Role of the von Hippel-Lindau Tumor Suppressor Protein during Neuronal Differentiation

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Abstract

The von Hippel-Lindau (VHL) tumor suppressor protein down-regulates transcription by transcriptional elongation enhanced by antagonizing elongin B and C. Transcriptional regulation is an important control mechanism for embryogenesis and tumorigenesis. The VHL gene and protein are expressed in neuronal cells of the fetal and adult brain. However, the role of the VHL gene in the central nervous system (CNS) has not been elucidated. The VHL gene might modify the expression of various genes during embryogenesis and tumorigenesis in CNS. We investigated the role of the VHL gene in CNS development using rodent CNS progenitor cells. Here we show that expression of the VHL protein is correlated with neuronal differentiation but not with glial differentiation in CNS progenitor cells, and we also show that VHL gene transduction induces neuronal differentiation. In addition, a VHL mRNA antisense oligonucleotide inhibits differentiation of CNS progenitor cells and up-regulates their cell cycle. In conclusion, the VHL gene plays an essential role in neuronal differentiation as well as transcription.

Introduction

Germ-line mutations of the VHL2 tumor suppressor gene (1) constitute a predisposition to the development of retinal angiomas, CNS hemangioblastomas, renal cell carcinomas, and pheochromocytomas. Loss or inactivation of the remaining wild-type allele is required for tumor development in VHL disease. In addition, somatic mutations of the VHL gene are frequently detected in sporadic renal cell carcinomas (2) and hemangioblastomas (3), and somatic inactivation of both VHL alleles is a critical step in the pathogenesis of sporadic renal cell carcinoma and hemangioblastomas. Despite the positional cloning of the VHL gene (1), the function of the VHL gene is not well defined. However, because abnormalities of this gene are occasionally associated with transformation of cells in the nervous system (4), it is possible that the normal function of the VHL gene is involved in CNS development.

Elongin B and C bind to elongin A to form a heterotrimeric transcription factor. This complex increases RNA transcription by the suppression of polymerase II pausing. A frequently mutated region of the VHL gene is involved in a proximal segment of neuronal differentiation (9). The adenomatous polyposis coli (APC) gene protein is also up-regulated in association with neuronal differentiation (10). The retinoblastoma gene product (pRb) plays an important role not only in controlling entry into the cell cycle, but also in the distal differentiation of many different cell types (11). The expression of tuberous sclerosis gene 2 (TSC2) is up-regulated on induction of neuronal differentiation at the posttranscriptional level (12). In contrast, both p53 and p21 block neuronal differentiation, and suppression of p53 and p21 induces neuronal differentiation (13, 14). In terminal neuronal differentiation, basic helix-loop-helix transcriptional factors such as MASH-1 (15), NeuroD (16), NeuroD-related factor, and HES-1 (17) also play critical roles. Recently SOX-1, a HMG-box protein related to SRY, was shown to be one of the earliest transcription factors involved in an embryogenic stage transition from ectodermal cells to neural fate (18).

Pluripotential neural progenitor cells demonstrate the ability to differentiate over time when exposed to the appropriate environmental signals. These progenitor cells eventually give rise to the full complement of cell types found in the mature brain and spinal cord (19) CNS stem cells, which exist in embryonic (20) and adult brains (21). During the course of cellular differentiation, cell type-specific antigens are expressed at specific times. Nestin, a marker for CNS stem cells (22), is expressed early, when cells are presumed to possess pluripotentiality. Recently, it has been reported that POU transcription factors bound by the enhancer of nestin control expression of the nestin gene (23). The progressive expression of GFAP, a marker for astrocytes (24), is considered to represent glial differentiation of CNS stem cells. On the other hand, MAP-2, a marker for neurons (25), is expressed along with neuronal differentiation. The VHL gene and protein are expressed in neuronal cells in the fetal and adult CNS (6, 7, 26). However, the relationship between the differentiation of neuron or glia from CNS stem cells and the function of the VHL gene is not well defined.

Materials and Methods

Progenitor Cell Culture. Embryonic E12 fetuses were obtained from anesthetized pregnant Sprague-Dawley rats (Harlan, San Diego, CA). Tissues from the forebrain and hindbrain were dissected, digested with 0.05% trypsin (Life Technologies, Inc.) and 0.02% ethylenediamine tetraacetic acid (Life Technologies, Inc.) at 37°C for 10 min, dissociated by gentle trituration in DMEM (Life Technologies, Inc.) and 10% (FCS Life Technologies, Inc.) and...
define the conditions that promote optimum cell survival and differentiation.

10 EGF and bFGF were added either alone or in combination at concentrations of

cold acetone (4°C), cooled quickly in a deep freezer (2

glucose (0.6%), glutamine (2 mM), NaHCO3 (3 m M), insulin (25

tation of 106 cells/35-mm culture dish. After 24 h, the medium was changed

filtered through a sterile 60-mesh membrane. Cells in suspension were plated

on poly-L-ornithine-coated coverslips in DMEM and 10% FCS at a concen-

primary culture of E12 neural progenitor cells. Day 14, 14 days after the primary culture

of E12 neural progenitor cells.

Double Immunocytochemical Study. Cells on coverslips were washed

with PBS before fixation with acetone for 30 s, cooled quickly, and maintained at

−70°C until the study. On days 1, 5, and 14, the cells were blocked with 5%

NGS in PBS and exposed to one of the following primary antibodies for 60

min at 37°C: (a) a mouse mAb specific for MAP-2 (1:100; Sigma); (b) a rabbit

polyclonal antibody (pAb) specific for GFAP (1:100; Sigma); (c) a mouse

mAb specific for nestin (1:100; PharMingen); (d) mouse mAb VHL40 (28),

specific for pVHL (1:100; kindly provided by Dr. N. Sakashita; Department of

Pathology, Kumamoto University School of Medicine, Kumamoto, Japan).

Controls consisted of staining with 5% NGS in PBS from which the primary

antibody was omitted. Secondary antibody staining consisted of exposure to

swine antimouse or antirabbit immunoglobulin conjugated to rhodamine (1:40)

for 30 min at 37°C. Coverslips were mounted onto slides and viewed. Expression

of marker proteins was evaluated by fluorescence immunocytochemical

study with a positive rate in cultured rodent progenitor cells.

Western Blotting. Cultured cells were washed twice with PBS and ho-
mogenized in a lysis buffer (0.1 mol/liter NaCl, 0.01 mol/liter Tris-HCL, 0.01

mol/liter EDTA, and 1 μg/ml aprotinin). Assays to determine the protein

concentration of the lysate were subsequently performed by comparison with

known concentrations of bovine serum albumin. SDS-gel electrophoresis was

performed in 12% polyacrylamide gels under nonreducing conditions. Lysates
equivalent to 15 μg of protein were electrophoresed on each gel, together with

prestained molecular weight markers (Sigma). The electrophoresis running
buffer contained 25 mmol/liter Tris base, 250 mmol/liter glycine, and 0.1%

SDS (pH 8.3). Proteins on the gel were subsequently transferred to a nitrocel-
lulose transfer membrane (Sigma) in transfer buffer containing 25 mmol/liter

Tris base, 250 mmol/liter glycine, and 20% methanol (pH 8.3). The membrane

was placed in 5% skim milk in 25 mmol/liter Tris-buffered saline for 1 h to

block nonspecific binding. The membrane was then incubated with the fol-

VHL PROTEIN AND NEURONAL DIFFERENTIATION

Fig. 1. Expressions of cell-specific marker protein in cultures of rodent neural pro-
genitor cells at day 1 and day 14 treated with bFGF or EGF. Bar graphs show the ratios
of positive cells with fluorescence immunocytochemical study. The percentage of positive cells
was calculated based on a count of 1000 cultured progenitor cells. Expression of the
nestin decreased for either bFGF or EGF treatment. There is a significant difference
(P < 0.01) between bFGF and EGF treatments. Expression of GFAP increased with bFGF
or EGF treatment. The ratio of GFAP-positive progenitor cells was significantly higher
with EGF treatment than with bFGF treatment (P < 0.01). Expressions of MAP-2 and
pVHL increased for either bFGF or EGF treatment. There is no significant increase in the
positive proportion of MAP-2 or pVHL reactivity at day 14. Day 1, one day after the
primary culture of E12 neural progenitor cells. Day 14, 14 days after the primary culture of E12 neural progenitor cells. *, P < 0.05; **, P < 0.01.

Fig. 2. Double fluorescence immunocytochemical study on CNS progenitor cells at day 14 using a laser scanning confocal microscope. A, coexpression of pVHL and MAP is found at the same CNS progenitor cells. pVHL is detected with FITC (green), and MAPs are detected with rhodamine (red). B, expressions of pVHL and GFAP are found in different CNS progenitor cells. pVHL is detected with FITC (green), and GFAP is detected with rhodamine (red). Scale bar, 10 μm.
lowing primary antibodies for 60 min at 37°C: (a) a mouse mAb specific for MAP-2 (1:100; Sigma); (b) a rabbit pAb specific for GFAP (1:100; Sigma); (c) a mouse mAb specific for nestin (1:100; Pharmingen); and (d) mouse mAb VHL-40. The dilution solution was TBS-T [50 mmol/liter Tris-HCl (pH 7.6), 150 ml of NaCl, and 0.05% Tween 20]. After thorough washing with TBS-T, biotinylated antiserum IgG (1:400; Vector Laboratories, Burlingame, CA) or biotinylated antirabbit IgG (1:400; Sigma) was applied for 60 min. An additional series of washes was followed by incubation with biotin-streptavidin complex conjugated with horseradish peroxidase (1:400; Vector Laboratories). Proteins were detected with diaminobenzidine tetrahydrochloride (Sigma). Membranes were finally washed in distilled water and air dried.

**Labeling with BrdUrd.** Bromodeoxyuridine (Sigma) was added at 10 mm to progenitor cell cultures 24 h before fixation. Cells were washed with PBS, fixed in cold acetone, and preserved at −70°C until the study. After incubation with 2 mM HCL washing with borate buffer (pH 9), and blocking with 0.1% Triton X-100 in 2% NGS, primary mAb to BrdUrd (1:100; DAKO) was added for 60 min at 37°C. The secondary antibody was a rabbit antiserum immunoglobulin conjugated to biotin (1:100; Sigma), which was added for 30 min at 37°C. The third antibody was avidin-biotin complex conjugated to horseradish peroxidase (1:100; Vector Laboratories), which was added for 30 min at 37°C. The reaction was initiated with 0.02% diaminobenzidine tetrahydrochloride (Sigma) and 0.02% hydrogen peroxide in distilled water. Coverslips were mounted onto slides and viewed.

**VHL Gene Transduction.** VHL cDNA was amplified with the PCR method. The primers used were as follows: (forward, 5′-CTGAATTCAC-CATGGAGGCCGGGCGGCCG-3′; and (backward, 5′-GAGAATTCT-CAATCTCCCATCCGTTGATG-3′). A defective herpes simplex virus vector expressing pVHL (dvHSV/VHL) was generated as described previously (29). The VHL gene was driven by the cytomegalovirus immediate early promoter. Amino acids 54-213 of pVHL were expressed with transfection of dvHSV/VHL. The VHL gene transduction into rodent progenitor cells was performed with 1 × 10^6 plaque-forming units of dvHSV/VHL at day 1. As the control vector, a defective herpes simplex virus vector containing the bacterial LacZ gene was used, with the same plaque-forming units. Expressions of pVHL and MAP-2 were observed at 3 and 24 h after transduction, respectively. The expression rate of pVHL and MAP-2 were observed at 3, 6, and 24 h after VHL gene transduction. Significant expressions of pVHL and MAP-2 were observed at 3 and 24 h after the transduction, respectively. The expression rate of pVHL was significantly higher than that of MAP-2 at 3 and 6 h after VHL gene transduction (P < 0.001). In addition, both expressions were observed significantly earlier than the control (P < 0.0001).  

**Results.** When cultures were exposed to serum-free medium supplemented with N2 (Life Technologies, Inc.) and bFGF (Life Technologies, Inc.), progressive stereotypic development of cell type-specific markers was observed. On initial plating (day 1), cultured cells from embryonic E12 fetuses of Sprangue-Dawley rats assumed a small round shape with short processes, consistent with their identity as progenitor cells. This was confirmed by positive immunostaining for nestin, an inter-
mediated filament present in pluripotent CNS progenitor cells. At the same time, staining for MAP-2, a marker for neurons, or GFAP, a marker for astrocytes, was rarely observed, whereas staining for pVHL was detected in the nucleus of some cells. With progressive culture, the cell soma increased in size and developed elongated processes, consistent with the attainment of a more mature phenotype. Starting from 5 days after primary culture (day 5), the number of GFAP-positive cells increased, whereas the expression of MAP-2 and VHL was also increased (data not shown). A decline in nestin expression occurred concomitantly with the increase in MAP-2, pVHL, and GFAP expression at day 14. By day 60, nestin immunoreactivity was barely detectable. Together, these data indicate that the cultured E12 cells were behaving as pluripotential stem cells, differentiating over time into cells demonstrating neuronal and glial properties. When the serum-free medium was supplemented with N2 and EGF, cells preferentially developed GFAP immunoreactivity, and MAP-2- or pVHL-positive cells were rarely seen at day 14. These data indicated that EGF selectively induced glial marker expression (Fig. 1).

To identify pVHL-positive cells, a double immunocytochemical study was performed using a laser scanning confocal microscope. On day 14, coexpressions of MAPs and pVHL were detected in the same cells, whereas expressions of GFAP and pVHL were detected in different cells (Fig. 2). MAPs were detected distinctly in the perinuclear cytoplasm and dendrites as well as in the nucleus, whereas expression of pVHL was detected in both the nucleus and cytoplasm. The pVHL staining in the cytoplasm was more distinct than that in the nucleus. The staining was scarcely observed in the dendrites. The double immunocytochemical studies on cultured embryonic rodent cells at day 14 revealed that MAP-positive cells were identified as pVHL positive.

Western blotting studies revealed that lysates of the cultured embryonic rodent cells on day 1 showed a distinct expression of nestin, a distinct expression of VHL protein on day 1, and a weak expression of MAP-2 on day 14, but no expression of GFAP. Lysates of the cells cultured with bFGF on day 14 showed potent expressions of GFAP, MAP-2, and VHL proteins but had a reduced expression of nestin. On the other hand, lysates of the cells cultured with EGF on day 14 showed potent expression of GFAP but little expression of MAP-2 and pVHL and a more reduced expression of nestin than cells cultured on days 1 and 14 with bFGF (Fig. 3). These data were supported by the fluorescence immunocytochemical studies on cell-specific proteins of CNS progenitor cells.

The effect of VHL gene transduction was examined at 3, 6, 24, 48, 72, 120, and 168 h after the transduction. Significant expressions of pVHL and MAP-2 were observed at 3 and 24 h after the transduction, respectively. The expression rate of pVHL was significantly higher than that of MAP-2 at 3 and 6 h after VHL gene transduction. The pVHL expression was shown earlier than that of MAP-2. In addition, both expressions were observed significantly earlier than the control (P < 0.0001). These results revealed that VHL gene transduction into neural progenitor cells induced rapid neuronal differentiation (Fig. 4).

The effect of VHL mRNA antisense oligonucleotides on marker expression was examined with expressions of nestin, MAP-2, VHL, and GFAP proteins on day 10. The study with the control oligonucleotide revealed that the protein expression pattern was similar to that observed in cells without exposure to the oligonucleotide. The proportions of nestin and GFAP-positive cells (positive cells/all cells in the random 400 cells counted) with the control oligonucleotide were 24.2 ± 3.4% and 15.7 ± 2.9%, respectively. In addition, the proportions of MAP-2- and pVHL-positive cells were 60.1 ± 5.4% and 72.0 ± 5.3%, respectively. The protein expression pattern with VHL mRNA antisense oligonucleotide A852T was similar to that seen using the control oligonucleotide. On the other hand, the protein expression with VHL mRNA antisense oligonucleotide A617T showed a pattern different from those seen using the control oligonucleotide or the A852T antisense oligonucleotide. The expressions of nestin (37.9 ± 4.7%) and GFAP (20.1 ± 3.6%) were at higher levels than those seen when cells were exposed to the control oligonucleotide or antisense A852T oligonucleotide. In contrast, the expressions of MAP-2 (8.0 ± 2.0%) and pVHL (8.3 ± 1.7%) were suppressed. These immunocytochemical results revealed that VHL mRNA antisense oligonucleotide A617T suppressed the expression of neuronal markers. In addition, the BrdUrd labeling index on days 1, 5, and 9 was examined. The BrdUrd index of rodent E12 culture cells treated with A617T was significantly higher (P < 0.001) than those of nontreated cells, cells treated with the control oligonucleotide, and cells...
treated with A852T on days 1 and 5. These results suggest that inhibition of neuronal markers with VHL mRNA antisense oligonucleotide A617T promoted transition of the cell cycle of rodent progenitor cells. The protein expression pattern was similar to the expression pattern with serum-free culture medium containing EGF, but the nestin expression showed higher than the culture with EGF (Fig. 5).

Discussion

Expression of pVHL was progressively observed in cultured CNS progenitor cells. pVHL was initially undetectable in freshly harvested E12 cells, which were mostly nestin positive. By day 5 and day 14, pVHL expression was increasingly evident. In addition, pVHL expression was correlated with expression of MAP-2, a neuronal marker. Double immunocytochemistry revealed that pVHL and MAPs were expressed in the same cells. High-power observation, however, revealed that pVHL expression was shown in the nucleus and the cytoplasm, whereas MAP expression was shown in the cytoplasm and the dendrites. Their expression in the same cell type suggested that the VHL gene might be involved in neuronal differentiation from CNS stem cells. In addition, the present VHL gene transduction study revealed that the VHL gene induced neuronal differentiation. In contrast, the VHL mRNA antisense oligonucleotide study revealed that suppression of the VHL gene inhibited neuronal differentiation and promoted cell cycle transition in rodent E12 cultured progenitor cells. These results suggest that the VHL gene plays an essential role not only in neuronal differentiation but also in the proliferative state of CNS development. Because VHL modulates transcription, it may modulate CNS development through its effects on the transcription of developmentally related genes. This is similar to other tumor suppressor genes such as Rb (10), TSC2 (11), APC (9), and DCC (8), which are also implicated in neuronal differentiation at the posttranscriptional state. The Rb gene is implicated in neuronal differentiation, probably through its effects on gene transcription (10). The Rb gene product is detected in normal retinal nerve cells, but its expression in CNS neuron has not been demonstrated. The expression of the TSC2 gene product is identified in cerebral and cerebellar nerve cells, and the TSC2 gene plays a role in distal-neuronal differentiation. On the other hand, VHL mRNAs and the VHL gene product are readily demonstrated in CNS neurons (7, 8, 26). These observations and the results from the present study suggest that the VHL gene plays an important role in neuronal differentiation, probably through its effects on gene transcription. In this regard, the VHL gene product is similar to the proneural gene product, a basic helix-loop-helix transcription factor (30), which is essential for neuronal differentiation, probably through its effects on gene transcription. In this study, the VHL gene product forms a stable complex with human CUL-2, a member of the cdc 53 family of proteins. Proc. Natl. Acad. Sci. USA, 94: 2156–2161, 1997.


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