Short Communication

Up-Regulation of Vascular Endothelial Growth Factor in Stromal Cells of Hemangioblastomas Is Correlated with Up-Regulation of the Transcription Factor HRF/HIF-2 α

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Hemangioblastomas, the most frequent manifestation of the hereditary von Hippel-Lindau disease (VHL), are highly vascularized tumors of the central nervous system. In previous studies, the endothelial-specific mitogen vascular endothelial growth factor (VEGF) was shown to be up-regulated in the stromal cells, the putative neoplastic cells in hemangioblastomas. Therefore, it was suggested that secretion of VEGF by stromal cells is the pathogenetic cause of the vascular lesions in hemangioblastomas. The novel basic helix loop helix transcription factor HRF/HIF-2 α is a candidate regulator of VEGF expression during development. We therefore investigated expression of HRF/HIF-2 α in hemangioblastomas and found the overexpression of VEGF mRNA in stromal cells to be highly correlated with elevated expression levels of HRF/HIF-2 α mRNA. This finding is suggestive for a role of HRF in VEGF-dependent vascular growth in hemangioblastomas and could provide a link between transcriptional activation of the VEGF gene and loss of function of the VHL gene product. (*Am J Pathol* 1998, 153:25–29)

Hemangioblastomas, vascular tumors of the central nervous system, are the most frequent manifestation of the autosomal dominantly inherited von Hippel-Lindau (VHL) disease. These tumors consist of a dense network of capillaries interspersed with stromal cells. The nature and the origin of the stromal cells is unclear up to now. It was hypothesized that stromal cells represent the neoplastic cell type in hemangioblastomas.¹ This view was substantiated by the finding that stromal cells produce high amounts of vascular endothelial growth factor (VEGF).²

VEGF is a mitogen and survival factor specific for endothelial cells.³ The specificity of VEGF for endothelial cells is due to the fact that the receptors for VEGF are almost exclusively expressed by endothelial cells.^{4,5} There are several lines of evidence that the VEGF/VEGF receptor signal transduction system is the major physiological regulator of vascular growth *in vivo*.⁶ In addition, VEGF appears to be a major regulator of tumor angiogenesis. In glioblastomas, which are malignant brain tumors of astrocytic origin, the expression level of VEGF has been shown to be correlated with vascular density of the tumors. However, unlike hemangioblastomas, which show a uniform up-regulation of VEGF in almost all stromal cells, glioblastomas show up-regulation only in those tumor cells that are adjacent to necroses.^{7–9} Consistent with this finding, it was demonstrated that VEGF is up-regulated in glioma cells in response to hypoxia.¹⁰ Recently, a heterodimeric transcription factor that was designated hypoxia inducible factor 1 (HIF-1), was identified as a putative link between oxygen tension in a given tissue and expression of hypoxia-sensitive genes such as VEGF and erythropoietin.¹¹ Such genes share the presence of hypoxia-responsive elements in their 5' and 3' flanking regions, which are binding sites for HIF.¹² As this system was based on the reciprocal relationship of oxygen tension and growth factor expression, it was unclear whether it is involved in the high expression levels of VEGF in stromal cells of hemangioblastomas. More recently, a homologue of the α -subunit of HIF-1 was cloned by our and other groups and was designated HRF/EPAS-1/HLF (which stands for HIF-related factor/endothelial PAS protein-1/HIF-like factor)^{13–15} and re-

Supported by grant C4 from the Center for Clinical Research I, Freiburg University Medical School, and by grant P1158/3-1 from the Deutsche Forschungsgemeinschaft to K. H. Plate.

Accepted for publication April 7, 1998.

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cently renamed HIF-2 α .¹⁶ In the mouse embryo, this new factor is expressed by endothelial cells, neurons, smooth muscle cells, and a variety of epithelia. Endothelial expression of HRF could be correlated to the *in vitro* transactivation of the promoter of the receptor tyrosine kinase tie-2,¹⁵ which is essential for signal transduction during embryonic angiogenesis.^{17,18} In epithelia, the level of mRNA expression is highly correlated with the expression of VEGF. In addition, HRF acts as a transactivator of VEGF promoter constructs *in vitro*.¹⁴ Thus, HRF is a novel candidate regulator of angiogenesis. To elucidate whether HRF could also be involved in the high VEGF expression observed in stromal cells of hemangioblastomas, we examined a series of cerebellar hemangioblastomas for the expression of VEGF and HRF and compared these data with normal brain and cerebellum. We found high expression levels of HRF mRNA in VEGF-producing stromal cells and the surrounding capillaries. This finding is suggestive for a role of this transcriptional regulator in VEGF-dependent vascular growth in hemangioblastomas.

Materials and Methods

Specimens

Tumor specimens were immediately snap-frozen after their removal and stored at -70°C until further use. Fourteen hemangioblastomas (from patients both with and without clinically confirmed VHL disease) and four glioblastomas were taken from the brain tumor bank of the Department of Neuropathology, Freiburg University Medical Center. For control purposes, normal cerebellum and normal cerebrum from two patients without neurological disease were obtained postmortem and snap-frozen in liquid nitrogen.

RNA Extraction and Northern Analysis

Total RNA was isolated using the guanidinium thiocyanate method.¹⁹ Aliquots of 20 μg were separated on 1% agarose gels containing 0.66 mol/L formaldehyde in 1 $\times$ MOPS buffer (40 mmol/L 3-(*N*-morpholino)propanesulfonic acid, 10 mmol/L sodium acetate, and 1 mmol/L EDTA (pH 7.2) and transferred to nylon membrane (Duralon-UV, Stratagene, La Jolla, CA) by standard procedures in 20 $\times$ SSC (1 $\times$ SSC is 150 mmol/L NaCl, 15 mmol/L sodium citrate). Probes for the mouse HRF cDNA (nt 1688-2344), human VEGF cDNA (kindly provided by Dr. H. Weich, Braunschweig), human HIF-1 α cDNA (500-bp *Hind*III fragment, kindly provided by Dr. H. Marti, Bad Nauheim), and β -actin were labeled with [³²P]dCTP using a random primer labeling kit (Stratagene). Hybridizations were carried out as described previously.²⁰

In Situ Hybridization

In situ hybridization was performed as described previously.¹³ Single-stranded sense and antisense [³⁵S]-labeled RNA probes were generated from a *Pst*I-*Hind*III

fragment of HRF subcloned in pBluescript and a hVEGF121 pBluescript plasmid using T3 and T7 polymerases (Stratagene) and [³⁵S]UTP. Sections were hybridized with 2.5×10^4 cpm/ μl probe overnight in hybridization buffer (50% formamide, 10% dextran sulfate, 10 mmol/L Tris/HCl, pH 7.5, 10 mmol/L sodium phosphate, pH 6.8, 5 mmol/L EDTA, 150 $\mu\text{g}/\text{ml}$ *Escherichia coli* tRNA, 0.1 mmol/L UTP, 1 mmol/L ADP β S, 10 mmol/L γ S-ATP, 1 mmol/L dithiothreitol, 1 mmol/L 2-mercaptoethanol) at 42 $^{\circ}\text{C}$. After washes at high stringency (2 $\times$ SSC, 50% formamide, 10 mmol/L 2-mercaptoethanol, 48 $^{\circ}\text{C}$) slides were coated with Kodak NTB-2 emulsion and exposed for 2 to 4 weeks in the dark. Slides were developed in Kodak D-19 developer and counterstained with Giemsa's solution (Merck, Darmstadt, Germany).

Immunohistochemistry

To identify endothelial cells, sections were incubated with anti-PECAM/CD31 mouse monoclonal antibody (BioGenex, San Ramon, CA), and binding of the antibody was visualized using the Peroxidase LSAB Kit (Dako, Glostrup, Denmark), according to the manufacturer's instructions.

Results and Discussion

The high expression of VEGF in stromal cells of capillary hemangioblastomas is considered to be an important pathogenetic determinant for the formation of the vascular tumor phenotype.² HRF/EPAS-1, a novel member of the basic helix loop helix family of transcription factors, was recently shown to be a candidate regulator of VEGF gene expression in the mouse embryo.¹⁴ We therefore examined the expression pattern of HRF in a series of seven hemangioblastomas and found striking correlation of VEGF expression with HRF expression in the stromal cells. Specimens were examined by *in situ* hybridization of parallel sections with HRF and VEGF antisense probes and sense probes as a control. Hybridization with sense probes did not show any specific signal (Figure 1D). By hybridization with VEGF antisense, VEGF expression was seen in areas of clustered stromal cells as described previously (Figure 1, A and B). These areas were overlapping with those expressing HRF in parallel sections (Figure 1C). At higher magnification, HRF expression was detected in both stromal and endothelial cells (Figure 1F). In contrast to previous studies in mouse embryos, in which HRF message was found in all capillary endothelia of the brain, no significant signal could be recorded on the tumor capillaries unless they were located in the vicinity of VEGF-producing stromal cells. The identity of endothelial cells and their discrimination from stromal cells could be confirmed by immunostaining of parallel sections with an anti-CD31 (PECAM) antibody (Figure 1G). In the normal cerebellum, Purkinje cells exhibited noticeable hybridization with HRF antisense probe. The signal for HRF was less pronounced in endothelial cells and in neurons of the granular layer (not shown). In normal cerebrum, neurons, but not the glial cells of the

Figure 1. Expression of VEGF in stromal cells of capillary hemangioblastomas is correlated with expression of the transcription factor HRF/HIF-2 α as shown by *in situ* hybridization. Parallel sections of a representative capillary hemangioblastoma were hybridized with 35 S-labeled VEGF antisense RNA probe (A, dark-field illumination; B, bright-field view), 35 S-labeled HRF/HIF-2 α antisense RNA probe (C), or 35 S-labeled sense control RNA probe (D). Whereas VEGF is expressed exclusively by stromal cells E, HRF/HIF-2 α mRNA is detected on both stromal and endothelial cells (F). Endothelial cells are distinguished from stromal cells by the shape of their nuclei and positive staining for CD31 (G), bv, large blood vessel. Scale bars, 100 μ m (A–D) and 50 μ m (E–G).

gray matter, revealed hybridization signals for HRF. Less pronounced signals were seen on capillary endothelial cells as described previously for the mouse adult brain¹³ (data not shown).

The results from Northern hybridization confirmed the data from *in situ* hybridization. Whereas in normal brain

and cerebellum only low levels of HRF and VEGF mRNAs were detectable, strong hybridization signals were obtained in tissue from seven different hemangioblastomas (Figure 2). Interestingly, the expression level of VEGF mRNA, which significantly varied among the different hemangioblastoma specimens, was highly correlated

Figure 2. Northern blot analysis of total RNA from different human tissues. Lane 1, normal cerebrum; lane 2, normal cerebellum; lane 3 to 9, seven different capillary hemangioblastomas; lanes 10 to 13, four different glioblastomas. The membrane was hybridized with a ^{32}P -labeled human VEGF cDNA and reprobbed with a mouse HRF/HIF-2 α probe, a human HIF-1 α probe, and a β -actin cDNA as loading control.

with the expression level of HRF. In contrast, the expression level of HIF-1 α mRNA did not differ significantly between normal tissues and hemangioblastomas. In four glioblastomas that were analyzed for comparison, no elevated levels of HRF could be detected, although in three of these the VEGF mRNA was clearly up-regulated. The level of HIF-1 α mRNA message was essentially the same in all tumors and normal tissues examined (Figure 2).

These findings suggest that the mechanisms of VEGF up-regulation in hemangioblastomas differ from those in glioblastomas. In glioblastomas, high VEGF expression is seen in the so-called palisading cells in the vicinity of necrosis. It was therefore assumed that metabolic factors related to the necrosis play an important role for expression of the VEGF gene in these cells.^{8,9} Hypoxia is believed to be the most important factor among these, and the transcription factor HIF-1 α is the most likely mediator of gene activation in response to hypoxia.²¹ *In vitro*, the HIF-1 α transcription factor has been shown to transactivate the VEGF promoter via binding to a hypoxia-responsive element.²² Several groups observed an increase in the protein level of HIF-1 α via stabilization of the protein under hypoxic conditions whereas no induction of mRNA was observed.^{12,23} In addition, all *in situ* data on HIF-1 α mRNA that are available so far do not reveal a correlation between expression of HIF-1 α mRNA and VEGF. In contrast, mRNA expression of HRF, the close relative of HIF-1 α , is correlated with expression of VEGF mRNA in the lung and in other organs.^{13,14} This also holds true for hemangioblastoma stromal cells. On the other hand, no such striking correlation between HRF and VEGF mRNA expression was observed in glioblastomas. Only a slight elevation of HRF mRNA level was observed in the palisading cells (data not shown), which is consistent with the data from Northern blot hybridization.

Hemangioblastoma, which may serve as a model for pathological angiogenesis in response to overexpression of VEGF, is the most frequent manifestation of VHL disease.¹ It may be speculated that loss-of-function mutations of the VHL gene result in increased gene expression of HRF in stromal cells of hemangioblastomas. VHL has recently been shown to control transcriptional elongation

by interaction with elongin B and C.^{24,25} One possibility, therefore, is that VHL controls transcriptional elongation of HRF in stromal cells. As the histogenesis of stromal cells is undefined, and no stromal cell line is available at present, this hypothesis requires additional evaluation. Whether this proposed dysregulation could hold true for other VHL-associated lesions, such as kidney and adrenal gland tumors, remains also to be examined. It has been shown that VHL mutations in certain cell lineages lead to an increased stability of VEGF mRNA.^{26,27} This mechanism is different from transcriptional activation of VEGF, which is expected to occur in response to HRF. Additional experiments on the molecular interaction of VHL, HRF, and the VEGF gene are necessary.

The increased expression of HRF in capillary endothelial cells located in close vicinity of VEGF-overexpressing stromal cells in contrast to more distant endothelial cells may result from activation of endothelium by VEGF or other stromal-cell-derived factors. As HRF was found to be a transactivator of *tie-2* promoter *in vitro*, it may be assumed that this transcription factor is involved in the transcriptional control of the *tie* genes, which are expressed in capillary endothelial cells in hemangioblastomas²⁸ (our own unpublished observations). Thus, it may be speculated that VEGF receptors that are up-regulated in hemangioblastoma capillaries² are also under the control of HRF.

In conclusion, our data show for the first time a correlation of HRF expression in a human tumor with overexpression of VEGF. The fact that this particular tumor is highly vascular underlines the central role this novel transcription factor may have for the regulation of vascular growth and differentiation.

Acknowledgments

We thank Dr. Werner Risau (Max-Planck-Institut Bad Nauheim) for continuous support and critical reading of the manuscript.

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