Reversion of Deregulated Expression of Vascular Endothelial Growth Factor in Human Renal Carcinoma Cells by von Hippel-Lindau Tumor Suppressor Protein

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Abstract

Mutations or loss of both alleles of the von Hippel-Lindau (VHL) tumor suppressor gene has been documented in sporadic renal cell carcinomas and neoplasms that arise in individuals having the VHL syndrome. The well-vascularized phenotype of tumors that form in VHL disease let us consider vascular endothelial growth factor (VEGF) as a mediator of tumor growth in VHL disease. Human renal carcinoma cells that either lacked endogenous wild-type VHL or were transfected with an inactive mutant VHL showed deregulated expression of VEGF on the mRNA and protein level that was reverted by introduction of wild-type VHL. Stimulation of proliferation of endothelial cells by conditioned medium of cells expressing mutant VHL was almost abolished by neutralizing the VEGF. In contrast, expression of basic fibroblast growth factor and of c-myc proto-oncogene was not affected by VHL. Our data suggest VEGF as the key tumor angiogenesis factor in VHL disease.

Introduction

Individuals with germ line mutations in the VHL gene (1) are predisposed to multiple forms of cancer, including renal carcinoma, hemangioblastoma of the central nervous system, retinal capillary angiomia, and pheochromocytoma. In addition, mutations in the VHL gene were found in sporadic forms of renal carcinoma (2). Very recently, the VHL gene product was discovered as a negative regulator of the transcriptional elongation factor elongin (3-5). Elongin, a heterotrimeric protein complex, consists of the catalytic subunit elongin A and the regulatory subunits B and C. Elongin B and C form a complex, which binds to and activates elongin A. By competing with elongin A for binding to elongin B/C, VHL protein disrupts elongin function. In patients with the VHL syndrome, forms of VHL were found which were mutated in the region of elongin B/C binding. These findings suggested the regulation of elongin’s transcriptional elongation activity by VHL as the basis of VHL’s tumor suppressor function. As can be deduced from the viability of cells expressing mutant VHL, VHL is obviously not a global regulator of transcriptional elongation. Several genes, which are implicated in the pathogenesis of malignancies and which are regulated on the level of transcript elongation, such as the members of the myc proto-oncogene family or c-fos, were supposed to be candidates for targets of VHL (6).

The development of well-vascularized tumors, such as renal cell carcinoma and pheochromocytoma, and frequent cyst formation, which may be due to increased vascular permeability, is characteristic for VHL disease. Among the various angiogenic factors, VEGF plays a pivotal role in tumor angiogenesis: it is expressed by most solid tumors, it is secreted, and VEGF was identified as an endothelial specific mitogen that induces angiogenesis and vascular permeability in vivo. The VEGF receptors FLT1 (VEGFR-1) and FLK/KDR (VEGFR-2) are almost exclusively expressed in vascular endothelial cells, preferentially in vessels lining and penetrating the tumors (for recent review, see Ref. 7). Growth factors, such as platelet-derived growth factor and epidermal growth factor, activation of protein kinase C by tumor promoters, and activated ras and raf oncogenes, have been shown to induce VEGF expression by promoter activation most likely mediated via AP-1 transcription factors (8-11). Hypoxia was identified as another major stimulus for VEGF expression (12) acting preferentially via mRNA transcript stabilization (11, 13).

The well-vascularized phenotype of tumors that form in VHL disease let us consider VEGF as a key mediator of tumor growth in VHL disease. We investigated the expression of VEGF, bFGF, and proto-oncogene c-myc in human renal carcinoma cells that either lacked endogenous wild-type VHL, or were transfected with expression plasmids that encoded inactive mutant VHL or wild-type VHL.

Materials and Methods

Cell Culture. The generation of stably transfected VHL cells VHL(−/−) (=VHL pRC9), VHL(mt) (=VHL(1-115)ARZ4), and VHL(wt) (=VHL WT) has been described elsewhere (14). VHL cells were grown in DMEM, 10% FCS, and G418 (1 mg/ml). Cells were serum arrested by incubation in DMEM, 0.5% FCS, and G418. Growth of cells under hypoxic conditions was performed using GasPak pouches (BBL Microbiology Systems). For stimulation of HUVE cells by conditioned media, VHL cells were adapted to growth in endothelial basal medium (Promocell), 1.3% FCS, and G418. Primary cultures of HUVE cells were purchased (Promocell) and grown in endothelial growth medium, 5% FCS.

Northern Blot Analysis. RNA was isolated from cultured cells, separated in formaldehyde-containing agarose gels, transferred to nylon membranes, and hybridized to 32P-labeled DNA fragments using standard methods (15). DNA probes used were human VEGF 165 cDNA, human bFGF cDNA, mouse c-myc genomic DNA fragment covering exon 2, intron 2, and exon 3, and human GAPDH cDNA.

VEGF-ELISA. VEGF protein concentration in conditioned media of VHL cells was determined using a commercial VEGF-ELISA following the instructions of the manufacturer (R&D Systems).

Thymidine Incorporation Assay. HUVE cells were serum arrested for 24 h by incubation in endothelial basal medium supplemented with 1.3% FCS, followed by stimulation of the cells with either 5 ng/ml VEGF, or prepared from an Escherichia coli expression system7 or conditioned medium of VHL cells grown for 24 h in endothelial basal medium. For VEGF neutralization experiments, 100 ng/ml recombinant sFLT17 was included. After 18 h of incubation, [3H]thymidine (0.5 μCi) was added, and incorporation was continued for an additional 6 h. The cells were washed, and incorporation of radioactivity was determined by scintillation counting.

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1 This work is dedicated to Professor Dr. Gerd Nagel on occasion of his 60th birthday.
2 To whom requests for reprints should be addressed. Phone: 49-761-206-1700; Fax: 49-761-206-1705.
3 The abbreviations used are: VHL, von Hippel-Lindau; bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVE, human umbilical vein endothelial; sFLT1, soluble extracellular domain of VEGF receptor 1 (VEGFR1/FLT1); TPA, 12-O-tetradecanoylphorbol-1-3-acetate; VEGF, vascular endothelial growth factor.

4 G. Siemeister, G. Martiny-Baron, and D. Marmé, unpublished results.
5 B. Barleon, F. Totzke, D. Marmé, and G. Martiny-Baron, unpublished results.

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Repression of VEGF by Von Hippel-Lindau Protein

Figure 1. Northern blot analysis of VEGF, c-myc, and bFGF mRNA levels in VHL renal carcinoma cells. Total RNA (5 μg) from 786-O cells lacking wild-type VHL stably transfected with an expression vector encoding wild-type VHL [VHL(wt)] or mutant VHL lacking amino acids 116–213 [VHL(mt)] of VHL protein, or transfected with the pRC vector alone [VHL(-/-)] were blotted and probed with 32P-labeled cDNA fragments of VEGF, c-myc, bFGF, and GAPDH (loading control) as indicated. VHL cells were serum arrested for 24 h (A), serum-arrested for 24 h followed by TPA stimulation (100 ng/ml) for the indicated periods of time (B), and subjected to hypoxic conditions for 12 h or 24 h (C). VEGF and c-myc blots were subjected to autoradiography for 72 h on BioMax films, whereas bFGF blots were exposed for 7 days using more sensitive X-Omat films and two intensifying screens.

Results

Northern blot analysis revealed high VEGF mRNA levels in 786-O human renal carcinoma cells (14) lacking wild-type VHL [VHL(-/-)] or expressing an inactive mutant form of VHL [VHL(mt)] lacking the C-terminal amino acids 116–213, a region frequently altered in renal cell carcinomas. Expression of wild-type VHL [VHL(wt)] dramatically decreased VEGF mRNA level (Fig. 1A). In contrast, mRNA expression of a growth factor which also induces angiogenesis, bFGF, showed no significant differences comparing VHL(-/-), VHL(mt), and VHL(wt) cells, and bFGF mRNA was at a very low level as compared to VEGF. In addition, c-myc mRNA expression, which has been shown to be regulated by transcript initiation and by transcript elongation (16, 17), showed no significant differences comparing VHL(-/-), VHL(mt), and VHL(wt) cells. Phorbol ester (TPA) stimulation of the cells resulted in VEGF mRNA induction in VHL cells, which peaked around 8 h of induction. The level of VEGF mRNA was still considerably higher in VHL(-/-) and VHL(mt) cells as compared to VHL(wt) (Fig. 1B). The time course of the c-myc mRNA level showed the characteristic shape of an immediate early gene with a rapid and transient induction within the first 4 h of incubation. Remarkably, we detected no significant differences in c-myc mRNA induction comparing the VHL cells (the smaller hybridization signals in the VHL(wt) lanes in Fig. 1B at time points 1 and 8 h were due to reduced gel loading as shown by the GAPDH and 28S rRNA controls). Subjecting the cells to hypoxic growth conditions resulted in VEGF transcript accumulation at a similar level in VHL(-/-), VHL(mt), and VHL(wt) cells within 24 h (Fig. 1C). Interestingly, the c-myc mRNA level rose approximately 5-fold during hypoxia.

On the protein level, serum-arrested VHL(mt) and VHL(-/-) cells produced within 24 h 200 and 120 pg VEGF/10⁶ cells, respectively, whereas VHL(wt) cells secreted only 3 pg VEGF/10⁶ cells. Upon phorbol ester stimulation, VEGF secretion of VHL(-/-) and VHL(mt) rose to 400 pg/10⁶ cells and to 170 pg/10⁶ cells in VHL(wt) cells (Fig. 2).

Conditioned medium of VHL(mt) cells, which produced under serum-arrested conditions the highest VEGF level in comparison to the other VHL cells, was used to stimulate proliferation of HUVE cells, which was measured as growth factor-stimulated DNA synthesis. Stimulation of HUVE cells by the VHL(mt) medium, which contained 0.5 ng/ml VEGF as determined by the VEGF-ELISA, was strongly decreased by addition of VEGF-neutralizing sFLT1, as was, for control, VEGF-stimulated proliferation of HUVE cells (Fig. 3).

Discussion

Human renal carcinoma cells lacking wild-type VHL tumor suppressor gene or expressing an inactive VHL mutant showed deregu-
lated expression of VEGF, which is reverted by reintroduction of wild-type VHL. bFGF, another angiogenic growth factor, is expressed in the VHL cells examined only at very low levels as compared to VEGF, and its expression is not elevated in VHL(−/−) and VHL(mt) cells, making bFGF unlikely to be a mediator of neovascularization in VHL disease. Furthermore, stimulation of endothelial cells by conditioned medium of VHL cells was almost completely abolished by addition of VEGF-neutralizing sFLT1. Our data correspond to the finding of up-regulation of VEGF and of its receptors in VHL disease-associated and sporadic hemangioblastomas (18).

As shown by Iliopoulos et al. (14), transfection of VHL(wt), VHL(mt), or the expression vector alone [VHL(−−)] into 786-O renal carcinoma cells had no demonstrable effect on cell growth in vitro. In contrast, the presence of wild-type VHL dramatically reduced the ability of 786-O cells to form tumors in nude mice. It was suggested that the inability of VHL(wt) expression to suppress cell growth in vitro might reflect the dependence of VHL action on cell-cell and/or cell-matrix interaction in vivo. Focusing on the cells used in the present study, the tumors that did arise from VHL(mt) cells were about 1.7 times larger in size than the tumors that did arise from VHL(−/−) cells, whereas VHL(wt) cells were essentially nontumorigenic. These observations correlate closely with our data on VEGF production by the cells with VHL(mt) secreting 1.7-fold more VEGF than VHL(−/−) under identical growth conditions, and VHL(wt) producing less than one-fourth of the amounts that were produced by VHL(−/−) and VHL(mt).

Transcriptional regulation of VEGF mRNA had been demonstrated on the level of transcript initiation and on the level of mRNA stabilization. Although mRNA levels rose in VHL(wt) as well as in VHL(−/−) and VHL(mt) cells during the time course of phorbol ester induction of VEGF promoter activity, the repression of VEGF mRNA in VHL(wt) cells was maintained at a low level as compared to VHL(−/−) and VHL(mt). This result argues against the possibility that VHL acts on VEGF expression at the level of transcript initiation.

Upon subjection of the VHL cells to hypoxic growth conditions, VEGF mRNA level rose in VHL(−/−) as well as in VHL(mt) cells, but mRNA accumulation was most prominent in VHL(wt) cells, resulting in approximately equivalent mRNA levels within 24 h of hypoxia. It had been shown that hypoxia acts on VEGF mRNA expression preferentially via RNA stabilization (11, 13). Our data cannot exclude mRNA destabilization as a hypothetical mode of action of wild-type VHL protein on reversion of deregulated VEGF expression in VHL cells, but such an interpretation would hardly fit with the strong VEGF mRNA accumulation in VHL(wt) cells under hypoxic growth conditions. However, VHL tumor suppressor protein was recently identified as a negative regulator of transcriptional elongation factor elongin (3–5). This finding suggests loss of control of transcriptional elongation as the molecular mechanism underlying VEGF overexpression in VHL renal carcinoma cells.

Comparison of VEGF and c-myc mRNA expression in VHL cells revealed a distinct specificity of VHL action. Whereas VEGF mRNA is repressed by VHL(wt), c-myc seems not to be regulated by VHL, at least in the renal cells examined. Although c-myc expression had been shown to be regulated on the level of transcript elongation (16, 17), our results suggest elongation factors other than the elongin/VHL system as more favorite candidates for c-myc regulation.

The studies presented suggest VEGF as the key tumor angiogenesis factor resulting in well-vascularized tumors that are characteristic for VHL disease. Although the definite mechanism of VHL action on VEGF expression remains to be elucidated, our data suggest VEGF as a prime candidate for a target of VHL, a regulator of transcript elongation.

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References

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