Biochemical Pharmacology, Vol. 59, pp. 47–53, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00292-0

Expression of Hypoxia-inducible Factor 1: Mechanisms and Consequences

*Gregg L. Semenza**
THE JOHNS HOPKINS SCHOOL OF MEDICINE, CMSC-1004, BALTIMORE, MD 21287-3914, U.S.A.

ABSTRACT. Hypoxia-inducible factor 1 (HIF-1) is a basic-helix-loop-helix transcription factor that plays essential roles in mammalian development and physiology. HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits. The expression and activity of the HIF-1α subunit are tightly regulated by cellular O_2 concentration. Under hypoxic conditions, HIF-1 activates the transcription of genes encoding erythropoietin, glucose transporters, glycolytic enzymes, vascular endothelial growth factor, and other genes whose protein products increase O_2 delivery or facilitate metabolic adaptation to hypoxia. HIF-1 is essential for embryonic vascularization and survival, neovascularization in ischemic myocardium, hypoxia-induced pulmonary vascular remodeling, and tumor vascularization. HIF-1α is overexpressed in the majority of common human cancers and their metastases, due to the presence of intratumoral hypoxia and as a result of mutations in genes encoding oncoproteins and tumor suppressors. Pharmacologic manipulation of HIF-1 levels may provide a novel therapeutic approach to diseases that represent the most common causes of mortality in Western society, including cancer, chronic lung disease, and myocardial ischemia. BIOCHEM PHARMACOL **59**;1:47–53, 2000.

KEY WORDS. angiogenesis; cancer; ischemia; oxygen homeostasis; pulmonary hypertension

HYPOXIA-INDUCIBLE FACTOR 1: STRUCTURE AND FUNCTION

Cellular O_2 concentrations in the human body are precisely regulated to maintain adequate substrate for oxidative phosphorylation and other essential metabolic reactions while minimizing the production of ROS† capable of damaging cellular DNA, lipids, and proteins. HIF-1 is a transcription factor that is expressed in most, if not all, cells in response to hypoxia [1, 2]. HIF-1 activates transcription of genes whose protein products function either to increase O_2 delivery or to provide metabolic adaptation under conditions of reduced O_2 availability. Examples of the former category include erythropoietin and VEGF, which stimulate erythropoiesis and angiogenesis, respectively; examples of the latter category include the glucose transporters and glycolytic enzymes [reviewed in Ref. 3].

HIF-1 is a heterodimeric protein (Fig. 1) consisting of HIF-1α and HIF-1β subunits [4, 5]. Both subunits contain amino-terminal basic-helix-loop-helix-PAS (bHLH-PAS) domains that are required for dimerization and DNA binding [5, 6]. The consensus DNA sequence for HIF-1 binding is 5′-RCGTG-3′ [7]. HIF-1β, which was originally identified as the aryl hydrocarbon receptor nuclear translocator [8], is a subunit of several different bHLH-PAS heterodimers. Expression of the HIF-1α subunit is tightly regulated by the cellular O_2 concentration, increases exponentially as O_2 concentration declines, and determines the level of HIF-1 activity [5, 7, 9]. The half-life of HIF-1α in posthypoxic cells is less than 5 min [5, 10]. Under non-hypoxic conditions, HIF-1α is subject to ubiquitination and proteasomal degradation which is blocked under hypoxic conditions [11–14].

In addition to an increase in HIF-1α protein stability, the activity of the HIF-1α transactivation domains is dramatically increased in hypoxic cells [15–17]. Both the stability and transcriptional activity of HIF-1α are under negative regulation in non-hypoxic cells [6, 11–15], such that the factor is poised for rapid activation in response to hypoxia [5, 10]. Recently, cysteine 800 in the carboxyl-terminal transactivation domain (Fig. 1) has been shown to play a critical role in the interaction of HIF-1α with the coactivator CBP/p300, and stimulatory effects of thioredoxin and REF-1 suggest that a free (reduced) thiol group is required [17]. HIF-1 activity is also modulated by carbon monoxide and nitric oxide, which can inhibit HIF-1 DNA-binding activity in hypoxic cells without affecting HIF-1α protein expression [18, 19].

The molecular and physiological roles of HIF-1 were illustrated by a study in which near-term fetal sheep were subjected to chronic anemia *in utero* [20]. To compensate for the reduced O_2-carrying capacity, the anemic fetuses

Presented at Oxford, 10–12 June 1999.
* Corresponding author: Gregg L. Semenza, M.D., Ph.D., The Johns Hopkins University School of Medicine, CMSC-1004, 600 North Wolfe Street, Baltimore, MD 21287-3914. Tel. 1-410-955-1619; FAX 1-410-955-0484; E. Mail: gsemenza@jhmi.edu

† *Abbreviations:* HIF-1, hypoxia-inducible factor 1; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; and ES, embryonic stem cells.

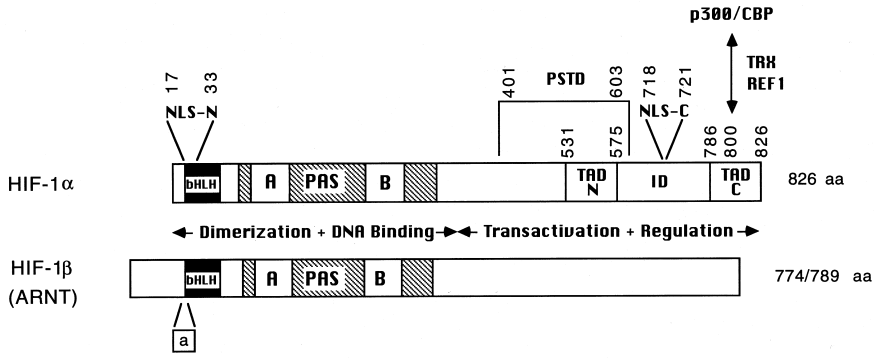

FIG. 1. Structure of HIF-1. Important functional domains of the HIF-1α and HIF-1β subunits are indicated as follows: a, alternate exon encoding 15 amino acids (aa) in HIF-1β; bHLH, basic-helix-loop-helix domain; ID, inhibitory domain; NLS-N and NLS-C, amino- and carboxyl-terminal nuclear localization signal; PAS, Per-ARNT-Sim homology domain with internal A and B repeats; PSTD, proline-serine-threonine-rich protein stability domain; TAD-N and TAD-C, amino- and carboxyl-terminal transactivation domain; REF-1, redox factor 1; TRX, thioredoxin.

increased their cardiac output by 50%, which was associated with myocardial hypertrophy/hyperplasia as evidenced by a 30% increase in the heart:body weight ratio. A consequence of increased myocardial work and mass was increased O_2 consumption, resulting in myocardial hypoxia that stimulated 3- to 5-fold increases in the expression of HIF-1α protein, VEGF mRNA, and VEGF protein, leading to significantly increased capillary diameter and capillary density in the hearts of anemic compared to control fetuses [20]. These results suggest that gene therapy aimed at overexpression of HIF-1α in ischemic myocardium may stimulate neovascularization via induction of VEGF and possibly other angiogenic factors (Fig. 2).

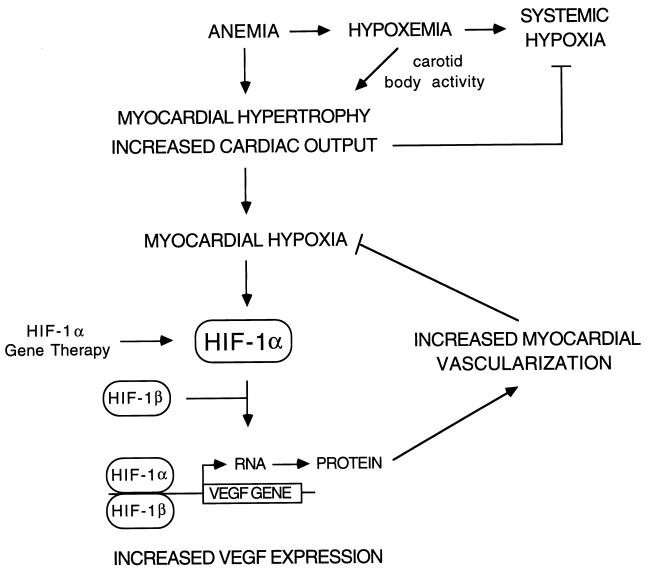

FIG. 2. Involvement of HIF-1 in physiologic responses to chronic anemia in fetal sheep. The potential use of HIF-1α gene therapy to treat myocardial ischemia in humans is also indicated.

MODELS OF HYPOXIA SIGNAL TRANSDUCTION

The mechanisms by which human cells sense decreased O_2 concentration and transduce this signal to induction of HIF-1 activity are not well understood. Two models have received particular attention and there are experimental data for and against each of them. According to model I (Fig. 3), an NADPH oxidase of unknown subcellular localization converts O_2 into superoxide, which is subsequently reduced by superoxide dismutase (SOD) to hydrogen peroxide [21, 22]. A reduction in O_2 concentration would result in reduced production of ROS (superoxide and/or hydrogen peroxide), which would affect cellular signal transduction pathways leading to HIF-1 induction. Spectrophotometric data have suggested involvement of a non-mitochondrial cytochrome b_{558} in this process [22, 23]. Exposure of cells to medium containing catalase or the antioxidant *N*-mercaptopropionyl-glycine induced expression of a hypoxia-inducible reporter gene under non-hypoxic conditions [14], data which are consistent with this

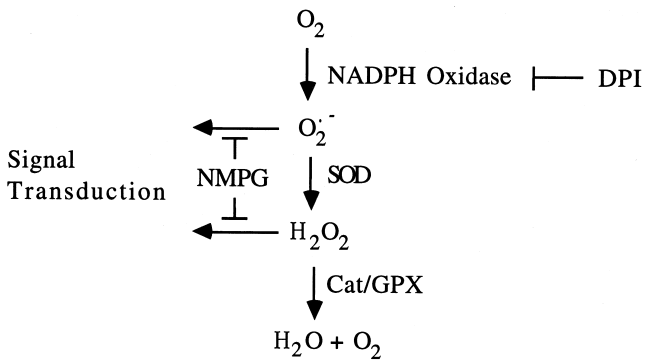

FIG. 3. Mechanisms of cellular oxygen sensing (model I): decreased ROS generation under hypoxic conditions. Decreased levels of superoxide and/or hydrogen peroxide signal hypoxia. Cat, catalase; DPI, diphenylene iodonium; GPX, glutathione peroxidase; NMPG, *N*-mercaptopropionyl-glycine; SOD, superoxide dismutase.

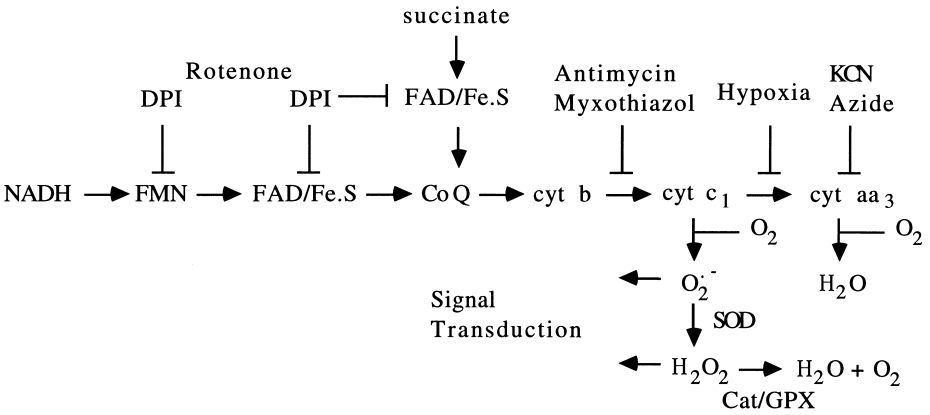

FIG. 4. Mechanisms of cellular oxygen sensing (model II): increased mitochondrial ROS generation under hypoxic conditions. Increased levels of superoxide and/or hydrogen peroxide signal hypoxia. DPI, diphenylene iodonium; Cat, catalase; SOD, superoxide dismutase; GPX, glutathione peroxidase; and cyt, cytochrome.

hypothesis. However, diphenylene iodonium (DPI), an inhibitor of NADPH oxidases, blocked the induction of HIF-1 in response to hypoxia [24], an effect opposite to what is predicted by model I.

According to model II (Fig. 4), hypoxia results in increased generation of ROS in the mitochondria [25], possibly resulting from decreased activity of cytochrome oxidase [26]. DPI and other inhibitors of complex I or III of the mitochondrial electron transport chain (ETC) blocked the induction of HIF-1 activity [25] and HIF-1α protein expression* in hypoxic cells. In contrast, inhibitors of complex IV, such as cyanide or azide, did not block HIF-1 induction [25; Agani *et al.*, see footnote], which is consistent with the generation of ROS at complex III but difficult to reconcile with the hypothesis that inhibition of cytochrome oxidase (complex IV) is responsible for this ROS generation. In response to hypoxia, there was no ROS generation or induction of HIF-1 activity in ρ^0 cells lacking mitochondrial ETC activity [25]. These data suggest that mitochondrial ETC activity is necessary for induction of HIF-1 in hypoxic cells, but do not rule out the possibility that signal transduction may require events in addition to mitochondrial ROS generation. HIF-1 was induced by exposure to cobalt chloride or desferrioxamine in cells with or without electron transport chain activity [25, 27; Agani *et al.*, unpublished], indicating that other cellular pathways that do not involve mitochondrial ROS generation can lead to HIF-1 activation. Given the fundamental importance of O_2 homeostasis, it seems unlikely that hypoxia signal transduction occurs via a simple linear pathway.

INVOLVEMENT OF HIF-1α IN CELLULAR AND DEVELOPMENTAL O_2 HOMEOSTASIS

To investigate the essential biological functions of HIF-1α, mouse ES cells were generated in which the *Hif1a* gene was inactivated by homologous recombination [28–30]. Analysis of *Hif1a*$^{+/+}$, *Hif1a*$^{+/-}$, and *Hif1a*$^{-/-}$ ES cells revealed that HIF-1α was required for the induction of at least 13 different genes encoding glucose transporters and glycolytic enzymes in response to hypoxia, which represents the most extensive example of coordinate transcriptional control of a metabolic pathway in mammals [29]. HIF-1α was also required for induction of the *Vegf* gene in response to hypoxia [28–30]. These results confirmed the role of HIF-1α as an essential transcriptional activator of these genes, as first demonstrated by reporter gene cotransfection assays [7, 31]. As a measure of cellular physiology, *Hif1a*$^{+/+}$ and *Hif1a*$^{-/-}$ ES cells were cultured under hypoxic (1% O_2) and non-hypoxic (20% O_2) conditions for 24–48 hr. The proliferation of *Hif1a*$^{-/-}$ cells was significantly reduced, especially under hypoxic culture conditions [29].

Injection of *Hif1a*$^{+/-}$ ES cells into mouse blastocysts resulted in germline transmission. Although *Hif1a*$^{+/-}$ mice were viable, *Hif1a*$^{-/-}$ embryos arrested in development by E9.0 and died at E10.5 with cardiac, vascular, branchial arch, and neural tube defects and extensive cell death, especially in the branchial and cephalic regions [29, 30, 32]. Initial vasculogenesis of *Hif1a*$^{-/-}$ embryos occurred normally, but was followed by dramatic vascular regression and remodeling by E9.25 that was preceded by the death of premigratory and postmigratory neural crest cells in the neurosomatic junction and cephalic mesenchyme, respectively [32]. Cranial neural crest cells are the progenitors of pericytes which are required for maintenance of vascular integrity, and the loss of these cells appears to have played a major role in the observed vascular regression. *Hif1a*$^{-/-}$ embryos also manifest hyperplasia of the presumptive myocardium and outflow tract obstruction [29]. It is therefore unlikely that a functional circulatory system was established and the embryos were therefore deprived of O_2 and glucose. *Vegf* expression was induced by glucose deprivation but not by hypoxia in HIF-1α-deficient ES cells [29], and glucose deprivation was shown to induce VEGF mRNA without inducing HIF-1α protein in wild-type cells, which provided

* Agani F, Feldser D and Semenza GL, unpublished data.

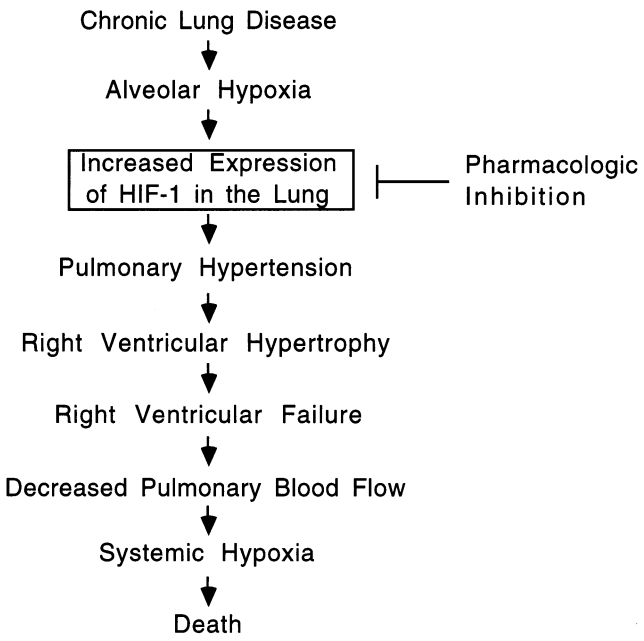

FIG. 5. Pathophysiology of hypoxic pulmonary hypertension in patients with chronic lung disease. Potential therapeutic intervention via pharmacologic inhibition of HIF-1 activity is also indicated.

an explanation for the discovery of increased, rather than decreased, VEGF mRNA levels in *Hif1a*$^{-/-}$ embryos [32].

INVOLVEMENT OF HIF-1α IN POSTNATAL PHYSIOLOGY

The physiologic effects of complete HIF-1α deficiency could not be ascertained due to the death of *Hif1a*$^{-/-}$ embryos at mid-gestation [29]. However, analysis of adult *Hif1a*$^{+/+}$ and *Hif1a*$^{+/-}$ mice revealed that partial HIF-1α deficiency was associated with significantly impaired responses to chronic hypoxia such as the development of polycythemia, right ventricular hypertrophy, pulmonary artery hypertension, and pulmonary vascular remodeling [33]. Expression of HIF-1α was induced by hypoxia in a variety of pulmonary cell types, including vascular endothelial and smooth muscle cells, both *in vivo* and in cell culture [10]. These results suggest that local pharmacologic inhibition of HIF-1 activity in the lungs of at-risk individuals with chronic obstructive lung disease might prevent the development of pulmonary hypertension, which is a major cause of morbidity and mortality in this patient population (Fig. 5).

INVOLVEMENT OF HIF-1α IN CANCER

A hallmark of cancer is dysregulated cellular proliferation. Increased cell numbers result in increased O_2 consumption and hypoxia. In order for tumors to grow beyond a volume of several mm^3, the tumor must become vascularized [reviewed in Ref. 34]. There is also a direct correlation between intratumoral vessel density and metastasis [reviewed in Ref. 35]. The important role of VEGF as a mediator of tumor vascularization is well established [34, 35]. However, the tumor vasculature is often unable to maintain a normal level of oxygenation, due to the high rate of cell proliferation and the dysfunctional nature of the vessels that form [36]. Adaptation to hypoxia appears to be an important step in tumor progression. Cervical cancers with $pO_2 < 10$ mm Hg exhibited larger tumor extensions, more frequent invasion of adjacent structures, greater resistance to therapy, and higher rates of patient death relative to tumors with $pO_2 > 10$ mm Hg [37]. Soft-tissue sarcomas with $pO_2 < 10$ mm Hg had a 2-fold greater probability of metastasis than tumors with $pO_2 > 10$ mm Hg [38]. One important adaptation to hypoxia is the Warburg effect, an increased rate of glycolysis even under aerobic conditions, which represents one of the most universal characteristics of solid tumors [39].

Recent immunohistochemical studies using a monoclonal antibody specific for HIF-1α indicate that overexpression of HIF-1α occurs in most common human cancers as a response to physiologic (hypoxia) and non-physiologic (genetic alterations) stimuli [40]. HIF-1α overexpression was detected in >90% of colon, lung, and prostate cancers analyzed, whereas no expression was detected in the corresponding normal tissues. For breast cancer, <1/3 of primary tumors but >2/3 of metastases overexpressed HIF-1α, as did cases of ductal carcinoma *in situ*, which represents the earliest detectable malignant lesion, suggesting that HIF-1α may represent an early biomarker for aggressive disease. In contrast, non-malignant tumors such as breast fibroadenoma and uterine leiomyoma did not overexpress HIF-1α [40].

Among brain tumors, HIF-1α overexpression was detected in high-grade, highly vascularized tumors but not in low-grade, poorly vascularized tumors. Among high-grade brain tumors, two patterns of expression were detected. In glioblastoma multiforme, HIF-1α was detected in pseudopalisading cells surrounding areas of necrosis [40] in a pattern consistent with hypoxia-induced expression that was identical to that previously described for VEGF mRNA [41, 42]. These results were also consistent with the analysis of hepatoma cell lines which were wild-type or deficient for HIF-1 expression [43, 44]. When injected into nude mice, the wild-type cells formed larger and more vascularized tumors than the mutant cells. In tumors derived from wild-type cells, areas of necrosis were surrounded by intense VEGF mRNA expression, which was absent around necrotic areas of tumors derived from mutant cells [44]. Thus, in these human cancers and mouse xenografts, hypoxia-induced expression of HIF-1 appears to be essential for vascularization and other aspects of tumor progression.

A second pattern of expression emerged from the analysis of hemangioblastomas, which are among the most well-vascularized human tumors. HIF-1α expression was detected in most tumor cells including those immediately adjacent to blood vessels, indicating that a stimulus other

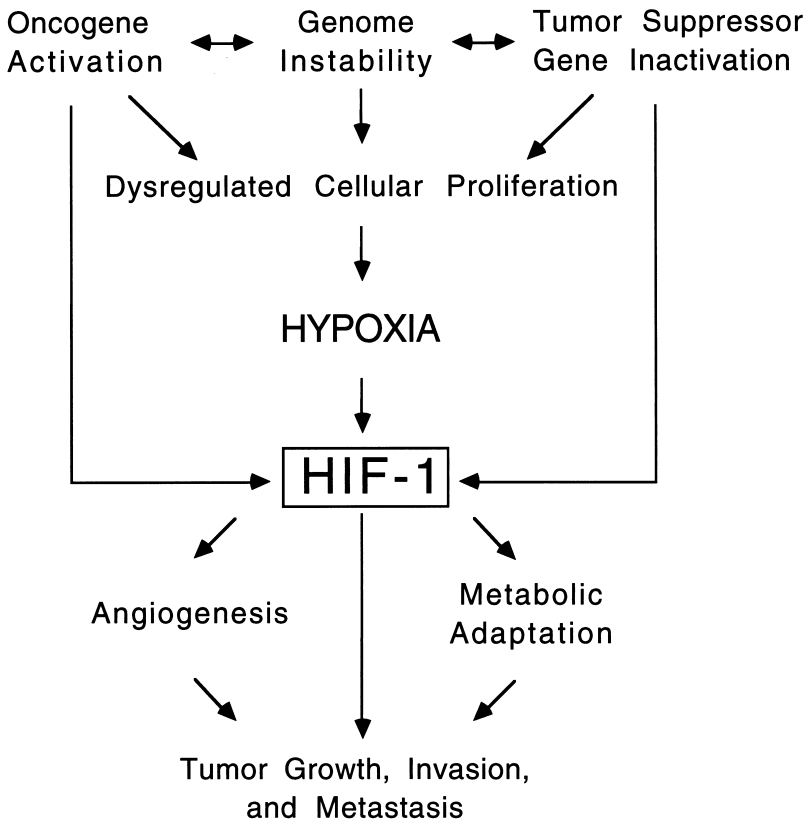

FIG. 6. Mechanisms and consequences of HIF-1α overexpression in cancer.

than hypoxia was driving its expression [40]. A similar pattern was observed in clear cell renal carcinoma. Recent studies indicate that loss of the VHL (von Hippel–Lindau) tumor suppressor is directly responsible for overexpression of HIF-1α in renal carcinoma cell lines [45], and VHL loss-of-function has also been detected in hemangioblastoma [46]. HIF-1α overexpression was also correlated with expression of mutant p53 in human cancers, again suggesting that loss of tumor suppressor activity may contribute to HIF-1α overexpression [40].

Among a variety of human tumor types analyzed, there was a strong correlation between HIF-1α overexpression and Ki67 labeling index as a measure of cell proliferation [40]. HIF-1α expression was induced by treatment of cultured cells with insulin, insulin-like growth factors 1 and 2, fibroblast growth factor 2, or epidermal growth factor, and induction in response to insulin occurred at low, but not high, cell density, suggesting that HIF-1α expression was associated with growth factor-induced cell proliferation [47]. HIF-1α was in turn required for expression of IGF-2, suggesting its participation in an autocrine growth factor loop. *IGF2* was the most highly up-regulated gene in colon cancer [48] and HIF-1α was overexpressed in all 22 colon cancers analyzed [40]. The expression of HIF-1α in human prostate cancer lines was also greater when cultured at low, as compared to high, cell density [49]. The correlation between cell proliferation and HIF-1α expression may relate to the observation that when quiescent thymocytes are stimulated to divide they switch from oxidative phosphorylation to glycolysis as a means of ATP production, perhaps in order to reduce generation of ROS that might damage replicating DNA [50]. In addition to the IGF-2 autocrine loop described above, other oncogene products may induce HIF-1α overexpression. V-SRC, the tyrosine kinase oncoprotein of Rous sarcoma virus, was shown to induce expression of HIF-1α under non-hypoxic conditions, leading to increased transcription of target genes encoding VEGF and the glycolytic enzyme enolase 1 [43]. Thus, a variety of oncogene products that function in growth factor signal transduction pathways may induce HIF-1α expression.

Taken together, these studies suggest that HIF-1α overexpression may be important for tumor vascularization and metabolic adaptation to hypoxia, which are in turn essential for tumor progression to the malignant metastatic phenotype (Fig. 6). Further studies are required to determine whether HIF-1α overexpression in breast and possibly other cancers might identify a subpopulation at high risk of metastasis for whom particularly intensive therapy would be indicated. The fact that intratumoral pO_2 is significantly lower than in normal tissues suggests that inhibitors of HIF-1 might provide a novel pharmacologic approach to cancer therapy, especially in combination with anti-angiogenic agents [51] that would further reduce tumor oxygenation.

CONCLUSIONS

Hypoxia is a critical pathophysiological component of the major causes of mortality in Western cultures. HIF-1 appears to play a major role in a wide variety of responses to hypoxia. It therefore represents a novel therapeutic target, provided that appropriate means of delivery and dosage can be established to prevent undesirable side effects. In the case of myocardial ischemia, local overexpression of HIF-1α via coronary catheterization–gene therapy appears a promising approach to promoting neovascularization, whereas in chronic lung disease local pharmacologic inhibition of HIF-1 activity via inhalation therapy might be beneficial in preventing hypoxia-induced pulmonary vascular remodeling. In cancer, combination systemic therapies that include pharmacologic inhibitors of HIF-1 might also prove efficacious. The critical involvement of hypoxia in human pathophysiology has been remarkably underappreciated to date, and it is hoped that as greater resources are brought to bear on this problem exciting scientific advances will be translated rapidly into clinical practice.

G. L. S. is an Established Investigator of the American Heart Association and work in his laboratory is supported by grants from the American Heart Association National Center and Maryland Affiliate, the National Institutes of Health (R01-DK39869 and R01-HL55338), and Genzyme Corporation. Under licensing agreements between Johns Hopkins University and Genzyme Corporation and Novus Biologicals, Inc., G. L. S. is entitled to a share of royalties received from sales of technologies described in this article. G. L. S. is also a consultant to Genzyme. The terms of this arrangement are managed by the University in accordance with its conflict of interest policies.

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