Inhibition of Ocular Angiogenesis by an Adenovirus Carrying the Human von Hippel-Lindau Tumor-Suppressor Gene In Vivo

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PURPOSE. The purpose of this study was to investigate the effect of the von Hippel-Lindau (VHL) protein on VEGF gene expression in vitro and to determine whether adeno-virus-mediated VHL intraocular gene transfer inhibits the development of angiogenesis in a monkey model of multiple branch retinal vein occlusion (BRVO).

METHODS. A recombinant adeno-virus vector adVHL was constructed to deliver the human VHL gene. Total RNA prepared from various kinds of cells transduced with adLacZ (control) or adVHL under normoxic or hypoxic conditions was subjected to Northern blot analyses. Either adLacZ or adVHL was delivered by preretinal injection in monkeys. The effects of adLacZ or adVHL on ocular neovascularization in laser-induced multiple BRVO was evaluated in color photographs and with fluorescein angiography (FA).

RESULTS. VHL expression in adVHL-transduced cells was confirmed at the transcript and protein levels. VHL overexpression significantly decreased the levels of VEGF transcripts in human aortic endothelial cells (HAECs); retinal pigment epithelium (RPE) cells; and RCC 786-O cells, renal carcinoma cells lacking VHL expression under normoxia. In contrast, VHL had no effect on the hypoxia-mediated increase in VEGF expression in these cells, although basal levels of VEGF expression were substantially reduced. Color photographs and FA revealed that retinal neovascularization and iris rubeosis accompanied by multiple BRVO in a monkey model were obviously suppressed by VHL overexpression. Northern blot analysis and immunostaining for VHL and VEGF indicated that VHL transfer obviously suppressed VEGF gene expression in VHL-transduced tissues such as retina and RPE.

CONCLUSIONS. The results showed that adeno-virus expressing VHL led to a significant reduction in VEGF expression in vitro under normoxic or hypoxic conditions. adVHL effectively inhibited angiogenesis in retina and iris in laser-induced multiple BRVO in monkey eyes. These data suggest that gene therapy based on VHL gene delivery has potential in the treatment of human ocular neovascularization. (Invest Ophthalmol Vis Sci. 2004;45:1289–1296) DOI:10.1167/iovs.03-0282
presence of oxygen. pVHL, in association with elongin B and C, binds directly to hypoxia inducible factor (HIF)-1α subunits and targets them for polyubiquitination and destruction.15 In hypoxic cells, HIF-1α contributes to the stabilization and mediation of the transcriptional activation of the VEGF gene.16 Mukhopadhyay et al.17 and Pal et al.18 also have reported that pVHL physically interacts with the Sp1 transcription factor in vitro and in vivo and that it suppresses Sp1-mediated activation of the VEGF promoter.17,18 Thus, pVHL plays an important role in suppressing VEGF expression through an interference with HIF-1α and Sp1 function.

In this study, we showed that VEGF transcripts were decreased by transduction of retinal pigment epithelium (RPE) and human aortic endothelial cells (HAECs) with adenovirus-expressing pVHL and attempted to determine the effects of VHL gene transfer on ocular angiogenesis, using experimental multiple branch retinal vein occlusion (BRVO) in a monkey model. We found that VHL gene overexpression effectively inhibited angiogenesis in the retina and iris in laser-induced multiple BRVO in monkey eyes. We showed that VHL gene therapy may be useful for inhibiting ocular neovascularization.

**METHODS**

**Materials**

Affinity-purified mouse polyclonal antibodies for VHL were purchased from PharMingen (San Diego, CA); [α-32P]dCTP (3000 Ci/mmole) from Amersham (Piscataway, NJ); anti-human VEGF antibody from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-human von Willebrand Factor (vWF) antibody from Dako (Carpinteria, CA). We used an Anaero Pack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) to induce hypoxia, as previously described.19

**Production of Recombinant Adenovirus**

The replication-deficient adenovirus was prepared as described elsewhere.20 Briefly, flag-tagged human VHL cDNA was inserted into the cassette-cosmid vector (pAXCAwt) with CAG (chicken β-globin poly(A)) signal sequences, driven by the cytomegalovirus (CMV) promoter (adVHL), by using an adenovirus expression vector kit (TaKaRa Biomed, Otsu, Shiga, Japan). The recombinant viruses were obtained by in vitro recombination in 293 cells identified as human embryonic kidney cells. Adenovirus encoding βgal sequences (pAXCAIαLacZ) were used as a negative control in all experiments (adlacZ). The adenovirus was purified twice by cesium chloride ultracentrifugation, as described.20

**Cell Culture and Infection**

COS, renal carcinoma cells (RCC786-O), and RPE cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and HAECs from cell culturing kits (Toyobo, Osaka, Japan). RCC786-O cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in 5% CO2. COS cells were also cultured in DMEM, and HAECs were cultured in endothelial cell basal medium with endothelial cell growth supplement (Cell Application, Inc., San Diego, CA) with 10% FBS and antibiotics at 37°C in 5% CO2. Cells were infected with either adlacZ or adVHL. After 24 hours of incubation, cells were conditioned under normoxia or hypoxia for 12 hours and harvested for Northern blot analysis.

**cDNA Probes and Northern Blot Analyses**

A 642-bp fragment of human VEGF cDNA sequence was used as a probe for Northern blot analyses. VHL cDNA, used as a probe for Northern blot, was synthesized by RT-PCR with total RNA prepared from A549 cells (human lung carcinoma cells). The sequences of oligonucleotides were as follows: VHL forward, 5'-GGGCGATCCAT-GCCCGGAGGGGAGGAC-3'; VHL reverse, 5'-CCCTCGAGATC-TCCCATCGGTGATGGTCG-3'. The β-gal probe was generously provided by Taku Iwami. The cDNA probes were labeled with [α-32P]dCTP, using a random primer method.21 Preparation of total cellular RNA and Northern blot analysis was performed as previously described.22 The tissues including retina, RPE, choroid, ciliary body, and iris were homogenized in extraction reagent (Isogen, Nippon Gene Co., Tokyo, Japan), and total RNA was extracted by the aforementioned method.

**Western Blot Analyses**

Whole extracts from adlacZ- or adVHL-overexpressing COS cells (20 multiplicities of infection (MOI)) were directly subjected to immunoblot analysis for VHL. For Western blot analysis, after incubation for 48 hours after adenovirus infection, cells were harvested. SDS-PAGE was performed with a 15% gel, according to a standard procedure, after boiling with sample buffer, and protein in the gel was transferred electrophoretically to a nitrocellulose membrane at 400 mA for 3.5 hours. VHL was visualized by using an affinity-purified mouse polyclonal antibody and a horseradish-peroxidase-linked anti-mouse IgG secondary antibody (Amersham). The complexes were detected by autoradiography, using a chemiluminescence detection system (ECL, Amersham).

**ELISA for VEGF**

The concentration of VEGF produced was measured with a commercially available ELISA kit (Immuno-Biological Laboratories Co., Gunma, Japan). The culture supernatants were collected after stimulation for 24 hours, and the absorbency was measured at 450 nm. VEGF production was normalized to the volume of the medium and cell number.

**Multiple BRVO and Preretinal Space Injection**

After a monkey was anesthetized,22 pupils were dilated with 1% tropicamide, and eyes were gently protruded using a rubber sleeve. The eyes were then covered with sodium hyaluronidase (Healon; Pharmed, New York, NY). We performed laser-induced occlusion of both major temporal and nasal retinal veins of a monkey’s eyes and produced neovascularization in the iris and retina.22 Laser photocoagulation was delivered with a superfield lens (+90 D, noncontact type) through a slit lamp. Laser (Coherent; Carl Zeiss Meditec, Jena, Germany) parameters were as follows: spot size of 300 μm, power of 240 to 280 mW, and exposure duration of 0.3 second. After FA to confirm venous congestion, an incision was made 1.5 mm behind the limbus with the tip of a 25-gauge needle. The needle was inserted tangentially toward the posterior pole of the eye, and 0.2 mL of viral suspension containing 1010 viral particles was injected to the preretinal space. Preretinal injection was confirmed with an indirect ophthalmoscope. In a similar fashion, the contralateral eye was injected with adlacZ. The animal experiments were conducted according to Animal Use and Care Guidelines, were approved by Animal Investigations Committee of Gunma University, and were consistent with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Fluorescein Angiography**

Neovascularization of multiple BRVO was demonstrated by FA at 0 and 7 days after laser photocoagulation. Fluorescein sodium (10% 0.1 mL/kg) was injected into the femoral vein of the anesthetized monkey. To evaluate the angiographic features of the entire fundus, we produced panoramic fluorescein angiograms and combined them with regional angiograms.

**Immunohistochemistry**

Immunohistochemistry was performed as previously described.23 Enucleated eyes were fixed (4% paraformaldehyde in 0.1 M PBS) for 48 hours, and 10-μm paraffin-embedded sections were cut. Immunohis-
In these experiments, we set out to confirm whether adVHL, which we constructed, would express VHL in vitro. COS cells were infected with adVHL at 20 MOI and were harvested after 36 hours, including 12 hours of normoxic or hypoxic conditions for Northern blot analyses. As expected, VHL transcripts were highly expressed in cells infected with adVHL, under either normoxic or hypoxic conditions (Fig. 1A). Western blot analysis using whole extracts from LacZ- or VHL-overexpressing COS cells showed that infections increased VHL protein levels, which correspond to the two distinct bands with a molecular mass of either 19 or 30 kDa, owing to splicing of its mRNA or degradation of pVHL (Fig. 1B).

To confirm that adVHL is functional in infected cells, we determined the effects of adVHL on VEGF gene expression by Northern blot analysis using RNA prepared from RCC786-O (VHL null) cells. VHL overexpression (50 or 100 MOI) in RCC786-O cells resulted in significant reduction of VEGF transcripts in normoxia, compared with LacZ-infected cells. In 12 hours of hypoxia, however, AV-VHL did not inhibit the induction of VEGF gene expression, although basal levels of VEGF expression were substantially reduced (Figs. 1C, 1D).

**Effect of VHL Gene Transfer on VEGF mRNA in HAECS and RPE**

To determine whether the expression of VEGF mRNA is regulated by adVHL gene delivery in VHL-positive cells, HAECS and RPE cells were transduced with either adLacZ or adVHL and were cultured in normoxia or hypoxia for 12 hours and subjected to Northern blot analyses. As shown in Figure 2A, VEGF mRNA levels were substantially reduced in cells transduced with adVHL under normoxic conditions, compared with cells transduced with adLacZ. In hypoxia, VEGF mRNA levels were increased 1.8- to 2.0-fold in adLacZ- and adVHL-transduced cells, although the relative levels of VEGF expression in adVHL-transduced cells were approximately 70% less than those in adLacZ-transduced cells (Fig. 2B). Northern blot analyses using total RNA extracted from RPE transduced with either adLacZ or adVHL were performed in a similar fashion (Fig. 2C), and the results showed the same tendency: adVHL potently inhibited the levels of VEGF expression in the hypoxic condition. This effect was largely due to the inhibition of the basal expression of the VEGF gene, not to the inhibition of the inducible expression of the VEGF gene by hypoxia.

**Effect of Overexpression of VHL on VEGF Production in HAECS**

To assess whether the observed decrease in VEGF transcripts represents downregulation of VEGF production, we performed specific ELISA of the conditioned medium of confluent cultures of COS cells that had been transduced with adLacZ or adVHL at 50 MOI. As shown in Figure 2D, overexpression of VHL reduced the VEGF protein levels produced in the normoxic conditions.
condition by 2.1-fold. The VEGF protein levels were increased by 2.2- and 2.1-fold in adLacZ- and VHL-transduced cells, respectively, in response to hypoxia.

**Prevention of Neovascularization by VHL Overexpression**

We next investigated the effect of AV-VHL on in vivo ocular angiogenesis by using the monkey multiple BRVO model. Laser irradiation was performed to occlude both major temporal and nasal retinal veins in a monkey’s eyes (Fig. 3A). Immediately after laser irradiation, FA showed the same levels of venous congestion in the right and left eyes (Fig. 3B). The adLacZ virus (10^{10} particles per eye) was injected into the preretinal space of the right eye of the monkey all at once, and adVHL virus (10^{10} particles per eye) was injected into the left eye (Fig. 3C). Seven days after adenovirus injection, color photographs and FA revealed that VHL overexpression significantly prevented retinal edema and neovascularization compared with the LacZ-overexpressing eye with optic disc neovascularization (NVD) and neovascularization elsewhere (NVE; Fig. 4). In addition to the suppression of NVD and NVE, an iris photograph examination at 7 days after infection showed that VHL gene transfer considerably suppressed iris rubeosis (Fig. 5A). To investigate whether we successfully delivered adenovirus to the tissues including retina, RPE, choroid, ciliary body, and iris, Northern blot analyses were performed. Analyses of total RNA extracted from both adLacZ- and adVHL-infected eyes showed that adLacZ and adVHL were obviously expressed in ocular tissues.
and VEGF mRNA levels were reduced in adVHL-transduced tissues compared with that in adLacZ-transduced tissues (Fig. 5B).

**Localization of VHL Expression and Examination of VEGF Expression**

To investigate the expression of the VHL and VEGF protein in the adVHL-transduced ocular tissue, we performed immunocytochemistry. VHL protein expression was detected in the retina and RPE but not in the ciliary body (Fig. 6). VEGF expression was reduced in the retina and RPE by VHL gene transfer compared with that in LacZ-transduced eye (Figs. 7A–D). Integrity of vascular structure after adVHL transduction was confirmed by immunostaining with vWF, which is specifically expressed in endothelial cells in choroidal vessels but not in sclera (Fig. 7E). These results are consistent with the observation in vitro that adVHL-transduced cells express reduced levels of VEGF mRNA and protein.

**DISCUSSION**

In this study, adenovirus expressing VHL led to a significant reduction in VEGF expression in vitro under a normoxic or hypoxic condition and effectively inhibited angiogenesis in retina and iris in laser-induced multiple CRVO. We used the monkey model of laser-induced multiple CRVO because the pathologic features of monkey multiple CRVO are similar to that of human multiple CRVO: Retinal neovascularization and rubeosis are remarkable in both species, and VEGF most likely plays a key role in mediating iris or retinal neovascularization in response to retinal ischemia in both human and monkey multiple CRVO. To the best of our knowledge, this is the first report demonstrating that VHL gene transfer effectively inhibits ocular neovascularization in vivo.

Inhibition of angiogenesis is a promising strategy for the treatment not only of ocular neovascular disease but also of cancers. Although numerous endogenous angiogenesis inhibitors have been identified, the clinical evaluation of these...
agents has been hindered by high magnification requirements, manufacturing constraints, and relative instability of the corresponding recombinant proteins. Previous studies have reported that the gene therapies with endostatin,\textsuperscript{26} angiostatin,\textsuperscript{27} tissue inhibitor of metalloproteinase (TIMP)-3,\textsuperscript{28} pigment epithelium-derived factor (PEDF),\textsuperscript{29,30} and TGF-\textbeta type II receptor\textsuperscript{31} are effective for the inhibition of experimental CNV in vivo. In contrast to the genes encoding these extracellular substances, we delivered the VHL gene to repress the function of the transcription factor HIF-1\textalpha, a transcription factor that drives several hypoxia-inducible genes of diverse functions, including angiogenic factors and enzymes regulating oxygen homeostasis. Therefore, it is conceivable that VHL gene therapy would be more advantageous in suppressing angiogenesis than delivery of the other genes described.

In experiments in vitro, we found that VEGF expression was induced in response to hypoxia to a comparable extent (approximately 1.8–2.0-fold) in both adLacZ- and adVHL-infected cells, although basal levels of VEGF expression were substantially reduced in adVHL-infected cells compared with that in adLacZ-infected cells. Based on the current understanding of the metabolisms of pVHL,\textsuperscript{32} the most plausible explanation for the inability of pVHL to inhibit hypoxia-induced VEGF expression is as follows: Under normoxic conditions, human pVHL interacts with a specific domain of the HIF-1\textalpha subunit through hydroxylation of a proline residue of HIF-1\textalpha. Then, HIF-1\textalpha is rapidly destroyed by a mechanism that involves ubiquitylation by a pVHL complex consisting of pVHL and elongin-B and -C. Thus, pVHL acts as a potent inhibitor of the function of HIF-1\textalpha in the presence of oxygen molecules. Under hypoxic conditions, the pVHL cannot form complex with HIF-1\textalpha because the proline residue of HIF-1\textalpha is not hydroxylated. Thus, pVHL allows HIF-1\textalpha to translocate into the nucleus, where it binds to HRE in the VEGF promoter, which results in the activation of VEGF expression in hypoxia.

We found that adVHL gene transfer inhibits neovascularization in laser-induced multiple BRVO, assessed by color photographs and FA. In accordance with the in vitro data, VEGF mRNA expression was significantly reduced in the adVHL-treated retina and iris. Because VEGF is known to increase vascular permeability, these changes may account for the absence of retinal edema, NVD, NVE, and rubeosis in vivo. Adenovirus itself did not appear to induce neovascularization, because neovascularization was comparable between adLacZ-infected and uninfected eyes (data not shown).
Although we have examined only the VEGF expression levels in adVHL-infected ocular tissue, we should take into account that pVHL acts as a negative regulator of extracellular matrix (ECM) production, cell cycle progression, morphogenesis, cellular adhesion, cytoskeletal organization, and motility. pVHL has been described to induce cyclin-dependent kinase inhibitor p27Kip1. From these published data, we assume that the reduction in angiogenesis observed in AV-VHL-infected ocular tissue is not only due to the inhibition of VEGF expression but also to the inhibition of ECM production or endothelial cell migration and proliferation. Further studies are obviously needed to test this hypothesis.

Problems related to the use of adenoviral vectors include immunologic and inflammatory reactions. Immunologic reactions may be at least partly explained by the fact that high-titer adenovirus induces expression of NF-κB and activates a cytotoxic T lymphocyte response. Some of the problems in relation to the use of adenoviruses may be related to impurities or replication-component viruses in the virus lots. Immunostimulatory properties of adenoviruses may limit the use of very high-titer viruses or repeated gene transfer. An additional limitation is that this study was not designed to determine a statistically significant difference in the duration of observation, the route of injection, or various titers, in that we used only two monkeys because of ethical considerations.

In conclusion, this study provides evidence that the VHL gene can be successfully introduced into intraocular tissues by means of adenovirus-mediated gene delivery and is effective for inhibiting neovascularization associated with multiple BRVO in monkeys. Because VEGF expression is inhibited in adVHL-infected cells and ocular tissue, the favorable effects of VHL gene delivery are partly due to the inhibition of VEGF expression. These data suggest that VHL gene therapy represents a potential treatment for neovascular diseases in human eyes.

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References


ERRATUM


The name of the second author was inadvertently misspelled. The correct spelling is Masato Kobayashi.

The online version of the article was corrected on March 29, 2004.