A Peptide Derived from Type 1 Thrombospondin Repeat–Containing Protein WISP-1 Inhibits Corneal and Choroidal Neovascularization

Marisol del Valle Cano,1,2 Emmanouil D. Karagiannis,2,5 Mohamed Soliman,1 Belal Bakir,1 Wenjuan Zhuang,1 Aleksander S. Popel,5 and Peter L. Gehlbach1

PURPOSE. Ocular neovascularization is the primary cause of blindness in a wide range of prevalent ocular diseases including proliferative diabetic retinopathy, exudative age-related macular degeneration, and retinopathy of prematurity, among others. Antiangiogenic therapies are starting to give promising results in these diseases. In the present study the antiangiogenic potential of an 18-mer peptide derived from type 1 thrombospondin repeat-containing protein WISP-1 (wispostatin-1) was analyzed in vitro with human retinal endothelial cell proliferation and migration assays. The peptide was also tested in vivo in the corneal micropocket and the laser-induced choroidal neovascularization (CNV) mouse models.

METHODS. Human retinal endothelial cells were treated with the WISP-1 peptide and in vitro migration and proliferation assays were performed. Also evaluated was the antiangiogenic effect of this peptide in vivo using the corneal micropocket assay and the laser-induced CNV model.

RESULTS. Wispostatin-1 derived peptide demonstrated antimigratory and antiproliferative activity in vitro. Wispostatin-1 completely abolished bFGF-induced neovascularization in the corneal micropocket assay. The peptide also demonstrated significant inhibition of laser-induced CNV.

CONCLUSIONS. An inhibitory effect of Wispostatin-1 on ocular neovascularization was found in vitro and in vivo. The identification of novel and potent endogenous peptide inhibitors provides insight into the pathogenesis of corneal and choroidal neovascularization. The results demonstrate potential for therapeutic application in prevalent ocular disease. (Invest Ophthalmol Vis Sci. 2009;50:3840–3845) DOI:10.1167/iovs.08-2607

From the Departments of 1Ophthalmology, Wilmer Eye Institute, and 2Biomedical Engineering, Johns Hopkins University, School of Medicine, Baltimore, Maryland.

1Contributed equally to the work and therefore should be considered equivalent authors.

Supported by Fight for Sight (MdC), the Sheila West Research Grant Award (MdC), the JG Foundation (PLG); an unrestricted grant from Research to Prevent Blindness (Wilmer Eye Institute); a research grant to Prevent Blindness Career Development award (PLG); the Jack and Gail Baylin Philanthropic Fund; the Johns Hopkins University Fund for Medical Discovery (PLG); a gift from Kenneth and Brenda Richardson (PLG); and a gift form Mr. and Mrs. George Laniado.

Submitted for publication July 28, 2008; revised December 7, 2008; and January 10, 2009; accepted June 15, 2009.

Disclosure: M. del V. Cano, None; E.D. Karagiannis, P.; M. Soliman, None; B. Bakir, None; W. Zhuang, None; A.S. Popel, P; P.L. Gehlbach, None.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Peter L. Gehlbach, Retina Division, Wilmer Eye Institute, Johns Hopkins University, School of Medicine, 1550 Orleans Street, Baltimore, MD 21231; pgelbach@jhmi.edu.

Ocular neovascularization is the primary cause of blindness in a wide range of prevalent ocular diseases, including proliferative diabetic retinopathy, exudative age-related macular degeneration, retinopathy of prematurity, ischemic central and branch retinal vein occlusions, infectious keratitis, trauma, and various inflammatory ocular diseases.1

In the normal and developmentally mature ocular vascular system, angiogenic stimulating factors, such as vascular endothelial growth factor (VEGF) and angiogenic inhibitors exist in a homeostatic balance.2 In a variety of pathologic conditions, such as hypoxia, ischemia, inflammation, infection, and trauma, the balance between angiogenic stimulators and inhibitors is disrupted, leading to formation of pathologic vessels. Abnormal growth of new vessels in the eye can limit light transmission and affect physiological function.3

During the past two decades, the mechanisms of endogenous suppression of angiogenesis have been elucidated by studying several antiangiogenic proteins and derivative peptides including pigment endothelium–derived factor (PEDF), kallistatin, angiostatin, and thrombospondin-1 (TSP-1). PEDF has been identified as a potent antiangiogenic factor.4,5 It is a potent inhibitor of endothelial cell (EC) proliferation and migration.6 Kallistatin levels are significantly reduced in the vitreous from patients with proliferative diabetic retinopathy and in the retina of diabetic rats, suggesting that it is implicated in diabetic retinopathy.7,8 Kallistatin inhibits retinal neovascularization and reduces vascular leakage in the retina.9 In patients with diabetic retinopathy significant elevation of vitreous angiostatin and decreased VEGF concentrations are observed in eyes previously treated with laser photocoagulation.10 The TSP-1 protein has been identified as an important ocular angiogenesis inhibitor.11,12 The protein is primarily expressed in the limbal area where it is speculated that it creates a natural barrier to vascular invasion into the corneal stroma.13

We have identified numerous peptides derived from proteins containing TSP-1 repeats that share similarities with the known angiogenesis inhibitors Mal-II and -III.14-16 In the present study, one of these peptides, wispostatin-1 (WISP-1), composed of 18 amino acids, derived from TSP-1 repeat-containing protein WISP-1 (human sequence: SPWSPCSTSCGL-GVSTRI, and its orthologous mouse sequence: SPWSPCST-TGGGLGVRRII) inhibited proliferation and migration in human retinal EC assays (human sequence). WISP-1 also significantly inhibited neovascularization in the corneal micropocket assay (mouse sequence) and the laser-induced choroidal neovascularization (CNV) model (both human and mouse sequences). WISP-1 is 52% identical with the known antiangiogenic peptides (Mal-II and -III) derived from the TSP-1 domains of the TSP-1 protein (Fig. 1A). It is a small peptide with molecular mass of 1837 Da. It is composed of mostly hydrophilic amino acids, apart from a small stretch of five amino acids near the C-terminus that are hydrophobic (Fig. 1B). WISP-1 is slightly positively charged at neutral pH (charge +1.9) and has an isoelectric point of 9.2, which means that under neutral pH environments the peptide retains its positive charge.
MATERIALS AND METHODS

Peptide Identification

Using a bioinformatics algorithm, we searched within the human proteome for antiangiogenic peptides and identified a set of peptides 17 to 20 amino acids in length that are derived from the TSP-1 repeats of human proteins and share similarities with the known angiogenesis inhibitors Mal-II and -III.14–16 We named the predicted peptides according to the names of the proteins of origin. We have demonstrated that these peptides inhibit the proliferation and migration of human umbilical vein ECs (HUVECs) in vitro. In the present study, we experimentally tested the ocular antiangiogenic potency of WISP-1 derived from the protein WISP-1 (Wnt-1 induced secreted protein 1) in vitro using human retinal EC (HREC) proliferation and migration assays and an in vivo corneal micropocket and a CNV mouse model. The properties of the peptide were calculated with the ProtScale tool.17

Peptide Synthesis and Handling

The peptides were produced by a commercial provider (Abgent, San Diego, CA) using a solid-phase synthesis technique. HPLC and mass spectrometry analyses of each peptide were performed. In each case, the synthetic procedure yielded >95% pure peptide. Scrambled peptides were also produced to be used as a negative control.

Cell Culture

HRECs from a single donor were obtained from The Applied Cell Biology Research Institute (Kirkland, WA). The cells were propagated in EGM-2 medium, consisting of a basal cell medium with 2% FBS, growth factors (hbfFGF and VEGF) and antibiotics (gentamicin/amphotericin B). All the cells used were from passages 3 to 6.

In Vitro Cell Viability Assay

We assessed the effects of WISP-1 on the proliferation of ECs by measuring the metabolic activity of the live cells using a colorimetric cell proliferation reagent (WST-1; Roche, Indianapolis, IN). Approximately 2000 cells/well were seeded in a 96-well microplate without any extracellular matrix substrate and exposed for 3 days to different peptide concentrations: 0.01, 0.1, 1, and 10 μg/mL and 20, 30, and 40 μg/mL. The molecular mass of the peptide is approximately 2 kDa; thus, the aforementioned concentrations are equivalent to 5 nM, 50 nM, 500 nM, 10 μM, 15 μM, and 20 μM. Each of the concentrations was tested simultaneously in quadruplicate, and each of the experiments was repeated three times. As a positive control, we applied 100 ng/mL (0.22 μM) TNP-470. As a negative control (normal viability) the cells were cultured without any agent in full medium containing growth factors and serum.

In Vitro Cell Imaging

Two thousand cells/well were seeded in a 96-well microplate without any extracellular matrix substrate and were allowed to attach overnight, as previously described, and exposed for 3 days to different peptide concentrations: 10, 20, 30, and 40 μg/mL. The medium with the peptide was removed and a Dil cell-labeling solution (Vybrant; Invitrogen, Carlsbad, CA) was added according to the manufacturer’s protocol and allowed to stand for 15 minutes. The cells were then washed three times with medium and imaged by 549 nm absorbance and 565 nm emission.

In Vitro Cell Migration Assay

A modified Boyden chamber migration assay (BD Biosciences, San Jose, CA) was used to examine EC migration in the presence of an activator and the peptide solution. In our case, we used 20 ng/mL VEGF (Invitrogen) and 1, 10, and 50 μg/mL of the tested peptide solution. A serum- and growth factor-free medium was used as a negative control, and 20 ng/mL VEGF was used as a positive control. The chambers were then incubated for 20 hours at 37°C. The cells that had migrated into the lower chamber were stained with calcein (Invitrogen–Molecular Probes, Eugene, OR) for 90 minutes before termination of the experiment and counted.

Animals

All animal studies were conducted in accordance with an animal protocol reviewed and approved by the Johns Hopkins University Animal Care and Use Committee and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Normal C57BL/6 mice were used in these studies. Six- to 8-week-old male C57BL/6 mice were obtained from Harlan (Indianapolis, IN) and...
were housed on a 12-hour light-dark cycle, with food and water provided ad libitum. All mice were fed with a standard caloric diet for their age.

**Preparation of Pellets**

Hydron pellets (polyhydroxyethyl-methacrylate [polyHEMA], Intercepton, New Brunswick, NJ) were made so that each one contained approximately 40 ng of bFGF (Peprotech Inc., Rocky Hill, NJ), and 30 μg of sucrose aluminum sulfate (Sucraltate; Sigma-Aldrich, St. Louis, MO). Briefly, in this modified technique, we prepared 6 μL of a suspension containing 4.1 μL of 12% Hydron in ethanol, 1250 ng of bFGF (1 μL of a 1250 ng/μL solution), and 900 μg of sucralfat (0.9 μL of a 1 mg/μL solution). Subsequently, 2 μL of this suspension was taken with a pipette (0.1 to 2.5 μL; Eppendorf North America, Westbury, NY) and deposited onto an autoclaved 15×15-mm piece of nylon mesh (3–300/50, approximate pore size 0.4 μm; Tetko, Lancaster, NY). Ten holes of this mesh were filled with each 2 μL of our suspension, which resulted in a pellet volume of approximately 0.2 μL. Under the aid of a surgical microscope (Carl Zeiss Meditec, Inc.), the fibers of the mesh were pulled apart, and uniformly sized pellets were selected for implantation. In the case of a peptide application the Hydron pellets were made in a similar manner as described for bFGF. In this case, we prepared 8 μL of a suspension containing 3.5 μL of 12% Hydron in ethanol, 1625 ng of bFGF (1.3 μL of a 1250 ng/μL solution), 200 μg of peptide (2 μL of a 100-μg/μL solution), and 1200 μg of sucralfat (1.2 μL of a 1-mg/μL solution). The suspension was deposited onto the nylon mesh as just described. Each pellet of approximately 0.2 μL contained approximately 40 ng of bFGF, 100 μg/μL of peptide, and 30 μg of sucralfat.

**Corneal Micropocket and Pellet Implantation**

Mice received general anesthesia with an intraperitoneal injection of ketamine/xylazine (45 and 4.5 mg/kg, respectively), and topical anesthetia (1 drop of proparacaine 0.5%) before each intervention. Surgery was performed by the same surgeon (WZ) with the use of an ophthalmic operating microscope (Carl Zeiss Meditec, Inc.). Using a modification of the technique described by Kenyon et al., a 1-mm stromal linear keratotomy was performed with a disposable 15° surgical blade (OR Specialties, Inc., Baltimore, MD). The incision was made parallel to the insertion of the lateral rectus muscle, approximately 1.5 mm away from the temporal limbus. An intrastromal micropocket (1×0.5 mm) was dissected toward the limbus with a lamellar dissecting blade originally designed for deep anterior lamellar keratoplasty (Dutch Ophthalmics, Kingston, NJ). A single pellet was implanted and advanced toward the temporal corneal limbus, within 0.7 to 1.0 mm. A drop of levofloxacin 0.5% (Quixin, Santen Inc., Napa, CA) was instilled in each eye immediately after the surgical procedure. In addition, topical levofloxacin eye drops were also administered three times daily, for 6 days. For analgesia, SC buprenorphine (0.1 mg/kg) was administered to each animal every 12 hours for 4 days.

**Design of the Corneal Micropocket Experiment**

Before the surgery, the animals were randomly distributed to receive one of three pellet types: bFGF, hFGF with peptide, or empty. The eyes were photographed at day 6 after pellet implantation with a digital camera (Power Shot S2 IS; Canon, Tokyo, Japan) with the aid of a dissecting microscope (Steamini 2000 C; Carl Zeiss Meditec) and then the animals were killed. The neovascularization emerging from the limbal vessel was evaluated. Two parameters were used to assess corneal neovascularization (NV): the maximal vessel length (VL) extending from the limbal vasculature toward the pellet, and the contiguous circumferential zone of NV (clock hours [CN] of NV, where 1 clock hour equals 30° of arc). The area (in square millimeters) of NV was then calculated, as described by Kenyon et al. The areas of NV are expressed in percentage, with the growth factor control (bFGF) representing 100% of neovascularization, and the empty pellet control representing 0% of neovascularization. One week after pellet implantation, the animals were killed, and the neovascularization emerging from the limbal vessel was evaluated. Two parameters were used to assess NV: the maximalVL extending from the limbal vasculature toward the pellet, and the contiguous circumferential zone of NV (CN of NV, where 1 clock hour equals 30° of arc). The area (in square millimeters) of NV response was then calculated using the formula of half an ellipse: 0.5 × π × R1 × R2, where the conversion factor for R2 (mm) = CN × 0.4 (mm) and R1 (mm) = VL (mm), and Area (mm²) = π × VL (mm) × CN × 0.2 (mm). The area of NV is expressed as a percentage, with the positive control (bFGF) representing 100% of neovascularization, and the negative control (empty pellet) representing 0% of neovascularization.

**Laser-Induced CNV**

Adult C57BL/6 mice were used for these experiments. The mice were anesthetized and a drop of tropicamide was applied for pupillary dilatation. Bruch’s membrane was ruptured with laser photocoagulation at three locations in each eye. Briefly, laser photocoagulation (400-nm wavelength, 50-μm spot size, 0.05-second duration, and 120-mW intensity) was delivered via the slit lamp delivery system with a handheld cover slide used as a contact lens. Burns were performed in the 9, 12, and 3 o clock positions two to three disc diameters from the optic nerve. Production of a vaporization bubble at the time of lasering, which indicates rupture of the Bruch’s membrane, is an important factor in obtaining CNV, and therefore only burns in which a bubble was produced were included in the study.

**Intravitreous Injection**

After laser rupture of the Bruch’s membrane, the mice were injected intravitreally with 100 μg/mL of the peptide studied. Intravitreous injections were performed with a pump microinjection apparatus (Harvard Apparatus, Holliston, MA) and pulled glass micropipettes. Each micropipette was calibrated to deliver 1 μL of vehicle containing the peptide on depression of a foot switch. The mice were anesthetized and the pupils dilated. Under a dissecting microscope, the sharpened tip of the micropipette was passed through the sclera, just behind the limbus into the vitreous cavity, and the foot switch was depressed. The injections were repeated 7 days after Bruch’s membrane rupture to replenish the peptide concentration in the vitreous cavity.

**Measurement of the Sizes of Laser-Induced CNV Lesions**

Two weeks after laser treatment, the sizes of CNV lesions were measured in choroidal flatmounts. Mice used for the flatmount technique were anesthetized and perfused with 1 mL phosphate-buffered saline (PBS) containing 50 mg/mL fluorescein-labeled dextran (500,000 average molecular weight; Sigma-Aldrich), as previously described. The eyes were removed and fixed in 10% phosphate-buffered formalin. The cornea and lens were removed, and the entire retina was carefully dissected from the eye cup. Radial cuts (four to seven; average, five) were made from the edge to the equator, and the eye cup was flattened in aqueous medium (Aqua Poly/Mount; Polysciences, Inc., Warrington, PA) with the sclera facing down. The flatmounts were examined by fluorescence microscopy (Axiovert 200M; Carl Zeiss Meditec, Thornwood, NY), and images were digitized with a CCD camera (Axiocam MRc5). Image analysis software (AxioVision Software; Carl Zeiss Meditec) was used to measure the total area of hyperfluorescence associated with each burn, corresponding to the total fibrovascular scar. The areas within each eye were averaged to obtain one experimental value, and mean values were calculated for each treatment group and compared by Student’s paired t-test.

**Statistical Analysis**

For the in vitro experiments, Student’s t-test was used. For NV, descriptive statistics were expressed as the mean ± SE. Nonparametric tests were used for the analysis of NV. A paired Student’s t-test was used to conduct a statistical analysis for CNV.
RESULTS

Peptide Effects: Proliferation and Migration of HRECs

Two key characteristics of the angiogenic process are the proliferation of ECs near a maternal vessel, where the novel sprouting bud emerges, and their coordinated migration along a chemotactic gradient. To evaluate the antiproliferative and antimigratory potency of WISP-1, we used in vitro proliferation and migration assays with HRECs and measured the peptide potency compared to positive and negative control experiments. We tested the ability of the peptide to inhibit the proliferation of HRECs in an in vitro assay (Fig. 2A). As a negative control, we added only the full medium to the cells; as a positive control, we added the full medium with 100 ng/mL of TNP-470 (fumagillin). TNP-470 is a microtubule-stabilizing agent that has the ability to induce EC apoptosis. The optical signal from the proliferation assay was scaled so that 0% represented the signal from the negative control and 100% represented the signal from the wells to which TNP-470 was added. We expressed this scaled result as peptide activity relative to the activity of TNP-470. The tested peptide reached a maximum efficacy of approximately 20% proliferation inhibition.

To observe whether the peptide induces any alterations to the morphology of the ECs we imaged the cells after applying increasing concentrations of WISP-1 (10, 20, 30, and 40 μg/mL). The cells were stained with Dil-LDL, and images were taken after 3 days of peptide application. From the images, it was apparent that the density of the cells that take up the dye is significantly reduced at increasing peptide concentrations. Also the round morphology of the cells at higher peptide concentrations suggests the decreased viability of the cells in the presence of WISP-1 (Fig. 2B).

FIGURE 2. In vitro screening of WISP-1 using human retinal ECs. (A) The activity of the peptide in the proliferation and migration assays. Insets, right: the positive and negative controls used to scale the results from the proliferation and migration experiments. (B) The morphology of the ECs was altered after increasing concentrations of WISP-1 were applied for 3 days. After 3 days of peptide application, the cells were stained with Dil-Ac-LDL and then imaged with phase microscopy. At increasing peptide concentrations, the cells were less dense and acquired a round morphology typical of apoptosing cells. Scale bar, 100 μm.

FIGURE 3. Inhibition of NV by WISP-1 (mouse sequence). Left: a representative mouse from each group, showing NV (arrows) and pellet (asterisk) at postoperative day 6. bFGF (positive control) showed abundant neovascularization (arrow), and the empty pellet (negative control) showed none. Right: the mean neovascularization area ± SE on day 6 after pellet implantation. WISP-1 reduced neovascularization by 96.6% compared with the untreated control (n = 10).
areas at the sites of Bruch’s membrane injury indicating the laser injury resulted in well-circumscribed hyperfluorescent parable to that induced by empty pellets. WISP-1 also significantly suppressed experimentally induced NV to levels comparable to that induced by empty pellets. WISP-1 inhibited laser-induced CNV (Figs. 4, 5). In control mice, laser injury resulted in well-circumscribed hyperfluorescent areas at the sites of Bruch’s membrane injury indicating the formation of CNV. These lesions predominantly represent outgrowths of the choroidal vasculature at sites of laser injury. In contrast, laser injury in mice treated with intravitreous WISP-1 (mouse sequence) showed a 43% inhibitory effect on the area of CNV ($P < 0.05$). Mice treated with a WISP-1 human sequence exhibited an approximately 28% reduction in the area of CNV ($P = 0.059$). Treatment with a scrambled amino acid sequence peptide resulted in a slight increase in the area of neovascularization (the human sequence was used; data not shown).

**DISCUSSION**

Several prevalent ocular diseases have been identified that are characterized by pathologic angiogenesis, including age-related macular degeneration, diabetic retinopathy, and retinopathy of prematurity. Discovery of angiogenesis inhibitors contributes to the development of therapeutic treatments of these diseases. Moreover, the identification of endogenous inhibitors also contributes to fundamental understanding of the angiogenic balance in the ocular tissue.

The TSP-1 protein has been identified as a potent angiogenic inhibitor that plays an important role in maintaining vascular homeostasis in the eye. TSP-1 is synthesized and secreted by cultured RPE cells and is upregulated by vitamin A. Immunohistochemistry-stained sections of human eyes indicate that in the macula region TSP-1 is found in Bruch’s membrane, the choriocapillaris, and the larger choroidal vessels. The aqueous humor and vitreous from normal human eyes also contain elevated levels of the TSP-1 protein. Of interest, the TSP-1 levels in the aqueous humor and vitreous are downregulated in some diabetic animal models. This association suggests that ocular vascular abnormalities in the diabetic animals result from decreased levels of the thrombospondin protein. TSP-1 is also implicated in the pathogenesis of CNV in the setting of macular degeneration. There is evidence suggesting a relationship between spatial localization of TSP-1 and disease in eyes with macular degeneration. Notably, expression of antiangiogenic TSP-1 is significantly decreased in Bruch’s membrane and choriocapillaris in AMD eyes and TSP-1 levels correlate inversely with severity of disease.

In the present study, we tested the activity of an endogenous peptide derived from a protein containing a thrombospondin repeat, in two in vivo ocular angiogenesis models, the corneal micropocket model and the laser-induced CNV model. The peptide was previously identified by using a bioinformatics computational approach, and the predictions were validated in human umbilical vein EC proliferation and migration assays. WISP-1 strongly inhibited bFGF-induced neovascularization in the corneal micropocket model. Qualitatively
similar results were reported when histidine-rich glycoprotein (HRGP) was used, which masked the antiangiogenic epitope of TSP-1.

Furthermore, addition of WISP-1 to bFGF-loaded pellets completely suppressed experimentally induced NV. Topically administered bevacizumab (Avastin; Genentech, San Francisco, CA), reduces NV by 40%.27 The effect of addition of bevacizumab to bFGF pellets in this model is not known. WISP-1 was also potent in suppressing angiogenesis in the laser-induced CNV model. In this murine model, the mouse WISP-1 peptide sequence reduced the mean area of CNV by 43%. By way of comparison, use of a mouse monoclonal antibody against VEGF in this model results in reduction of neovascularization by 69%.28 Intravitreous administration of the human instead of mouse WISP-1 peptide sequence in the mouse model of CNV resulted in a smaller (28%) reduction in CNV area that did not reach statistical significance in this study (P = 0.059). This interspecies differential effect potentially indicates an incomplete cross-reactivity between human and mouse forms of the ligand in vivo. This result also demonstrates the critical importance of peptide sequence to bioactivity.

Identification of novel endogenous antiangiogenic peptides that can play a role in both physiological and pathologic conditions, such as the peptide described herein, have the potential to increase our understanding of neovascularization in health and disease. They can also serve as a basis for novel, potent, and potentially synergistic therapeutics for various ocular neovascularization-related diseases.

References