonhypoxic conditions and a superinduction in response to hypoxia (Jiang et al., 1997a). Similarly, VEGF and ENO1 mRNA expression are increased in nonhypoxic and hypoxic *v-src*-transformed cells. The expression of reporter genes containing the hypoxia response element from the human *VEGF* or *ENO1* gene is also increased in *v-src*-transformed cells, whereas reporters in which these hypoxia response elements contain mutations that eliminate HIF-1 binding are unaffected by *v-src* transformation, demonstrating a requirement for HIF-1-dependent transcriptional activation (Jiang et al., 1997a). V-SRC also induces the expression of the STAT3 transcription factor, which is required for V-SRC-mediated transformation (Bromberg et al., 1999; Turkson et al., 1998). Thus, HIF-1 and STAT3 may represent two major transcriptional activators downstream of V-SRC and, by inference, mutant C-SRC in human cancers. V-SRC and activated C-SRC are constitutivelyactive tyrosine kinases. Whereas STAT3 is known to be directly activated by tyrosine phosphorylation, the connection between tyrosine kinase activity and HIF-1α expression has not been determined, although the tyrosine kinase inhibitor genistein blocks hypoxia-induced HIF-1α expression in Hep3B human hepatoblastoma cells (Wang et al., 1995b). V-SRC-mediated transformation is mediated via two of the major intracellular signal transduction pathways, the RAS-RAF-MEK-ERK and PI3K-AKT-FRAP pathways (Penuel and Martin, 1999), which are described in detail below.

B. PI3K-PTEN-AKT-FRAP

Recently, across tumor types, loss-of-function mutations in tumor suppressor genes or activating mutations in oncogenes have been shown to dysregulate signal transduction pathways leading from growth factors (e.g., epidermal growth factor [EGF]) and their cognate receptor tyrosine kinases (e.g., EGFR) to phosphatidylinositol (PI) 3-kinase (PI3K), which catalyzes the conversion of PI-4-phosphate and PI-4,5-bisphosphate to PI-3,4-bisphosphate and PI-4,5-trisphosphate, respectively (Cantley and Neel, 1999). These latter compounds function as allosteric activators of PI-dependent kinase 1 (PDK-1), which phosphorylates and activates AKT (also known as protein kinase B), a serine-threonine kinase (Figure 2). Targets of AKT include BAD, an inhibitor of apoptosis, and the mammalian target of rapamycin (mTOR, also known as FKBP12/rapamycin-associated protein [FRAP]), an activator of $p70^{s6k}$, a kinase that is required for translation initiation and cell cycle progression (Brown and Schreiber, 1996; Cantley and Neel, 1999). These findings have delineated mechanisms by which the PI3K-AKT pathway promotes cell proliferation and inhibits cell death. This pathway is negatively regulated by PTEN (phosphatase and tensin homolog deleted

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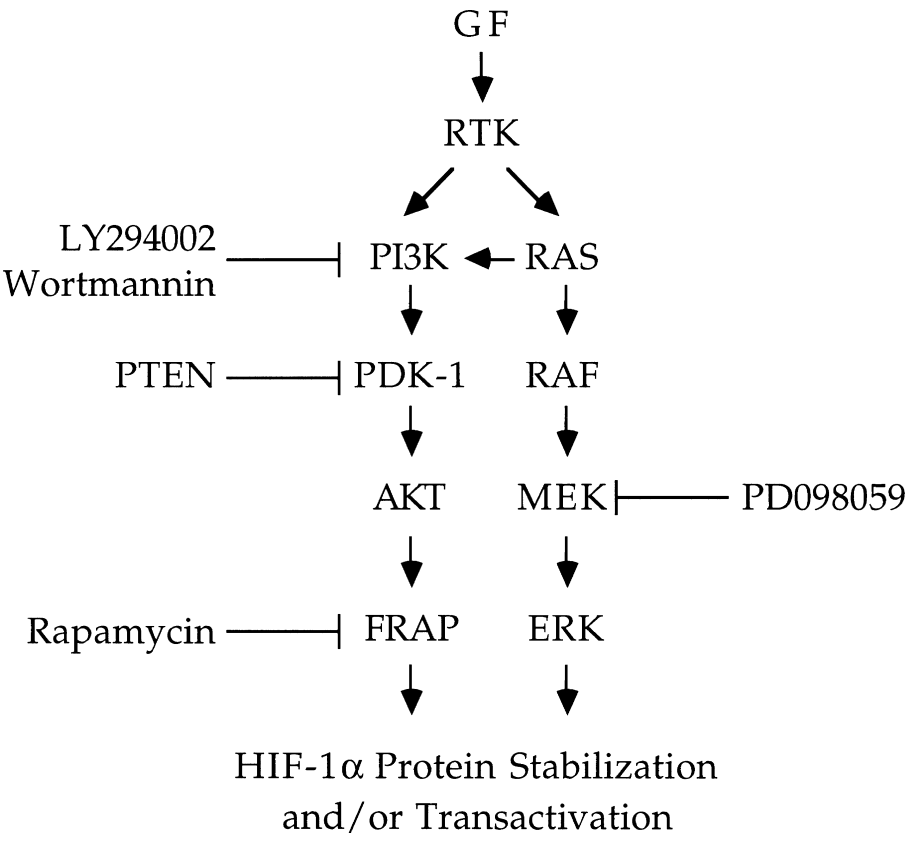

Figure 2. Growth factor-mediated signal-transduction pathways that induce HIF-1a expression and activity.

on chromosome ten), which dephosphorylates PI-3,4-bisphosphate and PI-3,4,5-trisphosphate (Cantley and Neel, 1999). PTEN is a tumor suppressor that is inactivated in brain, breast, prostate, and other human cancers (Li et al., 1997). Mutations of PTEN frequently occur in the progression from early to advanced prostate cancer (McMenamin et al., 1999) and correlate with increased angiogenesis (Giri and Ittmann, 1999).

In the human prostate cancer cell lines DU-145, PC-3, PPC-1, and TSU, basal-, EGF-, and mitogen-induced expression of HIF-1α is blocked by treatment with LY294002, or rapamycin (Figure 2), inhibitors of PI3K and FRAP, respectively (Zhong et al., 2000). The transcription of reporter genes containing a hypoxia response element from the human *ENO1* or *VEGF* gene is blocked by co-transfection of an expression vector encoding dominant-negative AKT or PI3K and by wild-type PTEN, whereas expression is stimulated by constitutivelyactive AKT or dominant-negative PTEN (Zhong et al., 2000). Treatment with LY294002 or rapamycin inhibits secretion of VEGF, the product of a known HIF-1 target gene, thus linking PI3K-PTEN-AKT-FRAP, HIF-1, and (via VEGF) tumor angiogenesis. Thus, within this pathway oncogene activation (*EGFR*, genes encoding other receptor tyrosine kinases, *PI3K*, or *AKT*) or tumor suppressor gene inactivation (*PTEN*) results in increased HIF-1 transcriptional activity. These data indicate that pharmacological agents that target PI3K, AKT, or

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FRAP in tumor cells inhibit HIF-1α expression, and that such inhibition may contribute to their therapeutic efficacy.

C. RAS

Expression of an activated *H-RAS* or *K-RAS* oncogene is associated with increases in both glycolysis (Racker et al., 1985) and VEGF-mediated angiogenesis (Feldkamp et al., 1999; Mazure et al., 1996; Okada et al., 1998; Rak et al., 1995). The effect of RAS on VEGF expression is mediated via the PI3K and MAP kinase pathways (Arbiser et al., 1997; Grugel et al., 1995; Mazure et al., 1997), with the relative involvement of the two pathways varying in different cell lines. Activated H-RAS^{V12} increases the expression of a reporter gene containing *VEGF* promoter sequences only when the reporter contains an intact HIF-1 binding site (Mazure et al., 1997). In NIH 3T3 cells, the effect of H-RAS^{V12} on transcription from the *VEGF* promoter can be blocked by expression of a dominant negative form of PI3K, whereas dominant negative forms of ERK1 or ERK2 MAP kinase have no effect (Mazure et al., 1997). Thus, H-RAS^{V12} appears to activate the PI3K-AKT pathway described above for prostate cancer cells. In support of this hypothesis, the PI3K inhibitor wortmannin blocked hypoxia-induced reporter gene expression in H-RAS^{V12}-transformed NIH 3T3 and Rat1 cells (Mazure et al., 1997). However, rapamycin has no effect on *VEGF* reporter gene expression in NIH 3T3 cells (Mazure et al., 1997), in striking contrast to the studies described above using prostate cancer cell lines (Zhong et al., 2000), suggesting that in NIH 3T3 cells H-RAS^{V12} may induce HIF-1 activity via a PI3K-dependent, FRAP-independent pathway. As in the case of V-SRC, RAS transformation is mediated by multiple transcriptional activators in addition to HIF-1, including CREB (Xing et al., 1996) and NF-κB (Finco et al., 1997).

D. RAF-MEK-ERK

RAS can transduce signals from growth factor receptor tyrosine kinases either via the PI3K pathway or via the RAF1 (MAP kinase kinase kinase)-MEK1 (MAP kinase kinase)-ERK (MAP kinase) pathway (Figure 2). In CCL39 hamster fibroblasts, VEGF mRNA and reporter gene expression are induced by expression of an activated form of RAS, RAF, or MEK1 (Milanini et al., 1998). Furthermore, p42 and p44 MAP kinases (ERK1 and ERK2) phosphorylate HIF-1α *in vitro* and expression of activated RAF1 amplifies HIF-1-mediated reporter gene expression in CCL39 cells (Richard et al., 1999). Whereas the PI3K-AKT pathway induces increased HIF-1α protein expression in prostate cancer cells (Zhong et al., 2000), the activated RAF1-MEK1-ERK pathway in CCL39 cells appears to stimulate HIF-1 transcriptional activity without affecting HIF-1α protein expression (Richard et al., 1999), suggesting an effect on transactivation domain function, which can be regulated independently of the protein expression (Jiang et al., 1997b). In PC12 rat pheochromocytoma cells, transcriptional activation mediated by HIF-2α (which shares conserved amino acid sequence identity with HIF-1α in the bHLH-PAS and transactivation domains) is blocked by the MEK1 inhibitor PD098059 without any change in HIF-2α protein expression (Conrad et al., 1999). Taken together, these studies suggest a synergistic effect of PI3K and MAPK signalling pathways, with the former inducing HIF-1α protein expression and the latter inducing HIF-1α transcriptional activity.

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An alternative hypothesis is that the effect of PI3K or MAPK signaling on HIF-1 activity is determined by the specific stimulus and/or the specific cell type that receives the stimulus. The organomercurial compound mersalyl induces HIF-1α protein expression, HIF-1 DNA-binding activity, HIF-1-dependent reporter gene transcription, as well as ENO1 and VEGF mRNA expression under nonhypoxic conditions (Agani and Semenza, 1998). Because mersalyl is a cell-impermeant compound with insulin-mimetic effects, it was hypothesized to act via a cell surface receptor. To test this hypothesis, mouse embryo fibroblasts that were wild type (W) or homozygous-null for a targeted mutation in the *Igf1r* gene encoding the IGF-1 receptor (R^-) were analyzed. In both W and R^- cells, expression of HIF-1α protein, HIF-1 DNA-binding activity, and VEGF mRNA expression are induced by hypoxia, cobalt chloride, or the iron chelator desferrioxamine. However, mersalyl induces these activities in W cells but not in R^- cells. Furthermore, in W cells the effect of mersalyl can be blocked by the MEK1 inhibitor PD098059, whereas the PI3K inhibitor wortmannin has no effect, indicating that the induction of HIF-1α protein expression by mersalyl is mediated via IGF-1R signaling to MEK1 (Agani and Semenza, 1998). Thus, it appears that within each cell type under investigation it will be necessary to determine which signalling pathways are activated by specific stimuli and whether they mediate HIF-1α protein stabilization or transactivation or both.

In many human solid tumors, autocrine growth factor loops are activated in which the cancer cells express both a growth factor and its cognate receptor-tyrosine kinase, such as EGFR or IGF-1R. The involvement of HIF-1α in the latter case is particularly striking (Figure 3): HIF-1α expression, which is induced by IGF-1R stimulation is in turn required for maximal *IGF2* gene expression (Agani and Semenza, 1998; Feldser et al., 1999). It is interesting to note that HIF-1α overexpression appears to represent a common occurrence in colon cancers (Zhong et al., 1999) and that *IGF2* is the most highly upregulated gene in colon cancer (Zhang et al., 1997).

E. VHL

The Von Hippel-Lindau (VHL) tumor suppressor gene was identified as the locus mutated in patients with the autosomal dominant von Hippel-Lindau syndrome (Latif et al., 1993). The occurrence of somatic mutations in the wild-type allele results in com-

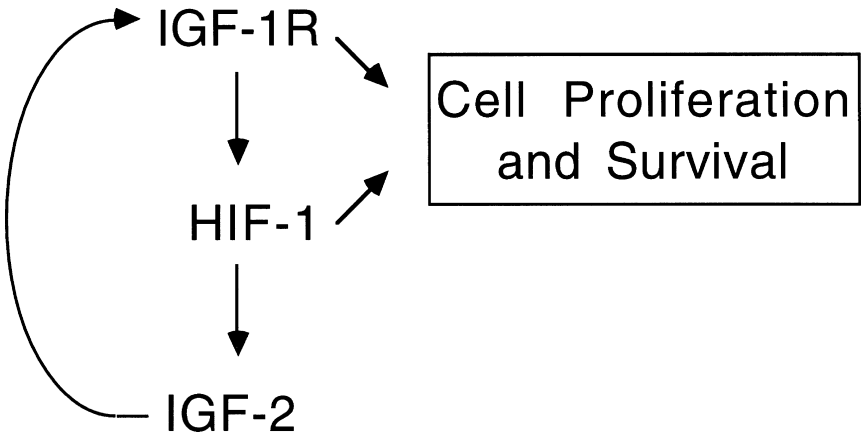

Figure 3. Involvement of HIF-1 in an autocrine growth factor loop in cancer cells.

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plete loss of VHL activity, which predisposes affected individuals to the development of retinal angioma, cerebellar and spinal hemangiomas, renal cell carcinoma, pheochromocytoma, pancreatic adenoma and islet cell tumor, epididymal cystadenoma, and endolymphatic sac tumors of the inner ear (reviewed by Ohh and Kaelin, 1999). In renal cell carcinoma lines lacking VHL activity, GLUT1 and VEGF mRNA was constitutively expressed at high levels in nonhypoxic cells, whereas introduction of a VHL expression vector results in low levels of GLUT1 and VEGF mRNA in nonhypoxic cells that increase in response to hypoxia (Gnarra et al., 1996; Iliopoulos et al., 1996). Expression of GLUT1 and VEGF mRNA is regulated by O_2 concentration at both the transcriptional (via HIF-1) and posttranscriptional levels, but these studies concluded that VHL specifically regulates mRNA stability in nonhypoxic cells. However, further analysis revealed that VHL-null renal cell carcinoma lines constitutively express HIF-1α and HIF-2α protein whereas, in VHL-transfected subclones, expression of these proteins is hypoxia inducible (Maxwell et al., 1999). Immunoprecipitation experiments demonstrate that VHL is associated with ubiquitin-protein ligase activity (Iwai et al., 1999; Lisztwan et al., 1999) and physically interacts with HIF-1α and HIF-2a (Maxwell et al., 1999), suggesting that VHL functions to target these proteins for ubiquitin-mediated proteasomal degradation under non-hypoxic conditions, as previously demonstrated (Huang et al., 1998; Kallio et al., 1999; Salceda and Caro, 1997).

These data provide a basis for the marked overexpression of VEGF and the highly vascular nature of cerebellar hemangioblastoma and renal cell carcinoma, as described above. What is less clear is the role of VHL in O_2-dependent regulation of HIF-1α protein stability, because VHL remains associated with HIF-1α and HIF-2α under hypoxic conditions (Maxwell et al., 1999). Thus, the mechanism by which hypoxia results in decreased ubiquitination of HIF-1α (Sutter et al., 2000) remains obscure. In contrast, treatment of cells with cobalt chloride or iron chelators, two other inducers of HIF-1α protein expression and HIF-1 DNA-binding activity (Jiang et al., 1997b; Wang et al., 1995a; Wang and Semenza, 1993), is associated with the disruption of the HIF-1α/VHL complex (Maxwell et al., 1999). These results support the hypothesis that loss of VHL binding leads to stabilization of HIF-1α under non-hypoxic conditions and demonstrate that cobalt chloride and iron chelators act via a mechanism distinct from that by which hypoxia induces HIF-1α expression. A second major unanswered question involves the number of different cell types in which loss of VHL activity is sufficient for constitutive expression of HIF-1α and/or HIF-2α. The limited number of relatively rare tumor types associated with the VHL syndrome suggests that other ubiquitin-protein ligases are involved in the regulation of HIF-1α protein expression in many cell types. This hypothesis is supported by recent data regarding the effect of p53 and MDM2, which are described below.

F. p53

In normal cells, p53 activity induced in response to DNA damage can lead to either apoptosis or cell cycle arrest at G_1 or G_2 (reviewed by Giaccia and Kastan, 1998; Levine, 1997; Semenza, 1998). Somatic mutations resulting in loss of p53 transcriptional activity occur in approximately 50% of all human tumors (Hollstein et al., 1991; Levine et al., 1991). Functional inactivation of p53 can also occur by overexpression of

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MDM2, a ubiquitin-protein ligase that targets p53 for ubiquitination and proteasomal degradation (Haupt et al., 1997; Kubbutat et al., 1997). The E6 protein of human papilloma virus (HPV) types 16 and 18 also binds to p53 and targets it for ubiquitination and degradation (Scheffner et al., 1990). However, E6 acts indirectly by recruiting the ubiquitin-protein ligase E6-AP (Scheffner et al., 1993).

Exposure of cells to hypoxia can also induce p53 activity and apoptosis in transformed cells (Graeber et al., 1994, 1996; Schmaltz et al., 1998). There is some debate as to whether p53 mediates apoptosis of tumor cells in response to hypoxia (Graeber et al., 1996) or hypoxia-induced lactic acidosis (Schmaltz et al., 1998). In either case, the extremely low O_2 concentrations within human tumors *in vivo* may select for cells that have lost p53 activity, as such cells would manifest increased resistance to hypoxia-mediated apoptosis (Graeber et al., 1996). *Hif1a*$^{-/-}$ mouse embryonic stem cells was resistant to apoptosis induced by complete O_2 and/or glucose deprivation (Carmeliet et al., 1998). Thus, HIF-1α shares in common with oncogenes, such as C-FOS, C-MYC, and CYCLIN D_1, proapoptotic properties that are counteracted by tumor-specific genetic alterations such as a loss of p53 activity (reviewed by Blagosklonny, 1999). The interaction between HIF-1α and p53 is physical as well as functional, as these proteins co-immunoprecipitate, and the binding of HIF-1α to p53 has been proposed to protect the latter from degradation, thus providing a potential mechanism for hypoxia-induced accumulation of p53 (An et al., 1998).

Loss of p53 activity is also associated with tumor angiogenesis (reviewed by Bouck, 1996). Vessel counts and VEGF expression are higher in colon cancers that express mutant p53 (Takahashi et al., 1998). Overexpression of p53 results in decreased VEGF expression in some cell lines (Bouvet et al., 1998; Mukhopadhyay et al., 1995), although such effects could be interpreted either as physiologic or an artifact of overexpression (Agani et al., 1997). *p53*$^{-/-}$ HCT116 human colon carcinoma cells, in which p53 expression was eliminated by homologous recombination (Bunz et al., 1998), demonstrate significantly increased growth, vessel density, vascular volume, and vascular permeability of tumor xenografts when compared with *p53*$^{+/+}$ *HCT116* cells (Ravi et al., 2000). The *p53*$^{-/-}$ cells express increased levels of VEGF mRNA and protein, as well as increased expression of HIF-1α protein, HIF-1 DNA-binding activity, and increased transcription of a reporter gene containing the *VEGF* hypoxia response element, under hypoxic culture conditions (Ravi et al., 2000). Thus, in addition to decreasing the expression of the antiangiogenic factor thrombospondin-1 (Dameron et al., 1994; Van Meir et al., 1994), loss of p53 activity also promotes angiogenesis by increasing expression of the angiogenic factor VEGF (Ravi et al., 2000).

HIF-1α mRNA expression is unaffected by p53 status, whereas the half-life of HIF-1α protein is increased in *p53*$^{-/-}$ cells and HIF-1α protein expression is markedly reduced in *p53*$^{-/-}$ cells transfected with an expression vector encoding p53. Expression of HPV16 E6 protein in PA-1 ovarian teratocarcinoma cells (that express wild-type p53) is associated with decreased p53 protein, increased HIF-1α protein and half-life, increased *VEGF* reporter gene expression, and increased VEGF protein levels. Ubiquitination of HIF-1α is reduced in *p53*$^{-/-}$ cells, whereas transfection with a p53 expression vector is associated with increased ubiquitination. MDM2 co-immunoprecipitates with p53 and HIF-1α and mutant forms of p53 that cannot interact with MDM2 do not affect HIF-1α expression. Finally, in *p53*$^{+/}$

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$^{+}$ cells, expression of mutant forms of MDM2 that can bind to p53 but are deficient in ubiquitin-protein ligase activity results in increased HIF-1α protein levels. These data indicate that p53 binding to HIF-1α targets the latter for ubiquitination by MDM2 and subsequent degradation (Ravi et al., 2000). Preferential ubiquitination of HIF-1α by MDM2 in the trimolecular complex may account for the HIF-1α-mediated stabilization of p53 described above. Most importantly, these data suggest that loss of p53 activity not only protects tumor cells from hypoxia-mediated apoptosis, but promotes metabolic adaptation and angiogenesis as a result of increased HIF-1 activity. Expression of mutant p53 is correlated with overexpression of HIF-1α in human cancers (Table 4). p53 has also been proposed to interfere with HIF-1-mediated transactivation by competitive binding to the coactivator p300 (Blagosklonny et al., 1998). However, p300 has been implicated in the MDM2-mediated degradation of p53 (Grossman et al., 1998), and therefore it is not clear whether there is an effect of p53 on HIF-1α transcriptional activation independent of its effects on protein stabilization.

VI. IMPLICATIONS FOR CANCER THERAPY

The studies reviewed above provide considerable evidence in support of the hypothesis that HIF-1 plays a critical role in the establishment and progression of common human cancers (Figure 4). HIF-1 activates the transcription of genes encoding proteins that mediate metabolic adaptation (glucose transporters and glycolytic enzymes), angiogenesis (VEGF), and cell survival (IGF-2). It is likely that only a fraction of the genes that are activated by HIF-1 have been identified thus far. HIF-1-regulated gene products are likely to play essential roles in invasion and metastasis as well as contributing to the increased resistance of hypoxic tumor cells to chemotherapy and radiation. Expression of the HIF-1α subunit, which determines HIF-1 activity, is markedly increased in human cancers and their metastases. Increased HIF-1α can be attributed in part to tumor hypoxia, but the basal level of HIF-1α expression in tumor cells is also markedly increased as a consequence of somatic mutations that activate oncogene products or inactivate tumor suppressors. The data summarized above suggest that a consequence of the clonal selection of cells that contain an increasing number of mutations in key oncogenes and tumor suppressor genes is a progressive increase in HIF-1α expression during tumor progression. Indeed, increased HIF-1α expression is likely to contribute significantly to the development of the lethal cancer phenotype.

The connection between alterations of key signal-transduction pathways and of HIF-1α expression underscores the essential role of HIF-1 in cellular physiology. Inhibitors of these signal transduction pathways, such as farnesyltransferase inhibitors, geldanamycin, rapamycin, wortmannin, and related compounds, are currently being evaluated as cancer chemotherapeutics (reviewed by Buolamwini, 1999; Cardenas et al., 1998; Oliff, 1999). It is possible that inhibition of HIF-1 activity may contribute significantly to their therapeutic efficacy. Screening for inhibitors that target HIF-1 directly may also lead to the development of novel anti-cancer drugs. The potential efficacy of combination therapy utilizing an angiogenesis inhibitor (Bergers et al., 1999; Boehm et al., 1997; reviewed by Jekunen and Kairemo, 1997; Keshet and Ben-Sasson, 1999) and a HIF-1 inhibitor is particularly appealing, because the former would deprive the tumor cells of O_2 and the latter would deprive them of adaptive mechanisms that would otherwise enable them to re-

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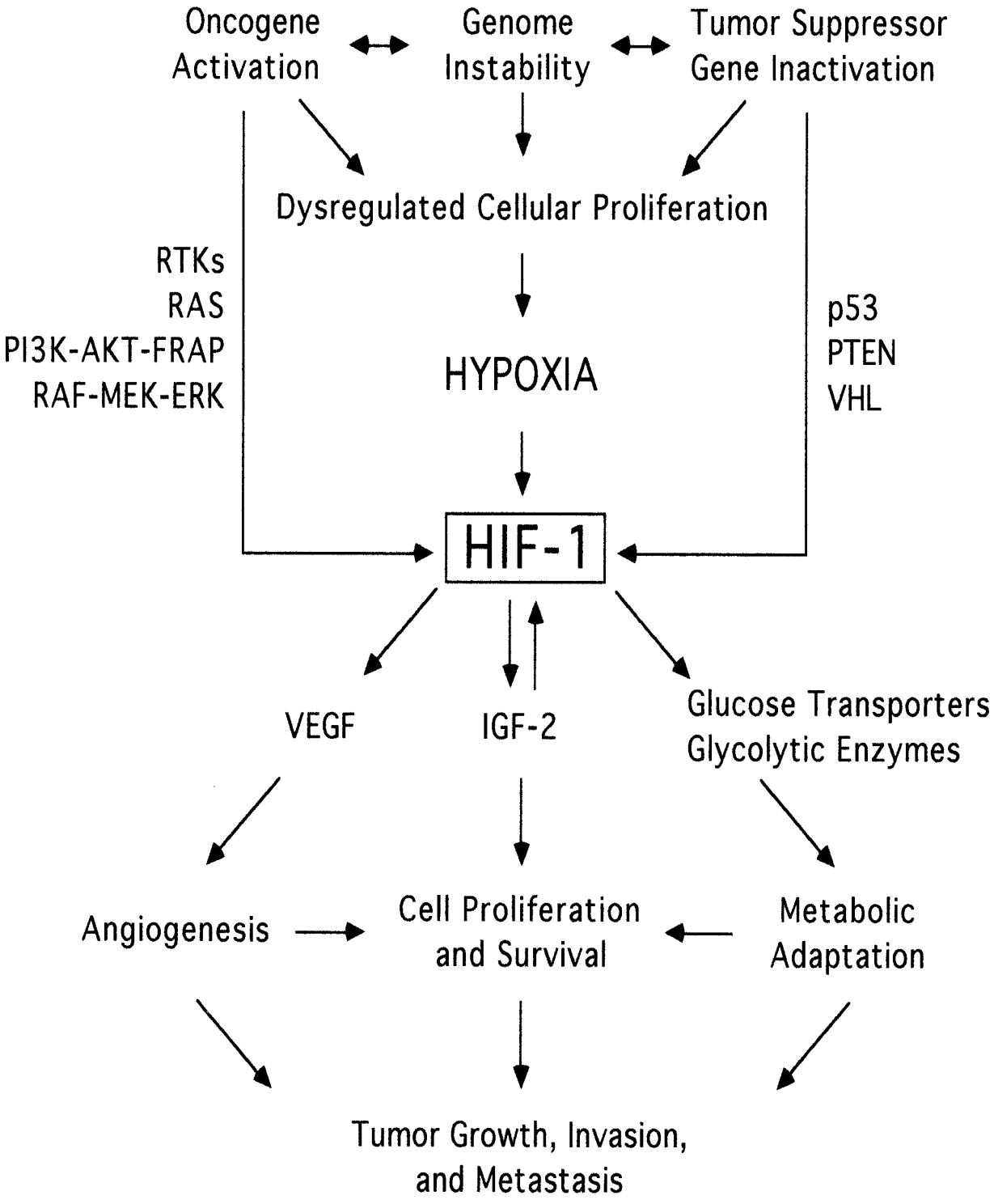

Figure 4. Expression of HIF-1 in human cancer: mechanisms and consequences.

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spond to the hypoxic stimulus. A gene therapy strategy utilizing vectors that contain hypoxia response elements in order to target expression of a chemotherapeutic gene product to HIF-1-overexpressing tumor cells has also been proposed (Dachs et al., 1997).

Since the purification of HIF-1 protein (Wang and Semenza, 1995) and cloning of HIF-1α cDNA sequences (Wang et al., 1995a) 5 years ago, rapid progress has been made in elucidating the role of this transcription factor in normal development and physiology as well as in cancer biology. The rate of knowledge acquisition in the field is now exponential and the accelerating pace of discovery hopefully will provide sufficient momentum for the transition from basic science to clinical application in the near future.

ACKNOWLEDGMENTS

Work in the author's laboratory is supported by grants from the Children's Brain Tumor Foundation and the National Institutes of Health.

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