Developmental Arrest of Angioblastic Lineage Initiates Tumorigenesis in von Hippel-Lindau Disease

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Hippel-Lindau Disease

Abstract

The nature of the cell responsible for von Hippel-Lindau (VHL) disease-associated tumor formation has been controversial for decades. We demonstrate that VHL disease-associated central nervous system tumors are composed of developmentally arrested angioblasts that coexpress erythropoietin (Epo) and Epo receptor. The angioblasts are capable of differentiating into RBCs via formation of blood islands with extramedullary hematopoiesis. Because of VHL deficiency, Epo receptor-expressing, developmentally arrested angioblasts simultaneously coexpress Epo, which may represent a crucial pathogenetic step in tumor formation.

Introduction

VHL disease is a tumor syndrome caused by germ-line mutation of the VHL gene (1). Tumors most frequently develop in the retina, cerebellum, spinal cord, and kidneys. Central nervous system tumors, hemangioblastomas, are characterized by complex morphological features and endocrine activity, and hemangioblastoma histogenesis is considered uncertain (2, 3). According to the hypothesis of Knudson, tumor formation is initiated by inactivation of the wild-type VHL allele (4). Subsequently, lack of VHL protein induces profound intracellular metabolic changes that closely resemble, if not mimic, changes observed in oxidative stress. In wild-type cells, the α subunit of HIF is controlled by the pVHL-elongin-cullin-2 complex (5, 6). In VHL protein-defective cells, however, HIF is up-regulated and further mediates the up-regulation of hypoxia-inducible genes, including Epo and VEGF (5, 6). It remains unclear, however, how these general and ubiquitous mechanisms of hypoxic response can be implicated in the tumorigenesis of specific subsets of cells.

In 1928, Cushing and Bailey (7) divided vascular malformations of the nervous system into two major groups, the angiomatous malformations and the hemangioblastomas. In 1931, Arvid Lindau (8) based the distinctness of central nervous system hemangioblastoma on a series of criteria including “unmistakable neoplasticity” with “composition of blood vessel elements” and a “tendency toward cyst formation.” He speculated that hemangioblastoma tissue may be derived from a “congenital anlage” and that the histological picture revealed an “...embryological type of the tumor cells.” As further evidence for an embryological origin, Lindau cites a paper by Roussy and Oberling (9), who “...have found evidence of hemopoiesis, a circumstance that Cushing and Bailey, as well as myself [Arvid Lindau], have sought for in vain.” In 1960, Stein et al. (10) suggested an angiomesenchymal origin of hemangioblastoma, based on original observations by Florence Sabin (11). On morphological grounds, Stein et al. (10) described embryonic blood and vessel formation in hemangioblastoma tissue and speculated the presence of “an arrest or defect in maturation of one or more tissues at a particular time in embryonic development.” During the last decades, however, the histogenesis of hemangioblastomas has been highly controversial, and contemporary texts characterize these tumors as “neoplasms of uncertain histogenesis” constituted of vascular and so-called “stromal” cells (3, 12). Recently, we have provided evidence in VHL disease-associated hemangioblastomas that these “stromal” cells are VHL-defective neoplastic cells (13). We here report that the “stromal” cells are the morphological correlate of neoplastic angiomesenchyme and capable of blood island formation. We thus confirm the original hypotheses of Lindau and Stein and provide new insight into the histogenesis and pathogenetic progression of hemangioblastoma and, possibly, other VHL disease-associated tumors.

Materials and Methods

Immunohistochemistry. Immunohistochemistry was performed on both paraffin-embedded and frozen sections. Routinely, multiple serial sections were taken from frozen and paraffin-embedded tissues to facilitate comparison of immunohistochemistry results with morphological phenotype. Frozen tissue sections were used after fixation in 80% ethanol. Sections from formalin-fixed paraffin-embedded tissue were fixed in xylene and then washed in decreasing concentrations of methanol. For antigen retrieval, sections were treated with DAKO Target Retrieval Solution (DAKO, Carpinteria, CA) and incubated at 95°C for 20–30 min. Sections were cooled at room temperature and washed three times in PBS. Both paraffinized and frozen sections were then quenched for 20 min in a solution of 3 ml of H2O2 and 180 ml of methanol. After three washes in PBS, sections were incubated in 10% horse serum for 1 h. The primary antibody was diluted in 2% horse serum, and the sections were then incubated in a humidified chamber at 4°C overnight. Primary antibodies used were as follows: mouse ascites antihuman HIF-1α, 1:200; rabbit polyclonal antihuman Epo, 1:100; sheep polyclonal antihuman EpoR; rabbit polyclonal antihuman neuron-specific enolase, 1:200; and monoclonal CD 34, 1:400. The sections were then incubated with secondary antibody and avidin-biotin complex for 1 h each. Diaminobenzidine was left on the sections for only the amount of time it took for the reactivity to become apparent; this time ranged from 20 s to 4 min. Tissue reaction with diaminobenzidine was stopped by dipping sections in tap water. A 10-min counterstaining with Mayer’s hematoxylin followed. The sections were then dehydrated by graded ethanol washes and xylene wash before being mounted. The presence and intensity of antibody expression were examined in conjunction with the various morphological features of hemangioblastoma.

Western Blot. For Western blotting, 20 μl of cell lysate were separated by electrophoresis on 10–20% gradient Tris-glycine gels (Novex, San Diego, CA). Proteins were electrotransfered onto Immobilon-P membranes (Millipore Corp., Milford, MA). Blots were blocked in PBS/0.05% Tween 20 containing 5% fetal bovine serum and incubated with anti-Epo antibodies.
(1:25; Oncogene, Cambridge, MA) and anti-EpoR [1:400 (Calbiochem, San Diego, CA) and 1:200 (Santa Cruz Biotechnology, Santa Cruz, CA)], respectively. Antibody binding was detected with horseradish peroxidase-conjugated secondary antibodies (Amersham, Arlington Heights, IL). Enhanced chemiluminescence reagent (Amersham Biosciences United Kingdom Ltd., Little Chalfont/Buckinghamshire, United Kingdom) was used for visualization.

**Total RNA Isolation and Epo/EpoR RT-PCR.** Frozen tissues were sectioned at 10 μm and microdissected under a light microscope. Total RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer’s instructions. During the RNA precipitation step, nucleic acid carrier (Novagen) was added. A first-strand cDNA from total RNA was synthesized as follows. To avoid amplification of possible contaminating genome DNA, total RNA was treated with RNase-free DNase I (0.2 unit, total volume of 10 μl; Invitrogen) at room temperature for 15 min, denatured at 65°C for 10 min, and subsequently reverse transcribed by SuperScript II (Invitrogen) with 0.5 μg of oligodeoxynucleoside triphosphate in a volume of 20 μl. The PCR amplification was performed following these cycling parameters: initial denaturing at 95°C for 1 min, 95°C for 15 s, and 68°C for 30 s for 35 cycles. Reaction mixture contained 2 μl of the cDNA template, 1.0 unit of Advantage-2 DNA polymerase (Clontech), 1/10 PCR buffer, 200 μM of each deoxynucleoside triphosphate, and 200 nM of each primer. The sequences of oligonucleotides used for RT-PCR were as follows: Epo, 5'-TCTATGCTTGGAGAGATGGAGGTGCG-3' and 5'-TGCGAAAGTTCGAGCTGATGTC-3'; EpoR, 5'-CACAAAGGCTACCTCAGCTGAGCTGTA-3' and 5'-CATTGTCC-AGCACCAGATAGATCCGG-3'; and glyceraldehyde-3-phosphate dehydrogenase, 5'-TGGCCAAGGTCATCCATGACAACTTTG-3' and 5'-GCCTGCTTCAACACTTTCTTGTGACA-3'. The PCR products were separated in 1.5% agarose gel and stained with ethidium bromide.

**Adult and Fetal Hemoglobin RT-PCR.** Frozen tissues were sectioned at 10 μm and microdissected under a light microscope. Quantitative real-time PCR assay of β- and γ-globin transcripts in tissue cDNA samples was carried out using gene-specific TaqMan probe/primer sets and platinum quantitative PCR SuperMix-UDG (Invitrogen) after RT using Superscript II reverse transcriptase (Invitrogen). Probes were labeled with FAM and TAMRA as reporter and quencher, respectively. The following primer/probe sets were used: β-globin forward primer, 5'-CTCATGGCAAGAAAGTCD-3'; β-globin reverse primer, 5'-GCCTGCTTCAACACTTTCTTGTGACA-3'; and γ-globin forward primer, 5'-CTCATGGCAAGAAAGTCD-3'; and γ-globin reverse primer, 5'-GCCTGCTTCAACACTTTCTTGTGACA-3'.

**Fig. 1.** Western blots revealing the presence of Epo (Mr 34,000) and EpoR (Mr 56,000) in six different hemangioblastomas with EMH. Bottom panel, RT-PCR consistently amplifies RNA fragments specific for Epo and EpoR in six hemangioblastomas with EMH (Lanes 1–6). Lane 7, normal brain control reveals amplification of Epo but not EpoR RNA.

**Fig. 2.** Different areas of blood island formation (#1–3) in hemangioblastoma; from each block, serial sections were prepared, and immunohistochemistry was performed on consecutive sections revealing strong immunoreactivity with anti-Epo and anti-EpoR antibodies in the same cellular structures. Highlighted structures [bottom drawing, modified after Shumway and Adamstone (24) and Patten (25)] resemble early blood island formation from angiogenic mesenchyme.
reverse primer, 5′-AATTCCTTGCCAAAGTGATGGG-3′; β-globin probe, 5′-FAM-CGTGATCTGAGAACTTCAGGCTCCT-TAMRA-3′; γ-globin forward primer, 5′-GGCAACCTGTCTCTGCTC-3′; γ-globin reverse primer, 5′-GAAATGGATTGCCAAAACGG-3′; and γ-globin probe, 5′-FAM-CAAGCTCTGGGAAATGTGCTGGTG-TAMRA-3′.

**DNA Analysis.** Unstained 5-μm sections on glass slides were deparaffinized with xylene, rinsed in ethanol from 100% to 80%, stained briefly with H&E, and rinsed in 10% glycerol in 10 mM Tris (pH 8)-1 mM EDTA buffer. Microdissection was performed under direct light microscopic visualization using a 30-gauge needle. Microdissected areas of interest included foci of EMH from different tumors, neoplastic angioblasts for positive control, and adjacent normal brain tissue for negative control. Procured cells were immediately resuspended in a 5-μl buffer containing Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1% Tween 20, and 0.1 mg/ml proteinase K and incubated at 37°C overnight. The mixture was boiled for 10 min to inactivate proteinase K, and 2 μl of the DNA solution were used for PCR amplification using markers D3S1038 and D3S1110 (Research Genetics, Huntsville, AL) flanking the VHL gene.

**Results and Discussion.**

From a series of hemangioblastomas that were surgically removed from VHL patients with known VHL germ-line mutations during the last 2 years, we collected six cerebellar tumors with evidence of EMH. In two of these tumors, EMH was multifocal and extensive; the four last 2 years, we collected six cerebellar tumors with evidence of EMH. In two of these tumors, EMH was multifocal and extensive; the four last 2 years, we collected six cerebellar tumors with evidence of EMH. In two of these tumors, EMH was multifocal and extensive; the four last 2 years, we collected six cerebellar tumors with evidence of EMH. In two of these tumors, EMH was multifocal and extensive; the four last 2 years, we collected six cerebellar tumors with evidence of EMH. In two of these tumors, EMH was multifocal and extensive; the four last 2 years, we collected six cerebellar tumors with evidence of EMH. In two of these tumors, EMH was multifocal and extensive; the four last 2 years, we collected six cerebellar tumors with evidence of EMH. In two of these tumors, EMH was multifocal and extensive; the four last 2 years, we collected six cerebellar tumors with evidence of EMH. In two of these tumors, EMH was multifocal and extensive; the four last 2 years, we collected six cerebellar tumors with evidence of EMH. In two of these tumors, EMH was multifocal and extensive; the four last 2 years, we collected six cerebellar tumors with evidence of EMH. In two of these tumors, EMH was multifocal and extensive; the four last 2 years, we collected six cerebellar tumors with evidence of EMH.

Early embryonic blood island formation is associated with EpoR protein expression at mouse embryonic day 8.0–9.5 (14). In full agreement with the hypothesis of angiomesenchymal histogenesis, Western blot and RT-PCR analysis revealed the presence of EpoR protein and mRNA in all six hemangioblastomas (Fig. 1). Similarly, in agreement with the VHL-deficient status of the tumor cells, Western blot and RT-PCR analyses of the same six tumors also revealed the presence of Epo expression. In contrast, expression of Epo and EpoR is temporally and spatially segregated during normal embryonic blood island development (14). Simultaneous expression of Epo and EpoR may therefore play a critical pathogenetic role in the progression of hemangioblastoma. Immunohistochemical analysis of serial sections taken from the same tissue blocks revealed intense coexpression of Epo and EpoR in all cells of the same neoplastic structures with striking resemblance to blood islands (Fig. 2).

Homozygous *VHL*+/− mouse embryos die in utero at 10.5–12.5 days of gestation because of failure of vasculogenesis, which implies that the *VHL* gene is critical for functional blood island formation (15). Because blood islands in VHL disease-associated hemangioblastomas are deficient of wild-type *VHL* gene, it appears that the developmental arrest of the neoplastic angioblasts is directly related to VHL gene dysfunction. Therefore, the coexpression of Epo and EpoR in neoplastic blood islands may be directly linked to VHL deficiency and may represent a key event associated with developmental arrest and further neoplastic proliferation of these tumors.

Another hypoxia-inducible protein implicated with angiogenesis, VEGF, is known to be expressed in hemangioblastoma (16, 17). VEGF is expressed in rat blood islands between days 8 and 11 (18), and mice deficient in VEGF (19) or its two receptors, Flt-1 (20) and Flk-1 (21), die in utero with severely impaired or absent blood island.
formation and absent angiogenesis. VEGF expression in hemangioblastomas is a contributor to the histogenetic controversy because one of its functions is the recruitment of abundant reactive vascular cells into the tumor. Immature vasformation derived from neoplastic angioblasts in hemangioblastomas, however, is easily distinguished from reactive vascularization by immunohistochemistry.

To provide further evidence that neoplastic angioblasts are pluripotent and capable of differentiation into RBCs, we microdissected foci of primitive EMH from hemangioblastomas and performed deletion analysis of the VHL wild-type allele. Deletion of the wild-type VHL allele was detected both in areas of blood island formation and in areas of EMH (Fig. 3). Therefore, at least a subset of the abundant RBCs in hemangioblastomas appears to be derived from neoplastic angioblastic differentiation. To further confirm the presence of primitive hematopoiesis, we detected fetal-type hemoglobin by Western blot from the same tumors after extracting protein from microdissected tumor tissue (Fig. 4). In addition, RT-PCR performed after RNA extraction of microdissected tumor tissue revealed elevated levels of embryonic (γ) versus adult (β) hemoglobin in two tumors with extensive EMH, as compared with tumors with spurious or absent EMH (Fig. 4).

In summary, VHL disease-associated hemangioblastomas are primarily comprised of developmentally arrested hemangioblasts with differentiation potential into primitive vascular structures and RBCs in analogy to embryonic angioblastic mesenchyme. VHL gene deficiency appears to be primarily responsible for the developmental differentiation arrest, possibly via up-regulation of Epo in EpoR-expressing angiomesenchyme at embryonic development. Coexpression of Epo and EpoR may not only mediate developmental stagnation but may also induce proliferation through autocrine/paracrine stimulation.

These results indicate that tumorigenesis in VHL disease and possibly in other tumor suppressor gene syndromes may be initiated during embryogenesis, as has been theoretically assumed by the highly specific set of organs that is consistently involved in affected patients (10). What needs further explanation is the highly unpredictable clinical course of individual tumors (22). A similar mechanism may underlie the origin of other VHL disease-associated neoplasms, in particular the biologically malignant renal carcinoma. VHL disease-associated renal clear cell carcinoma strikingly resembles hemangioblastoma, possibly reflecting similarities between angiomesenchyme and nephrogenic mesenchyme. We detected coexpression of Epo and EpoR by immunohistochemistry in two VHL disease-associated renal clear cell carcinomas (data not shown), suggesting developmental pathways similar to those demonstrated here. It will also be tempting to investigate to what extent pathological embryogenetic events in hereditary tumor disease are paralleled by stem cell pathology in sporadic tumorigenesis (23). Finally, specific targeting of the Epo/EpoR autocrine pathway may provide new therapeutic options for VHL disease-associated tumors and their sporadic counterparts.

References


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