HIF-1: An Oxygen Response System with Special Relevance to the Kidney

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Requirement of Cells for Oxygen

All metazoan organisms are committed to respiration, using oxygen as the terminal electron acceptor in a reaction that is carried out by cytochrome c oxidase. The consequence is that cells need a continuous supply of oxygen. In higher organisms such as humans, an extensive infrastructure is required to achieve this. It includes the airways, lungs, heart, and vascular network, but the “organ” most clearly devoted to this task is the erythron—consisting of 2 × 10^{12} cells, occupying 2 L, and carrying 99% of the oxygen in the blood. The size of this circulating organ is regulated through the action of erythropoietin (EPO), which acts as a survival factor for red blood cell precursors.

A fundamental challenge underlying much of the physiology and anatomy of higher organisms is avoiding local oxygen starvation. The efficiency with which we meet this challenge means that it is easy to take it for granted. Adding to the precision that is required, excess oxygen is potentially dangerous, with the ability to generate reactive species that may damage proteins and nucleic acid. Hypoxia-inducible factor-1 (HIF-1) is a transcription complex that was isolated as a factor controlling EPO gene expression (1) and is now recognized to be centrally involved in many aspects of meeting these challenges.

The HIF-1 system operates in all mammalian cell types examined to date but has specific relevance to the kidney in several ways. First, HIF-1 underlies regulation of EPO, and the kidney is the dominant site of production. Intriguing is that expression of EPO is regulated by oxygen and is unique to the oxygen response pathway.

How HIF-1 Responds to Oxygen

At the heart of the HIF-1 system, molecular oxygen reacts with HIF-α proteins in a way that switches their ability to interact with other proteins (Figure 1). The HIF-α chain is regulated by two enzymatic reactions in which molecular oxygen reacts with specific amino acid residues (7–9). The addition of a single 16-Da oxygen atom to a prolyl residue in the central region of these approximately 120 kD proteins leads to capture by the VHL tumor suppressor protein (7,8). Both of these reactions are catalyzed by 2-oxoglutarate–dependent dioxygenases, which belong to an extended family of enzymes with diverse biologic roles (10,11). The extended family includes collagen prolyl 4 hydroxylase (necessary for collagen chain assembly), AlkB (involved in DNA repair), and Phytanoyl Co-A 2-hydroxylase (defects in which underlie many cases of Refsum’s disease) (10–13).
several enzymes in the family (including the HIF asparaginyl hydroxylase FIH-1) have now been solved, and the enzymes are based on a β barrel jelly roll motif, coordinating a non-heme ferrous iron atom at the catalytic center (14–16). The iron is coordinated at three positions, by two histidines and a carboxylate, leaving the other three positions available to interact with oxygen, 2-oxoglutarate, and the prime substrate during the reaction process. In the HIF hydroxylase reactions, one atom of molecular oxygen is used to convert 2-oxoglutarate to succinate and carbon dioxide. The other atom from the oxygen is added to the HIF substrate. Several characteristics of EPO regulation can be explained by the properties of the enzymes. First, EPO production can be provoked by exposure to cobalt. In fact, accidental or experimental exposure of humans to cobalt increases red blood cell production sufficiently to cause erythrocytosis, and it seems that heavy metal exposure contributes to chronic mountain sickness in some areas (17,18). The explanation is that when other atoms are substituted for ferrous iron in the catalytic center, the enzymes can no longer oxidize HIF, which remains able to activate target genes despite the presence of oxygen. Second, depleting intracellular iron can also inactivate the enzymes, explaining why iron chelators can mimic the effect of hypoxia in cultured cells and why systemic administration of desferrioxamine in humans and mice can increase EPO production (19,20).

HIF transactivation is regulated by hydroxylation of an asparaginyl residue (9). In the absence of this modification, HIF-α interacts with the CH1 pocket of CBP/P300, which results in transactivation of target genes (21,22). However, in the presence of oxygen, the enzyme FIH-1 (for factor-inhibiting HIF) adds an oxygen to the β carbon of Asn803 of HIF-1α, or the equivalent residue in HIF-2α (23–25). This modification is highly unfavorable to the interaction with CBP/P300 and thus prevents HIF-α from recruiting the basal transcription machinery when cells are oxygenated.

HIF destruction is regulated by conversion of a prolyl residue in the middle portion of the molecule. The system is most clearly understood in the nematode Caenorhabditis elegans, where it is less complicated than in mammals and more genetic information is available. C. elegans has a single isoform of each of HIF-α, the hydroxylase enzyme eg9, and the E3 ligase recognition component vhl. In the presence of oxygen, eg9 converts Pro621 of hif1 to 4-hydroxyproline (26). The modified hif1 is then captured by vhl, which leads to ubiquitylation and destruction. In low oxygen, hif1 is not oxidized (because eg9 requires molecular oxygen as a co-substrate) and is stabilized. Worms that carry mutant alleles for vhl or eg9 show constitutive activation of hif1.

**Complexity of HIF-α Prolyl Hydroxylation in Mammals**

In mammals, the HIF system is more complicated than in the worm. This is not entirely surprising given that the worm has no specialized oxygen distribution systems. Thus, in mammalian cells, there are at least two different oxygen-responsive HIF-α chains, termed HIF-1α and HIF-2α, both of which are subject to VHL-dependent destruction by the proteasome (5). Homozygous genetic inactivation of either HIF-1α or HIF-2α in the mouse embryo is lethal, showing that in embryonic development, these proteins are not functionally redundant (27–30).

It is unclear to what extent HIF-1α and HIF-2α may be selective for particular subsets of HIF-1 responsive genes when the proteins are expressed at normal levels and the target genes are in their normal chromosomal setting. Although HIF-2α was initially thought to be endothelial specific, it is expressed by a very wide range of cell types (31–33). That intestinal cells in the kidney express HIF-2α, rather than HIF-1α, in response to hypoxia suggests that HIF-2α may be particularly involved in control of EPO production (34). This is further supported by the observation that decreasing HIF-2α expression decreases retinal EPO production in hypoxia (35).

Another homologous polypeptide, HIF-3α, has also been reported to be a target for oxygen-regulated prolyl hydroxylase and VHL-mediated ubiquitylation (36). An alternative splice variant of this gene, IPAS, which lacks the transactivation domain, can act as a dominant inhibitor of the HIF response and seems to be important in suppressing the hypoxic response in certain tissues, such as the cornea (37).

In mammalian HIF-1α and HIF-2α, two different prolyl residues can be subject to enzyme-mediated oxygen-dependent hydroxylation followed by VHL capture (38). Mutation of either site partially protects HIF-α chains from destruction, but mutation of both sites increases the effect.

Three different candidate prolyl hydroxylase genes were identified on the basis of homology to eg9 and other dioxy-
genes; they have been termed PHD1, PHD2, and PHD3 (for prolyl hydroxylase domain) (26). Alternative names are HIF-1 for PHD1, HIF-2 for PHD2, and HIF-3 for PHD3 (for homologues of eg9) (39,40). Each of the three PHD enzymes is capable of hydroxylating the target prolyl residues in mammalian HIF-α chains in vitro studies, and overexpression of each enzyme has been shown to downregulate HIF-1. As yet, the relative contributions of these three enzymes to the regulation of HIF-1 is not clear, but in a biochemical approach, purification of the prolyl hydroxylase activity from rabbit reticulocyte lysate isolated EGLN2/HIF-PH1/PHD2 (41). An additional candidate HIF prolyl hydroxylase gene has been termed PH-4 (42).

The amounts of the three different prolyl hydroxylase enzymes is variable. Because the hydroxylation reaction is not at equilibrium, it is anticipated that increasing the level of hydroxylase enzyme would downregulate the HIF response to moderate hypoxia. Expression of the prolyl hydroxylase genes can be increased in hypoxic cells, which is likely to provide an element of feedback inhibition on the HIF-1 response (26,43,44). Furthermore, it is clear that the relative expression of the three genes is variable from one cell type to another and that PHD3 expression is modulated by many stimuli (44–46).

In addition to the hydroxylation events, HIF is modulated in other ways. For example, HIF is phosphorylated and was recently shown to be acetylated, and these modifications likely contribute to regulating HIF (47,48). Overall, there is scope for considerable complexity in the way that HIF operates in mammalian cells, which is presumably important in tailoring the basic oxygen response system to different cellular requirements.

Role of the VHL Tumor Suppressor Protein in HIF Regulation

The VHL tumor suppressor protein is critical for the normal regulation of HIF-1 because it is required to ubiquitylate HIF-α subunits in the presence of oxygen. In this process, VHL acts as the recognition component of a ubiquitin E3 ligase complex that includes several other proteins—elongin C, elongin B, RBX1, and CUL2. The initial observations showing that VHL is required for HIF regulation were made in renal carcinoma cell lines (5). Importantly, it is now clear that VHL is necessary for regulated HIF destruction in many other (possibly all) cell types in metazoan organisms, because disabling VHL in C elegans, D melanogaster, CHO-K1 cells, mouse embryonic stem cells, myeloid cells, and hepatocytes results in constitutive HIF activation in each case (49–53).

After hydroxylation of either Pro564 or Pro402, HIF-1α is captured by the VHL tumor suppressor protein (38,54,55). A crystal structure of the overall complex of the hydroxylated HIF target peptide with elongin B and elongin C shows that the hydroxyl group fits into a surface pocket, with hydroxylation permitting the formation of two hydrogen bonds (56,57). After capture, HIF-α chains are ubiquitylated and destroyed by the proteasome. In cells that lack VHL, HIF-α chains are constitutively stable, and the HIF-1 system is activated even when oxygen is present (5). An interesting question is why asparaginyl hydroxylation of the transactivation domain by FIH-1 does not inactivate HIF-α effectively in the absence of VHL. Possibilities include another enzyme that reverses the HIF-1 hydroxylation reaction, that FIH-1 enzyme activity in these cells is too low to inactivate HIF-1, or that VHL has a role in inactivation of the C terminus (58).

Range of Processes Influenced by HIF-1

An early finding was that the oxygen responsive system underlying EPO regulation was not restricted to EPO-producing cells, suggesting that it was involved in regulating other target genes (59). HIF-1 actually operates in all cell types and has a key role in matching supply and demand for oxygen throughout the organism.

HIF-1 activation has a diverse range of effects. Illustrating this activation of HIF-1 in particular cells has consequences within the cell itself and in the local environment and can also be used to adjust parameters that affect the whole organism.

Cell Autonomous Responses. HIF-1 regulates glucose utilization in a very wide variety of cells. Activation of HIF-1 in response to hypoxia results in increased expression of glucose transporters and glycolytic enzymes (27). This adapts the individual cell to lower oxygenation by enhancing its ability to produce ATP through glycolysis if mitochondrial metabolism becomes compromised. HIF-1 activation also influences decisions concerning cell proliferation and apoptosis (60,61). Not surprising, this is more variable from one cell type to another, presumably reflecting integration of the HIF signal with other signals reflecting aspects of the internal and external environment.

Distant Control. HIF-1 controls production of the circulating hormone EPO. In this case, cells in the kidney sense a change in oxygen delivery and generate a circulating signal that indirectly affects the whole organism.

Local Coordination and Architecture. HIF-1 is a major regulator of angiogenesis. As a consequence of HIF activation, cells increase their production of angiogenic growth factors, such as vascular endothelial growth factor, in response to reduced oxygen. As with EPO regulation, monitoring oxygenation provides a feedback control system—because increased blood vessel growth will increase oxygen delivery. In this case, the sensing and signaling are general properties of parenchymal cells and result in a local signal.

As yet, our understanding of the role of HIF-1 is certainly incomplete, but an idea of the broad capabilities of the system is the range of HIF target genes summarized in Table 1.

At What Level of Oxygenation is HIF-1 Activated, and How Does This Relate to Values Experienced In Vivo?

In terms of understanding the role of the HIF-1 system in physiology and disease, an important question is the relationship between local pO2 and HIF activation. In cell culture under standard conditions, HIF-1 is usually barely detectable by electrophoretic mobility shift assay or by immunoblotting for HIF-α subunits. As the level of oxygen is reduced toward approximately 0.5%, the activity of HIF-1 progressively increases (62). An uncertainty in many experiments is that un-
The level of HIF-1 activity in cell lines cultured under standard normoxic conditions is variable from one cell type to another, and in a number of cancer cell lines there is appreciable activation of the HIF-1 system under basal conditions. HIF-1 thus can influence gene expression not only when oxygen is reduced but also under standard culture conditions. The extent to which this occurs is likely to vary between cell types. Evidence for such an effect comes from studies of cells with genetic defects in HIF-1 components that show that HIF-1 is contributing to basal levels of expression of genes encoding, for example, glycolytic enzymes (64). It is increasingly apparent that many pathways that enhance expression of genes encoding, for example, glycolytic enzymes comes from studies of cells with genetic defects in HIF-1 components that show that HIF-1 is contributing to basal levels of expression of genes encoding, for example, glycolytic enzymes (64). It is increasingly apparent that many pathways that enhance expression of genes encoding, for example, glycolytic enzymes (64).

Predicting from tissue culture experiments when HIF-1 is activated in vivo is complicated by uncertainties over the actual levels of pO2 experienced by cells in tissues. The best established method is using polarographic microelectrodes, but the spatial resolution is relatively low and the electrode disturbs and damages the tissue and also consumes oxygen (68). However, it is clear that within tissues, there is often great heterogeneity in pO2, and readings of 10 to 40 mmHg are common in normal tissues, with readings in malignant tumors generally being lower (69).

Taken together, these observations make it probable that HIF-1 influences gene expression in some cells in normally perfused tissues and will be activated in settings such as ischemia and solid tumors. A current challenge is to determine the precise circumstances in which HIF-1 is activated in tissues and what effect this has.

Assessing HIF-1 Activation In Vivo

In principle, several different approaches could be used to study when and where HIF-1 is activated and what the consequences of this activation are.

Immunolabeling of HIF-α. Because HIF activation correlates with the protein level of HIF-α subunits, immunolabeling these subunits should be useful in assaying when and where HIF activation is occurring. There are caveats. First, HIF-α protein could be present but could be transcriptionally inactive, for example because it has been subjected to hydroxylation of the C-terminal transactivation domain. Second, during the tissue handling and processing, HIF-α could be artificially destroyed or stabilized as the steps involved could expose the protein to higher or lower levels of oxygen than were present in the intact tissue. Using this approach has shown striking accumulation of HIF-α subunits in normal tissues in response to hypoxia and has shown high levels of HIF-α protein in malignant tumors and after ischemia (33,34,70–72).

Expression of HIF Target Genes. Another approach is to examine the expression of genes that are targets of HIF-1 and whose expression therefore reflects HIF activation. However, these genes will often be acted on by other regulators of gene expression besides HIF-1; furthermore, the range of genes subject to HIF-1 control varies from one cell type to another. Nevertheless, the expression of certain genes or combinations of genes could be used to examine HIF-1 activation. Furthermore, in experimental animals, transgenes linked to an HIF response element can be used to assess HIF activation. Endogenous genes that can be used to examine HIF activation in the

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<td><strong>Red blood cell production</strong></td>
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<td><strong>Vascular architecture and tone</strong></td>
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<td><strong>Energy metabolism</strong></td>
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<td><strong>Cellular proliferation, differentiation, and viability</strong></td>
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<td><strong>Negative feedback</strong></td>
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<td><strong>Miscellaneous</strong></td>
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VEGF, vascular endothelial growth factor.
kidney include EPO (fibroblasts) and carbonic anhydrase IX (CAIX; epithelial cells). CAIX is a surface membrane carbonic anhydrase whose expression is normally restricted to gastric epithelium. The protein is expressed at a high level in most CCRCC, and in renal carcinoma cells expression was shown to be effectively suppressed by VHL (73). Subsequently, CA9 was shown to be an HIF target gene, and immunohistochemical labeling for CAIX provides a useful marker for HIF activation in many tumors (74,75). In renal epithelium, CAIX provides a sensitive marker for individual cells in which VHL inactivation has occurred (76) (Figure 2).

Effects of HIF Inactivation. Comparison of animals with genetic inactivation of HIF-1 could be used to dissect the role of HIF-1 in vivo. Unfortunately, this is currently limited because homozygous inactivation of HIF-1α, HIF-2α, or HIF-1β/aryl hydrocarbon receptor nuclear translocator is lethal during embryonic development (28–30,64,77). At present, this therefore requires tissue and/or temporal control of the genetic inactivation. This approach has now been used in several cell lineages and clearly shows that HIF-1 is important in many aspects of the normal organism—for example, in inflammatory cells and cartilage (53,78). As yet, tissue-specific deletion of HIF-2α has not been studied, but it has been shown that HIF-2α is important in controlling aspects of lung maturation, sympathetic nervous system development, and vascularization of the retina (29,35,79). As yet, however, the consequences of HIF inactivation have not been described in cell populations in the kidney. The accompanying review by Abrahamson discusses the role of HIF-1 in renal development.

Oxygenation in the Kidney
A number of intriguing aspects relating to local oxygenation in the kidney are likely to be relevant to the role of HIF-1 in this organ. Furthermore, an exciting possibility is that we can use our understanding of the cells’ own HIF-1 oxygen-response pathway to gain new insight into the actual level of oxygen experienced by individual cells in the normal mammalian kidney and in pathologic settings. A caveat is that the relationship between oxygenation and HIF-1 activation may vary from one cell type to another and/or be substantially influenced by environmental changes such as an altered cytokine milieu.

The kidneys receive a higher blood flow per unit mass than other organs in the body. The fraction of oxygen extracted by the whole organ is relatively low compared with other organs. Despite this, the kidney is very sensitive to conditions of hypoperfusion (3). This relates to high levels of local oxygen consumption by tubular epithelial cells and also to aspects of the renal vasculature. The close apposition of the vessels taking blood to and from the medulla not only results in counter-concentration of osmolytes in the medulla but also prevents much of the oxygen in the descending vasa recta from reaching the inner medulla. Less well recognized is that diffusion of oxygen directly from arterial to venous vasa recta seems to remove approximately 50% of the oxygen contained in arterial blood before it arrives at the glomerulus. This probably occurs mainly at the level of the interlobular vessels (2).

Although there are uncertainties about physical measurements of oxygen in tissues, microelectrode studies indicate that low partial pressures are common not only in the medulla but also in the cortex. In one study, approximately 20% of all readings in the cortex were <5 mmHg (2).

Activation of HIF-1 in the Kidney
In one recent immunolabeling study, HIF-1α and HIF-2α were not detected in the rodent kidney in unstimulated adult animals, even in the renal medulla. Another study using a different antibody reported some nuclear HIF-1α in the distal convoluted tubule and did not detect any in other cell types, but it would be incorrect to conclude that HIF-1 is not influencing gene expression in the kidney under normal circumstances.

Figure 2. Identification of foci of HIF activation in the kidney of a patient with a germline mutation in the VHL gene. (Left) Labeling for carbonic anhydrase IX. Membrane labeling (brown) of a group of tubular epithelial cells in which the second copy of the VHL gene is inactivated. (Right) An adjacent section labeled for Tamm-Horsfall protein. The focus lies within the cortical thick ascending limb and illustrates that VHL inactivation is associated with loss of THP expression (courtesy of Dr. S. Mandriota, University Medical Center, Division of Oncology, Geneva, Switzerland).
because the relationship between the threshold for immunode-
tection and target gene activation is unknown.

When oxygen delivery was decreased by exposing the ani-
mals to 0.1% carbon monoxide, HIF-α subunits were detect-
able at the protein level in many cells (34). This stimulus
induces a functional anemia, with approximately 50% of the
hemoglobin being unavailable for oxygen transport. Under
these circumstances, HIF-1α and HIF-2α were detected in
different cell populations. In the cortex and outer medulla,
HIF-1α was expressed in epithelial cells, whereas HIF-2α was
expressed in fibroblasts and endothelial cells. Because the
fibroblasts of the outer medulla and the cortex produce EPO,
this suggests that HIF-2α may be responsible for regulating
EPO production (Figure 3). Another interesting aspect of this
study is that functional anemia activated HIF-1α more in
tubular cells in some parts of the nephron than in others, the
most marked induction being in the S2 segment of the cortical
proximal convoluted tubule and the collecting ducts. It is not
yet known to what extent this observation could relate to
different levels of local oxygenation and to what extent it
reflects differential sensitivity of the HIF system in different
cell types. Nevertheless, that proximal tubular profiles showed
HIF activation while adjacent distal tubular profiles did not
favor the possibility that cells in the proximal tubule activate
the HIF system more readily. One potential reason for this is
different levels of HIF hydroxylase activity, as a result of
different levels of expression of the enzymes or different
co-factor/co-substrate availability.

Renal ischemia is a more complex stimulus than reducing
oxygen delivery. An important question is the extent to which
compromising or interrupting renal perfusion will activate
HIF-1. The data available show that total ischemia in the rat
kidney does result in activation of HIF-1α but that this is
generally less extensive and less intense than is seen when
oxygen delivery is reduced (34). This suggests that HIF acti-
vation in renal ischemia is likely to be submaximal and there-
fore that increasing HIF activation should be feasible and may
be of therapeutic benefit in this setting.

EPO Production

EPO regulation has provided an important paradigm for
studying the physiology and molecular biology of adaptive
responses to changes in oxygenation (80). Important reasons
include the magnitude of the response and that changes in
oxygen delivery are the dominant variable influencing
expression.

The kidney is the major site of EPO production in the adult,
with the liver being the main site antenatally. Although se-
quences important in governing expression in the liver and in
the kidney have been identified using a transgenic approach
(81–83), our knowledge of how the switch from liver to kidney
production is regulated is limited. Because the timing of the
switch relative to birth is very different in different mammals,
it is clear that it is not primarily determined by the alterations
that occur in the circulation and oxygenation at the time of
birth. A potentially relevant observation is that the ability to
produce substantial amounts of EPO is greatly increased in
regenerating liver after partial hepatectomy or in the recovery
phase of viral hepatitis (84,85).

The EPO gene is expressed in other cells besides the kidney,
for example, in the brain, the eye, and the testis (86,87). In
these sites, EPO expression is also oxygen regulated. Furth-
more, the EPO receptor is not restricted to red cell precursors
and has been identified on endothelial cells and renal epithelial
cells (88,89). Together, these observations suggest that EPO
may have other roles besides regulating erythropoiesis, partic-
ularly in the central nervous system, where the action of local
EPO would be isolated from circulating levels of the hormone.
There has been considerable recent interest in the possibility
that EPO could be protective to neurons and photoreceptors
(87,90).

Our knowledge concerning the way in which EPO produc-
tion is regulated in the kidney and in particular why this fails
in renal disease remains limited. One reason for this is no renal
fibroblast cell line that expresses the EPO gene has yet been

Figure 3. Expression of erythropoietin (EPO) and HIF-2α by cells in
the interstitium in response to anemia. (A) Kidney from an acutely
anemic mouse bearing a knock-in marker gene at the EPO locus,
leading to expression of a nuclear antigen (SV40 large T) when the
EPO gene is expressed (82). In response to anemia, interstitial cells
throughout the cortex and the outer medulla accumulate the marker
(black nuclear labeling). (B) Double-labeling for SV40 large T
(brown, nuclear) and ecto-5′-nucleotidase (red), which labels fibro-
blasts. (C) Kidney from a rat exposed to CO, labeled with antibody to
HIF-2α. Nuclear signal (black) is seen in peritubular endothelial cells
(solid arrow) and peritubular fibroblasts (open arrow). (Reproduced
from Rosenberger et al. (34), courtesy of Dr. C. Rosenberger.)
generated. When hematocrit is in the normal range, the kidney produces a low level of EPO, with EPO expression limited to a small number of interstitial fibroblasts in the deep cortex and superficial outer medulla (82,91,92). Anemia results in a dramatic increase in EPO production, resulting in a rise in up to 3 orders of magnitude in the circulating level. EPO is not significantly stored before secretion, and increased production correlates with an increase in EPO mRNA in the kidney, largely achieved by increased gene transcription but likely to be contributed to by stabilization of its mRNA when oxygenation is reduced. The increased production involves progressive recruitment of additional interstitial fibroblasts in a pattern that spreads outward from the deep cortex toward the capsule and the inner medulla. Intriguing is that individual fibroblasts seem to be recruited in an all-or-none manner (91). At maximal stimulation, it seems that almost all of the fibroblasts of the cortex and the outer medulla activate expression of the EPO (82).

In most patients with substantially impaired renal function, EPO production is impaired at any given hematocrit. In principle, this could be because the EPO-producing cells are lost, because they experience higher levels of oxygen for a given hematocrit, or because the relationship between local oxygenation and EPO production is altered. Some clues as to how EPO is dysregulated in renal disease come from clinical observations that EPO production is commonly maintained in patients with inherited or acquired cystic disease and is impaired early in some patients with diabetes and in patients with primary autonomic neuropathy (93–95). Even in patients who are treated with dialysis, the remnant kidneys produce some EPO, and before the advent of recombinant EPO, it was widely recognized that removal of the native kidneys increased trans- 
fusion requirements. After transplantation, erythrocytosis may occur, and selective sampling suggests that this relates to dysregulated EPO production by the native kidneys (96). Intriguing is that angiotensin-converting enzyme inhibitors and AT1 receptor antagonists are effective in the treatment of erythrocytosis that follows transplantation or exposure to altitude (97).

Clinical Manifestations of VHL Loss of Function

The VHL tumor suppressor protein is required to regulate HIF-1, and VHL mutations occur in humans and are associated with disease. What can this tell us about the role of HIF-1 in humans and the effect of HIF activation? In considering this, it is important to appreciate that although HIF is the best characterized pathway that is affected by VHL loss of function, VHL status has been reported to have a range of effects within the cell that are not clearly related to HIF activation. Nevertheless, the clinical manifestations of VHL mutations are likely to give us important insights. Two inherited syndromes are associated with mutations in VHL: an autosomal dominant tumor syndrome and a more recently recognized autosomal recessive erythrocytosis.

Autosomal Dominant VHL Disease

Approximately 1 in 36,000 humans inherits a germline mutation in the VHL gene, giving rise to the classical hereditary tumor syndrome. This involves a very high lifetime risk of hemangioendothelioma (HAB) in the retina and central nervous system and of cysts and CCRCC in the kidney. The individuals are also at risk for developing phaeochromocytoma (PHE), endolymphatic sac tumors, and cysts and nonsecreting neuroendocrine tumors in the pancreas. Other recognized manifestations are papillary cystadenomas of the epididymis (men) and broad ligament of the ovary (women) (6). In the tumors, the remaining wild-type copy of the gene has been altered by a second hit, related to a somatic mutation or epigenetic inactivation. Importantly, inactivation of both copies of the VHL gene is not restricted to CCRCC in families with VHL disease; the great majority of sporadic CCRCC have loss of function of both VHL alleles (98). As first shown by Kaelin and colleagues (99), reexpression of a VHL gene product in cell lines from CCRCC has a dramatic effect on tumor growth as xenografts in nude mice. These findings establish that VHL is a tumor suppressor gene that conforms to Knudson’s two-hit hypothesis.

Autosomal dominant VHL disease has been subdivided on the basis of incidence of PHE, HAB, and CCRCC in kindreds into type I, IIA, IIB, and IIC. Interesting genotype-phenotype correlations are that deletions of the gene tend to be associated with a lower risk of PHE (type 1 disease), whereas certain missense mutations carry a reduced risk of CCRCC (type IIB disease). Challenges are to understand which cellular pathways link VHL loss to clinical manifestations and explaining why the consequences are restricted to specific organs. One way to address this is to compare the clinical consequences of subtle mutations in VHL with their effects on HIF regulation. For HAB, mutations associated with risk (types I, IIA, and IIB) impair HIF regulation, whereas other mutations (type IIC) do not (100,101). This would be consistent with dysregulated HIF underlying the development of HAB. PHE predisposition does not correlate with the effect on HIF regulation, because type IIC mutations do not seem to alter VHL’s ability to regulate HIF. For CCRCC, clinical risk and effect on HIF regulation are overlapping but not congruent, because type IIA mutations do affect the ability to regulate HIF. One possibility is that another target of VHL is important for CCRCC development. Alternatively, it may be that CCRCC development requires complete abrogation of HIF regulation given that the type IIA mutations seem to retain a partial ability to regulate HIF in renal carcinoma cells (100).

Two other approaches have investigated the role of HIF dysregulation in renal carcinoma. One route has been to ask whether a constitutively active HIF molecule can reverse tumor suppression by VHL in renal cancer cells. It was recently demonstrated that HIF-2α protein mutated at a residue that is a target site for hydroxylation and HIF destruction restores tumor growth (102). Although this does not test the role of HIF in the initiation of tumors in the kidney, it does suggest that suppression of HIF is required for the tumor suppressor action.
of VHL in fully transformed renal cancer cells. A second approach provides some insight into initiation. Immunolabeling of CAIX was used to detect HIF activation in kidney specimens from patients with VHL (76). This study showed that all morphologically abnormal areas, including cysts and dysplastic tubules, showed evidence of HIF activation, suggesting that HIF activation is an immediate consequence of VHL loss. Many other foci of VHL loss that were not morphologically abnormal were identified, most of which consisted of a single cell, suggesting that these foci were not associated with an increase in the rate of proliferation relative to cell death. Multicellular foci seemed to be restricted to the distal tubule, suggesting that the effect of VHL on net proliferation may be specific to this part of the nephron and that CCRCC in patients with VHL may arise from these cells (Figure 2). It is interesting that the earliest lesions contained HIF-1α, but the cysts and complex lesions also contained HIF-2α, which seems not to be present in hypoxic renal epithelial cells (34). An important possibility is that activating HIF-2α increases proliferation in these cells.

Autosomal Recessive VHL Disease

An interesting recent development is the recognition that some individuals and families with primary polycythemia are homozygotes or compound heterozygotes for mutations in the VHL gene (103). The main population in which this has been detected is in Chuvashia, in the former USSR. The Chuvash mutation affects a residue (Arg200) that is outside the surface that interacts with elongin C and that is distant from the region known to contact HIF-α. In functional assays, this mutation seems to have a more subtle effect on HIF regulation, consistent with its being tolerated as a homozygous mutation. Although phenotypic characterization of affected individuals is as yet incomplete, not only are EPO levels inappropriately elevated but also red cell precursors are more responsive to EPO (103). This suggests that in addition to anemia increasing EPO production by the kidney, activation of HIF-1 in hematopoietic cells in the marrow contributes to increased erythropoiesis. Whether individuals with these defects in both copies of the VHL gene are predisposed to HAB and CCRCC will clearly be of interest and is relevant to the safety of long-term therapeutic manipulation of the HIF pathway.

Therapeutic Potential of Manipulating HIF-1

Inhibiting the activity of the HIF hydroxylase enzymes offers a way of activating HIF-1 in normal tissues and of potentiating the HIF response in moderate hypoxia. Potential applications of this are to promote erythropoiesis and improve outcomes in ischemic conditions.

It is interesting that a compound developed as an inhibitor of collagen prolyl-4-hydroxylase, which also inhibits HIF hydroxylases, was recently shown to improve outcome in a rat model of myocardial infarction (104). A concern arises from the fact that such an approach would activate HIF-1 in all cells with widespread effects, but it is likely that selectivity among the different enzymes will be achievable, allowing a degree of targeting. Conversely, decreasing HIF-1 activation could be an attractive strategy in solid tumors if it could be achieved, especially in CCRCC.

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