Erythropoietin (Epo) is a glycoprotein hormone that is the principal regulator of red blood cell production. In the adult, it is produced primarily in the kidney in response to hypoxia or reduced hemoglobin. Hypoxic regulation of Epo production is due to activation of the Epo gene by HIF-1 (hypoxia Inducible factor-1), a heterodimeric transcription factor comprising HIF-1 α and HIF-1 β subunits. In the presence of oxygen, the HIF-1 α subunit is rapidly proteolyzed by a mechanism involving hydroxylation of specific prolyl residues and interaction with von Hippel-Lindau protein (pVHL). In reduced oxygen, HIF-1 α is stabilized, resulting in enhanced gene transcription. The Epo gene is but one of numerous genes, such as the pro-angiogenic VEGF, that are regulated in this manner. The inherited cancer syndrome von Hippel-Lindau disease is characterized by a mutated VHL gene leading to the absence of hypoxic gene regulation. The result is the constitutive expression of normally hypoxically regulated genes, including Epo. Some cases of renal cysts and renal cancer are caused by somatic mutations of the VHL gene in the renal tissue, and these cases often exhibit increased Epo production and the paraneoplastic syndrome of erythrocytosis. Those renal cancers resulting from other mutations but possessing normal VHL genes do not produce Epo. Also, there is heterogeneity of VHL gene deletions within individual renal cell carcinoma tumors, leading to a more complex clinical picture.

Key words: erythropoietin, von Hippel-Lindau, hypoxia, gene regulation.

Erythropoietin, von Hippel-Lindau protein and renal cancer: molecular basis for the paraneoplastic syndrome of erythrocytosis

Erytropoeytyna, białko von Hippel-Lindau i rak nerki: molekularne podstawy towarzyszące chorobie nowotworowej zespołu erytrocytozy

Arthur J. Sytkowski

Laboratory for Cell and Molecular Biology, Division of Hematology and Oncology, Beth Israel Deaconess Medical Center, Department of Medicine, Harvard Medical School, Boston, MA 02215 USA

A striking characteristic of neoplastic processes is the partial or total release of the involved tissue from control by those factors that regulate development and differentiation. A consequence of this release from control is the occurrence of several paraneoplastic syndromes due to secretion by the tumor cells of one or more substances. The secretion of these substances may be the result of the expression of a gene not normally expressed in the tissue, and, hence, a totally foreign molecule may be synthesized, e.g., ACTH secretion by bronchogenic carcinoma (“ectopic” hormone production). On the other hand, the substance may be secreted by the tissue normally, but the tumor, being independent of feedback processes, elaborates an abnormally large amount of the particular hormone and/or its precursors. One well-characterized example is the insulinoma, which secretes insulin at a rate often independent of plasma glucose levels and, moreover, may secrete large quantities of the precursor molecule proinsulin so as to achieve a plasma proinsulin/insulin ratio far greater than that resulting from normal secretion by the pancreatic islets.

Renal cancer, or more specifically adenocarcinoma or hypernephroma, frequently can be associated with such paraneoplastic syndromes [1], a point of substantial clinical importance since a significant fraction of patients with this tumor present with systemic but without specific genitourinary symptomatology. Like bronchogenic carcinoma, renal adenocarcinoma may secrete hormones or other substances not secreted by the normal organ parenchyma, i.e., ectopic production. Examples include parathyroid hormone, enteroglucagon and chorionic gonadotropin. But it must be emphasized that the kidney has several endocrine functions of its own, and excess production of renin, prostaglandins and erythropoietin (Epo) have all been reported in patients with renal adenocarcinoma. It is the molecular mechanism of Epo production by renal adenocarcinoma that will now be addressed.

Epo is a glycoprotein hormone that is the prime regulator of red blood cell production. It also has numerous non-hematopoietic actions (reviewed in [2]). In the adult, it is produced primarily by interstitial fibroblasts in the renal cortex. Its rate of secretion (corresponding to expression of the Epo gene) is regulated by hypoxia (see below). Renal tumors may secrete excess Epo by at least two different mechanisms. Firstly, the expanding mass may cause

**Słowa kluczowe:** erytropoetyna, von Hip-pel-Lindau, niedotlenienie, regulacja ekspresji genów.

partial obstruction of adjacent blood vessels resulting in localized areas of hypoxemia. This mechanism is the likely cause of increased Epo levels found in some patients with benign renal cysts. It may, of course, also be operative in some patients with malignant renal lesions. However, the second mechanism, that of true synthesis of Epo by the malignant tissue itself, is the more intriguing one, despite the difficulty in establishing it in a given case.

**Identification of hypoxia inducible factor-1 (HIF-1)— hypoxia regulates more than the erythropoietin gene**

In 1993, Wang and Semenza described hypoxia inducible factor 1 (HIF-1) [3, 4]. It is a nuclear factor from Hep3B cells, an Epo-producing hepatoma cell line, that was detected when cells were cultured in 1% oxygen but not in 20% oxygen. Hypoxia also induced it in several cell lines that did not express Epo. HIF-1 DNA binding activity disappeared rapidly when hypoxic cells were exposed to increased oxygen. HIF-1 rapidly associates with and dissociates from its DNA binding site in *vivo* (T_{1/2}<1 min for both processes). The authors also showed that the iron chelator desferrioxamine induced both Epo expression and HIF-1 binding activity [5]. Desferrioxamine induced Epo gene expression in the kidneys of mice in *vivo*. Importantly, desferrioxamine also induced HIF activity in Epo non-producing cells, suggesting “a common hypoxia signal transduction pathway leading to HIF-1 induction in different cell types.”

HIF-1 activates the transcription of numerous genes other than erythropoietin (see [6]). Semenza et al. showed that transcripts encoding the glycolytic enzymes aldolase A, phosphoglycerate kinase 1 and pyruvate kinase M were induced in cells by exposure to HIF inducers (1% oxygen, cobalt chloride or desferrioxamine). Partial gene sequences from these enzymes contained nucleotide sequences related to the HIF-1 binding site of the Epo enhancer and bound HIF-1 specifically. These glycolytic enzyme gene sequences containing HIF-1 binding sites were shown to mediate hypoxia inducible transcription, further demonstrating the importance of hypoxic regulation outside of the erythropoietin system.

HIF-1 is a heterodimer [7]. Both subunits of HIF-1 (α and β) are basic helix-loop-helix (bHLH) proteins containing a PAS domain. The PAS domain is common to the *Drosophila* PER and SIM proteins and the mammalian ARNT (aryl hydrocarbon receptor nuclear translocator) and AHR proteins. HIF-1α is related to SIM and HIF-1β is one of a series of ARNT gene products. Thus, the designations “HIF-1α” and “ARNT” are often used interchangeably throughout the literature.

The importance of HIF-1 action in hypoxic regulation of gene expression was further emphasized when it was shown that vascular endothelial growth factor (VEGF) gene transcription was activated by HIF-1. Forsythe et al. observed that VEGF induced angiogenesis in several clinically important situations including myocardial ischemia, retinal disease and tumor growth and that HIF-1 was responsible [8]. Interestingly, in demonstrating that a 500 nt region of the 3’ UTR of VEGF mRNA was critical for stabilization of VEGF mRNA [9, 10], Levy et al. observed that the protein mRNA complex was elevated in cells lacking the von Hippel-Lindau tumor suppression gene. This observation was to have great importance in the elucidation of the oxygen sensor.

**Other interacting proteins and the regulation Of HIF-1**

The regulation of Epo gene expression as well as other hypoxia inducible genes requires several regulatory proteins in addition to HIF-1 α and β. In a study of 11 different orphan nuclear receptors, Galson et al. screened their ability to bind to elements in the Epo promoter and enhancer by electrophoretic mobility shift assay [11]. Four of these receptors bound
specifically to the response elements in the Epo promoter and enhancer, namely, hepatic nuclear factor 4 (HNF-4), TR2-11, ROR α 1 and EAR3/COUP-TFI. By ectopically expressing HNF-4 in HeLa cells, the authors observed an 8-fold increase in hypoxic induction of a reporter gene construct containing the minimal Epo enhancer and promoter. Thus, HNF-4 is an important positive regulator in tissue specific and hypoxia inducible expression of the Epo gene.

The homologous transcription adaptors p300/CRB and CBP also play a role in hypoxic regulation. Arany et al. searched for specific p300 binding proteins and found that HIF-1 α interacted with p300 [12] and that hypoxia induced the formation of a DNA binding complex containing both HIF-1 α and p300/CBP. p300/CBP-HIF complexes are important in the regulation of hypoxia induced genes.

Ebert and Bunn provided an important insight into the complex role played by p300/CRB in hypoxic regulation [13]. They analyzed the protein multimer that binds to the lactate dehydrogenase A (LDH-A) promoter and demonstrated the involvement of HIF-1, p300/CRB and CREB-1/ATF-1. Also, Bunn and his colleagues demonstrated the mechanism of regulation of HIF-1 α. Huang et al. identified an oxygen dependent degradation domain (ODDD) within HIF-1 α that controlled degradation by the ubiquitin-proteasome pathway [14]. Deletional mutagenesis of the domain yielded a HIF-1 α that was stable and active in the absence of hypoxic induction.

The von Hippel-Lindau protein, proline hydroxylation and the oxygen sensor

The von Hippel-Lindau protein was the seminal clue that led to the identification of the oxygen sensor. Von Hippel-Lindau (VHL) disease is an inherited disorder in which individuals have a predisposition to develop clear cell renal carcinoma, pheochromocytoma, spinal cord cerebellar and retinal hemangioblastoma [15-21]. Hemangiomas of other organs (adrenals, lungs, liver) and multiple pancreatic and carcinomas, pheochromocytomas, pancreatic tumors and cysts in several organ systems [20]. Within the central nervous system, individuals may develop retinal hemangioblastomas, endolymphatic sac tumors and cranial spinal hemangioblastomas of the cerebellum, brain stem, spinal cord, lumbar sacral nerve roots and supratentorial structures. Disorders of the viscera include renal cell carcinoma and cysts, pheochromocytomas, pancreatic tumors or cysts, epididymal cystadenomas and broad ligament cystadenomas. Table 1 (modified from reference [20]) shows the age of onset and frequency of VHL disease lesions.

Cockman et al. demonstrated that pVHL was essential for an oxygen dependent degradation domain (ODDD) mediated destruction of HIF-1 α by the ubiquitin-proteasome pathway [14, 24]. HIF-1 α ubiquitylation was defective in VHL deficient renal carcinoma cells and that exogenous expression of pVHL complemented this defect. This effect was specific for HIF-1 α subunits. They went on to demonstrate that short sequences within the internal transactivation domains of HIF α were sufficient for recognition by pVHL. Mutagenesis studies delineated the structural requirement for this interaction. The authors concluded that pVHL regulated HIF α degradation by functioning as a "recognition component of a ubiquitin ligase complex".

In a critical discovery, Jaakkola et al. showed that HIF-1 α targeting to the pVHL ubiquitin E3 ligase complex was dependent upon oxygen regulated prolyl hydroxylation [25]. Studies of HIF-1 α demonstrated that proline 564 hydroxylation was critical for this interaction [26, 27]. The enzyme responsible for this reaction was designated as HIF α prolyl hydroxylase (HIF-PH). The absolute requirement for oxygen as a cosubstrate and iron as a cofactor suggested that HIF-PH functioned directly as a cellular oxygen sensor. Hydroxylation of proline 402 provides yet another site for pVHL binding [28].

In addition to prolyl hydroxylation that regulates the hypoxia inducibility/stabilization of HIF-1 α, hydroxylation at another site in HIF-1 α plays a distinct role. The C-terminal activation domain (CAD) of HIF-1 α and its regulation involve hydroxylase activity that is not dependent upon pVHL [29]. This CAD hydroxylation is on asparagine 803 of HIF-1 α. Indeed, a hypoxia inducible HIF asparagine hydroxylase, identical to a previously identified HIF interacting protein (HIP) [30], was shown to downregulate HIF α transactivation and was later shown to interact with HIF-1 α and pVHL, thus mediating repression of HIF-1 α transcriptional activity. This asparagine hydroxylation abrogated p300 binding to HIF-1 α. Figure 1 depicts the results of HIF-1 α proline and asparagine hydroxylation.

Von Hippel-Lindau (VHL) disease and acquired VHL mutations

Von Hippel-Lindau disease is an autosomal dominant cancer syndrome resulting from a germ line mutation in the VHL gene [16-21]. Mutation or loss of the VHL gene results in a propensity to develop numerous benign or malignant tumors, as well as cysts in several organ systems [20]. Within the central nervous system, individuals may develop retinal hemangioblastomas, endolymphatic sac tumors and cranial spinal hemangioblastomas of the cerebellum, brain stem, spinal cord, lumbar sacral nerve roots and supratentorial structures. Disorders of the viscera include renal cell carcinoma and cysts, pheochromocytomas, pancreatic tumors or cysts, epididymal cystadenomas and broad ligament cystadenomas. Table 1 (modified from reference [20]) shows the age of onset and frequency of VHL disease lesions.

Since VHL protein plays a critical role in the regulation of the Epo gene, it is not surprising that some VHL disease
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Tumors can produce Epo constitutively. Renal cell carcinomas or cysts and cerebellar hemangioblastoma are most frequently associated with elevated circulating Epo levels [31-39]. There is no clear reason why other VHL disease lesions may not also produce Epo. Perhaps they do, but not at levels high enough to be detected clinically. There appears to have been no systematic assessment of this phenomenon among VHL disease patients or their pathological tissues.

Acquired VHL mutations also occur most frequently in clear cell renal carcinoma [40-46]. Loss of chromosome 3p or loss of heterozygosity can result in inactivation of VHL. The incidence of erythrocytosis associated with renal adenocarcinoma has been reported to be between 1-5% [39, 47] and has not been correlated with the frequency of VHL mutations. Besides loss or mutation of VHL, other genes have been associated with renal cell carcinoma including FHIT, FOXP1 and others [40, 46, 48-53]. This potential

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**Table 1. Von Hippel-Lindau disease lesions**

<table>
<thead>
<tr>
<th>CENTRAL NERVOUS SYSTEM</th>
<th>Mean (range) age of onset, years</th>
<th>Frequency in patients (%)</th>
</tr>
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<tbody>
<tr>
<td>Retinal hemangioblastomas</td>
<td>25 (1-67)</td>
<td>25-60%</td>
</tr>
<tr>
<td>Endolymphatic sac tumors</td>
<td>22 (12-50)</td>
<td>10%</td>
</tr>
<tr>
<td>Craniospinal hemangioblastomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cerebellum</td>
<td>33 (9-78)</td>
<td>44-72%</td>
</tr>
<tr>
<td>Brain stem</td>
<td>32 (12-46)</td>
<td>10-25%</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>33 (12-66)</td>
<td>13-50%</td>
</tr>
<tr>
<td>Lumbosacral nerve roots</td>
<td>Unknown</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>Supratentorial</td>
<td>Unknown</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>VISCERA</th>
<th>Mean (range) age of onset, years</th>
<th>Frequency in patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal cell carcinoma or cysts</td>
<td>39 (16-67)</td>
<td>25-60%</td>
</tr>
<tr>
<td>Pheochromocytoma</td>
<td>30 (5-58)</td>
<td>10-20%</td>
</tr>
<tr>
<td>Pancreatic tumor or cyst</td>
<td>36 (5-70)</td>
<td>35-70%</td>
</tr>
<tr>
<td>Epididymal cystadenoma</td>
<td>Unknown</td>
<td>25-60%</td>
</tr>
<tr>
<td>Broad ligament cystadenoma</td>
<td>Unknown</td>
<td>25-60%</td>
</tr>
</tbody>
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**Fig. 1.** Regulation of HIF-1 α by hydroxylation of proline 402 and 564 and asparagine 803

**Ryc. 1.** Regulacja HIF-1 alfa przez hydroksylację proliny 402 i 564 oraz asparaginy 803
multiplicity of causes of renal cell carcinoma may account for the relatively low incidence of Epo production by this tumor. Also, there is heterogeneity of VHL gene deletions within individual renal cell carcinoma tumors [41].

Other erythropoietin-producing tumors

Other, non-VHL associated tumors, may also produce Epo. One of the most common is hepatocellular carcinoma [54-56]. Epo gene expression by these cells may simply reflect “de-differentiation” from the adult to a more fetal phenotype (the liver is the principal source of Epo production in the fetus). Also, Wilms’ tumors may produce Epo [57-62]. More unusual types of Epo-producing tumors include pancreatic ductal carcinoma [63], renal capillary hemangioma [64-79] and uterine leiomyoma [65-79]. In most of these more unusual situations, an investigation for VHL mutations was not carried out.

Summary

Our present understanding of the mechanism of hypoxic regulation of the Epo gene (and numerous other genes) provides a direct link to the cause of some forms of renal cancer, namely, the von Hippel-Lindau protein. Mutation or loss of this gene, either in an inherited fashion or in a tissue-specific acquired fashion, can lead to renal cysts and carcinoma as well as neoplasms of several other organs. Loss of hypoxic regulation of several genes plays a role in the neoplastic process, and increased production of Epo is recognized as the paraneoplastic syndrome of erythrocytosis.

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Author for correspondence

Arthur J. Szytkowski, MD
Laboratory for Cell and Molecular Biology
Beth Israel Deaconess Medical Center
330 Brookline Ave. – W/BL 548
Boston, MA 02215
phone +1 (617) 632-9980
fax +1 (617) 632-0401
e-mail: asytkows@bidmc.harvard.edu