A short peptide derived from pigment epithelial-derived factor exhibits an angioinhibitory effect

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Abstract

Background: Pigment epithelial-derived factor (PEDF), a 50 kDa secreted glycoprotein, exhibits distinct effects on a range of cell types. PEDF has been shown to inhibit vascular endothelial growth factor (VEGF)-mediated angiogenesis and widely accepted as a promising agent for treatment eye diseases related to neovascularization. A pool of short peptide fragments derived from PEDF reportedly manifests angioinhibitory activity. This study aims to determine the minimal PEDF fragment which can exert the anti-VEGF effect.

Methods: A series of shorter synthetic peptides, derived from the 34-mer (PEDF amino acid positions Asp44-Asn77), were synthesized. An MTT assay was used to evaluate the ability of the 34-mer-derived peptides to inhibit VEGF-induced proliferation of multiple myeloma RPMI8226 cells. Cell apoptosis was monitored by annexin V-FITC staining. Western blot analysis was used to detect phosphorylated kinases, including c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK), and the expression of apoptosis-associated proteins, including p53, bax and caspase-3. VEGF-mediated angiogenesis of human umbilical vein endothelial cells (HUVECs), rat aortic ring and mouse cornea were used to detect the angioinhibitory activity of the PEDF-derived peptides.

Results: The MTT assay showed that the anti-VEGF effect of a 7-mer (Asp64-Ser70) was 1.5-fold greater than the 34-mer. In addition, massive apoptosis (37%) was induced by 7-mer treatment. The 7-mer induced JNK phosphorylation in RPMI8226 cells. Cell apoptosis and apoptosis-associated proteins induced by the 7-mer were blocked by pharmacological inhibition of JNK, but not p38 MAPK. Moreover, the 7-mer prevented VEGF-mediated angiogenesis of endothelial cells (ECs), including tube formation, aortic EC spreading and corneal neovascularization in mice.

Conclusions: This is the first study to show that the PEDF 7-mer peptide manifests anti-VEGF activity, further establishing its potential as an anti-angiogenic agent.

Keywords: Pigment epithelial-derived factor, Vascular endothelial growth factor, Peptide, Corneal neovascularization, Apoptosis

Background

Pigment epithelial-derived factor (PEDF) was initially identified in the culture supernatant of human fetal retinal pigment epithelial cells, with the ability to promote neuronal differentiation of retinoblastoma Y79 cells [1]. Further studies showed PEDF to be a potent angiogenic inhibitor and the pathological neovascularization in retina is associated with low levels of PEDF in the ocular vitreous humor [2, 3]. Presently, full-length PEDF is considered to be less suitable as an anti-angiogenic agent than PEDF-derived active fragments because of several issues, including antigenicity, stability, production cost and, perhaps, its multifunctional nature [4, 5].
this regard, the 34-mer and a shorter peptide within the 34-mer (an 18-mer, Asn60-Asn77) were investigated for the anti-angiogenic activity of PEDF, including induction of endothelial cell apoptosis, blockage of VEGF-induced neovessel formation, analyzed by a mouse corneal pocket assay, and reduction of vascular density in tumor tissues [4, 6]. In addition, the 34-mer and 18-mer have been shown to have no neurotrophic activity, suggesting that they posses only PEDF anti-angiogenic function [4, 6, 7].

VEGF is an important proangiogenic factor responsible for the development and formation of blood vessels. VEGF binds to the tyrosine kinase receptors of vascular endothelial cells to induce cell proliferation, migration and sprouting angiogenesis [8]. In addition, VEGF is important for maintenance of the integrity of pericyte-endothelial interactions in the retinal microvasculature [9]. VEGF is strongly involved in several angiogenesis-driven eye diseases. For example, anti-VEGF drugs, including pegaptanib (Macugen) and ranibizumab (Lucentis), have been approved for treating retinal and choroidal vascular diseases, such as diabetic macular edema and exudative age-related macular degeneration (AMD). Notably, it has been found that many exudative AMD patients have a persistent hemorrhage and progressive fibrosis at the retinal fovea, following long-term treatment with current anti-VEGF drugs, rendering significant unmet medical needs [10]. VEGF also has a particular angiogenesis-independent effect. For example, VEGF can promote proliferation of multiple myeloma cells in culture [11, 12].

Development of PEDF-based angioinhibitory therapies may be helpful for resolving the problem of persistent side effects associated with current anti-VEGF drugs. In this study, a series of shorter peptides covering the 34-mer were synthesized. We investigated their anti-VEGF activity using an established model of multiple myeloma RPMI8226 cell growth induced by VEGF. The 7-mer had effective anti-VEGF activity. Moreover, we demonstrated for the first time that the 7-mer was able to abolish VEGF-induced angiogenesis in vitro and in a mouse model of corneal neovascularization.

Methods
Materials
VEGF and TACS annexin V-FITC kits were purchased from R&D Systems (Minneapolis, MN, USA). Matrigel was purchased from BD Biosciences (New Bedford, MA, USA). HUVECs were purchased from Cascade Biologies, Inc. (Portland, OR, USA). Hydron (529265), sucrose octasulfate–aluminum complex (S0652) were from Sigma-Aldrich (St. Louis, MO, USA). Culture medium, low-serum growth supplement (LSGS) and fetal bovine serum (FBS) were from Gibco-BRL.
a Cannon camera on a Zeiss inverted microscope with magnification × 40.

**Western blot analysis**

Cell lysis and SDS–PAGE were as described previously [13]. Antibodies used in this study were for active p38 MAPK and active JNK (Promega, Madison, WI), p38 MAPK/SAPK2, JNK (Upstate Biotechnology, Lake Placid, NY), p53 (Chemicon, Temecula, CA), bax, p21, cleaved caspase-3 and β-actin (Abcam Ltd., Cambridge, UK). Proteins of interest were detected using the appropriate IgG-horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology) and enhanced chemiluminescence reagent (Amersham, Arlington Heights, IL). X-ray films were scanned on a model GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA) and analyzed with Labworks 4.0 software. For quantification, blots from at least three independent experiments were used.

**Annexin-V/propidium iodide (PI) double-staining assay**

Cell apoptosis was examined using a TACS annexin V-FITC kit according to the manufacturer’s instructions for in situ staining. The cell numbers were monitored by counterstaining with Hoechst 33342. Afterward, apoptosis was examined and photographed by a Zeiss epifluorescence microscope (×100, 10 fields/sample). Images were recorded on Zeiss software.

**Animal studies**

All animals were housed in an animal room under temperature control (24–25°C) and a 12:12 light-dark cycle. Standard laboratory chow and tap water were available ad libitum. Experimental procedures were approved by the Mackay Memorial Hospital Review Board (project code: MMH-A-S-103-10; New Taipei City, Taiwan). Animals in ophthalmic research were performed in compliance with national animal welfare regulations (Council of Agriculture, Taiwan, ROC), the ARVO Statement and the ARRIVE Guidelines. C57BL/6 mice with an age of...
6 weeks were anaesthetized by an intraperitoneal injection of a mixture of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg). All animals were euthanized by CO₂ inhalation prior to dissection.

Rat aortic ring assay
Thoracic aortas were removed from euthanized 10-week-old male Sprague Dawley rats and gently stripped of periaortic fibroadipose tissue. Aortas were sectioned into ~1 mm length rings, being manipulated in Medium 200. Matrigel (120 μl) mixed with 24 μl LSGS, 72 μl medium, 200 ng/ml VEGF and 100 μM 7-mer was used to embed the aortic ring and then polymerized in 12-well plates (37 °C for 30 min). Subsequently, 1 ml culture medium supplemented with 100 units/ml penicillin, and 100 ng/ml streptomycin was added. The cultures were propagated at 37 °C in a humidified incubator for up to 5 days. Sprouts were recorded using an inverted microscope platform (Leica DMI6000B) with bright-field optics. Each ring was scored by three independent observers on a scale 0–3, depending on the degree of vessel sprouting observed (0 = no sprouting, 3 = profuse sprouting).

Mouse corneal micropocket assay
The assay was performed according to procedures described previously [14]. Briefly, corneal micropockets were created in 6-week-old C57BL/6 mice with the needle of 1-ml injector. A micropellet of sucrose aluminum sulfate and hydron polymer containing 200 ng/ml VEGF, with or without 2.5 μg 7-mer peptide, was delivered into each corneal pocket. Eyes were photographed by a slit-lamp biomicroscope on day 7 after micropellet implantation. Ingrowth of the blood vessels into the avascular cornea, towards the pellet, was scored as a positive response. The results are reported as the fraction of positive corneas of the total implanted.

Statistics
The data were generated from at least three independent experiments. All numerical values are expressed as the mean ± SD. Comparisons of two groups were made using the Mann–Whitney test. P < 0.05 was considered significant.

Results
Identification of an active core region in the PEDF 34-mer, in terms of its anti-VEGF activity
We used an MTT assay to measure the growth and survival of multiple myeloma RPMI8226 cells induced by VEGF. PEDF blocked the VEGF effect (93 ± 3.8% versus 120 ± 3.9%; untreated cells set as 100%; Fig. 1b), similar to previous reports [11, 15] and the 34-mer displayed a stronger inhibitory effect than PEDF (84 ± 4.0%). The 14-mer, missing the 20 amino acids at the N-terminal end of the 34-mer, had a weaker anti-VEGF effect (97 ± 3.6%). We deleted the amino acid residues at the C-terminal end of the 14-mer to generate a 10-mer, 7-mer, 6-mer, and 5-mer and these yielded values of 116 ± 2.64%, 58 ± 4.1%, 99 ± 6.2% and 113 ± 4.2%, respectively, in the MTT assay. The 10-mer and 5-mer failed to suppress the VEGF effect, but the 6-mer had the same anti-VEGF activity as the 14-mer. Unexpectedly, the 7-mer exhibited more potent anti-VEGF activity than the 34-mer (~1.5-fold).

The D-form 7-mer peptide exerts higher anti-VEGF effect in vitro than its L-form isomer
Natural L-amino acids are unstable in vivo because they are susceptible to protease degradation. To improve the resistance of the 7-mer to proteolysis, D-amino acid substitution was used for peptide modification. Using D-amino acids for peptide modification have been suggested potentially to promote the peptide therapeutic activity [17]. To investigate whether 7-mer modified by D-amino acids can enhance its anti-VEGF activity in the RPMI8226 cells, an MTT assay revealed that replacement of the D64 and V68 residues of the 7-mer by D-amino acids (~105–98%) at the tested concentration (25 μM; Fig. 1d) Next, the VEGF-treated
RPMI8226 cells were exposed to various doses of the 7-mer peptide (1, 5, 10, and 25 μM). An MTT assay showed that treatment with the 7-mer, 7-mer DD and 7-mer DV decreased cell viability in a dose-dependent manner (Fig. 1e). We noted that the 7-mer DD and 7-mer DV had a greater anti-VEGF effect than the 7-mer at the lowest concentration (1 μM; 75 ± 2.8% and 78 ± 1.7% versus 101 ± 1.2%). In contrast, cell viability decreased significantly with the 34-mer treatment at 10 and 25 μM, but not at 1 and 5 μM. Taken together, these results indicate that the D-form 7-mer peptide has a greater anti-VEGF activity than its L-form.

The 34-mer and 7-mer induce RPMI8226 cell apoptosis

We next investigated whether the 34-mer and 7-mer can induce apoptosis of VEGF-treated RPMI8226 cells by annexin V-FITC/PI double staining. This technique has been used to detect viable cells (annexin V⁻/PI⁻), cells undergoing early apoptosis (annexin V⁺/PI⁻), late apoptotic cells (annexin V⁺/PI⁺) and necrotic cells (annexin V⁻/PI⁺; Fig. 2a). The simultaneous detection of early and late apoptosis (annexin V⁺ cells) revealed that withdrawal of serum from the culture medium caused apoptosis of RPMI8226 cells, whereas VEGF could prevent this serum deprivation-induced apoptosis (annexin V⁺ cells: 14.0 ± 1.7% versus 6.5 ± 1.1%; Fig. 2b). Importantly, there was a significant increase of cell apoptosis after treatment with the 34-mer and 7-mer for 24 h (25.1 ± 2.2% and 38.6 ± 2.1%, respectively). In addition, Necrosis (annexin V⁻/PI⁺) was rare after cells received these treatments. In addition, we noted that part of apoptotic RPMI8226 cells displayed morphologic changes, including shrinkage and membrane blebbing, resulting in different sizes of green dots. A previous report also indicates that PPARγ overexpression in RPMI8226 cells induces cells undergoing apoptosis with morphologic hallmarks, such as shrinkage and membrane blebbing [18]. Taken together, these findings provide evidence that the 34-mer and 7-mer are able to suppress the VEGF-mediated cell protective effect by inducing cell apoptosis.

The 7-mer triggers JNK-dependent apoptotic signaling in VEGF-treated RPMI8226 cells

To investigate whether the 7-mer affects JNK and p38 MAPK signaling in RPMI8226 cells, western blot analysis was performed using antibodies against the active phosphorylated forms of JNK and p38 MAPK. An increase in p-p46 JNK was detected 20 min after 7-mer treatment, reaching a peak value at 2 h, and the phosphorylation was sustained for up to 10 h. Treatment with the
7-mer had no significant effect on p38 MAPK phosphorylation (Fig. 3a and b). In this regard, pretreatment of RPMI82226 cells with a JNK inhibitor (10 μM SP600125), but not in cells pretreated with a p38 MAPK inhibitor (10 μM SB203580), altered 7-mer-induced RPMI8226 cell apoptosis from 38.6 ± 2.1% to 11.6 ± 1.5%, assayed by annexin V-FITC staining (Fig. 3c).

Overexpression and activation of p53 play a critical role in PEDF-induced endothelial cell apoptosis [19]. As shown in Fig. 4a, western blot analysis showed that treatment with the 7-mer for 8 h caused an increase of the protein levels of p53 and its transcriptional target genes, including Bax (an apoptosis inducer) and p21Waf1 (a cyclin-dependent kinase inhibitor) compared to solvent control (3.6 ± 0.39, 4.5 ± 0.40 and 5.5 ± 0.43 -fold; Fig. 4b). Moreover, an increase in p53 expression corresponded to the elevation of cleaved/active caspase-3, a frequently activated death protease in the apoptosis pathway, was observed following 7-mer treatment for 24 h. We determined whether JNK activation induced by the 7-mer can regulate the level of p53 protein. Western blotting revealed that increases in the levels of induced p53, bax, p21, and cleaved caspase-3 were significantly inhibited in cells pretreated with SP600125, but not in cells pretreated with SB203580, suggesting p-JNK engagement in RPMI8226 cell apoptosis or cell cycle arrest induced by the 7-mer. Taken together, our results reveal that apoptosis and the p53/Bax/p21Waf1 pathway induced by the 7-mer were mediated by JNK activation.

The 7-mer exhibits the angioinhibitory activity in HUVECs

ECs are converted to capillary tube-like structures in response to VEGF. We observed the VEGF effect on HUVECs using a matrigel-based tube formation assay. As depicted in Fig. 5a, exposure of HUVECs to VEGF for 6 h formed a massive tube-like network as described previously [20] (Fig. 5a). Treatment with the 7-mer and 34-mer resulted in a marked decrease of the VEGF-stimulated tube-like network. Next, we investigated whether the 7-mer can induce apoptosis of HUVECs. The annexin V-FITC staining showed that treatment of HUVECs with the 34-mer and 7-mer for 16 h increased the percentage of apoptotic cells from 6.3 ± 1.6% (VEGF-treated) to 20.1 ± 4.4% and 43.0 ± 5.5%, respectively (Fig. 5b). A control peptide (the 5-mer) had no such effect. These results indicate that the 7-mer has anti-angiogenic activity through arresting VEGF-mediated capillary morphogenesis and inducing endothelial cell apoptosis.

The 7-mer inhibits VEGF-induced microvessel sprouting ex vivo and corneal neovascularization

The effect of the 7-mer on VEGF-induced microvessel sprouting was evaluated using a rat aortic ring assay.
As shown in Fig. 6a, outgrowth of sprouts from the aortic rings was observed after VEGF treatment for 5 days, whereas this outgrowth was suppressed when the VEGF-treated aortic rings were exposed to the 7-mer (P = 0.001). In addition, aortic rings exposed to multiple angiogenic factors (LSGS and VEGF) produced profuse sprouts around the ring that were blocked by the 7-mer. Furthermore, in a mouse corneal model of neovascularization, VEGF-induced neovessels in the corneas of C57BL/6 mice were prevented by the 7-mer (Fig. 6b). The results imply that the 7-mer is active against angiogenesis in vivo.

Discussion
Identification of angioninhibitory peptides derived from PEDF may be a promising strategy for improving the treatment of angiogenesis-related diseases. Previous studies have identified the 34-mer (D44-N77) and 18-mer (N60-N77) that preserve the anti-angiogenic and antitumor activities of human PEDF, but the 23-mer (A55-N77) and 14-mer (D64-N77) are devoid of PEDF activity [4]. For example, the 14-mer is unable to induce EC apoptosis in vitro. Here, we found for the first time that the 7-mer peptide (D64-S70), representing a minimal region within PEDF, reproduced the anti-VEGF effect, comparable to the 34-mer. Also, the longer peptides (34-mer, 14-mer and 10-mer) with N- and C- terminal sequences near the 7-mer region, displayed a rather poor inhibitory effect on VEGF, compared to the 7-mer peptide.

It has been reported that the vitreous humors of exudative AMD patients contain lower levels of PEDF [3]. This reduction of PEDF levels leads to patients’ vitreous insufficiency in suppressing EC migration, compared to vitreous humor from age-matched control donors [3]. These findings suggest that loss of PEDF may create a permissive environment for the pathogenesis of exudative AMD. Overexpression of PEDF in mice by intravitreous injection of an adenoviral vector encoding PEDF (AdPEDF) and PEDF transgenic mice have been reported to block laser-induced choroidal neovascularization (CNV) and VEGF-induced retinal neovascularization [21, 22]. This suggests that elevated levels of intraocular PEDF are beneficial in suppressing ocular neovascularization. However, treatment of ocular neovascular diseases with the wild-type (WT)-PEDF may generate additional unexpected effects through its multifunctional activities. These functional effects could come from the interaction of different PEDF domains with an individual receptor on the surface of different types of cells. PEDF is known that contributes to antiangiogenesis, antitumor, antiinflammation, neuroprotection and tissue repair by stem cells [23]. In addition, For example, in comparison with WT-PEDF, it has been found that mutation of putative phosphorylation sites in PEDF (phosphomimetic mutants) leads to more profound anti-angiogenic activity and may avoid the induction of tumor growth [24]. In addition,
recombinant PEDF may create complex antigenicity [4]. In this regard, it has been proposed that synthetic anti-angiogenic peptides derived from PEDF are more practical as agents for treating neovascularization in tumors and the eye [4, 5].

In the present study, the 7-mer induced massive apoptosis of RPMI8226 cells and it is plausible to propose apoptosis as the major anti-VEGF mechanism of the 7-mer in culture, rather than cell cycle arrest alone. In addition, we found that the 7-mer was able to activate JNK signaling in RPMI8226 cells. Pharmacological inhibition of JNK with SP600125 suppressed the 7-mer induced RPMI8226 cell apoptosis. Meanwhile, SP600125 also suppressed the effect of the 7-mer on induction of p53, Bax, p21^waf1 and active caspase-3 protein expression. Several studies have shown that the PEDF receptor (37/67 kDa laminin receptor; LR), JNK, PPARγ and p53 and effectors, including FasL and Bax, are associated with PEDF-induced EC apoptosis [16, 17, 25, 26]. In particular, it has been demonstrated that a neutralizing antibody against LR can block the effect of PEDF on VEGF-induced multiple myeloma
cell proliferation [15]. These results suggest that PEDF-mediated apoptotic signaling acts on ECs and myeloma cells in a similar manner.

Conclusions
In summary, identification of PEDF-derived angioinhibitory peptides may facilitate the development of more effective therapeutic strategies for angiogenesis-related eye diseases and potentially with less side effects. VEGF-activated myeloma cells and ECs are responsive to the 7-mer, providing evidence for the 7-mer as an anti-VEGF agent. Further preclinical animal studies of ocular neovascular diseases are required to evaluate the therapeutic efficacy of the 7-mer.

Abbreviations
PEDF: Pigment epithelial-derived factor; VEGF: Vascular endothelial growth factor; JNK: c-Jun N-terminal kinase; MAPK: Mitogen-activated protein kinase; HUVECs: Human umbilical vein endothelial cells; ECs: Endothelial cells; AMD: Age-related macular degeneration; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DMSO: Dimethyl sulfoxide; bFGF: Basic fibroblast growth factor; EGF: Epidermal growth factor; SDS–PAGE: Sodium dodecyl sulfate polyacrylamide gelelectrophoresis; PI: Propidium iodide; CNV: Choroidal neovascularization.

Supplementary Information
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Additional file 1.

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Authors’ contributions
YPT and TCH participated in the design of the experiment, analysed the data, and manuscript writing. TCH, SIY and TWC participated in creating the animal model, specimen collection, experimental manipulation, data collection and analysis. SIY and SLC participated in analyzed the results and modified the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
The Mackay Memorial Hospital Review Board approved the animal study. All animal procedures were conducted in accordance with the ARVO Statement and the ARRIVE Guidelines for the use of animals in ophthalmic research. The animals were purchased from Lasco Co., Ltd. (Taiwan). Experimental procedures were approved by the Mackay Memorial Hospital Review Board (New Taipei City, Taiwan) and were performed in compliance with national animal welfare regulations (Council of Agriculture, R.O.C).
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