Effective Treatment of Ocular HSK with a Human Apolipoprotein E Mimetic Peptide in a Mouse Eye Model

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PURPOSE. To evaluate efficacy of the small apolipoprotein E (apoE) mimic dimer peptide (apoEdp) in the treatment of herpetic stromal keratitis in a mouse ocular model and determine its therapeutic effects against HSV-1-induced inflammatory cytokines.

METHODS. Female C57Bl/6 mice were cornally infected with HSV-1 strain KOS-GFP; topical treatment was initiated 24 hours after infection and continued for 10 consecutive days. Treatment groups were 1% apoEdp, 1% triflurorothymidine (TFT), and phosphate-buffered saline (PBS). The incidence and severity of stromal keratitis were monitored by slit lamp examination in a masked fashion. Infectious HSV-1 titer in eye swabs and alteration in inflammatory cytokines were determined in the early postinfection period by real-time RT-PCR.

RESULTS. One percent apoEdp treatment, which significantly reduced the incidence and severity of HSK, was equal to the effect of 1% TFT; both groups had significantly lower incidence and severity than the placebo treatment group. In the in vivo mouse ocular model results of apoEdp therapy correlated with accelerated clearance of virus from eye swabs. Topical 1% apoEdp treatment in mice significantly downregulated gene expression of mouse proinflammatory cytokines.

CONCLUSIONS. These results suggest that topical treatment with apoE peptide has efficacy against HSK through anti-HSV-1 and anti-inflammatory activities. (Invest Ophthalmol Vis Sci. 2008; 49:4263–4268) DOI:10.1167/iovs.08-2077

Herpetic stromal keratitis (HSK) is a chronic, immunoinflammatory disease of the cornea caused by HSV-1. The current antiviral and anti-inflammatory therapies fail in approximately 33% of patients with necrotizing HSK,1,2 resulting in the necessity of corneal transplantation. Currently, topical triflurorothymidine (TFT, 1% wt/vol) is the most widely used and effective topical antiviral for treating HSV-1 corneal infections. However, TFT has been reported to be cytotoxic with long-term use.3 The therapeutic agents that are currently available consist of a small number of nucleoside analogues. A nucleoside analogue, acyclovir (ACV) as ointment has demonstrated efficacy against superficial herpes keratitis but has not been approved in the United States because of side effects associated with ointment therapy.4 Therefore, the development of new drugs with reduced toxicity and different mechanisms of action is needed.

Human apolipoprotein E (APOE [gene]; apoE [protein]) is a 34-kDa glycoprotein. In humans, APOE is polymorphic with three major allelic types: e2, e3, and e4. The most common allelic type is APOE e3 (77%–78%) followed by APOE e4 (14%–15%), and APOE e2 (7%–8%), which is the least frequent genotype.5,6 The apoE e4 genotype has also been strongly implicated as a risk factor in Alzheimer’s disease7–11 and in viral infections such as HSV-112 and HIV.13 APOE e4 carriers have an increased risk of cold sores caused by HSV-1 and genital herpes caused by HSV-2.14 The molecular basis by which apoE exerts its isofrom-specific effects on the outcome of infection is unknown. The potential mechanisms responsible for the influence of apoE allotype on HSV-1 infection are (1) the alteration of viral transport through hematogenous routes,15 (2) inflammatory modulation during infection,16 (3) repair of tissues after infection,17 and (4) a direct antiviral effect in vitro.18

Evidence suggests that apoE e4 gene carriers are more susceptible to ocular herpes and other viral infections and inflammatory diseases than carriers of the apoE e3 isoform.19,20 While ApoE e4 is not a disease-causing gene, it has been shown to be a susceptibility gene for these inflammation-associated diseases. The hypothesis that the apoE e3 allele could be anti-inflammatory, whereas apoE e4 is proinflammatory, has resulted in a search for therapeutic agents that mimic the apoE role and act to reduce or block this increased inflammation, thereby reducing or blocking the course of the diseases. It has been well established that carriers of the apo e4 gene are more susceptible to viral infections and inflammation-related diseases than their apo e3-carrying counterparts.12,13,14,19 Therefore, we reasoned that apoE e3 gene products could manifest potent anti-inflammatory and antiviral action against HSK.

Both HSV-1 and apoE can bind to the extracellular matrix (often heparan sulfate proteoglycans, HSPGs)21–23 and then to specific cellular receptors.24 Both apoE and HSV have been hypothesized to compete for the same binding sites or other receptors.21 One receptor binding region of apoE, which has been critical in its biological activity, is located at residues 142-147 (the heparin-binding domain) and mediates the attachment of apoE to cellular HSPGs (Fig. 1).24–26 A tandem-repeat dimer peptide, apoEdp, derived from the apoE residues 141-149, has been reported to exhibit antiviral activity against HSV-1, HSV-2, and HIV in vitro.18 The tandem-repeat design

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could reflect an increased adaptation of an α-helical structure and better stability.27,28 This peptide (apoEdp) has not been tested in vivo against any infectious disease.

**MATERIALS AND METHODS**

**Mice**

All experimental procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the LSUHSC Institutional Animal Care and Use Committee. Female C57Bl/6 mice (Charles River Laboratories Inc., Wilmington, MA), 5 to 6 weeks of age, were used.

**Cells and Virus**

CV-1 cells (American Type Culture Collection, Manassas, VA) were propagated in Eagle’s minimum essential medium (EMEM) containing 0.15% HCO3 supplemented with 10% fetal bovine serum (FBS), penicillin G (100 U/mL), and streptomycin (100 mg/mL). HSV-1 strain KOS-GFP29 was used and titered in CV-1 cells.

**Ocular Infection**

Before HSV-1 inoculation, mice were anesthetized by intraperitoneal administration of xylazine (6.6 mg/kg of body weight) and ketamine (100 mg/kg). The eyes were scarified in a 2 × 2 cross-hatch pattern and inoculated with 5 × 105 plaque forming units (PFU) of virus (in a volume of 4 μL) in each eye.

**Quantification of Viral Titer from Eye Swabs**

Plaque assays to quantify infectious HSV-1 in tears were performed using CV-1 cells as indicator cells. Briefly, eye swabs were collected using sterile filter paper strips and placed in 1 mL of cold EMEM containing 10% FBS. Swab samples were serially diluted and plated on CV-1 cells for 1 hour at 37°C. Finally, the medium was aspirated, EMEM was added to each well, and viral plaques in each well were quantified after 2 days.

**Treatment Groups**

The apoE mimetic peptide was synthesized (Genemed, Arlington, TX) with a purity of greater than 95%. The 18 amino acid (Ac-LRKLKRKLL-LRKLKRKLL-amide) tandem-repeat dimer peptide (apoEdp) was derived from the apoE receptor binding region between residues 141 and 149 (Fig. 1). In vivo treatments started 24 hours after viral infection (24 hours PI) and continued for 10 consecutive days. Before starting topical treatment, all mice eyes were checked for corneal fluorescence (Fig. 2A) as a measure of HSV-1 replication. A subjective score of 0 to 4 per eye was recorded: 0 indicates no fluorescence; 1, approximately 1 quadrant of the corneal circle; 2 half circle; 3, three quadrants; and 4264 Bhattacharjee et al. IOVS, October 2008, Vol. 49, No. 10

**FIGURE 1.** Human apoE consists of 299 amino acid residues. The heparin binding domain is located between residues 142 and 147 in the receptor binding region of apoE, which mediates its attachment to cellular HSPGs.24-26 ApoE dimer tandem repeat peptide (apoEdp) is reported to contain anti-HSV-1 activity in vitro.18

**FIGURE 2.** Representative photographs of (A) HSV-1 KOS replication (GFP expression) in the cornea at 1 day PI. (B) ApoEdp-treated and (C) mock-treated eyes on day 7 PI.
4, full circle. Mice were placed into balanced treatment groups based on the corneal fluorescence score. Topical treatment consisted of a five times daily application of 1% apoEdp solution made in PBS applied as 1 drop of 5 µL per eye. Control groups received either 1% TFT (Falcon Pharmaceuticals, Fort Worth, TX) or placebo treatment with PBS. All treatments were applied in a blind fashion. Before the start of the experiment, we determined the safety and tolerance of the peptide in the eyes of naive mice. We found that up to 5% of this peptide concentration applied five times a day for 10 consecutive days was well tolerated. No ocular toxicity was observed.

**Extraction of RNA, Reverse Transcription, and Real-Time PCR**

Total cellular RNA was isolated from corneas (RNasy Mini Kit; Qiagen, Santa Clara, CA), as specified by the manufacturer. For in vivo mRNA analysis, at 24 hours after treatment, three mice (six corneas) from each group were killed. The corneas were harvested and were placed immediately in preservative (RNA-later; Qiagen). The RNA sample was reverse transcribed into cDNA by using a high-capacity cDNA reverse-transcription kit (Applied Biosystems [ABI], Foster City, CA). Two microliters (100 ng) of each cDNA sample were added to 10 µL of 2X PCR reaction buffer (Applied Biosystems) and 50 ng each of sense and antisense primer in a final reaction volume of 20 µL. Quantitative real-time PCR was performed in the following conditions: 95°C for 3 minutes, followed by 35 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and a melting curve at 60 to 95°C at a heating rate of 0.5°C per second followed by cooling. The primer sequences for target genes are given in Table 1.

Real-time PCR was performed (MylQ; Bio-Rad, Hercules, CA) using SYBR Green I reagent (Bio-Rad) according to the manufacturer’s protocol. Relative mRNA quantitation was calculated using the 2^−ΔΔCt method by normalizing the value of the target gene for each sample to its endogenous housekeeping gene (mouse β-actin) and then normalizing these values to a baseline sample, called the calibrator. The average of naïve mice cornea (scarified but not infected) samples served as the calibrator.

**Slit Lamp Examination and Quantitation of Corneal Opacity and Neovascularization**

After corneal HSV-1 infection, the eyes were monitored with a slit lamp microscope (Eye Cap; Haag-Streit International, Mason, OH) in a masked fashion. HSK severity was quantified by measuring two clinical parameters, corneal opacity, and neovascularization. Corneal opacity was graded on a scale as follows: 0, no opacity; 1, mild cloudiness with visible iris; 2, moderate cloudiness with obscured iris; 3, total corneal cloudiness with invisible iris; and 4, total opacity with no posterior view. Mice with corneal opacity scores of ≥0.5 and limbal sprouting with a neovessel length of ≥0.1 mm were deemed positive for incidence determinations. Aided by an eye piece reticule, we scored neovascularization as the area of neovascularization using the following formula: \( A = (C \times 0.4 \times I \times \pi) / 2 \), where \( C \) is clock hours of neovascularization; 30° arc is 1 (360° circle with a total of 12); \( I \) is the length of the longest neovessel (length of longest neovessel of a mouse eye was 1.6 mm); and, \( A \) is the area of neovascularization.

**Statistics**

(1) The therapeutic efficacy against HSV-1-induced corneal disease (opacity and neovascularization) and HSV-1 titer in the eyes were expressed as the mean ± SEM and considered significant at \( P < 0.05 \), as determined by the Student’s t-test. (2) Results of gene expression analysis by quantitative real-time RT-PCR were reported as the mean ± SEM. The magnitude of differences between groups was evaluated using a two-way analysis of variance (ANOVA). The independent factors were the drug treatments and the genes being assayed; the interaction of these two terms was included in the model. Separation of treatment by gene interaction levels was performed with t-tests on the least-square means derived from the ANOVA model. Alpha levels were adjusted for the number of comparisons by using a simulation-based method. The significance criterion for the adjusted α levels was the standard \( P < 0.05 \), which was the overall experiment-wise α level for the entire set of comparisons conducted.

**RESULTS**

Treatment of the corneal lesions with apoEdp began 24 hours PI and continued for 10 consecutive days, significantly reducing the incidence and severity of HSK. All mice received topical application of the assigned drug 1 day after ocular infection. Treatment continued five times daily through day 10 PI. Clinical evaluation of HSK severity was monitored for progression of corneal opacity and neovascularization (Figs. 2B, 2C). Corneal opacity was evident in PBS-treated mice starting on day 7 with a gradual progression until day 21 PI (Fig. 3A). The peptide-treated mice had significantly less (\( P < 0.05 \)) corneal opacity, mimicking 1% TFT-treated mice (Fig. 3A). In both apoEdp- and TFT-treated groups, significant (\( P < 0.05 \)) inhibition of neovascularization was detected throughout the experiment (Fig. 3B). In the PBS-treated group, neovascularization continued up to day 21 PI. PBS-treated mice eyes had a 60% incidence of corneal opacity and a 90% incidence of corneal neovascularization compared with the 10% incidence (corneal opacity and neovascularization) in both apoEdp- and TFT-treated mice. Our results show that treating HSV-1 corneal lesions beginning 24 hours after infection and continuing for 10 consecutive days with apoEdp effectively blocked the incidence and severity of corneal opacity and neovascularization (Figs. 3A, 3B).

**Table 1. Primer Sequences Used for Real-Time RT-PCR Assay**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IL-1α</td>
<td>Forward</td>
<td>5′-ATG GCC AAA GTC CTC GAC TGG TTT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>3′-GCT TGA GCA AGA CGG CCT GGT C</td>
</tr>
<tr>
<td>Mouse IL-1β</td>
<td>Forward</td>
<td>5′-ATG GCA ACT GTC CTC GAA CTC AGC T</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>3′-CAG GAC AGG TAT AGA TGG TGT CTT TT</td>
</tr>
<tr>
<td>Mouse IL-6</td>
<td>Forward</td>
<td>5′-ATG AAG TTC CTC GTC GCA AGA GAC T</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>3′-CAG TAT GGT TGC CGA GTA CTC CTC</td>
</tr>
<tr>
<td>Mouse TNF-α</td>
<td>Forward</td>
<td>5′-TTG TGT CTA CTC AAC TTC GGG GTG ATG GGT CC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>3′-GTA TGA GAT AAC AAA TGC GAC GGT GTG GG</td>
</tr>
<tr>
<td>Mouse IFN-γ</td>
<td>Forward</td>
<td>5′-TGA ACG CTA CAC ACT GCA TCT GCA TCT TGG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>3′-CGA CTC CTT TTC CTC GCG CTC CTC AGC</td>
</tr>
<tr>
<td>Mouse VEGF</td>
<td>Forward</td>
<td>5′-GCC GGC TTC TGC GCA GTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>3′-CTA CCG CCT TGG CTT GTC AC</td>
</tr>
<tr>
<td>Mouse β-actin</td>
<td>Forward</td>
<td>5′-GTG GCC GCC CGC TTC AGG CAGCAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>3′-CTG TTT GAT GTG AGG CAG GAT TTT</td>
</tr>
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A direct antiviral role of apoEdp was observed through topical application to the mouse cornea. To determine whether diminished severity of HSK correlated to better virus control and a decrease in viral shedding, we examined the titers from tear film in HSV-1–infected eyes. Viral titers of the tear film from apoEdp-and TFT-treated eyes were significantly ($P < 0.05$) lower than in mock-treated eyes examined on days 4 and 6 PI (Fig. 4).

Reduced corneal disease in apoEdp-treated mice correlates with downregulated expression of mouse proinflammatory cytokines. IL-1α, IL-1β, IL-6, TNF-α, IFN-γ, and VEGF produced as a consequence of corneal HSV infection serve as potential molecules to induce the early inflammatory process. Selected groups of treated mice were killed 24 hours after treatment (48 hours PI), and their corneas were analyzed for gene expression at mRNA specific to proinflammatory cytokines known to be upregulated in mouse corneas after HSV-1 infection (IL-1α, IL-1β, IL-6, TNF-α, IFN-γ, and VEGF). Treatment with apoEdp significantly reduced proinflammatory cytokine gene expression at 24 hours after treatment (48 h PI) compared with mock-treated eyes, as revealed by real-time reverse transcription-PCR, similar to the result of TFT treatment (Table 2).

**TABLE 2. Cytokine mRNA Changes in Mock-Treated Eyes and Those Treated with apoEdp and TFT**

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Mock-Treated</th>
<th>1% apoEdp-Treated</th>
<th>1% TFT-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α</td>
<td>78 ± 15.54</td>
<td>1.53 ± 0.99</td>
<td>0.35 ± 0.1</td>
</tr>
<tr>
<td>IL-1β</td>
<td>14.65 ± 1.7</td>
<td>2.88 ± 1.32</td>
<td>0.65 ± 0.26</td>
</tr>
<tr>
<td>IL-6</td>
<td>12.88 ± 5</td>
<td>0.94 ± 0.16</td>
<td>0.18 ± 0.1</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>11 ± 2.77</td>
<td>0.55 ± 0.27</td>
<td>0.22 ± 0.1</td>
</tr>
<tr>
<td>TNF-α</td>
<td>9.03 ± 1.39</td>
<td>0.33 ± 0.05</td>
<td>0.22 ± 0.1</td>
</tr>
<tr>
<td>mVEGF</td>
<td>2.45 ± 0.27</td>
<td>0.26 ± 0.09</td>
<td>0.01 ± 0.0</td>
</tr>
</tbody>
</table>

Data are the mean multiples of change ($x$-fold) in mRNA ± SEM.

**DISCUSSION**

We reported the effect of human apolipoprotein E genotype on the pathogenesis of HSK. In that study, using transgenic mice, we found increased pathogenesis of ocular herpes in human apoE isoforms $e4$ knockin compared with isoform $e3$ knockin. We have also reported that mouse apoE affects survival and HSV-1 DNA load in trigeminal ganglia in the highly neurovirulent HSV-1 strain 17Syn+ through an ocular route of infection. Mouse apoE did not appear to have a role in ocular virus titer and acute corneal disease of epithelial keratitis.

This study is the first to demonstrate the beneficial effect of human host-derived peptidomimetic apoE (apoEdp) in an in vivo mouse model of HSK. ApoEdp blocked the development...
and progression of HSK severity, as demonstrated by slit lamp, virological, and immunologic evaluations.

From our observations, apoEdp has the potential for antiviral effects because viral shedding in the tears of infected mice eyes was significantly reduced at 4 and 6 days PI in the apoEdp-treated eyes compared with mock-treated eyes. This result was similar to mice treated with 1% TFT.

The mechanism by which apoEdp suppresses stromal keratitis is not clear. ApoE is known to compete for one of the receptors, cell-surface heparan sulfate; therefore, if the apoE occupied these sites, the result would be reduced HSV uptake of VEGF and HSV.

FIGURE 5. Possible mechanism of interaction among apoE, HSV, and VEGF. ApoE, HSV, and VEGF compete for binding to the HSPG molecule on the cell surface. ApoEdp treatment may block the binding of VEGF and HSV.

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