Up-Regulation of Vascular Endothelial Growth Factor and Its Receptors in von Hippel-Lindau Disease-associated and Sporadic Hemangioblastomas

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ABSTRACT

Capillary hemangioblastoma is the most frequent manifestation of the autosomal dominantly inherited von Hippel-Lindau (VHL) disease but also presents as a nonfamilial, sporadic vascular tumor. Hemangioblastomas are characterized by a dense network of capillaries in association with cysts. To investigate the mechanisms underlying neoangiogenesis and cyst formation, we analyzed eight VHL disease-associated and five sporadic hemangioblastomas. Histologically, both tumor types showed a similar phenotype. The capillaries expressed the endothelial cell markers von Willebrand factor and CD31 antigen. We investigated the expression of vascular endothelial growth factor (VEGF), an endothelial cell-specific mitogen which is also known to induce vascular permeability in vivo, and its high affinity tyrosine kinase receptors flt-1 and KDR. Northern blot and in situ hybridization analysis revealed significant up-regulation of VEGF and VEGF receptor expression in VHL disease-associated and sporadic hemangioblastomas compared to normal brain and tumor stromal cells as sites of abundant VEGF transcription. Endothelial cells did not express detectable amounts of VEGF mRNA but coexpressed flt-1 and KDR. By immunohistochmistry, VEGF protein was detectable in the tumor interstitium and was found to be concentrated around capillaries. Performing reverse transcription-PCR, we demonstrated that VEGF, and VEGF145 were the splice variants predominantly expressed, whereas mRNA encoding VEGF185 was present at smaller amounts. Our findings suggest that, in VHL disease-associated and sporadic hemangioblastomas, VEGF121 and VEGF165 are secreted by stromal cells and interact with the corresponding VEGF receptors expressed on tumor endothelial cells. This paracrine mechanism may mediate neoangiogenesis and cyst formation in capillary hemangioblastomas.

INTRODUCTION

VHL disease is an autosomal dominantly inherited disorder that predisposes affected persons to a variety of lesions in different organs. The major manifestations of VHL disease are hemangioblastomas of the CNS, renal cysts, renal cell carcinomas, pheochromocytomas, pancreatic cysts, and epididymal cystadenomas (1). Genetic linkage analysis mapped the VHL disease gene to chromosome 3p25-p26 (2, 3). Frequent loss of heterozygosity for chromosome 3p in tumors derived from patients with VHL disease suggests that the VHL gene acts as a tumor suppressor gene (2, 4). Recently, the VHL gene has been identified by positional cloning and has been shown to be mutated in the germline of VHL disease family members as well as in familial and sporadic renal cell carcinoma (5, 6).

The most frequent tumor type that forms in VHL disease is the capillary hemangioblastoma (7). Hemangioblastomas occur in the retina and cerebellum and to a lower extent in the area postrema and the spinal cord (1). Besides VHL disease-associated hemangioblastomas, sporadic, nonfamilial hemangioblastomas occur in the CNS. Histologically, familial and sporadic hemangioblastomas consist of a dense network of capillaries. By electron microscopy, three cell types are usually distinguished: (a) endothelial cells and (b) pericytes, which form the microvasculature; and (c) stromal cells, which are clustered between the capillaries (8, 9). Thus far, the stromal cells have no known counterpart among normal cells, and their histogenesis is unclear.

In addition to the development of vascular tumors in the CNS and formation of well-vascularized tumors, such as renal cell carcinoma and pheochromocytoma, frequent cyst formation, which may be due to increased vascular permeability, is characteristic for VHL disease. Recently, VEGF, an endothelial cell-specific mitogen, which induces angiogenesis and vascular permeability in vivo, has been described (10–12). Four isoforms of VEGF are known, which arise by alternative mRNA splicing. The smaller forms (VEGF121 and VEGF165) are secreted proteins, whereas VEGF189 and VEGF206 are cell-associated isoforms (13, 14). Additionally, VEGF has been shown to bind with high affinity to the cognate tyrosine kinase receptors VEGFR-1 (flt-1) (15) and VEGFR-2 (KDR/flk-1; Refs. 16 and 17).

We considered VEGF as a candidate regulator of neoangiogenesis and cyst formation in capillary hemangioblastomas. Therefore, we investigated the expression of VEGF and its two receptors VEGFR-1 (flt-1) and VEGFR-2 (KDR) in VHL-associated and sporadic cerebellar hemangioblastomas. In addition, we examined the distribution of VEGF protein by immunohistochmistry and compared the relative abundance of the different VEGF mRNA isoforms in tumor specimens and normal brain tissue by RT-PCR.

MATERIALS AND METHODS

Specimens. Surgically removed human specimens (eight capillary hemangioblastomas from five patients with VHL disease and five solitary capillary hemangioblastomas) and two specimens of normal brain tissue were routinely formalin fixed and paraffin embedded or frozen directly and stored at −70°C.

RNA Extraction and Northern Analysis. Total cytoplasmic RNA was isolated using the guanidinium thiocyanate method (18). Aliquots of 12.5 μg RNA were electrophoresed in a 0.8% agarose gel containing 0.66 M formaldehyde in 1× MOPS buffer (20 mM 3-(N-morpholino)propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA (pH 7.0)) and transferred to nylon membrane (Hybond N; Amersham) in 20× SSC (1× SSC = 150 mM NaCl-15 mM sodium citrate). RNA was cross-linked to the membrane with UV light (0.5 J/cm2) and hybridized overnight in 50% formamide, 5× SSC, 5× Denhardt’s (0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% BSA), 0.5% SDS, and 50 μg/ml tRNA at 42°C with 1–3 × 106 cpm/ml of [32P]dCTP-labeled cDNA probes (random primed labeling kit; Boehringer Mannheim). The following cDNA templates were used for random priming: a 517-bp cDNA encoding human VEGF (19); a 1080-bp BglII/SalI cDNA fragment of the human flt-1 gene (corresponding to nucleotides 3233–4313; Refs. 20 and 21); and a 1350-bp BamHI fragment of human KDR (corresponding to nucleotides 370–1720; Refs. 16 and 22). Filters were washed in 2× SSC at room temperature, in 2× SSC with 0.5% SDS at 42°C, and finally with 0.3× SSC-0.5% SDS at 42°C and exposed with intensifying screens at −70°C.

RT-PCR Analysis. For reverse transcription, 4 μg of total RNA were used in a 20-μl reaction volume containing 25 μM hexamer random primer (Boehringer Mannheim), 17 units human placental RNase inhibitor (Pharmacia), 50 μM of each dNTP, 1 unit of 200 U/μl Taq DNA polymerase (Perkin-Elmer). The following pairs of primers were used: 5′-CAGGTAGCCAAGCCAAAGTTCTC-3′ and 5′-TTTGGATGGTTGTGGCTTCTTT-3′ for VEGF; 5′-CTGGTGGGGTTGTGTGTATGAAT-3′ and 5′-TATCAACTGGCTACCAACTGCTAC-3′ for flt-1; 5′-CTGGCTATTTCTGCCATGCTC-3′ and 5′-CTGGTTTCACTCAGACATCTTCT-3′ for KDR; and 5′-CACGGATCTGAACCGTTATGG-3′ and 5′-CTGCTGAAGGCCTCTGAAGAG-3′ for GAPDH. Reverse transcription was performed in a 20-μl reaction volume containing 3 μg of total RNA, 50 μM of each dNTP, 1 μM of each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.5 U RNase inhibitor (Promega), and 1 μl of 200 U/μl M-MLV reverse transcriptase (Promega). A positive control for reverse transcription-PCR (RT-PCR), 25 μg of the human placental RNA, was added to each reaction as a control for RNA integrity and RT-PCR. Amplification of GAPDH cDNA copy in each sample was used to verify equal RNA input. A 25-μl reaction volume containing 0.2 μM of each primer, 200 μM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.5 U RNase inhibitor, and 1 μl of 200 U/μl Taq DNA polymerase was used for amplification. The following PCR conditions were used: 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 5 min. Amplification of the VEGF and GAPDH cDNA was performed in a total reaction volume of 5 μl. The PCR products were analyzed by electrophoresis of 2% agarose gel containing 0.5 μg/ml ethidium bromide in 0.5× TBE buffer and visualized under ultraviolet light. A commercial cDNA probe (4313 bp, Roche) was used as a positive control. The size of the amplified product was determined by direct comparison with a DNA molecular weight standard (123 bp, Gibco/BRL). The amplified products were analyzed using a STRANALYSIS program (Molecular Dynamics) for densitometric analysis.
VEGF AND VEGF RECEPTORS IN CAPILLARY HEMANGIOBLASTOMAS

We analyzed 13 surgically removed human hemangioblastomas from 10 patients. Eight tumors were derived from patients with the clinical diagnosis for VHL disease, and five tumors were classified as sporadic hemangioblastomas. All specimens were analyzed by immunohistochemistry for the expression of CD31 antigen (Ref. 25; Fig. 1a) and von Willebrand factor (data not shown), since these proteins are known to be highly expressed in endothelial cells. Histologically, familial and sporadic hemangioblastomas could not be distinguished. Both tumor types showed the typical dense network of blood-filled capillaries in association with stromal cells and cystic areas. A distinct boundary between highly vascularized tumor and normal brain tissue could be observed (Fig. 1a).

To investigate whether overexpression of the endothelial cell-specific mitogen VEGF underlies the proliferation of endothelial cells observed in capillary hemangioblastoma, we performed Northern blot analysis. Total RNA isolated from a hemangioblastoma which was derived from a patient with VHL disease and from normal human brain tissue was analyzed. Highly increased amounts of VEGF mRNA with transcript lengths of about 4.0 kb were present in capillary hemangioblastoma tissue, whereas in normal brain tissue, no VEGF transcripts could be detected by Northern analysis (Fig. 2). The cognate VEGF receptors, VEGFR-1 (flt-1) and VEGFR-2 (KDR), were coexpressed in capillary hemangioblastoma, but no signal could be detected in normal brain tissue (Fig. 2). The corresponding transcript lengths of approximately 7.7 kb for flt-1 and 7.0 kb for KDR, respectively, are in agreement with earlier reports (20, 26).

We then performed in situ hybridization to localize the VEGF-producing cells in capillary hemangioblastomas. To test the suitability of the surgically obtained material for in situ hybridization, the tissues were analyzed for expression of von Willebrand factor. Since we found by immunohistochemistry that von Willebrand factor protein was highly expressed in endothelial cells of the tumors, we used the von Willebrand factor mRNA expression pattern as an indicator for RNA degradation in the tissue specimens (data not shown).

By in situ hybridization, high levels of VEGF mRNA expression could be detected in all 13 capillary hemangioblastomas examined. The transcripts were evenly distributed throughout the tumor tissue, and the expression pattern was restricted to highly vascularized areas. Expression of VEGF was barely detectable in the adjacent normal cerebellar cortex, and no VEGF transcripts were detectable in meningeal blood vessels (Fig. 1, b and c). High magnification in Fig. 3a shows that the VEGF transcripts were localized in stromal cells clustered between the capillaries. No VEGF expression was observed in endothelial cells. The specificity of the signal was confirmed by control hybridization with a sense RNA probe which showed no specific labeling (Fig. 3b).

VEGF protein distribution was analyzed by immunostaining of frozen sections of four representative familial and sporadic cases of capillary hemangioblastomas using a polyclonal antiserum directed against human recombinant VEGF. Immunoreactivity was strongest in the microvasculature of capillary hemangioblastomas. The antisera stained tumor cells and the interstitium to a lesser extent (Fig. 3c). No immunoreactivity was detectable in the tumors when the primary antibody was omitted.

To identify the mRNA species coding for the different VEGF isoforms in capillary hemangioblastomas, we performed RT-PCR. We analyzed RNA isolated from two human hemangioblastomas, a human glioblastoma (known to express high amounts of VEGF; Ref. 21) and normal human brain. The oligo(dT)-primed cDNAs were amplified using oligonucleotide primers derived from the 5'- and 3'-ends of the protein coding region of the human VEGF gene, which are shared by all VEGF mRNA species. In all tissues analyzed, amplification products of 452, 584, and 656 bp corresponding to VEGF121, VEGF165, and VEGF189 were found (Fig. 4). This was also true for normal human brain, where only faint bands of VEGF121 and VEGF189 were visible. However, amplification products with 707 bp corresponding to VEGF206 were not detectable. This result was
Fig. 1. VEGF mRNA is highly expressed in human capillary hemangioblastoma but is barely detectable in normal brain tissue in situ. a, immunostaining for human CD31, a cell adhesion molecule expressed by endothelial cells, showing abundant microvasculature characteristic for capillary hemangioblastoma. b, in situ hybridization with antisense 35S-labeled RNA probe specific for human VEGF, counterstained with Toluidine blue. VEGF is highly expressed in the tumor tissue but is barely detectable in the adjacent normal brain tissue. c, dark field illumination of (b). Arrowheads, boundary between tumor (lower half) and adjacent normal brain tissue (upper half). MV, meningeal vessels; CC, cerebellar cortex. Bars, 50 µm. Exposure time (b and c) 11 days.
confirmed by Southern blot hybridization using a full-length human VEGF cDNA fragment (data not shown). To compare the amount of cDNA in the reactions, PCR amplification was performed for human GAPDH in parallel under the same conditions. The GAPDH-specific oligonucleotide primers generated a single 412-bp fragment (Fig. 4). The total amount of all amplified VEGF isoforms was much higher in capillary hemangioblastoma as compared to normal brain tissue, which is consistent with the in vivo levels of VEGF mRNA expression in these tissues. mRNA encoding VEGF165 was the most prominent one in normal brain tissue and glioblastoma. In contrast, in capillary hemangioblastoma, the mRNA encoding isoform VEGF121 was amplified to almost the same amount as VEGF165.

In parallel with VEGF, we analyzed the expression of the VEGF receptors VEGFR-1 (flt-1) and VEGFR-2 (KDR) at the cellular level in VHL disease-associated and sporadic cerebellar hemangioblastomas. We performed in situ hybridization with radiolabeled RNA probes specific for each receptor on serial sections of all 13 cases and of two normal brain tissues. As compared to normal brain, where a very weak expression of both receptors in endothelial cells could be observed (data not shown), their expression was much stronger in the capillary hemangioblastomas. VEGFR-1 and VEGFR-2 were coexpressed in endothelial cells of the same capillary vessels (Fig. 5, a-d). However, the transcription level of VEGFR-2 was higher than that of VEGFR-1. This differential pattern of expression was observed in familial as well as sporadic forms of capillary hemangioblastoma (Table 1). The expression of both genes was significantly higher in capillaries than in larger vessels (Fig. 5, a and c). No specific labeling could be detected in control hybridizations with sense RNA probes (data not shown). Table 1 summarizes the results of the in situ hybridization experiments on capillary hemangioblastomas.

DISCUSSION

VEGF, also known as vascular permeability factor (10, 11), is a dimeric glycoprotein that was found to specifically bind to endothelial cells (27). VEGF has been shown to stimulate endothelial cell growth in vitro and angiogenesis in vivo (12) and to induce microvascular
permeability (28). In human and rat glioblastomas, we suggested VEGF as the main regulator of tumor-induced angiogenesis (21, 29). We showed by in situ hybridization that VEGF is produced by malignant glioma cells and may act via a paracrine mechanism on tumor endothelial cells, which express VEGF receptors. Recently, up-regulation of VEGF and its receptors has also been reported in other tumor types such as human kidney and bladder carcinomas (30), indicating that VEGF may be a tumor angiogenesis factor in various tumors.

Hemangioblastoma, the most frequent manifestation of VHL disease, also presents as a nonfamilial, sporadic form. Both sporadic and VHL disease-associated hemangioblastomas are blood vessel tumors characterized by abundant endothelial cell proliferation and cyst formation. We hypothesized that overexpression of VEGF may underly endothelial cell proliferation and increased vascular permeability in hemangioblastomas. Therefore, we investigated the expression of VEGF and its receptors in 13 specimens of VHL disease-associated and sporadic hemangioblastomas. Histologically, familial and sporadic hemangioblastomas showed a similar phenotype. Tumors were characterized by abundant capillaries which expressed von Willebrand factor in VHL disease-associated and sporadic hemangioblastomas (Fig. 1a). Intratumoral cyst formation was frequent, and no obvious difference was observed between VHL-associated and sporadic hemangioblastomas. In addition, no difference regarding the spatial distribution and the expression level of VEGF could be observed by in situ hybridization. In contrast to normal brain tissue, where little VEGF expression could be observed, abundant amounts of VEGF transcripts were present in tumor stromal cells (Fig. 1 and 3), which is consistent with previous reports (31, 32). We could not detect VEGF transcripts in the tumor endothelium, thus excluding an autocrine control of endothelial cell proliferation.

According to Knudson’s tumor suppressor theory (33), hemangioblastoma formation in patients suffering from VHL disease implies germline transmission of one mutated allele of the VHL gene and inactivation of the balancing wild-type allele by a secondary somatic mutational event. The model further predicts that sporadic tumors would arise in the same cell type after somatic inactivation of both copies of the gene responsible for the hereditary cancer. Comparing the age incidence curves of familial and sporadic hemangioblastoma, the VHL gene behaves as a typical tumor suppressor gene (34). Loss of the wild-type VHL allele from chromosome 3p was detected in different tumors from VHL patients, including spinal and cerebellar hemangioblastomas (4), and somatic mutations of the VHL gene in sporadic hemangioblastomas of the CNS have also been reported (35).

The familial and sporadic cases of capillary hemangioblastoma in this study have been classified by familial history and clinical examination of predisposed organs. (Genetic analysis for VHL gene mutations is in progress). Little is known about the function of the VHL gene product since the partial nucleotide and amino acid sequences showed no apparent homology to known sequences (5), but our data suggest that inactivation of the VHL tumor suppressor gene directly or indirectly induces VEGF transcription in stromal cells of cerebellar hemangioblastomas. Another example for the regulation of angiogenesis by proteins which are under control of a tumor suppressor gene has been described recently. In two reports, it has been demonstrated that endogenous angiogenesis inhibitors are under control of the tumor suppressor gene p53 (36, 37).

One known mechanism of VEGF up-regulation is by hypoxia. Increased VEGF mRNA levels have been observed in cultured cell lines grown under anoxic conditions in vitro and in palisading cells in vivo, which are located adjacent to necrotic areas in glioblastomas (21, 29, 38). In hemangioblastoma, however, hypoxia is unlikely to be a major mechanism of VEGF up-regulation, since this tumor is very well vascularized, and necroses do not occur.

The majority of lesions in VHL patients are cystic. Beside renal and pancreatic tumors, cysts occur in the same organs. Hemangioblastomas of the CNS have been reported to be cystic in 75% of the cases (39). The mechanism which underlies cyst formation is unclear, but it is assumed that increased vascular permeability leads to extravasation of serum, which then accumulates in the interstitium. VEGF induces microvascular permeability (28) and is, therefore, a possible candidate for mediating cyst formation. In the human, four different VEGF isoforms, VEGF(121), VEGF(165), VEGF(189), and VEGF(206), respectively, have been identified which arise by alternative splicing of mRNA (13). VEGF(121) and VEGF(165) are secreted proteins, whereas VEGF(189) and VEGF(206) remain mostly cell associated (13, 14). VEGF(165) is the most abundant isoform in all human tissues except placenta, where VEGF(121) is predominant (40). In contrast to the cellular localization, little is known about the biological function of the four isoforms. We performed RT-PCR to identify the mRNA species coding for the different isoforms in cerebellar hemangioblastomas, human glioblastoma, and normal brain. We observed that the three smaller forms of VEGF mRNA are present in all tissues. VEGF(165) was the

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Table 1

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*a* Age at surgery.
*b* Specimens derived from the same patient.
*c* Specimens derived from the same patient.
*d* +, weak expression; ++, moderate expression; ++++, strong expression; ++++, abundant expression; ND, not determined.
most prominent splice variant in all tissues examined with the exception of cerebellar hemangioblastomas, where VEGF<sub>121</sub> and VEGF<sub>165</sub> were found in similar amounts. These data are consistent with earlier reports and suggest that VEGF<sub>121</sub> and VEGF<sub>165</sub> may be mediators of both neovascularization and tumor-associated cyst formation in capillary hemangioblastomas.

Two tyrosine kinase receptors, VEGFR-1 (flt-1; Ref. 15) and VEGFR-2 (KDR/flk-1; Refs. 16 and 17) have been described to bind VEGF with high affinity. We have shown that the expression of both VEGF receptors correlates with angiogenesis in the mouse embryo (17) and with tumor-induced angiogenesis in glioblastomas (21, 29). Under physiological conditions, vascularization of the brain is confined to embryonic and early postnatal development, whereas endothelial cell proliferation is low in the adult brain (41). By in situ hybridization, we could show that both VEGF receptors are specifically expressed by proliferating endothelium: (a) during brain angiogenesis in endothelial cells which invade the developing neuroectoderm (17); and (b) during tumor-induced angiogenesis in vessels which invade the tumor (29). These results are consistent with our findings in capillary hemangioblastomas, where VEGFR-1 and VEGFR-2 expression was up-regulated in the tumor compared to normal brain as shown by Northern analysis (Fig. 2). In situ hybridization revealed that both receptors were expressed in endothelial cells of the same capillary vessels and to a lesser extent in larger vessels (Fig. 5). This expression pattern may represent the proliferation status of the capillaries. We observed a higher expression level of VEGFR-2 compared to VEGFR-1 in all sporadic and VHL-associated hemangioblastomas. Although this finding may represent a different expression level of the receptors, it may also be due to different hybridization efficiencies.

Besides the expression of VEGF mRNA, we show by immunohistochemistry that VEGF protein is present in the interstitium throughout the tumor and is concentrated on capillary vessels (Fig. 3c). This suggests that VEGF is secreted by the stromal cells and interacts in a paracrine fashion with the corresponding receptors on the surface of the tumor endothelium, thereby inducing neovascularization and cyst formation.

Capillary hemangioblastomas are benign, nonmetastatic tumors. But their localization in the cerebellum, medulla, and spinal cord, and their multifocal distribution in VHL disease patients, makes conventional therapy such as tumor resection difficult to achieve. A tumor-specific antiangiogenic therapy might, therefore, be an alternative approach to inhibit hemangioblastoma growth in vivo. Inhibiting VEGF-VEGF receptor interaction by using neutralizing anti-VEGF antibodies (42) or by introducing a signaling-defective flk-1/VEGFR-2 receptor mutant into tumor endothelial cells (43) leads to significant inhibition of tumor growth in nude mice. The elucidation of a possible role of the VHL tumor suppressor gene in VEGF/VEGFR
regulation may bring insights into whether a similar approach is useful for hemangioblastoma treatment.

In summary, our findings of up-regulation of VEGF in stromal cells and of the corresponding VEGF receptors in tumor endothelial cells suggest that VEGF may act as a regulator of neovascularization and cyst formation in VHL disease-associated and sporadic hemangioblastomas. It is at present unclear whether inactivation of the VHL gene is involved in VEGF and/or VEGF-receptor up-regulation.

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