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Molecular Targeting of Antiangiogenic Factor 16K hPRL Inhibits Oxygen-Induced Retinopathy in Mice

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ABSTRACT

PURPOSE. To examine the ability and mechanism of the 16 kDa N-terminal fragment of human prolactin (16K hPRL) in the inhibition of abnormal retinal neovascularization.
METHODS. The 16K hPRL-encoding sequence was inserted into an adenoviral vector (16K-Ad). Western blot analysis verified the expression of 16K hPRL and inhibition of proliferation, confirming functional activity of the 16K hPRL in virus-infected adult bovine aortic endothelial (ABAE) cells. 16K hPRL inhibited retinal neovascularization in a mouse model of oxygen-induced retinopathy. The ability of recombinant 16K hPRL expressed in *E. coli* (r16K hPRL) was compared to that of endostatin in inducing apoptosis of cultured human retinal endothelial cells (HREC).
RESULTS. 16K was expressed in virus-infected ABAE cells and resulted in a dose-dependent inhibition of cell proliferation. Eyes injected with 16K-Ad showed a reduction in preretinal neovascularization of 82.3 ± 9.3% ($P < 0.00001$) when compared to uninjected controls. rl6K hPRL was 100 times more potent than endostatin in inducing apoptosis in HRECs.
CONCLUSIONS. Intravitreal administration of 16K hPRL inhibited neovascularization in the mouse model of oxygen-induced retinopathy. 16K hPRL stimulated apoptosis in HRECs and inhibited cell proliferation in ABAE cells. These results suggested a potential therapeutic role for 16K hPRL in the treatment of proliferative retinopathies.

INTRODUCTION

Proliferative retinopathies, which include retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and "wet" age-related macular degeneration (ARMD), are the leading causes of blindness in the western world. The underlying etiology common to these diseases is the proliferation of aberrant blood vessels.[1] To date, photocoagulation is the mainstay of management of patients with PDR. While effective at reducing severe vision loss, there can be serious side effects, including diminished night vision, reduced peripheral vision, and decreased visual acuity. Moreover, in some patients the disease progresses despite costly maximal laser therapy. ROP therapies are limited to laser and cryosurgery of the avascular zone. Clearly, the development of an affordable and efficacious drug therapy for the proliferative retinopathies would be a major breakthrough in medicine.

Vascular endothelial growth factor (VEGF) is currently viewed as the major effector for retinal neovascularization in all proliferative retinopathies. One approach in inhibiting neovascularization associated with eye disease is the use of antiangiogenic peptides. This concept has been extensively tested in animal models. The antiangiogenic peptides shown to inhibit retinal neovascularization in rat and mouse models include: angiostatin,[2,3] plasminogen kringle 5 fragment,[4] thrombospondin type 1 repeat fragments,[5] carboxyl-terminal fragment of tryptophanyl-tRNA synthases,[6] and pigment epithelium-derived factor.[5]

The study of inhibition of retinal vascularization by antiangiogenic peptides was extended to the antiangiogenic factor 16K hPRL for several reasons. 16K hPRL is a naturally occurring, specific antiangiogenic factor that has been evaluated in multiple in vitro and in vivo systems.[7] 16 K hPRL is endothelial cell specific and extremely potent. 16K hPRL inhibits VEGF-and fibroblast growth factor-2 (FGF-2)-induced capillary endothelial cell growth by blocking activation of the mitogen-activated protein kinase (MAPK) signaling cascade and induces apoptosis by inhibiting NF-κB activation.[8]

An adenovirus vector expressing 16K hPRL was developed to test the possibility that this well-characterized angiogenic factor could be used to prevent retinal neovascularization.[9] Primary cultures of human retinal endothelial cells (HREC) were used to test the efficacy of rl6K hPRL to induce apoptosis, a well-characterized action of antiangiogenic factors.[10] Primary cultures of ABAE cells were infected with the 16K-Ad to show that sufficient 16K hPRL was expressed after infection to inhibit endothelial cell proliferation. In vivo studies were carried out in the mouse model of oxygen-induced retinopathy.[11] This model is well established and resembles the human disease with extra retinal neovascularization. Increases in VEGF and VEGF receptors are closely correlated with neovascularization in this model.[12] l6K-adenoviral infection of the eye dramatically inhibited neovascularization in the mouse model of oxygen-induced retinopathy.

MATERIALS AND METHODS

Cell Culture

Human embryonic kidney (HEK) 293 cells, adenoviral E1-transformed HEK cells (BD Biosciences, San Diego, CA) were grown in Minimal Essential Medium (MEM) supplemented with 10% fetal calf serum (FCS), 1% nonessential amino acids and 100 U/mL penicillin/streptomycin and 2.5 μg/mL fungisome.

ABAE cells were isolated as previously described.[13] The cells were grown and serially passaged in low-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS and 100 U/mL penicillin/ streptomycin and 2.5 μg/mL fungisome (10% FCS/DMEM medium). Recombinant human FGF-2 (Promega, Madison, WI) was added (1 ng/mL) to the culture every other day. Confluent cells corresponding to passages 7 to 12 were used in the experiments.

Primary cultures of HRECs were prepared and maintained in DMEM/Hams F12 with 10% plasma-derived serum (PDS), 5 μg/mL transferrin, 2 μg/mL selenium, 1 μg/mL insulin, 0.584 mg/mL glutamine, and 15 mg/mL endothelial growth supplement (Sigma, St. Louis, MO)[10] and cells in passages 3 to 6 were used in the studies. The identity of endothelial cells in cultures was validated by demonstrating endothelial cell incorporation of fluorescence-labeled acetylated LDL and by flow cytometry analysis, as previously described.[14] To maintain purity of HRECs, the cells were grown in PDS, which is free of platelet-derived growth factor and does not promote the growth of pericytes (the contaminating cell type in these preparations). r16K hPRL was produced as previously described.[15] Endotoxin levels in the preparations were 100 times lower than amounts needed to mimic the actions of 16K hPRL. Endostatin was obtained from Sigma.

Construction and Generation of Adenovirus Vectors

All recombinant adenovirus was prepared using Adeno-X expression system purchased from BD Biosciences. The cDNA encoding 16K hPRL was excised from the pRC/CMV-hPRL 16K vector (Fig. 1A).[9] This construct was engineered to produce a secreted peptide consisting of the first 139 amino acids of the PRL. Codon 140 was replaced by a stop codon using PCR-directed mutagenesis.[16] Cysteine 58 (TGG) was replaced by a serine (TCC) to avoid non-native disulfide bridges. The rabbit β-globin intron was inserted 5' of the 16K hPRL signal peptide. The restriction sites *Nhe*I and *Kpn*I were added respectively to 5' and 3' ends by PCR. The sense primer was 5'-GGGCTAGCGATCCT-GAGAACTTCAGGGT-3' and the antisense primer, containing a stop codon (underlined), was *5'-CGGTACC[UNDERLN]TCA[/UNDERLN]*-AGGATGAACCTGGCTGAC-3'.

A replication defective adenoviral vector based on human Ad 5 serotype was used for the study. The 16K hPRL coding sequence was inserted in the expression cassette into the adenoviral shuttle vector, pShuttle, at the *Nhel* and *Kpnl* restriction sites and was verified by sequencing. The cytomegalovirus (CMV) promoter drives the expression of the expression cassette. The expression cassette was then excised from pShuttle and inserted to Adeno-X Viral DNA via the I-*Ceu*I and PI-*Sce*I restriction sites. The recombinant Adeno-X vector (16K-Ad) was propagated in HEK 293 cells, purified by cesium chloride density gradient ultracentrifugation, and stored at -80 °C. Adenovirus vector titer was determined by tissue culture infectious dose 50 ($TCID_{50}$). Adenovirus vector carrying an empty expression cassette was used as control (Null-Ad).

Western Blot Analysis

ABAE cells (1.5×10^4) were plated in 24-well plates in 0.5 mL 10% FCS/DMEM medium. On the following day the cells were infected with 16K-Ad or Null-Ad at a multiplicity of infection (MOI) of 200 pfu/cell, and the conditioned media were collected 48 hours postinfection. Since the cDNA for 16K hPRL contains a signal peptide, 16K hPRL is secreted into the culture medium. Conditioned media (20 μL) was heated in sample buffer

containing 1% 2-mercaptoethanol for 5 minutes at 95°C and separated by SDS/PAGE 15%/4% before being transferred to a nitrocellulose membrane (Hybond ECL; Amersham, Piscat-away, NJ). The membrane was saturated overnight in TBS-8% dry milk, followed by 1 hour of incubation with a 1/200 dilution of polyclonal antiserum directed against 16K hPRL (SB30) and 1 hour of incubation with a 1/5000 dilution peroxidase-conjugated goat antirabbit serum (Gamma; BioWhittaker, Verviers, Belgium). 16K hPRL detection was then carried out by chemiluminescence using the ECL Plus kit (Amersham). For the deglycosylation experiments, the N-Glycosidase F de-glycosylation Kit was used according to the manufacturer's instructions (Roche, Palo Alto, CA). Briefly, 20 μL of conditioned medium from Ad-16K hPRL-infected ABAE cells were incubated with 10 μL of denaturation buffer for 3 minutes at 95°C. Twenty microliter of reaction buffer and 15 μL of N-glycosidase F (1.2 units/μL) were added and incubated for 1 hour at 37°C. The deglycosylated product was precipitated with two volumes of cold methanol and analyzed by Western blotting as described above.

Experimental Animals

All animal procedures used were in agreement with the *NLH Guide for the Care and Use of Laboratory Animals,* with the ARVO Statement for the Use of Animals and with institutional guidelines, and approved by the University of Florida Institutional Animal Care and Use Committee. Timed pregnant C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Intravitreal Injection into Mouse Model

In the mouse model of oxygen-induced retinopathy,[11] mice at postnatal day (P)7 are placed with their nursing dams in a 75% oxygen atmosphere for 5 days. On return to normal air, these mice develop retinal neovascularization, with peak development occurring 5 days (P17) after their return to normoxia. Newborn mice (P1) were chilled on ice for 30 to 45 seconds to reduce their activity. Using a 32G needle attached to a 10 μL Hamilton syringe, 0.5 μL of a mixture containing the adeno-construct and 0.1% (v/v) fluorescein sodium (Angiofluor: Alliance Pharmaceuticals, Richmond, TX) was injected into the vitreous OD (Ad-16k = 9 x 10^9 pfu/mL) or OS (Ad-Null = 2.2 x 10^{10} pfu/mL); 0.1% fluorescein sodium was used to visualize the injection. After the fifth day following return to normoxia, the animals were killed, the eyes removed, fixed in 4% paraformaldehyde, and embedded in paraffin. Three hundred serial sections (6 μm) were cut sagitally through the cornea parallel to the optic disc. Every thirtieth section was placed on a slide and stained with hematoxylin-eosin (H&E). This resulted in ten sections from each eye being scored in a masked fashion using light microscopy to count endothelial nuclei extending beyond the inner limiting membrane into the vitreous as previously described.[11] The efficacy of treatment with a particular plasmid was then calculated as the percent average nuclei per section in the injected eye versus the uninjected eye.

For qualitative analysis the retinas from some mice were dissected and flatmounted, as described by D'Amato et al.[17] These animals were perfused with 3 mL of fluorescein-isothiocynate (FITC)-dextran (FD-2000S; Sigma) in 4% paraformaldehyde. The eyes were enucleated and the retinas removed and flatmounted for fluorescence microscopic analysis.

In Vitro Endothelial Cell Proliferation Assay

On day 1, ABAE cells were plated at a density of 1.5 x 10^4 cells per well in a 24-well plate, in 0.5 mL 10% FBS/DMEM medium. On day 2, cells were infected for 4 hours with increasing MOI of adenovirus vector. On day 3, the cells were stimulated with FGF-2 (1 ng/mL) for 16 hours. On day 4, the cells were incubated with 5 x 10 cpm of thymidine for 4 hours,[18] washed in 5% trichloroacetic acid, solubilized in NaOH, and counted as previously described.[16]

DNA Fragmentation ELISA Assay

The levels of mono- and oligonucleosomal DNA released in the cytosol of apoptotic cells were measured using the Cell Death Detection ELISA kit (Boehringer Mannheim, Mannheim, Germany). This is a quantitative sandwich-enzyme-immunoassay using antibodies against DNA and histones. By isolating nucleosomes in the cytosol with antibodies to histones, this assay specifically measures apoptosis and not necrosis.

For these studies, 100,000 HREC were plated per well in 12-well plates. Twenty-four hours later, rl6K hPRL, r23K hPRL, endostatin or endotoxin were added for 24 hours. The Cell Death Detection assay was performed on cytosolic fractions prepared using reagents supplied with the kit according to instructions. Levels of DNA

fragmentation were expressed as an enrichment factor, calculated by dividing the absorbance of a given sample by the absorbance of the corresponding 10% FCS control.

Statistical Analysis

All statistical analysis was done using the student t-test in Microsoft Excel. *P*-values < 0.001 are indicated with an asterisk on the graphs.

RESULTS

16K-Ad Infected ABAE Cells Express and Secrete Biologically Active 16K hPRL.

To extend findings with 16K hPRL to an in vivo setting, a 16K-Ad vector was engineered. To validate the usefulness of the 16K-Ad vector, ABAE cells were infected to determine that the infected cells expressed and secreted substantial amounts of biologically active 16K hPRL. ABAE cells were infected with a MOI of 200 pfu/cell of 16K-Ad or Null-Ad as a control for viral infection. ABAE cells infected with the 16K-Ad vector expressed 16K hPRL (Fig. 1A). Western blot analysis revealed a band running higher than that seen for recombinant 16K hPRL produced in *Escherichia coli* (Fig. 1B), possibly the result of N-glycosylation of Asn31 within 16K hPRL. After deglycosyla-tion of the conditioned media with N-Glycosidase F, the band co-migrated with the rl6K hPRL made in *E. coli* (Fig. 1C).

The amount of 16K hPRL produced in 16K-Ad infected ABAE cells, as estimated by Western blot analysis, appeared sufficient to obtain a biologically active concentration to inhibit cell proliferation. Consistent with this, FGF-2-induced ABAE proliferation was inhibited in a dose-dependent manner with increasing MOI of 16K-Ad while Null-Ad had no effect (Fig. 2).

Reduced Neovascularization by 16K hPRL in Mouse Model of Oxygen-Induced Retinopathy

The oxygen-induced model of retinopathy[11] was used to test the effect of the 16K hPRL in vivo. Mice, at P1, were injected in the right eye with the adeno-constructs (Null-Ad or 16K-Ad). The extent of preretinal neovascularization was scored by counting the average number of preretinal nuclei per section of eye (Fig. 3). Injection of the Null-Ad construct exhibited no significant difference from control ($P = 0.41$), as expected. The 16K-Ad construct showed a reduction in the average number of preretinal nuclei per section when compared to control left eyes of 66 ± 8% ($P = 3 \times 10^{-8}$). Cross-sections from representative eyes (Fig. 4) confirm the reduction in preretinal vessels in mice injected with the 16K-Ad construct. Perfusion of uninjected eyes from mice that underwent the time course of the OIR model demonstrated the leaky nature of the preretinal blood vessels that resulted from the hypoxic stimulus of this model (Fig. 5A). Injection of the 16K-Ad construct significantly reduced the number of leaky vessels in mice subjected to the OIR model (Fig. 5B). No difference in vasculature was found between uninjected eyes (Fig 5C) and eyes injected with the 16k-Ad construct (Fig. 5D) from mice that had not been subjected to the OIR model.

16K hPRL-Induced Apoptosis in HRECs

Increasing concentrations of rl6K hPRL progressively increased the level of DNA fragmentation (Fig. 6). Although the stimulation of DNA fragmentation was similar with 20 nM rl6K hPRL and 10 U of endotoxin, there was <0.01 U of endotoxin in the 20 nM rl6K hPRL preparation. Endostatin (10 μg/mL, 500 nM) gave the same response as 5 nM rl6K hPRL (80 ng/mL); that is, the potency of rl6K hPRL was 100 times greater. Although intact 23 kDa prolactin (10 nM) contained similar amounts of endotoxin to the rl6K hPRL preparation, it had no effect on DNA fragmentation, as previously reported in human umbilical vein endothelial cells (HUVEC) and bovine brain endothelial (BBE) cells.[15]

FIGURE 1: *Construction and characterization of 16K hPRL adenovirus vector:*
(A) Schematic representation of the 16K hPRL expression vector, the I-CeuI/PI-SceI restriction fragment cloned in adenovirus5 genome is shown. CMV IE, human cytomegalovirus immediate early promoter/enhancer; β-globin intron, rabbit β-globin intron; 16K hPRL, 16K hPRL coding sequence including the signal peptide; polyA, SV40 polyadenylation signal. (B) Analysis of protein expression by Western blot analysis from ABAE cells uninfected or infected at an MOI of 200 pfu/cell with 16K-Ad or Null-Ad. *Lane 1: E. coli* 16K hPRL; *lane 2:* protein from cells infected with 16K-Ad; *lane 3:* protein from cells infected with Null-Ad. Twenty µL of conditioned media were subjected to SDS-PAGE and probed with an anti-PRL antibody. The band corresponding to 16K hPRL (16 kDa) is labeled. Thirty nanograms of recombinant 16K hPRL produced in *E. coli* was loaded as a control. (C) Analysis of glycosylation. *Lane 4: E. coli* 16K hPRL; *lane* 5: protein from cells infected with 16K-Ad; *lane 6:* deglycosylated protein from cells infected with 16K-Ad. Twenty microliters of conditioned media from 16K-Ad-infected ABAE cells were incubated or not with N-glycosidase and subjected to Western blotting as in B. The *band*

corresponding to 16K hPRL (16 kDa) is labeled.

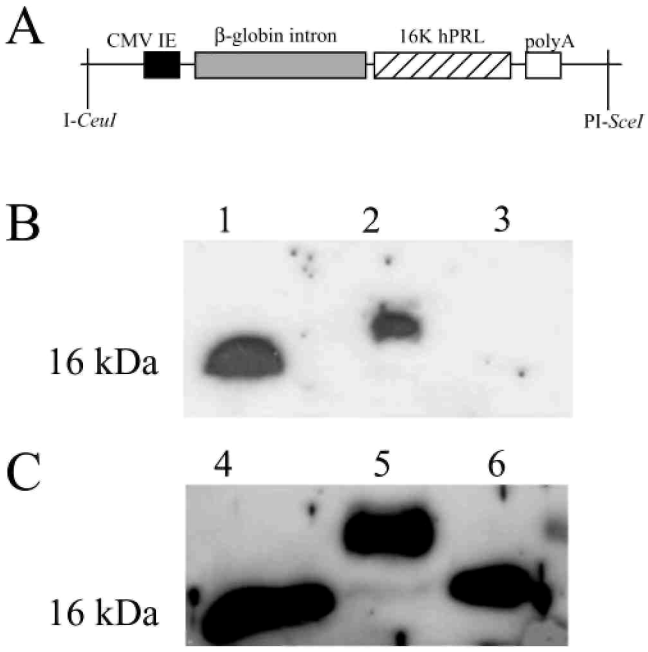


FIGURE 2: *Inhibition of endothelial cell proliferation by direct infection of 16K hPRL adenovirus vector*.
ABAE cells were infected with indicated MOI (pfu/cell) of 16K-Ad or Null-Ad and treated, 24 hours later, with FGF-2 (1 ng/mL). Proliferation was assessed by 3H-thymidine incorporation 48 hours postinfection. The data are expressed as percentages of the stimulation obtained with FGF-2 alone, 0% being the basal growth level. Data are the mean of triplicate wells; bars ± SE. The experiments were repeated at least three times, with similar results.

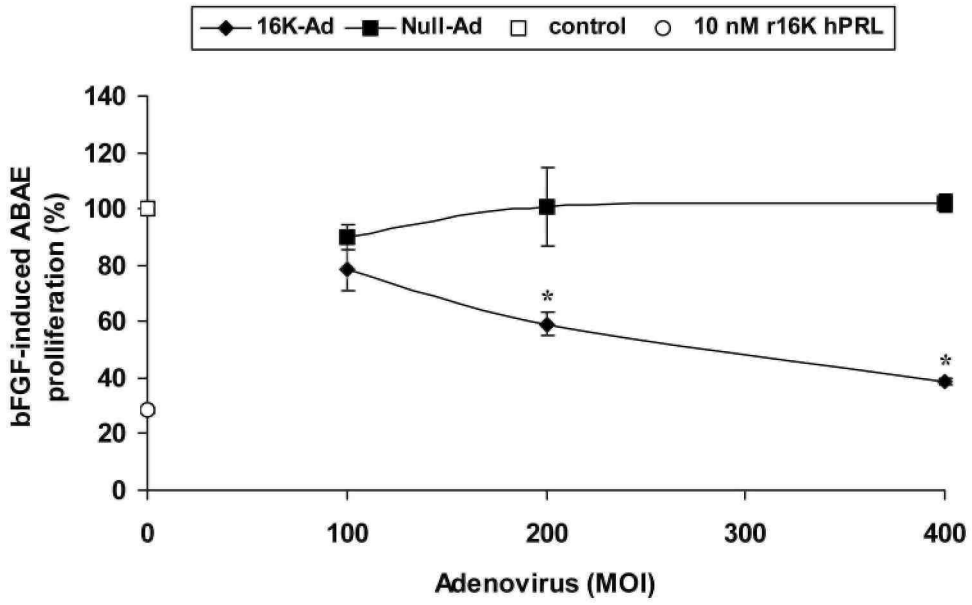


FIGURE 3: *Results of the injection of the adeno-constructs in the oxygen-induced mouse model of retinopathy.*
The *y-axis* indicates the average number of nuclei of preretinal endothelial cells per section. For each mouse the right eye (OD) was injected with the adeno-construct and the left eye was uninjected. For the 16K-Ad eyes (n = 11) and for the Null-Ad eyes (n = 4). Neovascularization was reduced by 66 ± 8% ($P = 3 \times 10^{-8}$) in the 16K-Ad injected eyes, while there was no significant reduction in the Null-Ad injected eyes (P = 0.3)

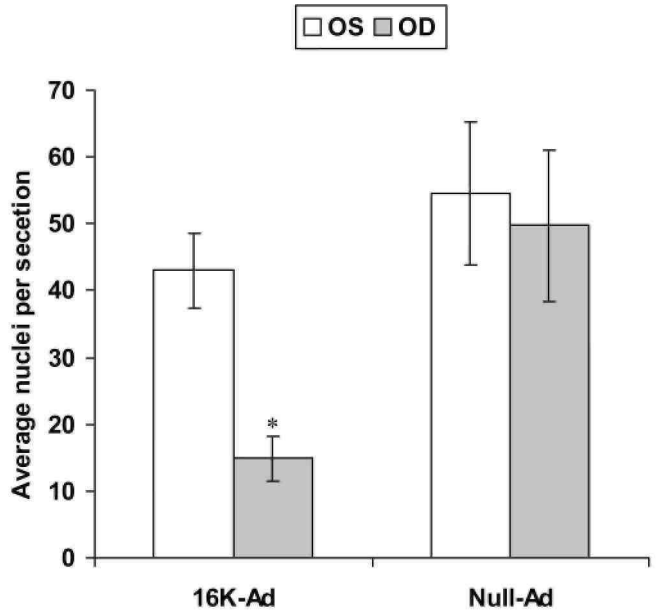


FIGURE 4: *Retinal cross-sections of eyes from mice that have undergone the oxygen-induced model of retinopathy.*
(A) [OS] and (B) [OD] paired eyes from a mouse that received no injection OS and was injected OD with the Ad-Null virus. (C) [OS] and (D) [OD] paired eyes from a mouse that received no injection OS and was injected OD with the Ad-16K virus. Only the eye injected with the Ad-16K virus shows significant reduction in preretinal nuclei (*). Original magnification, x5

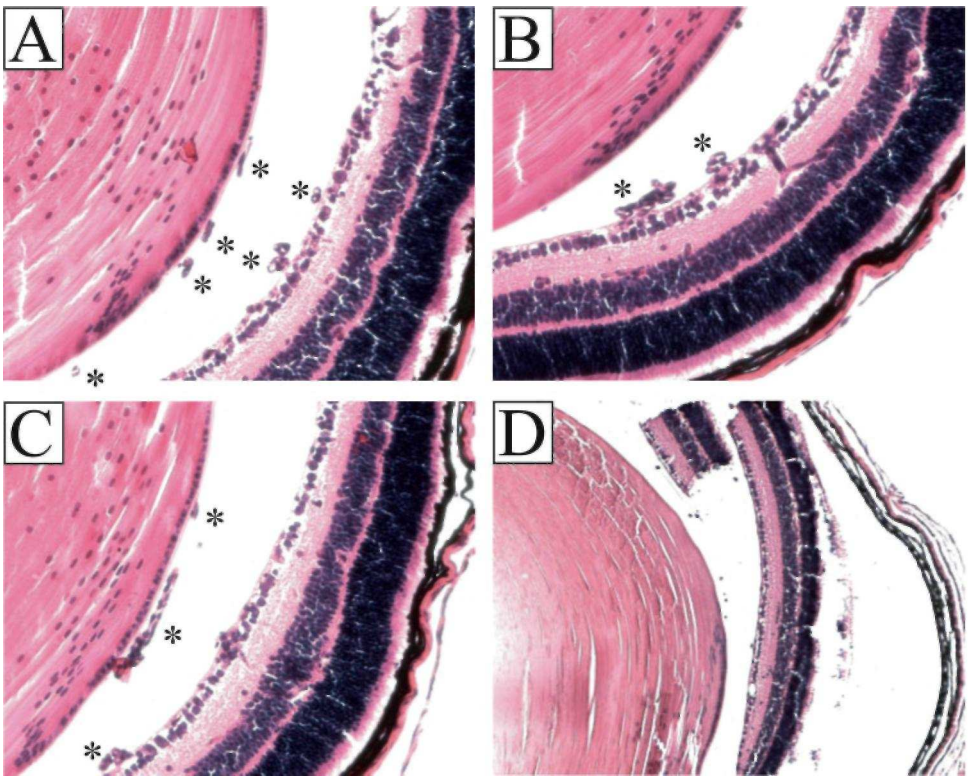


FIGURE 5: *Flatmounted retinas from mice perfused with FITC-dextran.*
(A) Retina from an uninjected mouse eye on postnatal day 17 after the OIR timecourse; (B) Retina from a mouse eye injected with the 16K-Ad construct on postnatal day 17 after the OIR timecourse; (C) Retina from an uninjected normoxic mouse eye on postnatal day 17; (D) Retina from a normoxic eye injected with the 16k-Ad construct on postnatal day 17.

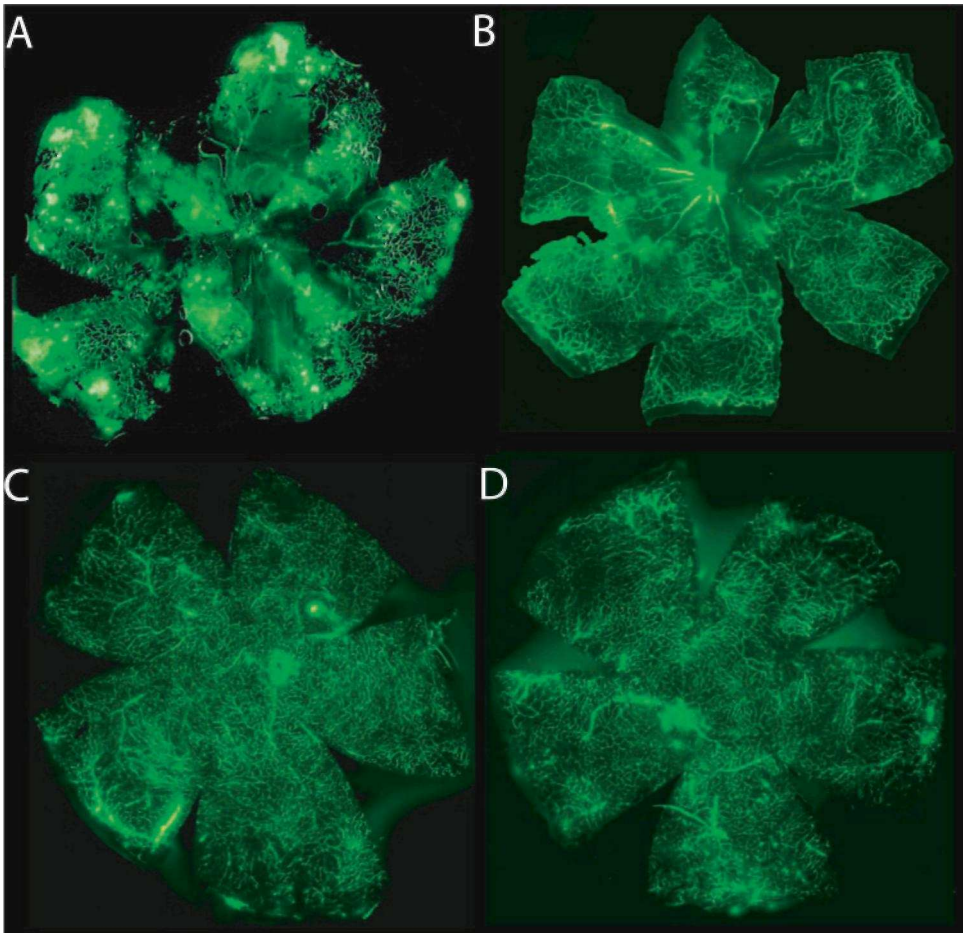
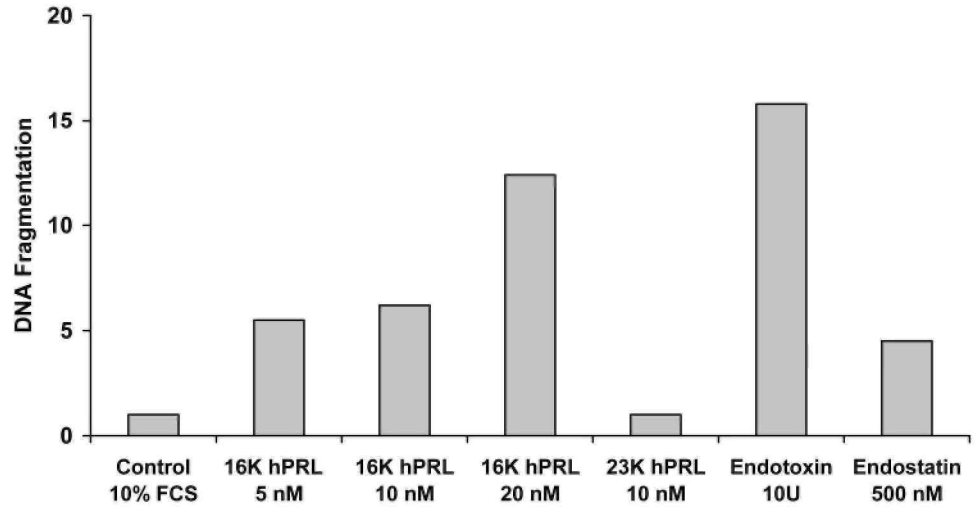


FIGURE 6: *Activation of apoptosis by recombinant 16K hPRL in HREC.*
The level of DNA fragmentation (cytosolic mono- and oligonucleosomes) was measured in HREC treated with increasing concentrations of recombinant 16K hPRL produced in *E. coli* (5, 10, and 20 nM), 10 U of endotoxin or 10 μg/mL endostatin.



DISCUSSION

Vascular retinopathies are significant vision-threatening diseases.[19] Occlusion of capillaries leads to retinal ischemia. Attempts to reperfuse areas of retinal ischemia with new, albeit aberrant, blood vessels lead to further vascular pathology. In all proliferative retinopathies the state of the nonperfused retina produces angiogenic growth factors such as VEGF and FGF-2, which stimulate this new, compensatory, blood vessel growth.

This study explored the effect of the antiangiogenic peptide 16K hPRL in both in vitro and in vivo systems relevant to the retina. Previously it was demonstrated that 16K hPRL, but not intact PRL, inhibits VEGF- and FGF-2-induced proliferation of a variety of capillary endothelial cells in nM concentrations[16,20] including endothelial cells isolated from the rat retina.[21] The signaling pathways for the antiproliferative action of 16K hPRL include the inhibition of VEGF- and FGF-2-induced Ras activation.[22] This is consistent with the idea that 16K hPRL might be particularly efficacious in inhibiting VEGF-driven retinopathy.

The inhibition of VEGF- and FGF-2-induced proliferation appears to be mediated through blocking of the MAPK signaling pathway. Activation and tyrosine phosphorylation of the Flk1 receptor by VEGF are not affected by the addition of 16K hPRL[23]; neither is the association of the receptor with Shc, and Grb2 or the recruitment of Sos.[22] However, activation of Ras is inhibited. When activated by VEGF alone, Ras is converted from the GDP- to the GTP- bound state. This conversion is blocked when 16K hPRL is added to VEGF. The blockade of Ras activation is consistent with the downstream inhibition of the VEGF-induced translocation of Raf-1 to the plasma membrane and the blockade of the activation of MEK and MAPK 42 and 44.[22]

16K hPRL activates apoptosis in capillary endothelial cells,[15] inhibits capillary endothelial cell migration, their organization into capillaries,[16] and urokinase activity.[24] Importantly, 16K hPRL inhibits neovascularization in the chick chorioallantoic assay,[16] rat corneal assay,[25] and in solid tumors in mice.[9] The 16 K hPRL fragment is naturally formed in rodents and in humans by proteolytic processing,[26-28] and PRL, the precursor molecule, is expressed by retinal endothelial cells.[21] 16K hPRL had been used successfully in gene therapy to inhibit the growth of the HCT116 human colon cancer cell line in immuno-incompetent mice.[9] 16K hPRL has no direct effect on the growth of HCT116 cells, but acts directly on the tissue vasculature.

The studies described here support the efficacy of this peptide in inhibiting growth of the relevant cell type (i.e., HREC). Furthermore, the 16 K hPRL fragment is unique in having endothelial cell specificity. Thus, unlike many other anti-angiogenic agents considered for possible therapy, the 16 K hPRL would not be expected to affect other cell types adversely in the retina.

These data demonstrate that 16K hPRL is potent in the nM range to activate apoptosis in HREC. 16K hPRL has also been reported to inhibit cell proliferation of rat corneal and retinal endothelial cells.[21,25] Clearly, 16K hPRL was considerably more potent than the preparation of endostatin tested. This is in close agreement with earlier findings that rl6K hPRL stimulates DNA fragmentation in BBE and HUVE cells[15] and that activation of 16K hPRL-induced apoptosis is dependent on activation of the caspase cascade and can be blocked by the addition of caspase inhibitors. The activity of the rl6K hPRL does not involve contamination with endotoxin. Heat denaturation of 16K hPRL by boiling, digestion with trypsin, or coincubation with the endotoxin blocker polymyxin-B has no effect on the action of 16K hPRL. Furthermore, immuno-neutralization of 16K hPRL with specific antibodies to 16K hPRL can block its ability to induce DNA fragmentation in BBE cells.

These findings with 16K hPRL were extended to an in vivo setting by using an engineered 16K-Ad vector. The usefulness of the 16K-Ad vector was validated by first infecting ABAE cells and demonstrating that the infected cells secreted substantial amounts of biologically active 16K hPRL. However, the 16K hPRL expressed in ABAE cells infected with the 16K-Ad vector was highly N-glycosylated. This is in agreement with observations made in HCT116 cells stably transfected with a 16K hPRL expression vector.[9] Glycosylated peptide produced in both cells was biologically active while the 16K hPRL expressed in *E. coli* as expected was not glycosylated.

In conclusion, the ability of 16 K hPRL to inhibit retinal neovascularization was studied because it is a potent and specific antiangiogenic factor.[20] 16K hPRL is a naturally occurring fragment found in rodents[26] and in humans,[27,28] and the parent protein prolactin is specifically expressed by retinal endothelial cells.[21] The signaling pathways regulating the inhibition of endothelial cell proliferation by 16K hPRL include the unique action of inhibiting Ras activation by both VEGF and FGF-2.[29] Previously, 16K hPRL was shown to activate apoptosis in BBCE.[15] But the present study demonstrated that rl6K hPRL was 100 times more potent than endostatin in inducing apoptosis in the most relevant cell type, HRECs.

Infection of ABAE cells with increasing amounts of 16K-Ad inhibited cell proliferation and eyes injected with 16K-Ad showed a dramatic reduction in preretinal neovascularization when compared to uninjected controls, while having no effect on animals exposed to normoxia. This combination of studies supports 16K hPRL as an endothelial cell-specific candidate therapeutic agent for the treatment of aberrant neovascularization of the retina.

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REFERENCES

1. Frank RN. On the pathogenesis of diabetic retinopathy. A 1990 update. *Ophthalmology.* 1991;98:586-593.

2. Meneses PI, Hajjar KA, Berns KI, Duvoisin RM. Recombinant an-giostatin prevents retinal neovascularization in a murine proliferative retinopathy model. *Gene Ther.* 2001;8:646-648.

3. Drixler TA, Rinkes IH, Ritchie ED, et al. Angiostatin inhibits pathological but not physiological retinal angiogenesis. *Invest Ophthalmol Vis Sci.* 2001;42:3325-3330.

4. Zhang D, Kaufman PL, Gao G, Saunders RA, Ma JX. Intravitreal injection of plasminogen kringle 5, an endogenous angiogenic inhibitor, arrests retinal neovascularization in rats. *Diabetologia.* 2001;44:757-765.

5. Stellmach W, Crawford SE, Zhou W, Bouck N. Prevention of ischemia-induced retinopathy by the natural ocular antiangiogenic agent pigment epithelium-derived factor. *Proc Natl Acad Sci USA.* 2001;98:2593-2597.

6. Otani A, Slike BM, Dorrell MI, et al. A fragment of human TrpRS as a potent antagonist of ocular angiogenesis. *Proc Natl Acad Sci USA* 2002;99:178-183.

7. Struman I, Bentzien F, Lee H, et al. Opposing actions of intact and N-terminal fragments of the human prolactin/growth hormone family members on angiogenesis: an efficient mechanism for the regulation of angiogenesis. *Proc Natl Acad Sci USA* 1999:96:1246-1251.

8. Tabruyn SP, Sorlet CM, Rentier-Delrue F, et al. The antiangiogenic factor 16K human prolactin induces caspase-dependent apoptosis by a mechanism that requires activation of nuclear factor-kappaB. *Mol Endocrinol.* 2003;17:1815-1823.

9. Bentzien F, Struman I, Martini JF, Martial J, Weiner R. Expression of the antiangiogenic factor 16K hPRL in human HCT116 colon cancer cells inhibits tumor growth in Ragl(-/-) mice. *Cancer Res.* 2001;61:7356-7362.

10. Grant MB, Guay C. Plasminogen activator production by human retinal endothelial cells of nondiabetic and diabetic origin. *Invest Ophthalmol Vis Sci.* 1991;32:53-64.

11. Smith LE, Wesolowski E, McLellan A, et al. Oxygen-induced retinopathy in the mouse. *Invest Ophthalmol Vis Sci.* 1994;35:101-111.

12. Suzuma K, Takagi H, Otani A, Honda Y. Hypoxia and vascular endothelial growth factor stimulate angiogenic integrin expression in bovine retinal microvascular endothelial cells. *Invest Ophthalmol Vis Sci.* 1998;39:1028-1035.

13- Gospodarowicz D, Cheng J, Lirette M. Bovine brain and pituitary fibroblast growth factors: comparison of their abilities to support the proliferation of human and bovine vascular endothelial cells. *J Cell Biol.* 1983;97:1677-1685.

14. Grant MB, Tarnuzzer RW, Caballero S, et al. Adenosine receptor activation induces vascular endothelial growth factor in human retinal endothelial cells. *Circ Res.* 1999;85:699-706.

15. Martini JF, Piot C, Humeau LM, Struman I, Martial JA, Weiner RI. The antiangiogenic factor 16K PRL induces programmed cell death in endothelial cells by caspase activation. *Mol Endocrinol.* 2000; 14:1536-1549.

16. Clapp C, Martial JA, Guzman RC, Rentier-Delure F, Weiner RI. The 16-kilodalton N-terminal fragment of human prolactin is a potent inhibitor of angiogenesis. *Endocrinology.* 1993;133:1292-1299.

17. D'Amato R, Wesolowski E, Smith LE. Microscopic visualization of the retina by angiography with high- molecular-weight fluorescein-labeled dextrans in the mouse. *Microvasc Res.* 1993;46:135-142.

18. Dong Z, Yoneda J, Kumar R, Fidler IJ. Angiostatin-mediated suppression of cancer metastases by primary neoplasms engineered to produce granulocyte/macrophage colony-stimulating factor. *J Exp Med.* 1998;188:755-763.

19. Klein R Klein BE, Moss SE, Davis MD, DeMets DL. The Wisconsin epidemiologic study of diabetic retinopathy. IV. Diabetic macular edema. *Ophthalmology.* 1984;91:1464-1474.

20. Ferrara N, Clapp C, Weiner R. The 16K fragment of prolactin specifically inhibits basal or fibroblast growth factor stimulated growth of capillary endothelial cells. *Endocrinology.* 1991;129: 896-900.

21. Ochoa A, Montes de Oca P, Rivera JC, etal. Expression of prolactin gene and secretion of prolactin by rat retinal capillary endothelial cells. *Invest Ophthalmol Vis Sci.* 2001;42:1639-1645.

22. D'Angelo G, Martini JF, Iiri T, Fantl WJ, Martial J, Weiner RI. 16K human prolactin inhibits vascular endothelial growth factor-induced activation of Ras in capillary endothelial cells. *Mol Endocrinol.* 1999;13:692-704.

23. D'Angelo G, Struman I, Martial J, Weiner RI. Activation of mitogen-activated protein kinases by vascular endothelial growth factor and basic fibroblast growth factor in capillary endothelial cells is inhibited by the antiangiogenic factor 16-kDa N-terminal fragment of prolactin. *Proc Natl Acad Sci USA.* 1995;92:6374-6378.

24. Lee H, Struman I, Clapp C, Martial J, Weiner RI. Inhibition of urokinase activity by the antiangiogenic factor 16K prolactin: activation of plasminogen activator inhibitor 1 expression. *Endocrinology.* 1998;139:3696-3703.

25. Duenas Z, Torner L, Corbacho AM, et al. Inhibition of rat corneal angiogenesis by 16-kDa prolactin and by endogenous prolactin-like molecules. *Invest Ophthalmol Vis Sci.* 1999;40:2498-2505.

26. Clapp C, Torner L, Gutierrez-Ospina G, et al. The prolactin gene is expressed in the hypothalamic-neurohypophyseal system and the protein is processed into a 14-kDa fragment with activity like 16-kDa prolactin. *Proc Natl Acad Sci USA.* 1994;91:10384-10388.

27. Kineman RD, Bullesbach EE, Schwabe C, Boggs KP, Frawley LS. Purification from human pregnancy serum of a low molecular weight mitogen similar to placental lactogen and growth hormone. *Proc Soc Exp Biol Med.* 1991;197:441-449.

28. Sinha YN, Gilligan TA, Lee DW, Hollingsworth D, Markoff E. Cleaved prolactin: evidence for its occurrence in human pituitary gland and plasma. *J Clin Endocrinol Metab.* 1985;60:239 -243.

29. D'Angelica M, Tung C, Allen P, et al. Herpes simplex virus (HSV)-mediated ICAM-1 gene transfer abrogates tumorigenicity and induces anti-tumor immunity. *Mol Med.* 1999;5:606-616.