Coexpression of Erythropoietin and Erythropoietin Receptor in Von Hippel-Lindau Disease–Associated Renal Cysts and Renal Cell Carcinoma

Youn-Soo Lee,1 Alexander O. Vortmeyer,1 Irina A. Lubensky,1 Timothy W.A. Vogel,1 Barbara Ikejiri,1 Sophie Ferlicot,3 Gérard Benoît,4 Sophie Giraud,5,7 Edward H. Oldfield,1 W. Marston Linehan,2 Bin T. Teh,8 Stéphane Richard,4,5,6 and Zhengping Zhuang1

1Surgical Neurology Branch, National Institute of Neurological Disorders and Stroke and 2Urologic Oncology Branch, National Cancer Institute, NIH, Bethesda, Maryland; 3Laboratoire de Génétique Oncologique EPHE-UMR 8125, Villejuif, France; 4Service de Néphrologie, Hôpital Edouard Herriot, Lyon, France; and 5Van Andel Research Institute, Grand Rapids, Michigan

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ABSTRACT

Von Hippel-Lindau (VHL) disease is characterized by multiple tumors in specific target organs. The tumors at different sites share distinct morphologic and genetic characteristics but their cell of origin is unknown. We show that VHL disease–associated renal clear cell carcinomas (RCC) consistently coexpress erythropoietin (Epo) and Epo receptor (EpoR). In addition, coexpression of Epo and EpoR is detected in many renal cysts, providing further evidence that renal cysts are potential precursors for RCC. In conjunction with VHL gene deficiency, coexpression of Epo and EpoR in renal cysts and tumors may reflect a developmental arrest in immature mesenchymal cells. Such arrest may lead to autocrine stimulation, cell proliferation, and renal tumor development, similar to tumorigenesis of VHL disease–associated hemangioblastomas.

INTRODUCTION

Von Hippel-Lindau (VHL) disease is an autosomal dominant disorder characterized by the development of multiple tumors in specific target organs. Retinal hemangioblastoma, central nervous system (CNS) hemangioblastoma, pancreatic microcystic adenomas and neuroendocrine tumors, pheochromocytomas, and renal cysts and carcinomas are the most frequent lesions in VHL disease (1). Consistent with Knudson’s hypothesis, tumor formation is initiated by a germ line mutation of the VHL gene followed by inactivation of the wild-type VHL allele (2, 3). Loss of VHL protein function up-regulates the hypoxia-inducible factor-1 signaling pathway, which in turn mediates up-regulation of hypoxia inducible genes including Epo and VEGF (4, 5). These genes are involved in managing cellular responses to oxidative stress and vasculogenesis.

Sixty-six percent of patients with VHL disease present with a spectrum of bilateral multifocal renal lesions including benign cysts, atypical cysts, and cystic and solid RCC (6–8). As with other tumors in patients with VHL disease with germ line mutation, renal tumor formation is initiated by inactivation of the wild-type VHL allele. Because loss of heterozygosity of the wild-type VHL allele (8) and up-regulation of hypoxia-inducible factor-1 (5) occurs in multifocal RCC as well as in many benign cysts in the kidney parenchyma of patients with VHL disease, it is assumed that some renal cysts are precursors of RCC. However, several critical questions in tumorigenesis of VHL disease remain unanswered. Why do VHL disease tumors develop only in certain organs? Why do VHL disease–associated tumors show specific histopathology? What is the cell of origin of VHL disease tumors?

Several authors hypothesized that VHL-associated hemangioblastoma is associated with an arrest in differentiation of angioblasts and angioblast precursor cells that are capable of differentiation into primitive vascular structures and red blood cells (9–11). The hypothesis has recently been supported by the demonstration of Epo and EpoR coexpression in hemangioblastoma tumor cells (9). The finding suggests that a developmental arrest of tumor cells is associated with coexpression of Epo and EpoR in hemangioblastoma (9). We here investigate whether coexpression of Epo and EpoR is present in VHL-associated renal neoplasms and whether the tumorigenesis pathway of RCC and hemangioblastoma is similar.

MATERIALS AND METHODS

Patients and Renal Lesions. Eleven RCCs and 16 renal cysts from eight patients with VHL were analyzed. The study was approved by the institutional review boards of the NIH, Bethesda, Maryland, of the Ethics Committee at Le Kremlin-Bicêtre University Hospital in France, and of the Van Andel Research Institute, Grand Rapids, Michigan. A summary of the clinical features of these patients is shown in Table 1.

Immunohistochemistry. Immunohistochemistry was done on formalin-fixed, paraffin-embedded tissue sections of RCC, renal cysts, and adjacent normal kidneys. Tissue sections were first soaked in xylene and then washed in decreasing concentrations of ethanol. For antigen retrieval, sections were treated with DAKO target retrieval solution and incubated

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at 95°C for 20 to 30 minutes. Sections were cooled at room temperature, washed thrice in PBS, and then quenched for 20 minutes in a solution of 3 % H2O2 and 180 mL methanol. After three washes in PBS, sections were drenched in 10% horse serum for 1 hour. The primary antibody was diluted in 2% horse serum, and the sections were incubated in a humidified chamber at 4°C overnight. Primary antibodies used were rabbit polyclonal anti-human Epo (1:100) and sheep polyclonal anti-human EpoR (1:200). Sections were then incubated with secondary antibody and avidin-biotin complex for one hour each. 3,3’-Diaminobenzidine was used for visualization, followed by counterstaining with hematoxylin. The sections were dehydrated with graded ethanol washes and xylene wash and mounted. The presence and intensity of antibody expression were examined in RCC, renal cysts, and normal kidney.

**Microdissection and DNA Analysis.** Formalin-fixed, paraffin-embedded tissue from 11 RCCs, and normal kidney in eight patients was used for loss of heterozygosity analysis. Unstained 5-μm tumor tissue sections on glass slides were deparaffinized with xylene, rinsed in 100% to 80% ethanol, briefly stained with H&E, and rinsed in 10% glycerin in Tris-EDTA buffer. Microdissection was done under direct light microscopic visualization using a 30-gauge needle. Control samples were obtained from the matched normal kidney tissue on the same histologic slide. Procured cells were immediately resuspended in 30 μL buffer containing Tris-EDTA-HCl (pH 8.0), 10 mmol/L EDTA (pH 8.0), 1% Tween 20, and 0.1 mg/mL proteinase K and were incubated at 37°C overnight. The mixture was boiled for 15 minutes to inactivate proteinase K and 2 μL of the DNA solution were used for PCR amplification using markers D3S2452 and D3S1110 (Research Genetics, Huntsville, AL) flanking the VHL gene. 32P-labeled amplified DNA was mixed with an equal volume of formamide loading dye (95% formamide, 20 mmol/L EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol). Samples were then denatured for 5 minutes at 95°C, loaded onto a 6% acrylamide gel, electrophoresed at 1,800 V for 90 minutes, and transferred to Whatman paper for autoradiography.

**Total RNA Isolation and Epo/EpoR Reverse Transcription–PCR.** Frozen RCC and normal kidney tissue from three patients with VHL was used for Western blot analysis for Epo and EpoR. One frozen hemangioblastoma from a patient with VHL was used as a positive control for Western blot analysis for both Epo and EpoR. For Western blotting, 20 μL of cell lysate were separated by electrophoresis on 4% to 12% gradient Bis-Tris gels (Invitrogen). Proteins were electrotransferred onto polyvinylidene difluoride membrane (Novex, San Diego, CA). Blots were blocked in PBS/0.05% Tween 20 containing 5% fetal bovine serum and incubated with anti-Epo (1:100, Oncogene, Cambridge, MA) and anti-EpoR (1:100, Calbiochem, San Diego, CA) antibodies, respectively. Antibody binding was detected with horseradish peroxidase–conjugated secondary antibodies (Amersham Biosciences UK Ltd., Little Chalfont, United Kingdom). Enhanced chemiluminescence reagent (Pierce, Rockford, IL) was used for visualization.

**RESULTS**

Immunohistochemistry analysis of clear cell epithelium in VHL-associated RCC showed coexpression of Epo and EpoR in the cytoplasm of all 11 tumors (Figs. 1 and 2). Coexpression of Epo and EpoR was also detected in cells of 10 of 16 renal cysts (Figs. 1D and 2D). Six renal cysts stained positively for Epo but not for EpoR.

**Table 1 Genetic and clinical features of 8 patients with VHL.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>VHL mutation</th>
<th>RCC and cysts</th>
<th>Hemangioblastomas</th>
<th>Pancreatic cysts</th>
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<td>1</td>
<td>M</td>
<td>Large deletion</td>
<td>Bilateral</td>
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<td>Positive</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>c.499C&gt;T (Q96X)</td>
<td>Bilateral</td>
<td>Positive (retinal)</td>
<td>Positive</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>c.681T&gt;G (Y156X)</td>
<td>Unilateral</td>
<td>Positive (CNS)</td>
<td>Positive</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
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<td>Positive</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>c.564G&gt;T (W117C)</td>
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<td>Positive (CNS, retinal)</td>
<td>Positive</td>
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<tr>
<td>6</td>
<td>F</td>
<td>c.464?_642?</td>
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<tr>
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</tr>
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<td>M</td>
<td>c.446A&gt;C (N78T)</td>
<td>Bilateral</td>
<td>Positive (CNS)</td>
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NOTE. The VHL gene mutation position coordinates are according to Genbank submission (NM_000551) and Ensemble Genome Browser (www.ensembl.org).
All patients had known germ line VHL mutations. In addition, we detected loss of heterozygosity of a wild-type VHL allele in different RCCs with microsatellite markers, D3S1110 and D3S2452, flanking the VHL gene (Fig. 3A).

Reverse transcription–PCR consistently revealed the presence of Epo mRNA in VHL-associated RCC (Fig. 3B). In normal VHL kidney, Epo mRNA was expressed in low levels. EpoR mRNA was consistently detected by reverse transcription–PCR analysis in RCC but not in normal kidney of patients with VHL (Fig. 3B).

Coexpression of Epo and EpoR in VHL renal tumors was further confirmed by demonstrating both Epo and EpoR protein expression in VHL-associated RCC using Western blot analysis (Fig. 3C). VHL-associated hemangioblastoma was used as positive control and showed coexpression of both Epo and EpoR.

DISCUSSION

In this study we found coexpression of Epo and EpoR in 11 RCCs and in 10 benign renal cysts in a spectrum of VHL renal
that may result in negative scoring. The intensity of EpoR staining may be weak for technical reasons of neoplasia and not yet express EpoR. Second, in some cysts stained for Epo but not EpoR. There are at least two possibilities why EpoR stain was not detected in these cysts.

Fig. 3 A, representative four VHL-associated RCCs (T1-4) show marked allelic imbalance (loss of heterozygosity) after amplification with VHL gene flanking primers D3S1110 and D3S2452. Matched adjacent normal kidney (N1-4) shows preservation of both alleles of the VHL gene. B, expression of Epo and EpoR mRNA in VHL-associated RCC abnormal kidney by reverse transcription-PCR. Lane 1, molecular weight marker; lane 2, normal VHL kidney; lane 3, VHL-associated RCC. Epo mRNA is expressed in RCC. Normal kidney shows low intensity of Epo mRNA expression. EpoR mRNA is expressed in VHL-associated RCC but not in normal kidney. C, Western blot analysis for Epo and EpoR expression in VHL-associated RCC. Epo and Epo-R proteins are expressed in three VHL-associated RCCs (lanes 1-3) and in VHL-associated hemangioblastoma (lane 4) used as positive control. Adrenal gland tissue is used as negative control (lane 5).

disease suggesting that Epo/EpoR coexpression is present in both benign and malignant stages of renal neoplasia. Six benign cysts stained for Epo but not EpoR. There are at least two possibilities why EpoR stain was not detected in these cysts. First, some renal cysts may represent different progression stages of neoplasia and not yet express EpoR. Second, in some cysts intensity of EpoR staining may be weak for technical reasons that may result in negative scoring.

The histogenesis of VHL-associated tumors remains controversial. In hemangioblastoma, several different cell types have been implicated as the cell of origin (12–15), and in RCC an uncertainty persists between proximal versus distal tubular cell of origin (16–18). Studies suggest that VHL-associated hemangioblastoma derives from primitive embryonal angiomesenchymal cells capable of differentiating into hemangioblasts (9–11). Primitive blood islands express EpoR (19) and coexpression of Epo because VHL deficiency may facilitate uncontrolled cell growth.

We chose to study RCC in VHL disease because RCC and hemangioblastoma have similar phenotype and genotype, and, therefore, could share a similar cell of origin. VHL-associated hemangioblastoma and RCC are strikingly similar morphologically, and they are composed of clear cells intermixed with numerous small vessels (Fig 4A and B). Similar to hemangioblastoma, renal lesions are multifocal and share the same genetic abnormality (8). Loss of heterozygosity of the wild-type VHL allele has been detected in multiple RCCs as well as in many benign cysts in kidney parenchyma of patients with VHL, suggesting that some renal cysts are precursors of RCC (8). Activation of the hypoxia-inducible factor-1 pathway as a mechanism of angiogenesis is present in hemangioblastoma and RCC (20).

Although hemangioblastoma and low-grade RCC have almost identical morphology on H&E staining, the presence of epithelial markers in RCC suggested commitment to epithelial differentiation. Interestingly, some hemangioblastomas also express epithelial markers (21). Recent evidence suggests that VHL-associated hemangioblastoma derives from primitive embryonal angiomesenchymal cells capable of differentiating into hemangioblasts (9). Analogous to hemangioblastoma, RCC may contain extramedullary hematopoiesis (22), a possible indicator of retained angiomesenchymal differentiation in RCC. Most importantly, in the kidney there is evidence that both nephrogenous mesenchyme and angiomesenchyme are derived from the same mesodermal precursor (refs. 23, 24; Fig. 4C). Furthermore, nephrogenic mesenchyme has a pluripotent capacity and gives rise to renal epithelia, mesenchymal, and stromal elements (24).

Expression of Epo in RCC and renal cysts may result from VHL gene deficiency through hypoxia-inducible factor-1 pathway (20, 25). EpoR expression normally occurs in the angioblast stage of embryonic development as part of hypoxia response (19). During normal development such EpoR expression in cells is transient (19). However, because of inactivation of VHL protein in cells of a patient with VHL, Epo and EpoR coexpression may persist in tumor precursor cells (9). The retention of Epo and EpoR coexpression in primitive mesenchymal cells may lead to cell proliferation via the autocrine stimulation (26) and become a critical pathogenetic step in tumor formation. Therefore, coexpression of Epo and EpoR in VHL-associated RCC and cysts suggests the possibility that a precursor cell of renal lesions is a developmentally arrested, pluripotent embryonal cell derived from nephrogenous mesenchyme.

In conclusion, similarities between angiomesenchyme and nephrogenous mesenchyme suggest that tumorigenesis in VHL disease may be initiated during embryogenesis and effect highly specific organs. VHL gene deficiency causes precursor cells in nephrogenous mesenchyme to arrest early in the developmental stage and to coexpress Epo and EpoR; Epo and EpoR coexpression persists and is detectable in benign renal cysts and RCC. Although further developmental biological evidence is necessary, striking analogies between hemangioblastoma and
RCC, including Epo and EpoR coexpression, morphology, and mesenchymal derivation strongly suggest a similar developmental origin for both VHL tumor types. How angiomesenchymal precursor cells develop into neoplastic cells and why some renal cysts develop into malignant tumors, whereas many others do not, need to be investigated further.

ACKNOWLEDGMENTS

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REFERENCES


Fig. 4 Histogenesis of VHL tumors. Similar histopathology of hemangioblastoma (A) and low-grade RCC (B) in VHL disease (H&E): both tumors are composed of clear cells intermixed with numerous small vessels. C, principal events of early nephrogenesis. Modified after Horster et al. (24). Ureteric bud, which is derived from Wolffian duct, invades nephrogenous mesenchyme and initiates reciprocal signaling between epithelial (ductal) and mesenchymal cell types. Nephrogenous mesenchyme expresses stem cells of several cell lineages including mesenchymal, epithelial, and stromogenic ones.


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