Constitutive Activation of Hypoxia-inducible Genes Related to Overexpression of Hypoxia-inducible Factor-1α in Clear Cell Renal Carcinomas


Departments of Nephrology and Medical Intensive Care [M. S. W., P. M. M., A. S., J. S. J., G. J. G., U. F., K.-U. E.] and Urology [J. R., S. A. L.]; Charité, Humboldt University, 13355 Berlin, Germany; German Cancer Research Center, 69120 Heidelberg, Germany [I. B., H.-J. G.]; Department of Paediatrics, Section of Medical and Molecular Genetics, University of Birmingham B15 2T, United Kingdom [N. V. M., E. R. M.]; Wellcome Trust Centre for Human Genetics, Oxford OX3 7BN, United Kingdom [P. H. M.]

ABSTRACT

The transcription factor hypoxia-inducible factor (HIF)-1α is an important mediator of hypoxic adaptation of tumor cells and controls several genes that have been implicated in tumor growth. Oxygen-dependent degradation of HIF-1α, the regulatory subunit, requires binding to the von Hippel Lindau (VHL) protein. Because functional inactivation of the VHL tumor suppressor gene occurs in up to 70% of clear cell renal carcinomas, we investigated whether this results in overexpression of HIF-1α and its target genes. Immunoblotting revealed increased expression of HIF-1α in 24 of 32 (75%) clear cell renal carcinomas but only 3 of 8 non-clear cell renal tumors. Somatic mutations of the VHL gene were detected only in clear cell renal carcinomas that overexpressed HIF-1α. None of the HIF-1α-negative tumors displayed a VHL mutation. The level of HIF-1α mRNA was not different between tumors and adjacent kidney tissue. Immunohistochemistry revealed distinct patterns of nuclear staining for HIF-1α, depending on histological type and overall abundance of HIF-1α. In those clear cell renal carcinomas that showed increased expression on immunoblots, HIF-1α was expressed in almost all cells. In the remaining clear cell and in non-clear cell renal tumors, staining was focal; these different patterns thus were compatible with genetic stabilization in contrast to microenvironmental stimulation of HIF-1α as the primary mechanism. The mRNA expression of two known target genes of HIF-1α, vascular endothelial growth factor and glucose transporter 1, increased progressively with increasing amounts of HIF-1α in tumor extracts. In addition, glucose transporter 1 protein levels correlated with HIF-1α abundance. In conclusion, the data provide in vivo evidence for a constitutive up-regulation of HIF-1α in the majority of clear cell renal carcinomas, which leads to more widespread accumulation of this transcription factor than hypoxic stimulation. These observations are most likely linked to functional inactivation of the VHL gene product. Increased expression of HIF-1α is associated with alterations in gene expression patterns that are likely to contribute to tumor phenotype and progression.

INTRODUCTION

Limited availability of oxygen and nutrients impairs the proliferation of tumor cell clusters. The ability of tumors to adapt to a hypoxic microenvironment is increasingly recognized as an important mechanism that promotes tumor growth (1). The molecular basis of this adaptation, however, is still not completely understood.

The basic-helix-loop-helix Per-Arnt-Sim domain transcription factor HIF-1α plays a critical role in oxygen homeostasis. It is composed of two subunits, HIF-1α and HIF-1β. HIF-1α is the oxygen-regulated component that determines HIF activity (2, 3). Its accumulation during hypoxia because of inhibition of its proteolytic degradation through the ubiquitin proteasome pathway (4, 5). HIF-1 transactivates a large number of genes whose protein products either increase oxygen availability or mediate metabolic adaptation to reduced oxygen tensions. Included among these are erythropoietin, glucose transporters, glycolytic pathway enzymes, VEGF, heme oxygenase, and inducible nitric oxide synthase (2, 3, 6). Experimental evidence indicates an important impact of HIF-dependent gene expression on tumor growth. Tumors derived from cells lacking HIF-1α or HIF-1β expression manifested significantly reduced vascularization and, in most studies, reduced growth rates as compared with parental cells (7–12).

Several HIF-responsive genes are known to be up-regulated in human malignancies (1). In addition, two studies using immunohistochemistry for HIF-1α have recently shown an accumulation of the protein in several types of common human cancers (13, 14). The strongest staining was usually found at the margin between necrotic and viable tumor tissues and at the invading edges of tumors. These localizations are consistent with the known regulation of HIF-1 through hypoxia. In addition, however, in certain tumors, genetic alterations may also be involved in the activation of HIF and its target genes. Most strikingly, cells derived from RCCs express high levels of hypoxia-inducible genes in cell culture independent of oxygenation (15, 16). Following this observation, recent work has linked an up-regulation of HIF to inactivation of the VHL gene (17, 18). RCCs in patients affected by the VHL syndrome and most sporadic RCCs are associated with loss of function of both alleles of the VHL gene (19). Experimental introduction of an intact VHL gene into cells derived from renal carcinomas suppresses HIF under normoxic conditions and restores its hypoxic induction, indicating that VHL is essential for stabilization of HIF-1α (20–23). The hypothesis derived from these in vitro findings is that stabilization of HIF-1α may occur independently of regional hypoxia in the majority of clear cell RCCs.

The two recently published series of different human tumors screened for HIF-1α expression included single cases of primary and metastatic RCC (13, 14, 18). The frequency of nuclear staining for HIF-1α was variable. In one of the surveys staining was noted to be higher and more homogeneous than in other tumors (14), which would be consistent with a genetic rather than a microenvironmental stimulation. If functionally relevant, up-regulation of HIF-1α should also be associated with enhanced expression of hypoxia-inducible target genes. Proof of this association might not only be relevant for understanding the progression of RCCs, which are typically highly vascularized and aggressive, but could also exemplify the significance of HIF-dependent gene expression in cancer in general.

We report here an analysis of a series of 40 human renal tumors of different histological type, which provides data strongly supporting...
the concept of genetic stabilization of HIF and its functional relevance. High-level expression of HIF-1α was detected in the majority of clear cell RCCs and was found to be homogeneous on tissue sections. In contrast, in non-clear cell RCCs, HIF-1α was expressed less frequently, at lower levels, and focally. Furthermore, the expression of HIF-1α was strongly correlated with the induction of two genes known to be regulated by HIF-1 and important for tumor metabolism and angiogenesis: VEGF and GLUT1.

**MATERIALS AND METHODS**

**Collection of Human Tissue.** After radical nephrectomy, samples of renal tumor and nonmalignant kidney tissue were immediately frozen in liquid nitrogen. In seven cases, tissue blocks were kept at room temperature for defined periods to assess *ex vivo* stability of HIF-1α protein. All tissues were stored at −70°C until analysis. The collection of samples was approved by local ethics committees, and informed consent was obtained from each patient. Renal tumors were routinely processed and classified for their histological type and grade according to Thönes et al. (24) in institutes of histopathology at Berlin and Heidelberg without knowledge of the study’s intent or results.

**Cell Culture.** Protein extracts from the human hepatoma cell line Hep3B were used as a standard for hypoxic induction on each immunoblot for HIF-1α protein. Cells were grown to confluency and then exposed to 4 h of either normoxia or hypoxia (1% O₂, 5% CO₂, 94% N₂). Hypoxic experiments were performed in a NuAire incubator (Zapf, Sarstedt, Germany).

**Immunoblotting.** Protein extraction and blotting were performed essentially as described previously (25). Sections of the frozen tissue were fractionated, weighed, and homogenized into 20-fold excess of extraction buffer [7 M urea, 10% glycerol, 1 mM Tris-Cl (pH 6.8), 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mg/ml each of aprotinin, pepstatin, and leupeptin (all chemicals from Sigma Chemical Co., St. Louis, MO)] with an electric homogenizer (Ultra-Turrax; IKA, Staufen, Germany). Extracts were quantified with the Bio-Rad DC protein assay (Bio-Rad, Bedford, MA) overnight and probed with anti-HIF-1α monoclonal antibody (Transduction Laboratories, Lexington, KY) or with an anti-GLUT1 affinity-purified polyclonal antiserum (Alpha Diagnostics, San Antonio, TX) at concentrations of 1 μg/ml each. Signals were detected with horseradish peroxidase-conjugated antibodies (DAKO, Ely, United Kingdom) and enhanced chemiluminescence (SuperSignal West Dura Extended; Pierce, Rockford, IL). After analysis, membranes were stained with Coomassie Blue (Sigma Chemical Co.) to verify equal protein loading and transfer. Each blot contained 40 μg of extract from normoxic and hypoxic Hep3B cells for cross comparison. The abundance of HIF-1α was rated semiquantiatively in comparison with nonmalignant tissue and hypoxic Hep3B extract: −, no expression; +, low-level but definite overexpression; ++, intermediate expression; and ++++, high-level expression as similar to the abundance of hypoxic Hep3B extract. The amount of GLUT1 in tumor extracts was rated semiquantiatively in comparison with matched nontumorous kidney tissue: −, no overexpression; +, definite overexpression; ++, high-level overexpression.

**Immunohistochemistry.** Immunohistochemistry was carried out on 5-μm sections of paraformaldehyde-fixed tissue. HIF-1α was detected by a mouse monoclonal antibody (0.4 μg/ml; Novus Biologicals, Littleton, CO) with the help of the DAKO catalyzed signal amplification system (DAKO, Hamburg, Germany) as described (13). Sections were incubated at 95°C for 45 min with target retrieval solution (DAKO). Subsequent steps were performed according to the manufacturer’s instructions.

For single-label immunohistochemistry of GLUT1, the anti-GLUT1 polyclonal rabbit antiserum (Alpha Diagnostics) was used at 100 μg/ml after application of the antibody, the slides were incubated for 2 h at 25°C; a mouse antirabbit antibody was then added at a dilution of 1:50 (M737; DAKO) and incubated at 22°C for 1 h. This was followed by incubation with rabbit antimonooval antibody (Z529; DAKO) at a dilution of 1:40, again at 25°C for 1 h. Alkaline phosphatase-conjugated mouse monoclonal antibody diluted 1:40 was then added and incubated at 25°C for 1 h. All dilutions were in PBS (pH 7.6). For staining, slides were exposed for 15 min to a solution of sodium nitrite (28 mM), new fuchsin (21 mM), naphthol-AS-Bl-phosphate (0.5 mM), dimethylformamide (64 mM), and levamisole (5 mM) in 50 mM Tris-HCl buffer (pH 8.4) containing 146 mM NaCl. Sections were counterstained with aqueous hematoxylin before application of gelating mounting medium.

Double-label immunohistochemistry was performed for HIF-1α and keratin as tumor cell marker, or GLUT1. After the presence of HIF-1α was demonstrated as described, sections were washed in water and PBS and then incubated with 10% FCS. Polyclonal rabbit anti-GLUT1 antibody was used at 100 μg/ml and applied to the sections for 18 h at 4°C. Monoclonal mouse antihuman keratin AE1/AE3 (DAKO) was used at a dilution of 1:25. Sections were then washed three times with PBS at 22°C. After subsequent incubations, as described for GLUT1 except that for AE1/AE3 antibody, Z529 was omitted, and incubation with alkaline phosphatase mouse monoclonal antibody, sections were developed with Vector Blue R (Camon, Wiesbaden, Germany) to produce a blue color.

Control experiments entailed immunohistochemistry with nonimmune mouse or rabbit IgG, respectively, without primary antibody, or without avidin biotin peroxidase or alkaline phosphatase antibody.

**RNA Analysis.** Total RNA was extracted from frozen tissue samples with RnaZol B, according to the manufacturer’s instructions (Biogenesia, Poole, United Kingdom). RNase protection assays were performed essentially as described previously (26). 32P-labeled human riboprobes were generated using SP6 or T7 RNA polymerase (Roche Diagnostics, Mannheim, Germany), with following protected fragments: HIF-1α, 240 bp (accession no. U224311); VEGF, 140 bp (accession no. M63971); GLUT1, 136 bp (accession no. K03195); and U6 small nuclear RNA, 106 bp (U6sn; accession no. X01366). Radiolabeled riboprobes were protected from RNase digestion by hybridizing to the following amounts of total RNA: HIF-1α, 30 μg; VEGF, 20 μg; GLUT1, 30 μg; and U6sn, 1 μg. The latter was used as internal control for each sample, adding 1/10 (i.e., 100 ng) of each reaction to its appropriate sample after RNase digestion. After resolution on polyacrylamide gels, signals were quantified using a phosphor imager (Fuji, BAS 2000; Fuji, Japan). Values were normalized to the control (U6sn). These values were used to calculate the ratio of abundance in tumor to adjacent kidney tissue, shown as fold induction.

**VHL Mutation Analysis.** Genomic DNA was extracted with QIAamp purification columns (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Details of the VHL gene mutation analysis techniques have been described previously (27, 28). In brief, PCR was used to amplify genomic DNA for the VHL coding region (covering codons 54–213) and single-strand conformation polymorphism analysis was performed to detect intragenic mutations as described. Fragments producing an aberrant single-strand conformation polymorphism band were sequenced on the ABI 377 semiautomated sequencer. Direct sequencing of the relevant coding region was performed in a similar fashion for those tumors in which a mutation remained unidentified.

**RESULTS**

**HIF-1α Protein and mRNA Expression in Homogenates of Renal Tumors and Adjacent Nonmalignant Renal Tissue.** Of the 40 renal tumors investigated, 32 (80%) were clear cell, 4 were papillary, 2 were chromophob RCCs, 1 was an oncocytoma, and 1 was a squamous cell carcinoma. Protein extracts were prepared from all tumors and adjacent tumor-free kidney tissue. HIF-1α was barely detectable in the non-tumor samples by immunoblotting. In contrast, significant overexpression of HIF-1α was found in 27 of 40 (68%) tumor specimens. The frequency and intensity of HIF-1α expression varied with the type of RCC (Fig. 1A–C). Enhanced accumulation of HIF-1α was detected in 24 of 32 (75%) clear cell RCCs but only 3 of 8 (38%) of non-clear cell RCCs. HIF-1α abundance on immunoblots was categorized semiquantiatively by cross-comparison with the signal from the extract of hypoxic Hep3B cells into three groups: low (+), intermediate (++, and high (+++). With this scoring system, HIF-1α expression was intermediate (++) or high (+++) in >60% of clear cell RCCs but only 2 of 8 (25%) of non-clear cell RCCs (Table 1). There was no relationship between tumor grading and the abundance of HIF-1α (data not shown).

In all cases care was taken to snap-freeze tissue samples as soon as possible after nephrectomy. Because HIF-1α is usually degraded...
HIF-1α ACTIVATION IN RENAL CARCINOMAS

Fig. 1. HIF-1α expression in renal tumors and adjacent nonmalignant kidney tissue. A–C, immunoblots of matched samples from 12 patients with RCC. K, nonmalignant kidney tissue; T, tumor. Numbers identify individual patients. All tumors in A and B and two tumors in C were clear cell renal carcinomas (CCRC). One tumor in C was a chromophobic renal carcinoma (CRC), and one was classified as papillary renal carcinoma (PRC). For comparison, total cellular protein from human hepatoma cells (Hep3B) cultured under normoxic (N) and hypoxic (H) conditions were coanalyzed (Lanes 1 and 2 in A and B). In experiments shown in B, tissue samples of tumor and adjacent tissue were kept at room temperature for up to 60 min before snap-freezing. For semiquantitative analysis, expression of HIF-1α and hypoxic (H) conditions were coanalyzed (Lanes 1 and 2 in A and B). In experiments shown in B, tissue samples of tumor and adjacent tissue were kept at room temperature for up to 60 min before snap-freezing. For semiquantitative analysis, expression of HIF-1α was classified as not detected (−), low level (+), intermediate (++), or high level (+++). D, RNase protection assay for HIF-1α mRNA in corresponding samples of nonmalignant kidney (K) and tumor (T). In contrast to marked differences in protein expression, there was no change at the mRNA level.

Table 1. HIF-1α abundance in clear cell and non-clear cell RCC

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>HIF-1α protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear cell RCC</td>
<td>−</td>
</tr>
<tr>
<td>Non-clear cell RCC</td>
<td>++</td>
</tr>
</tbody>
</table>

*Semiquantitative categorization of HIF-1α abundance in tumors was performed by comparison with signal intensity of adjacent nonmalignant tissue and extracts from hypoxic Hep3B cells in immunoblots (see “Materials and Methods”).

within minutes and, on the other hand, renal artery clamping may itself lead to hypoxic induction of HIF-1α, we were concerned about the effect of inevitable variations in time until tissue freezing. To address this issue, different tissue blocks of seven tumors, together with blocks of adjacent nonmalignant kidney tissue were kept at room temperature for up to 30 or 60 min before transfer to liquid nitrogen. Six of these tumors were later identified as clear cell RCCs, and one was an oncocytoma. Five of the six clear cell RCCs showed HIF-1α overexpression, and the abundance of HIF-1α in these tumors remained constant during the time period investigated (Fig. 1B). In addition, storage ex vivo did not induce HIF-1α in the oncocytoma, the HIF-1α-negative clear cell RCC, or any of the nontumorous kidney samples.

To assay for changes in HIF-1α gene expression, total RNA from all specimens was analyzed by RNase protection. HIF-1α mRNA levels were very similar in all samples investigated, with no difference between tumors and nonmalignant kidney tissue, irrespective of histological type or degree of HIF-1α protein expression [Fig. 1D; mean ratio of normalized signal intensity between tumors and adjacent nontumorous tissue (± SE), 0.99 ± 0.12 and 1.28 ± 0.31 in clear cell and non-clear cell RCCs, respectively].

Mutation Analysis of the VHL Gene in Clear Cell Renal Carcinoma. To evaluate whether the HIF status of a tumor correlates with VHL gene inactivation, we performed mutation analysis of the VHL gene in each of the clear cell RCCs for which tumor DNA was available. In total, 27 clear cell tumors could be studied; 21 of these showed HIF-1α overexpression, of which 12 displayed a somatic mutation of the VHL gene (57%). None of the remaining six HIF-1α-negative clear cell RCCs showed a mutation of the VHL gene (Table 2). The level of HIF-1α expression in the tumors did not seem to correlate with the presence of a VHL mutation.

Immunohistochemistry for HIF-1α. In accordance with recently published immunohistochemical observations (13, 14), very little expression of HIF-1α was found in normal kidney tissue. In the different renal tumors examined, two distinct patterns of nuclear staining for HIF-1α were observed (Fig. 2). In the subset of clear cell carcinomas that revealed no overexpression of HIF-1α on immunoblots and in non-clear cell carcinomas, immunostaining for HIF-1α was usually weak and nuclear, with cytoplasmic staining at the tumor margins. In the set of tumors that showed HIF-1α overexpression, nuclear staining was intense, with abrupt transition to negative staining at the tumor margins. This distribution pattern appears to be similar to that of the RNA expression data and of immunohistochemical observations (13, 14). The staining intensity in tumors did not seem to correlate with the presence of a VHL mutation.

Table 2. Mutational analysis of the VHL gene in clear cell RCC in relation to HIF-1α protein expression

<table>
<thead>
<tr>
<th>Tumor no.</th>
<th>Mutation</th>
<th>Effect</th>
<th>Exon</th>
<th>HIF-1α protein*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>550 C→T</td>
<td>R113X</td>
<td>1</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>ND</td>
<td></td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>3</td>
<td>407 C→A</td>
<td>S65X</td>
<td>1</td>
<td>+++</td>
</tr>
<tr>
<td>4</td>
<td>ND</td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>447insA</td>
<td>Frameshift</td>
<td>1</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
<td></td>
<td></td>
<td>−</td>
</tr>
<tr>
<td>7</td>
<td>776 T→C</td>
<td>L188P</td>
<td>3</td>
<td>++</td>
</tr>
<tr>
<td>8</td>
<td>461delT</td>
<td>Frameshift</td>
<td>1</td>
<td>++</td>
</tr>
<tr>
<td>9</td>
<td>ND</td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>11</td>
<td>ND</td>
<td></td>
<td></td>
<td>−</td>
</tr>
<tr>
<td>12</td>
<td>679-687del</td>
<td>delYTL</td>
<td>3</td>
<td>+++</td>
</tr>
<tr>
<td>13</td>
<td>ND</td>
<td></td>
<td></td>
<td>−</td>
</tr>
<tr>
<td>14</td>
<td>ND</td>
<td></td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>15</td>
<td>636delT</td>
<td>Frameshift</td>
<td>2</td>
<td>++</td>
</tr>
<tr>
<td>16</td>
<td>ND</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>IVS1+2 T→G</td>
<td>Splicing</td>
<td>11</td>
<td>++</td>
</tr>
<tr>
<td>18</td>
<td>416 C→A</td>
<td>S68X</td>
<td>1</td>
<td>+</td>
</tr>
<tr>
<td>20</td>
<td>ND</td>
<td></td>
<td></td>
<td>−</td>
</tr>
<tr>
<td>21</td>
<td>454 C→T</td>
<td>P81S</td>
<td>1</td>
<td>++</td>
</tr>
<tr>
<td>22</td>
<td>ND</td>
<td></td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>23</td>
<td>630-633del</td>
<td>Frameshift</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>24</td>
<td>ND</td>
<td></td>
<td></td>
<td>++</td>
</tr>
<tr>
<td>25</td>
<td>ND</td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>26</td>
<td>621insAA</td>
<td>Frameshift</td>
<td>2</td>
<td>++</td>
</tr>
<tr>
<td>27</td>
<td>ND</td>
<td></td>
<td></td>
<td>−</td>
</tr>
<tr>
<td>28</td>
<td>ND</td>
<td></td>
<td></td>
<td>−</td>
</tr>
<tr>
<td>29</td>
<td>ND</td>
<td></td>
<td></td>
<td>−</td>
</tr>
</tbody>
</table>

*Semiquantitative categorization of HIF-1α abundance in immunoblotting as described in “Materials and Methods.”

ND, nondetectable.
focal, with either single cells (Fig. 2A) or clusters of cells (Fig. 2B) accumulating the protein in their nuclei. In some of these cases that had areas of intratumoral necrosis, expression was observed in the perinecrotic tissue. Occasionally, as illustrated in Fig. 2B, immunostaining for HIF-1α was associated with an apparently avascular tumor stroma of a papillary RCC. In sharp contrast, in those clear cell RCCs that showed increased amounts of HIF-1α protein on immunoblots, i.e., the majority of clear cell RCCs, the staining pattern for HIF-1α was rather homogeneous, with almost all nuclei staining positive in tumor cells, which are labeled by keratin (blue) in E and F. As demonstrated in D, the expression pattern of HIF-1α in these tumors is independent of the vicinity of tissue necrosis (N) or vessels (V). For staining techniques, see “Materials and Methods.” The sample in A is counterstained with hematoxylin. Magnifications: ×200 in A; ×400 in B; ×100 in C; ×200 in D; ×600 in E and F.

mRNA Expression of VEGF and GLUT1. Using RNase protection, we could detect mRNA for VEGF and GLUT1 in all tumors and samples of nonmalignant kidney tissue. Both genes were frequently overexpressed in tumors, but the incidence and amplitude of overexpression varied, depending on the type of RCC.

In clear cell RCCs, comparison of the mRNA abundance in matched samples revealed at least a 2-fold induction in 24 of 32 cases for both genes (Fig. 3A). Although the majority of tumors showed overexpression of either both or neither of the two genes, five tumors expressed significantly higher levels of VEGF or GLUT1 mRNA only.

In contrast to the findings in clear cell RCCs, only two of seven and one of six extracts of non-clear cell RCCs contained an at least 2-fold higher amount of VEGF or GLUT1 mRNA compared with the level in adjacent nonmalignant tissue. The median tumor/nontumor ratios of GLUT1 and VEGF mRNA expression were 7.7- and 6.2-fold higher in clear cell than in non-clear cell RCCs, respectively (Fig. 3B).

Relationship between Expression of HIF-1α Protein and Target Genes. When tumors were grouped according to whether they contained HIF-1α in amounts sufficient to be detected by immunoblotting, a clear association was found with the incidence of increased mRNA expression of the target genes (Table 3). Of 24 clear cell RCCs containing HIF-1α, 23 overexpressed VEGF and/or GLUT1 mRNA. In contrast, only half of the HIF-1α-negative clear cell RCCs showed up-regulation of at least one of these genes. None of the HIF-1α-negative non-clear cell RCCs overexpressed either target gene.
HIF-1α ACTIVATION IN RENAL CARCINOMAS

Fig. 3. Expression of VEGF and GLUT1 mRNA in renal tumors. A, two representative RNase protection assays of paired samples (tumor and adjacent kidney tissue) from the same patients for which the abundance of HIF-1α protein is depicted in Fig. 1A. For quantitative analysis, signal intensity in tumors was related to that in adjacent nontumorous tissue after normalization for U6sn RNA signals. B, comparison of GLUT1 and VEGF induction in clear cell RCCs (CCRC) and non-clear cell RCCs (other). Columns indicate median values; bars, interquartile ranges.

Table 3 Number and percentage of tumors overexpressing the two target genes analyzed, depending on the histological type and HIF-1α protein accumulation, as detected by immunoblotting

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>HIF-1α protein</th>
<th>GLUT1 mRNA</th>
<th>VEGF mRNA</th>
<th>VEGF or GLUT1 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear cell RCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 32)</td>
<td>Positive</td>
<td>24/32 (75%)</td>
<td>21/24 (88%)</td>
<td>21/24 (88%)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>8/32 (25%)</td>
<td>3/8 (38%)</td>
<td>3/8 (38%)</td>
</tr>
<tr>
<td>Non-clear cell RCC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 8)</td>
<td>Positive</td>
<td>3/8</td>
<td>1/3</td>
<td>2/3</td>
</tr>
</tbody>
</table>
|                  | Negative       | 5/8         | 0/3        | 0/4                | 0/3

DISCUSSION

The incidence of RCC is increasing steadily, and the current worldwide annual mortality is estimated to exceed 100,000 (30, 31). Clear cell or “common” renal carcinoma accounts for 75–80% of RCCs and is associated with the worst prognosis, with the exception of extremely rare collecting duct carcinomas. At the time of diagnosis, >30% of patients have overt metastasis, and the response to therapy is poor (30, 31). There is great interest, therefore, in identifying the molecular basis of the aggressive biological behavior of clear cell RCCs.

A major insight into the molecular events leading to this type of tumor was drawn from the high incidence in patients with germ line mutations of the VHL gene. Conforming to Knudsen’s two-hit hypothesis, the second allele is inactivated in these tumors. The great majority (up to 70%) of sporadic clear cell RCCs have inactivation of both copies of the VHL gene, through either somatic mutations or hypermethylation (19, 32). In contrast, VHL mutations or functional inactivation are not a feature of other types of renal cancer. Several mechanisms have been suggested by which the VHL protein may act as a tumor suppressor. One recently identified function of pVHL is the targeting of HIF-1α for ubiquitin-mediated proteasomal degradation.
In these cases, virtually every tumor cell, independent of its position in relation to vascular structures or tumor geometry, expressed the parallel HIF-1α abundance. C–F; immunohistochemistry for GLUT1. In clear cell RCC without overexpression of HIF-1α (C), cell membrane staining for GLUT1 is focal compared with the homogeneous immunostaining in another clear cell RCC with high abundance of HIF-1α, as determined on immunoblots (D). E, costaining for HIF-1α (brown) and GLUT1 (blue) in a tumor with HIF-1α positivity in the majority of tumor cells. For staining techniques, see “Materials and Methods.” The samples in C and D are counterstained with hematoxylin. Magnification: ×800 in C; ×600 in D and E.

(17). The β domain of pVHL binds directly to the oxygen-dependent degradation domain of HIF-1α, which is critically important for its destabilization in the presence of oxygen (20–23). Following these in vitro findings, the present study provides evidence that marked overexpression of HIF-1α protein is a frequent and functionally relevant feature of clear cell RCCs. Several of our observations support the concept that this up-regulation of HIF-1α is produced mainly by a genetic alteration rather than stimulation through a hypoxic microenvironment. The first observation is that the incidence and level of HIF-1α overexpression were much higher in clear cell than in non-clear cell RCCs (Fig. 1 and Table 1). The second observation is that, in the majority of clear cell RCCs, immunohistochemistry revealed nuclear staining for HIF-1α throughout the tumor. In these cases, virtually every tumor cell, independent of its position in relation to vascular structures or tumor geometry, expressed the protein (Fig. 2, C–F). In contrast, in clear cell RCCs in which the amount of HIF-1α was too low to be detected by immunoblotting of tissue homogenates (Fig. 2A) and in non-clear cell RCCs (Fig. 2B), HIF-1α staining was inhomogeneous and focal. In these tumors, preferential expression in perinecrotic and poorly vascularized areas resembled the expression in diverse types of non-renal human tumors, as recently reported by Zhong et al. (13) and Talks et al. (14). This focal expression pattern most likely reflects hypoxic stabilization. In addition, other genetic alterations, trophic stimuli, and microenvironmental conditions other than hypoxia may also play a role in enhancing regional expression of HIF-1α (1). Some of the tumors in our series showed regional activation of HIF-1α on histological specimens, but no signals on immunoblots. This is most likely attributable to the lower sensitivity for detecting a protein in whole-tissue homogenates than with high-sensitivity amplification immunohistochemistry.

The third observation is that the incidence of HIF-1α overexpression detectable by immunoblotting was 75% and thus consistent with the expected frequency of loss of function of VHL. To further pursue the relationship between overexpression of HIF-1α and alterations of the VHL gene, we performed mutation analysis. In total, 44% of the screened clear cell RCCs (12 of 27) displayed a somatic mutation of the VHL gene, which occurred at a frequency similar to that reported previously in primary RCC tumors (27). Mutations were found only in the pool of clear cell RCCs that showed overexpression of HIF-1α, and in none of the HIF-1α-negative clear cell tumors. The frequency of VHL mutations in RCCs showing up-regulation of HIF-1α was 57% (12 of 21; Table 2). In ~15% of all clear cell RCCs, DNA hypermethylation alone has been found to be the mechanism of VHL inactivation (19, 32). In the selected pool of HIF-overexpressing tumors, this frequency can be expected to be higher. A proportion of tumors in our study with HIF-1α overexpression but no identified somatic mutation of the VHL gene should be accounted for by inactivation by promoter hypermethylation (28). In addition, VHL-independent mechanisms for HIF dysregulation might have occurred in some tumors. To date, mutations of HIF-1α itself have not been detected in RCCs (33). In summary, the mutation analysis showed a good association between VHL inactivation of a tumor and its HIF status, further supporting the concept of a genetic mechanism for HIF-1α stabilization.

An additional observation compatible with genetically determined inhibition of HIF-1α degradation is its ex vivo stability for up to 60 min in five series of samples from clear cell RCCs (Fig. 1B). How-

<table>
<thead>
<tr>
<th>GLUT1 mRNA</th>
<th>GLUT1 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>--</td>
<td>1.6 (0.1–3.4)</td>
</tr>
<tr>
<td></td>
<td>5.1 (1.5–5.2)</td>
</tr>
<tr>
<td></td>
<td>8.2 (2.3–3.7)</td>
</tr>
</tbody>
</table>

Table 4 Relationship between the amount of GLUT1 protein, GLUT1 mRNA, and HIF-1α protein in tumor extracts

Number and percentage of cases. Semiquantitative categorization of HIF-1α and GLUT1 protein abundance in tumors was performed by comparison with signal intensity of adjacent nonmalignant tissue and extracts from hypoxic Hep3B cells (for HIF-1α) in immunoblots (see “Materials and Methods”). GLUT1 mRNA expression was assessed by RNase protection and is expressed as the ratio of mRNA abundance between matched tumor and nonmalignant kidney tissue after normalization for U6sn RNA (median and interquartile range).
ever, the interpretation of this finding is hampered by the lack of a
tumor sample expressing HIF-1α initially and degrading rapidly over
time, presumably under the influence of functional VHL-mediated
degradation. We did not find such a sample in our series of tumors.
Nevertheless, together with the finding that changes in the amount of
HIF-1α were not related to alterations at the mRNA level (Fig. 1D),
these observations are compatible with an abnormal stabilization of
the HIF-1α protein, which is usually degraded within minutes after
reoxygenation in vitro (2, 3, 6, 25). On the other hand, although the
oxygen tension within these samples can be assumed to be low, these
conditions were not sufficient to induce HIF-1α in similar-sized
blocks of adjacent nonmalignant kidney tissue or tumors that did not
initially overexpress HIF-1α. Artificial induction of HIF-1α through
renal artery clamping and variable time periods until freezing of
tissue, therefore, seems unlikely.

Others have recently reported findings in brain tumors analogous to
our observations in RCCs (13, 34). Although HIF-1α was highly
expressed in areas adjacent to necrosis in glioblastomas, hemangio-
blastomas exhibited homogeneous HIF-1α expression throughout
highly vascularized and nonnecrotic tumor tissue. Hemangioblasto-
mas are another common manifestation of the VHL syndrome, and
loss-of-function mutations in the VHL gene occur in sporadic heman-
gioblastomas (19, 35, 36). Taken together, these findings indicate a
functional significance of VHL for destabilization of HIF-1α in di-
verse tumors in vivo.

An additional important finding in our study is the close correlation
between HIF-1α protein levels and the expression of two target genes
with entirely different physiological functions (Figs. 4 and 5; Tables
3 and 4). VEGF plays a central role in tumor neoangiogenesis (37–
39). Several previous studies demonstrated overexpression of VEGF
in RCCs at the protein and mRNA levels and correlations with microvessel density (e.g., see Refs. 40–44). The glucose transporter
GLUT1 mediates cellular glucose uptake and thus facilitates anaero-
bic glycolysis, which is a prerequisite for clonal expansion of cancer
cells (1). Immunostaining for GLUT1 was demonstrated previously at
the plasma membrane of tumor cells in 85% of clear cell RCCs, but
not in association with other renal cancer cell types (29).

Our findings confirm these observations and strongly suggest that
the heterogeneity in the expression pattern of both VEGF and GLUT1
between and within tumors is at least in part governed by the expres-
sion of HIF-1α. HIF-1α transactivates the promoters of both genes via
binding to hypoxia-responsive elements (45, 46) and has been shown
to regulate GLUT1 and VEGF expression in both cultured cells and
experimental tumors (2, 3, 6). The progressive increases of VEGF and
GLUT1 mRNA with increasing abundance of HIF-1α protein, as
observed in this study (Fig. 4), indicate that this regulative pathway
has a dominant influence within the diversity of potential stimuli in
vivo. Interestingly, the incidence of overexpression and amplitude of
induction for VEGF and GLUT1 mRNA were very similar (Fig. 3
and Table 3). Of note, however, is that single tumors in our survey
were also observed in which only one of the two genes was over-
expressed, pointing to additional regulatory mechanisms.

In the present study, we focused on HIF-1α as the regulatory
component of HIF-1. HIF-2α is a structurally related alternative
dimerization partner of HIF-1β, which can also transactivate reporter
genes via HIF DNA recognition sites (6). In vitro it is regulated in an
oxygen-dependent fashion very similar to that for HIF-1α (25). In
solid tumors, however, different but overlapping subsets of tumor
cells stained high for HIF-1α and -2α (14). In vitro studies showed that
VHL deficiency also stabilizes HIF-2α (17). Moreover, overexpression
of HIF-2α mRNA has been reported in hemangioblastomas (47).
It will be interesting, therefore, to address in future studies the extent
to which HIF-2 is induced in RCCs, which may contribute to and
amplify the induction of HIF-responsive genes.

The activation of those genes investigated in the present study and
other HIF-1-dependent genes as a consequence of overexpression of
HIF-1α may have a significant impact on tumor progression. In
support of this concept, expression of HIF-1α in cervical carcinomas
was recently found to be associated with adverse prognosis (48). In
RCCs, VEGF expression is associated with poor survival (40), and
VEGF receptor inhibition reduces the growth of human tumors, in-
cluding RCCs in experimental models (49). Suppression of GLUT1
expression in tumor cells also inhibits growth in a nude mouse model
(50). Nevertheless, therapeutic attempts to inhibit tumor growth by
interfering with the function of any single target gene usually are
limited by the role of this particular gene within the complexity of
tumor biology and hampered by the redundancy of processes such as
neoangiogenesis. In this situation, HIF-1α, which has several down-
stream targets that potentially promote tumor growth, is an attractive
candidate for therapeutic exploitation. We propose on the basis of our
findings that such attempts may be particularly worthwhile in patients
with clear cell RCCs.

ACKNOWLEDGMENTS

We thank P. Ratcliffe (Oxford) for the riboprobes and helpful discussions.
The technical assistance of S. Schulz and the help of B. Eisele in preparing the
figures are gratefully acknowledged.

REFERENCES

3. Semenza, G. L. Regulation of mammalian O2 homeostasis by hypoxia-inducible
4. Salceda, S., and Caro, J. Hypoxia-inducible factor 1α (HIF-1α) protein is rapidly
degraded by the ubiquitin-proteasome system under normoxic conditions. J. Biol.
5. Huang, L. E., Gu, J., Schau, M., and Bunn, H. F. Regulation of hypoxia-inducible
factor 1α is mediated by an O2-dependent degradation domain via the ubiquitin-
6. Wengen, R. H., and Gassmann, M. Oxygen(s) and the hypoxia-inducible factor-1.
7. Maxwell, P. H., Dachs, G. U., Gladele, J. M., Nicholls, L. G., Harris, A. L., Stratford,
I. J., Hankinson, O., Pugh, C. W., and Ratcliffe, P. J. Hypoxia-inducible factor-1 modulates
of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular
endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression.
9. Ryan, H. E., Lo, J., and Johnson, R. S. HIF-1α is required for solid tumor formation
10. Ryan, H. E., Poloni, M., McNulty, W., Elson, D., Gassmann, M., Arbeit, J. M., and
Johnson, R. S. Hypoxia-inducible factor-1α is a positive factor in solid tumor growth.
11. Carmeliet, P., Dor, Y., Herbert, J.-M., Fukumura, D., Brusselmans, K., Dewerchin,
M., Neemann, M., Bono, F., Abramovitch, R., Maxwell, P., Koch, C. J., Ratcliffe, P.,
Moons, L., Jain, R. K., Collen, D., and Keshet, E. Role of HIF-1α in hypoxia-
mediated apoptosis, cell proliferation, and tumor angiogenesis. Nature (Lond.), 394:
of tumor growth through disruption of hypoxia-inducible transcription. Nat. Med., 6:
hypoxia-inducible factor 1α in common human cancers and their metastases. Cancer
and Harris, A. L. The expression and distribution of the hypoxia-inducible factors
HIF-1α and HIF-2α in normal human tissues, cancers, and tumor-associated macro-
Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein.

Downloaded from cancerres.aacrjournals.org on November 9, 2017. © 2001 American Association for Cancer Research.


Constitutive Activation of Hypoxia-inducible Genes Related to Overexpression of Hypoxia-inducible Factor-1 α in Clear Cell Renal Carcinomas


Cancer Res 2001;61:5215-5222.

Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/13/5215

Cited articles
This article cites 46 articles, 22 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/13/5215.full#ref-list-1

Citing articles
This article has been cited by 49 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/61/13/5215.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/61/13/5215.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.