VHL Gene Deletion and Enhanced VEGF Gene Expression Detected in the Stromal Cells of Retinal Angioma

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Objectives: Retinal angioma frequently occurs in von Hippel-Lindau (VHL) disease. However, VHL gene alterations have not been documented in retinal angiomas.

Methods: Using tissue microdissection and polymerase chain reaction amplification, we have analyzed 7 retinal angiomas associated with VHL disease for loss of heterozygosity of the VHL gene. In addition, vascular endothelial growth factor expression was evaluated in these tumors by immunohistochemistry and in situ hybridization.

Results: All 6 informative retinal angiomas showed loss of heterozygosity of the VHL gene. Loss of heterozygosity was detected in vacuolated “stromal” cells, but not in vascular cells or reactive glial tissue. Vascular endothelial growth factor protein and messenger RNA were also present in vacuolated “stromal” cells.

Conclusions: These findings suggest that vacuolated “stromal” cells represent the true neoplastic component in retinal angioma. These cells express vascular endothelial growth factor and therefore may be responsible for abundant neovascularization of retinal angioma.


VON HIPPEL-LINDAU (VHL) disease is a hereditary cancer syndrome in which affected individuals are at risk to develop tumors in multiple organs, including the eyes, cerebellum, spinal cord, kidneys, inner ear, adrenal glands, and pancreas. Retinal angioma and cerebellar hemangioblastoma are the most common and frequently the earliest manifestations of VHL disease. Retinal angioma has been reported in nearly 60% of patients with VHL disease. Although most VHL disease–associated tumors have been shown to have genetic alterations of the VHL gene, retinal angiomas have not been genetically studied.

Retinal angioma has been proposed to represent either a congenital, hamartomaous-type lesion or a benign vascular neoplastic process. Retinal angiomas consist mainly of thin-walled, capillary-like or slightly larger, blood vessels forming an anastomosing pattern separated by plump, vacuolated, foamy, “stromal” cells. The nature and origin of these cells remain elusive. Ultrastructural and immunohistochemical studies have suggested the “stromal” cells represent lipidized fibrous astrocytes or glial cells. Many “stromal” cells contain foamy vacuolated cytoplasm, which is strikingly similar to that of cerebellar hemangioblastoma and renal clear cell carcinoma in VHL disease. Recently, the “stromal” cells of the cerebellar hemangioblastoma and renal carcinoma cells in VHL disease have been shown to have VHL gene alterations using microdissection and molecular techniques.

The VHL gene is localized at chromosome 3p25.9 and has been identified as a tumor suppressor gene. Malignant neoplasms in VHL are believed to follow the “two-hit” model, in which one allele is constitutionally inactivated while the other allele is subsequently inactivated (second hit) at the somatic level. It has been hypothesized that most, if not all, VHL-associated tumors develop after inactivation of the second VHL tumor suppressor allele. The VHL gene product, VHL protein (pVHL), inhibits the accumulation of hypoxia-inducible messenger RNA (mRNA), including vascular endothelial growth factor (VEGF), under normoxic conditions. The mutant pVHL is unable to regulate transcription of VEGF mRNA, resulting in neovascu-
MATERIALS AND METHODS

SPECIMENS

Eight eyes obtained from 7 patients with family histories and clinical diagnoses of VHL disease were studied. Six cases were collected from the files of the W. R. Green Eye Pathology Laboratory, Wilmer Eye Institute, Johns Hopkins Hospital, Baltimore, Md. They were obtained from either surgical enucleation or autopsy, fixed in formalin, and processed and sectioned between 1965 and 1994. All slides of these 6 cases were thoroughly reviewed to identify the retinal lesions that were suitable for subsequent microdissection study.

The remaining case was a 35-year-old man with a family history of VHL disease and VHL germline mutation who developed ocular and central nervous system tumors. Both eyes were extensively involved and became blind and phthisical. The left eye underwent several procedures including laser photocoagulation, vitrectomy, and retinal re-attachment. Eventually bilateral enucleation was performed. The right eye was immediately snap frozen. The left eye was fixed in 70% alcohol before embedding in paraffin. Both eyes of this patient were sent to the National Eye Institute, Bethesda, Md, for processing.

MICRODISSECTION

Unstained 5-μm sections on glass slides were deparaffinized with xylene, rinsed in ethanol from 100% to 80%, briefly stained with hematoxylin-eosin, and rinsed in 10% glycerol in tromethamine hydrochloride-EDTA buffer. Microdissection was performed under direct light microscopic visualization using a 30-gauge needle as previously described.8,11 From each case, at least 2 areas of neoplastic tissue and 2 areas of “normal” tissue (retina, ciliary body, iris, or cornea) were selected. In each case, the entire tumor was screened and tumor cells were procured from areas with predominately “stromal” cells and a minimal amount of vessels. From 1 case we selectively procured vascular cells without contamination of “stromal” components or intravascular leukocytes.

DNA EXTRACTION

Procured cells were immediately resuspended in 20 μL of buffer containing tromethamine hydrochloride, pH 8.0; 10-mmol/L EDTA, pH 8.0; 1% polyoxyethylene(20) sorbitan monolaurate; and 0.3-μg/mL proteinase K, and were incubated at 37°C overnight. The mixture was boiled for 10 minutes to inactivate the proteinase K, and 1.5 μL of this solution was used for polymerase chain reaction (PCR) amplification of the DNA.

PRIMERS AND PCR CONDITIONS

All samples were examined for loss of heterozygosity (LOH) using the microsatellite markers D3S1038 and D3S1110 flanking the VHL gene (Research Genetics, Huntsville, Ala). Each PCR sample contained 1.5 μL of template DNA as described above; 10 pmol of each primer; 20 nmol each of deoxyadenosine 5’-triphosphate (dATP), deoxycytidine 5’-triphosphate (dCTP), deoxyguanosine 5’-triphosphate (dGTP), and deoxythymidine 5’-triphosphate (dTTP); 15-mmol/L magnesium chloride; 0.1 unit of Taq DNA polymerase; 0.05-μL [32P]dCTP (1.85 × 107 Bq); and 1 μL of 10 mM buffer in a total volume of 10 μL. Polymerase chain reaction was performed with 35 cycles: denaturing at 94°C for 1 minute, annealing at 55°C for 1 minute, and extending at 72°C for 1 minute. The final extension was continued for 10 minutes.

LOH ANALYSIS

Labeled amplified DNA was mixed with an equal volume of formamide loading dye (95% formamide, 20-mmol/L EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). Samples were then denatured for 5 minutes at 95°C, loaded onto a gel consisting of 6% acrylamide (ratio of acrylamide to bisacrylamide, 49:1), and electrophoresed at 1800 V for 90 minutes. After electrophoresis, the gels were transferred to 3-mm Whatman paper and dried. Autoradiography was performed with Kodak X-OMAT film (Eastman Kodak, Rochester, NY).

A case was considered informative for a polymorphic marker if normal tissue DNA showed 2 different alleles (heterozygosity). The criterion for LOH was complete or near complete absence of one allele in the tumor DNA as defined by direct visualization.

IMMUNOHISTOCHEMISTRY

Frozen and deparaffinized sections were stained using the avidin-biotin-peroxidase complex technique. The primary antibodies were polyclonal rabbit antibodies against VEGF (Santa Cruz Biotechnology, Santa Cruz, Calif), factor VIII and glial fibrillary acidic protein (GFAP) (Dako Corp, Santa Barbara, Calif) or control rabbit IgG. The secondary antibody was biotin-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, Calif). The substrate was avidin-biotin-peroxidase complex, and the chromogen was diaminobenzidine.

IN SITU HYBRIDIZATION

The presence of VEGF mRNA was detected by a digoxigenin–uridine-5c-triphosphate–labeled riboprobe. A 649-bp fragment of VEGF complementary DNA (a gift from Karl Csaky, MD, of the National Eye Institute15) was initially subcloned into the pBluescript II KS ± vector (Stratagene, La Jolla, Calif) and linearized prior to labeling. The mRNA sense and antisense probes were then prepared in the in vitro transcription with digoxigenin–uridine-5’-triphosphate (Dig RNA Labeling Kit, Boehringer Mannheim, Indianapolis, Ind). Following hybridization on the proteinase K and Triton X-100 (Sigma, St Louis, Mo) pretreated slides, immunodetection was conducted using a nucleic acid detection kit (Boehringer Mannheim).

RESULTS

All specimens contained classic retinal angiomas associated with VHL disease. They were composed of numerous small capillary-like vascular channels, inter-
mixed with vacuolated “stromal” cells (Figure 1, A and B). In general, the larger tumor (>60 mm) consisted of a higher percentage (>70%) of vacuolated “stromal” cells. Three tumors had been treated with laser photocoagulation therapy, resulting in reduced cellularity and an increased amorphous intercellular matrix (Figure 1, C and D). At the tumor margins, reactive gliosis was observed. In one case, so-called massive gliosis was observed, consistent with abundant reactive proliferation of retinal glia secondary to a variety of pathologic processes.

Glial fibrillary acidic protein stain was negative in vacuolated “stromal” cells. In contrast, positive staining for GFAP was detected in adjacent areas of gliosis (Figure 2, A). Factor VIII (specific for vascular endothelial cells and megakaryocytes) was positive in the vascular component, especially in the capillary hemangioma-like channels (Figure 2, B and D). All large vacuolated “stromal” cells in the tumors stained strongly positive for VEGF protein (Figure 2, C) and mRNA (Figure 2, E and F), but negative for GFAP.

Because the vascular cells in retinal angiomas are intimately intermingled with the large vacuolated cells, we were unable to selectively microdissect the 2 types of cells separately. Instead, we procured areas of predominantly vacuolated “stromal” cells (Figure 1, A and B). From one case we were able to selectively procure a vascular structure (Figure 1, E and F). All samples were analyzed with the polymorphic markers D3S1038 and D3S1110. The areas that consisted of largely vacuolated “stromal” cells were found to have LOH with at least one marker in all 6 informative cases (Figure 3). One case was noninformative. Retention of heterozygosity was shown in normal control tissue, reactive glial tissue, and the selectively microdissected tumor vessels (Figure 1, A and B).
E and F). The 3 selected vascular components of the tumor did not contain leukocytes (Figure 1, E), which would mask LOH in these specimens.

According to the two-hit hypothesis, dominantly inherited predisposition to cancer entails a germline mutation, while tumorigenesis requires a second, somatic, genetic alteration. In patients with familiar cancer syndromes, inactivation would need to occur on the “wild-type” allele, the other allele already having an inactivating mutation transmitted in the germline (and so present in all cells). Theoretically, that “inactivation” can occur through a variety of mechanisms including chromosome loss, deletion, mitotic recombination, point mutation, and hypermethylation. Several hereditary disorders relevant to ophthalmology have been shown to be caused by tumor suppressor gene defects, including VHL disease, retinoblastoma, tuberous sclerosis, and neurofibromatosis 1 and 2. The associated tumors have been shown to carry somatic genetic alterations.

An autosomal dominant genetic disorder, Von Hippel-Lindau disease, is characterized by the development of a variety of neoplasms including hemangioblastomas of the central nervous system, renal cell carcinomas, pheochromocytomas, and pancreatic and renal cysts. The VHL tumor suppressor gene has been linked to chromosome 3p25 and subsequently identified. Recent genetic studies on renal cell carcinomas, pheochromocytomas, and central nervous sys-

Figure 2. Microphotographs showing protein and messenger RNA (mRNA) expressions in retinal angioma. A, Glial fibrillary acidic protein. B, Factor VIII. C, Vascular endothelial growth factor (VEGF) protein. D, Factor VIII. E, VEGF mRNA with antisense probe. F, VEGF mRNA with sense probe (avidin-biotin complex immunoperoxidase staining, A-D; in situ hybridization, E and F; original magnification A and B, ×200; C-F, ×400).
Recently, possible mechanisms by which pVHL—
the VHL suppressor gene product—may down-regulate VEGF expression have been demonstrated by both transcription and posttranscription via elongin, Sp1, and protein kinase C. The VHL gene product has been characterized as forming a stable trimolecular complex with 2 subunits of the highly conserved heterotrimeric transcription elongation factor elongin.13,30,37 Elongin is composed of 3 subunits (A, B, and C). The VHL gene product competes with elongin A for binding to elongin B and C, thereby inhibiting elongin activity. The VHL gene expression could be induced under normoxic conditions leading to a reduced transcriptional elongation rate of unknown target genes.30 The VHL gene product directly binds Sp1 and inhibits Sp1 activity.39 Endogenous Sp1 binds the guanosine-cytidine–rich region of the VEGF promoter. Thus, the VEGF gene is repressed by normal pVHL. Subsequently, the mRNA transcription of VEGF mRNA down-regulated by pVHL is reduced, resulting in reduced vascularization. In addition to Sp1 and elongin C and B, other proteins likely interact with pVHL. For example, the complexes of pVHL with protein kinase C isoforms ζ and δ in the cytoplasm are not able to translocate to the cell membrane where they otherwise would engage in downstream signaling of VEGF overexpression, and thereby inhibit mitogen-activated protein kinase phosphorylation.40 The mutant pVHL is thought to be unable to balance regulation. Vascular endothelial growth factor will be up-regulated, leading to an increased vascularization.

Vascular endothelial growth factor, a potent angiogenic factor, has been implicated in ischemic retinopathies.41,42 This growth factor has been shown to initiate production and release of collagenase by endothelial cells, and to stimulate endothelial-cell migration and proliferation in the eye. Introduction of the wild-type VHL gene into renal carcinoma cells with a VHL gene mutation can result in decreased VEGF mRNA expression.13,43 Furthermore, up-regulation of VEGF and expression of the VEGF receptor have been found in the “stromal” cells of cerebellar hemangioblastoma.44 These data correlate well with the results of the current study, which finds high expression of VEGF and mRNA in retinal angioma, particularly in the “stromal” cells of the tumor. The findings suggest that the genetic alteration of the VHL gene in the “stromal” cells is associated with the development of angioma in the retina. The prominent vascular elements may represent both preexisting vessels and neovascularization during tumor development.

In summary, allelic deletion at the VHL gene locus is identified in retinal angioma of patients with VHL disease. The vacuolated foamy “stromal” cells seem to represent the genetically altered, neoplastic component of retinal angioma. The mutated VHL protein may not be able to down-regulate VEGF expression via possible mechanisms of Sp1, protein kinase C, and elongin. The neoplastic cells will then produce high levels of VEGF and may be responsible for the prominent vascularization of retinal angioma. The up-regulation of VEGF transcription and the subsequent neovascularization presents an ideal target site for therapeutic intervention. Cur-

Figure 3. Autoradiographs showing loss of heterozygosity detected at the von Hippel-Lindau (VHL) gene locus in the foamy “stromal” cells of retinal angioma but not in normal ocular cells of the 6 cases with VHL disease. V indicates the sample of vascular cells depicted in Figure 1, E; T, tumor stromal cell DNA; and N, normal cell DNA. Cases 1, 3, 5, and 6 show reduced intensity of one allele due to “contamination” with somatic cells.

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rently, developing rational therapeutic approaches to prevent blindness in patients with VHL disease and other ocular neovascularization diseases are under way in our laboratory.

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