PEDF Reduces the Severity of Herpetic Simplex Keratitis in Mice

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Submitted: February 1, 2018
Accepted: May 8, 2018

PURPOSE. The purpose of this study was to explore the effects of pigment epithelium derived factor (PEDF) and PEDF-derived peptides Mer44 and Mer34 on the severity of herpetic simplex keratitis (HSK) in mice.

METHODS. Adult C57BL/6 mice were infected ocularly with the herpes simplex virus type 1 (HSV-1, McKrae strain) and injected subconjunctivally with PEDF, Mer44, or Mer34. Corneal nerve degeneration, neovascularization, sensitivity, neutrophils, macrophages and CD4+ T-cell infiltration, virus contents, and expressions of VEGF, PEDF, and proinflammatory factors were evaluated during acute period. The direct inhibitory effect of PEDF on HSV-1 replication was further evaluated in cultured monkey Vero cells.

RESULTS. Following HSV-1 infection, corneal PEDF expression decreased at 3 and 7 days postinfection (dpi) but increased at 15 dpi, and returned to the similar level of normal mice at 45 dpi, which was accompanied with the progress of corneal nerve degeneration and neovascularization. Exogenous PEDF application attenuated corneal nerve degeneration and neovascularization and improved the impaired corneal sensitivity. Moreover, PEDF attenuated the neutrophils, but not macrophage or CD4+ T-cell infiltration, with the reduced expressions of IL-1β, IL-6, TNF-α, and VEGF. In addition, PEDF inhibited the replication of HSV-1 both in vitro and in mice. Mer44 attenuated corneal nerve degeneration more significantly than Mer34, whereas Mer34 inhibited corneal neovascularization.

CONCLUSIONS. PEDF and its derived peptides reduce the severity of herpetic simplex keratitis in mice, representing the potential therapeutic approach to control HSK lesions.

Keywords: PEDF, HSV-1, nerve degeneration, neovascularization, virus replication

Herpetic simplex keratitis (HSK), a leading infectious cause of corneal blindness worldwide, results from the infection with the herpes simplex virus type 1 (HSV-1). Primary infection causes the corneal neovascularization, chronic inflammatory response, and impaired sensation, following which latency can be established principally in the trigeminal ganglion. Once stimulated by stress, UV light exposure, or immunosuppression, virus reacts from the latent state, which leads to the recurrent infection in cornea. Current therapies of HSK depend on the use of nucleoside analogue antiviral drugs to suppress virus replication, such as ganciclovir or acyclovir. However, the uncontrolled inflammation and neovascularization may lead to corneal opacity and scar formation. Therefore, the research of HSK pathogenesis is needed to provide the potential cue for the therapeutic development, especially alleviating corneal symptoms.

Pigment epithelium derived factor (PEDF) is a member of the serine protease inhibitor superfamily, which possesses antiangiogenic, antitumorigenic, and neurotrophic properties. Previous reports have confirmed the effect of PEDF on the suppression of neovascularization via inhibiting the proliferation and migration of vascular endothelial cells in choroid, retina, and cornea. In capsaicin-induced neotrophic keratouveitis, PEDF can promote the recovery of tear secretion and attenuate the corneal ulceration, neovascularization and leukocyte infiltration. Recently, PEDF has been reported to accelerate corneal epithelial wound healing, stimulate nerve regeneration, and inhibit pathologic neovascularization. Moreover, PEDF-derived peptide Mer44 induces nerve regeneration and promotes the survival and differentiation of spinal motor neurons. Meanwhile, Mer34 inhibits neovascularization by upregulating PPAR-γ in the ischemic heart. In addition, a recent report reveals that PEDF plus docosahexaenoic acid (DHA) administration plays important roles in inflammation resolution and nerve regeneration in rabbit cornea infected by HSV-1. However, the direct and independent therapeutic effects of PEDF on HSK still remain incompletely understood.

Considering to the multiple functions of PEDF in ocular diseases, our purpose was to explore the direct effects of PEDF, Mer44, or Mer34 on the severity of acute HSV-1 infection in mice. We found that the change of PEDF expression was consistent with the progress of corneal nerve degeneration and neovascularization, whereas subconjunctival injection of PEDF, Mer44, or Mer34 relieved the clinical symptoms of HSK during the acute period.
TABLE 1. Antibodies for Immunofluorescence Staining and Flow Cytometry

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FC, flow cytometry; IF, immunofluorescence.

MATERIALS AND METHODS

Mouse HSK Model and Treatment

Male C57BL/6 mice (6 to 8 weeks old; Institute of Laboratory Animal Sciences, CAMS & PUMC, Beijing, China) were maintained in the animal facility of Shandong Eye Institute. All animal procedures were carried out in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. HSV-1 (Mckrae strain) was propagated and titrated in cultured Vero cells. The mouse HSK model was performed by the scalification of central cornea with a sterile 25-gauge needle and the application of 5 µL virus suspension containing 10^6 PFU HSV-1 using a dissecting microscope, according to previous description. For the treatment, mice were anesthetized and subconjunctivally injected with PEDF (R&D, San Diego, CA, USA; 16 ng/µL, 5 µL/eye), Mer 44 (Val78-Thr121), Mer34 (Asp44-Asn77; GL Biochem, Shanghai, China; 16 ng/µL, 5 µL/eye), or PBS (5 µL/eye) as vehicle control at 0 h after HSV-1 infection, according to our preliminary experiments and previous reports. All the dosages used in this study were evaluated and optimized in our preliminary experiments.

Clinical Evaluation

Mice infected with HSV-1 were evaluated for clinical symptoms by a slit-lamp microscope at 3 and 7 dpi after PEDF, Mer44, or Mer34 treatment. Corneal opacity and neovascularization were graded according to previous descriptions. Briefly, the cornea was divided into four equal quadrants. The length of neovessels was rated from 0 to 4 in each quadrant. The neovascularization score of each cornea was presented as the sum of the four quadrants scores.

Corneal Sensitivity Measurement

Corneal sensitivity was measured using a Cochet-Bonnet esthesiometer (Luneau Ophthalmologie, Chartres Cedex, France) in HSK mice after PEDF, Mer44, or Mer34 treatment according to our previous descriptions. The central area of cornea was touched with beginning with the full length of nylon filament. Nylon filament was shortened by 5 mm each time until a blink response was elicited. The corneal sensitivity threshold was calculated as the mean value of three longest filament lengths with positive response.

HSV-1 Replication and Plaque Assay

HSV-1 was amplified in Vero cells and assayed by plaque assay. Eye swab materials of vehicle- and PEDF-treated HSK mice were collected at 3 dpi. In vitro, Vero cells infected by HSV-1 were treated with recombinant PEDF with 0, 5, 25, and 50 ng/mL, with the 5 µg/mL acyclovir (Millipore, Billerica, MA, USA) as a positive control. Samples were assayed by plaque assay as previously described. The infectious specimens were performed in a 10-fold serial dilution and cultured for 6 days. The number of PFUs in each well was counted after the crystal violet staining. The viral titers were determined by analyzing the average number of plaques for a dilution and the inverse of total dilution factor.

Immunofluorescence Staining

Corneal whole-mount staining was carried out as previous description. In brief, mouse eyes were fixed in Zamboni’s fixative for 1 hour with the cornea dissected. The fixed corneas were treated with PBS with 0.1% Triton X-100, 2% BSA, and 2% goat serum for 2 hours and incubated with Alexa Fluor 488 rabbit anti-mouse βIII-tubulin (Millipore, Billerica, MA, USA) and FITC rat anti-mouse CD31 (BD Biosciences, Palo Alto, CA, USA) overnight at 4°C. The flat mounts were examined under an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan) after washing with incubation buffer for eight times. For the immunostaining of corneal frozen sections, all samples were fixed with 4% paraformaldehyde, permeabilized with 1% Triton X-100, blocked with 5% BSA, and incubated with the primary antibodies (Table 1) according to previous descriptions. Corneal nerve fiber density and neovascularization area were quantified by ImageJ software (National Institutes of Health, Bethesda, MD, USA) based on the whole-mount corneal βIII-tubulin and CD31 staining as previously described.

Flow Cytometry Analysis

Mouse corneas infected with HSV-1 were collected at 3 and 7 dpi after PEDF treatment. Infection samples involved three corneas were digested by liberase (Roche, Basel, Switzerland) and incubated with antibodies (Table 1) according to previous descriptions. Cells were analyzed using a FACSCalibur flow cytometer (BD Bioscience, Palo Alto, CA, USA). Macrophages were identified as CD45^-/CD11b^-/Ly6G^, and CD4^- T cells were identified as CD45^-/CD3e^-/CD4^+. Data analyses were performed using FLOWJO software (FLOWJO, Ashland, OR, USA).

Quantitative RT-PCR

Corneas infected by HSV-1 were collected with or without PEDF treatment. Total RNA was extracted using Nucleospin RNA Kits (Takara, Dalian, China). cDNAs were synthesized...
using the Primescript RT Master Mix (Takara) according to the manufacturer’s protocol. Real-time PCR was carried out using SYBR Premix Ex Taq II and the Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The cycling conditions were 30 seconds at 95°C followed by 40 two-step cycles (5 seconds at 95°C and 34 seconds at 60°C). The quantification data were analyzed with the Sequence Detection System software (Applied Biosystems), using RPL5 and β-actin as an internal control for mouse corneas and Vero cells, respectively (Table 2).

**ELISA**

Total protein was extracted from the three pooled corneas of normal and vehicle- and PEDF-treated HSK mice. The supernatants homogenized in PBS were further analyzed by ELISA according to the manufacturer’s instructions, including PEDF (USCN, Wuhan, China), VEGF (R&D), IL-1β (Ebioscience, San Diego, CA, USA), IL-6 (Ebioscience), and TNF-α (R&D). The concentrations of total proteins were calculated using the BCA kit (Beyotime, Shanghai, China).

**Statistical Analysis**

All the data in this study were representative of at least three independent experiments and presented as mean ± SD. Statistical analysis was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA), Student’s t-test, and 1-way ANOVA. Differences were considered statistically significant at \( P < 0.05 \).

**RESULTS**

**Changes of Corneal Nerve and Neovascularization During HSK**

To evaluate the effect of HSV-1 infection on corneal nerve and neovascularization, normal and HSV-1–infected corneas were examined by immunostaining with βIII-tubulin and CD31.
antibodies. As shown in Figure 1A, HSV-1-infected corneas showed apparent edema and neovascularization at 7 dpi (acute period), and the corneal transparency was partially recovered at 45 dpi (latent period). According to the results of whole-mount immunostaining, normal cornea had the dense subbasal and branched stromal nerve fibers, whereas the corneal nerve fibers of HSK mice underwent rapid degeneration from 3 dpi, completely disappeared at 7 dpi, and partially regenerated from 15 to 45 dpi (Fig. 1B). Corneal neovessels grew from the limbal area at 3 dpi, reached to the maximum level at 7 dpi, and gradually regressed and branched from 15 to 45 dpi (Fig. 1B). Quantitative analysis of three independent experiments confirmed that the maximum degradation of nerve fibers and ingrowth of neovessels occurred at 7 dpi and partially recovered at 45 dpi (Figs. 1C, 1D; n = 3 per time point).

Change of Corneal PEDF Expression During HSK

To explore the expression change of corneal PEDF during HSK, normal and HSV-1-treated corneas at 3, 7, 15, and 45 dpi were collected for immunostaining, quantitative RT-PCR, and ELISA experiments. According to the results of immunostaining, PEDF was mainly expressed in the corneal epithelium and endothelium of normal mice, were reduced in HSK corneas at 3 and 7 dpi, and then recovered from 15 dpi (Fig. 2A). The results of quantitative RT-PCR and ELISA further confirmed that the mRNA and protein levels of PEDF in HSK corneas were reduced at 3 and 7 dpi, whereas the PEDF expression was significantly elevated at 15 dpi and returned to the similar level as in normal mice at 45 dpi (Figs. 2B, 2C; n = 3 per time point). The change of PEDF expression was consistent with the progress of corneal nerve degeneration and neovascularization during HSK, which indicated that PEDF might be involved in the regulation of HSK pathogenesis.

PEDF Treatment Reduces HSK Severity

To assess the direct effect of PEDF on the alleviation of HSK severity, PEDF (80 ng/eye) was injected subconjunctivally in the HSK mice at 0 and 3 dpi after HSV-1 infection, with the same volume of PBS as vehicle control. The excised corneas were further stained with βIII-tubulin and CD31 to quantify the nerve distribution and neovascularization. As shown in Figure 3A, subconjunctival PEDF injection relieved the corneal edema and neovascularization at 3 and 7 dpi. According to the results of whole-mount immunostaining, PEDF treatment attenuated corneal nerve degeneration and inhibited neovascularization at both 3 and 7 dpi compared with vehicle (Fig. 3B), which was further confirmed by the quantified analysis of corneal nerve and neovessels (Figs. 3C, 3E; n = 3 per time point). Corneal sensitivity was also improved in the mice with PEDF treatment at both 3 and 7 dpi (Fig. 3D; n = 9 per time point). Overall, PEDF treatment decreased the corneal scores from 9.8 ± 1.5 at 3 dpi and 12.8 ± 2.0 at 7 dpi of vehicle-treated mice to 6.5 ± 2.0 at 3 dpi and 8.5 ± 1.5 at 7 dpi (Fig. 3F; n = 12 per time point).

PEDF Treatment Attenuates Corneal Immune Responses

To explore the effect of PEDF on immune responses and proinflammatory factor expression, the corneas of normal and vehicle- and PEDF-treated HSK mice were excised at 7 dpi for immunostaining, flow cytometry, and ELISA analysis. The influence of PEDF on corneal immune response was unapparent at 3 dpi compared with 7 dpi. As shown in Figure 4A, subconjunctival PEDF injection reduced the infiltration of Ly6G+ neutrophils, but not F4/80+ macrophages in cornea and anterior chamber, compared with vehicle treatment at 7 dpi. Immunostaining and flow cytometry results further confirmed that PEDF application mainly decreased the number of infiltrated neutrophils but not macrophages or CD4+ T cells (Figs. 4B–4D; n = 9 per time point). Moreover, ELISA analysis showed that the corneal contents of IL-1β, IL-6, and TNF-α were significantly downregulated after PEDF treatment at 7 dpi compared with vehicle treatment (Figs. 4E–4G; n = 3 per time point).

PEDF Treatment Suppresses Corneal VEGF Expression

To investigate the suppression mechanism of PEDF treatment on corneal neovascularization, corneas of vehicle- and PEDF-treated HSK mice were harvested at 3 and 7 dpi. According to the results of quantitative RT-PCR and ELISA, subconjunctival injection of PEDF reduced the mRNA and protein expression of VEGF in HSV-1–infected corneas both at 3 and 7 dpi (Figs. 5A, 5B; n = 3 per time point).

PEDF Treatment Reduces HSV-1 Replication In Vivo and In Vitro

To explore whether PEDF possesses the direct inhibitory capacity on HSV-1 replication, mouse HSK corneas and cultured Vero cells with vehicle or PEDF treatment were collected for the analysis of immunostaining, quantitative RT-PCR, and plaque assays. According to the results of corneal immunostaining, HSV-1 antigen showed a diffused distribution in PBS-treated HSK corneas, whereas the staining of HSV-1 in PEDF-treated HSK corneas were weaker both at 3 and 7 dpi (Fig. 6A). Moreover, the virus plaque numbers were significantly reduced in the eye swab samples of PEDF-treated HSK mice at 3 dpi compared with vehicle control, accompanied with the reduced expression levels of ICP0 and UL41 in PEDF-
treated HSK corneas both at 3 and 7 dpi (Figs. 6B–6D; n = 3 per time point). In cultured Vero cells, PEDF with 25 ng/mL concentration showed the maximal inhibitory activity on the replication of HSV-1 by quantitative RT-PCR and plaque assays, although significant less than the inhibition of 5 μg/mL acyclovir supplement (Figs. 6E–6G; n = 3 per time point).

**Mer44 and Mer34 Treatment Differently Reduce HSK Severity**

Based on similar activity of PEDF-derived peptides to PEDF, Mer44 or Mer34 (80 ng/eye) was injected subconjunctivally in the HSK mice at 0 and 3 dpi after HSV-1 infection, with the same volume of PBS as vehicle control. The excised corneas were stained with βIII-tubulin and CD31 antibodies to quantify the nerve distribution and neovascularization. The influences of Mer44 or Mer34 on HSK severity were unapparent at 3 dpi compared with 7 dpi. These results showed Mer44 or Mer34 treatment also alleviated the HSK clinical symptoms examined by the slit microscopy at 7 dpi (Fig. 7A). More specially, Mer44 attenuated HSV-1–induced nerve degeneration more significantly than Mer34 (Figs. 7B, 7C; n = 3 per time point), whereas Mer34 played a more efficient role on the inhibition of HSV-1–induced neovascularization than Mer44 (Figs. 7B, 7E; n = 3 per time point).
time point). Consistently, corneal sensitivity was significantly improved with Mer44 treatment at 7 dpi but not Mer34 treatment (Fig. 7D; \(n = 12\) per time point). Overall, the corneal scores were decreased in Mer44-treated mice (12.2 ± 2.6) or Mer34-treated mice (12.8 ± 2.8) compared with vehicle-treated mice (15.7 ± 2.0) at 7 dpi (Fig. 7F; \(n = 12\) per time point).

**DISCUSSION**

PEDF is a natural neurotrophin, vascular protective factor, and anti-inflammatory factor in ophthalmic disease. In corneal HSV-1 infection, a recent report showed that PEDF plus DHA treatment promoted inflammation resolution and induced nerve regeneration in rabbit model. In the present study, we first examined the change of corneal PEDF expression and explored the direct roles of exogenous PEDF treatment in mouse HSK model, focusing on the prevention of corneal neovascularization, nerve degradation, sensation impairment, acute inflammation, and viral replication. The results
demonstrated that the change of corneal PEDF expression was parallel with the progress of corneal nerve degeneration and neovascularization after HSV-1 infection, which was attenuated with the exogenous PEDF treatment. However, the PEDF-derived peptides Mer44 and Mer34 differently influenced the corneal nerve degeneration and neovascularization. PEDF treatment decreased the infiltration of neutrophils but not macrophages or CD4+ T cells in the cornea of HSK mice. More importantly, we found that for the first time PEDF directly inhibited the HSV-1 replication in mouse cornea and in cultured cell model.

Considering the neurotrophic and antiangiogenic properties, PEDF combined with or without the omega-3 fatty acid DHA has been described to stimulate nerve regeneration and inhibit neovascularization in various animal models of corneal diseases.38–41 In the mouse HSK model, we found that PEDF treatment more efficiently attenuated the corneal nerve degeneration and neovascularization than Mer44 and Mer34 peptides. More specially, Mer44 decreased the corneal nerve degeneration but assumed no significant inhibition on neovascularization. Mer34 inhibited corneal neovascularization more significantly but showed less significant inhibition on corneal nerve degeneration than Mer44. The results directly confirmed the differences of Mer44 and Mer34 on their neurotrophic and antiangiogenic properties and suggest the more suitable choice of the intact PEDF for the alleviation of HSK severity. Mechanistically, PEDF treatment decreased the expression levels of corneal VEGF and IL-6 in HSK mice, which may contribute to the attenuation of corneal neovascularization and nerve regression.42

Although the ocular viral content showed no direct correlation with HSK severity, the aggravated infection by the replicating virus may promote the ongoing inflammation. When checking the ocular viral content of HSK mice at 3 dpi, we found weaker HSV-1 antigen staining in corneal section and reduced virus plaque numbers in the eye swab samples of HSK mice with PEDF treatment, accompanied with the decreased expression of HSV-1 protein ICP0 and UL41. To further confirm whether PEDF has the direct antiviral properties, we added different concentrations of PEDF in the cultured Vero cells infected with HSV-1. The results showed that PEDF actually inhibited the viral replication with the maximal inhibitory activity at 25 ng/mL concentration, but the inhibitory activity was significant less than the acyclovir. Although the mechanism needs further study, PEDF may directly influence the
replication of HSV-1 during the immediate early infection in mice.

The severity of HSK in mice is primarily caused by the infiltration of neutrophils and CD4+ T cells. Compared with the biphasic regulation of PEDF plus DHA treatment on the infiltration of neutrophils in a rabbit HSK model, we found that PEDF treatment significantly reduced the numbers and percentage of infiltrated neutrophils, but not the macrophages or CD4+ T cells in the mouse HSK model, which was consistent with the previous description in the capsaicin-induced neurotrophic keratouveitis rat model. The accompanied reduction of proinflammatory cytokines IL-1β, IL-6, and TNF-α may contribute to the reduction of neutrophil infiltration by the PEDF treatment in HSK mice. The difference of inflammation regulation may be related with the absence/presence of DHA, different animal models (rabbit versus mice), or different treatments (topical application versus subconjunctival injection). Considering to the importance of neutrophil in the HSK severity and irresponsive infiltration of subconjunctival injection. Considering to the importance of neutrophil in the HSK severity and irresponsive infiltration of macrophage and CD4+ T cells, the inhibition of neutrophil infiltration may represent the major effect of PEDF treatment in the reduction of HSK severity in mice.

In summary, PEDF treatment reduces the corneal severity of HSK in mice, including corneal neovascularization, nerve degeneration, and neutrophil infiltration. The combination of PEDF and current antiviral drug treatment may represent a potential therapeutic approach to control HSK lesions.

Acknowledgments
Supported by Shandong Provincial Nature Science Fund for Distinguished Young Scholars Grant JQ201518 and National Natural Science Foundation of China Grant 81570820. QZ is partially supported by Taishan Scholar Program Grants 20150215 and 20161059 and the Innovation Project of Shandong Academy of Medical Sciences.

Disclosure: X. Tian, None; T. Wang, None; S. Zhang, None; Q. Wang, None; X. Hu, None; C. Ge, None; L. Xie, None; Q. Zhou, None

References