Published in final edited form as:

Exp Eye Res. 2008 August ; 87(2): 122–130. doi:10.1016/j.exer.2008.05.007.

Effect of human apolipoprotein E genotype on the pathogenesis of experimental ocular HSV-1

Partha S. Bhattacharjee, PhD^a, Donna M. Neumann, PhD^a, Timothy P. Foster, PhD^b, Sadallah Bouhanik, MS^a, Christian Clement, PhD^a, Dass Vinay, PhD^a, Hilary W. Thompson, PhD^{a,d,e}, and James M. Hill, PhD^{a,b,c,d}

a Department of Ophthalmology, Louisiana State University Health Sciences Center, New Orleans, LA, 70112

b Department of Microbiology, Immunology and Parasitology, Louisiana State University Health Sciences Center, New Orleans, LA, 70112

c Department of Pharmacology, Louisiana State University Health Sciences Center, New Orleans, LA, 70112

d LSU Neuroscience Center, Louisiana State University Health Sciences Center, New Orleans, LA, 70112

e Section of Biostatistics, School of Public Health, Louisiana State University Health Sciences Center, New Orleans, LA, 70112

Abstract

The isoform-specific role of human apolipoprotein E (apoE) has been assessed in a mouse model of ocular herpes. Female, age-matched transgenic mice knocked-in for the human allele apoE3 or apoE4 and their parent C57Bl/6 mice were inoculated corneally with HSV-1 strain KOS. Ocular HSV-1 pathogenesis was monitored through viral replication and clinical progression of stromal opacity and neovascularization by slit-lamp examination. Establishment of latency was determined by analysis of HSV-1 DNA (copy number) by specific real-time PCR in the cornea, trigeminal ganglia (TG), and brain. Representative groups of transgenic mice were sacrificed for the analysis of gene expression of vascular endothelial growth factor (VEGF) by reverse-transcription PCR, and apoE expression by Western blot analysis. At 6 days post-infection (P.I.), the ocular infectious HSV-1 titer was significantly higher ($p < 0.05$) in apoE4 mice compared with apoE3 and C57Bl/6 mice. Corneal neovascularization in apoE4 mice was significantly higher ($p < 0.05$) than apoE3 and C57Bl/6 mice. The onset of corneal opacity in apoE4 mice was accelerated during days 9–11 P.I.; however, no significant difference in severity was seen on P.I. days 15 and beyond. At 28 days P.I., infected mice of all genotypes had no significant differences in copy numbers (range 0–15) of HSV-1 DNA in their corneas, indicating that HSV-1 DNA copy numbers in cornea are independent of apoE isoform regulation. At 28 days P.I., both apoE4 and C57Bl/6 mice had a significantly higher ($p = 0.001$) number of copies of HSV-1 DNA in TG compared with apoE3. ApoE4 mice also had significantly higher ($p = 0.001$) copies of HSV-1 DNA in their TGs compared with C57Bl/6 mice. In brain, both apoE4 and C57Bl/6 mice had significantly higher numbers ($p \leq 0.03$) of copies of HSV-1 DNA compared with apoE3 mice. However, the number of HSV-1 DNA copies in the brain of C57Bl/6 mice was not significantly different than that of apoE4 ($p = 0.1$). Comparative molecular analysis between apoE3 and apoE4 mice on selected days between 7 and 28 P.I., inclusive, revealed that the corneas of apoE4 mice expressed VEGF. None of the corneas in the apoE3 mice expressed VEGF.

Corresponding author: James M. Hill, Ph.D., LSU Eye Center, 2020 Gravier Street, Suite B, New Orleans, LA 70112, USA, Telephone: (504) 568 2274, Fax: (504) 568 2385, E-mail: jhill@lsuhsc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

during this time. Western blot analysis showed proteolytic cleavage of the apoE protein in the corneas of the apoE4 mice. Through days 14 to 28 P.I., a ~29 kDa C-terminal truncated apoE fragment was present in the corneas of apoE4 mice, but not in apoE3 mice. ApoE4 is a risk factor for ocular herpes, in part, through increased replication of virus in the eye, an earlier onset in clinical opacity, significantly higher neovascularization, and increased HSV-1 DNA load in TG and brain than that of apoE3. Increased pathogenesis of ocular herpes in apoE4 mice was also mediated, in part through up-regulated expression of VEGF and apoE proteolysis in the cornea. This is the first report linking a human gene, apoE4, as a risk factor for ocular herpes pathogenesis in a transgenic mouse model.

Keywords

HSV-1; apolipoprotein E; brain; cornea; herpetic stromal keratitis; trigeminal ganglia; transgenic mouse model; vascular endothelial growth factor

INTRODUCTION

Herpetic stromal keratitis (HSK) is a serious ophthalmic problem despite the availability of intensive antiviral and anti-inflammatory therapy. Over 90% of the human population is seropositive for HSV-1 (Xu et al., 2006). Furthermore, a recent study from our laboratory (Kaufman et al., 2005) reports that 98% (49/50) of asymptomatic subjects tested shed HSV-1 DNA in tears or saliva at least once in a 30-day trial. Recurrent herpetic lesions have been reported to affect 10–20% of the population worldwide (Pepose et al., 2006). Thus, one can conclude that the majority of humans harbor latent HSV-1, and most secrete HSV-1 DNA frequently, despite the absence of herpetic lesions. Why? This paradox has been studied in animal models, and the genotype and phenotype of the virus appear to play significant roles in reactivation and recurrent disease. However, there have been few studies on specific human host genetic factors related to herpetic diseases. One hypothesis is that a human host factor, apolipoprotein E (apoE), could be an important risk factor for the development of recurrent herpetic lesions. Humans who carry the apoE4 allele are genetically more predisposed to recurrent herpes labialis (cold sores) than those who do not (Lin et al., 1995; Lin et al., 1998). Therefore, in this study, we investigated the genetic association of the human apoE allele with the onset and severity of HSK using a mouse eye model. To date, there have been no reports that establish a link between a human gene, such as apoE, and a risk factor for ocular HSV-1.

Human apoE is a member of the soluble lipoprotein family and has a chaperone role in the redistribution of lipids among cells throughout the body (Mahley, 1988; Mahley and Rall, 2000). Recent evidence suggests that apoE is more than a lipid transport protein and plays many important roles in biology and medicine (Hill et al., 2007; Mahley, 1988; Mahley and Rall, 2000; Mahley et al., 2006; Weisgraber et al., 1994). In humans, apoE has three major alleles: E2, E3, and E4, with six genotypes. In Caucasian populations the allele frequencies for the apoE3, apoE4, and apoE2 alleles are 70%–80%, 10%–15%, and 5%–10%, respectively (Mahley, 1988; Mahley and Rall, 2000) and can differ in populations of different ethnicity. In the homozygous alleles, apoE4/4 represents approximately 2% of the general population; apoE3/3, 60%; and apoE2/2, less than 0.5%. In the heterozygous alleles, apoE3/4 represents 21%; 2/3, 11%; and 2/4, 5% of the general population (Roses, 1996). The difference between the isoforms is a single amino acid substitution in either the 112 or 158 position, creating apoE2 (Cys₁₁₂Cys₁₅₈), apoE3 (Cys₁₁₂Arg₁₅₈), or apoE4 (Arg₁₁₂Arg₁₅₈). The apoE4 gene has been genetically linked to Alzheimer's disease (AD) and has a gene-dose effect on the risk and age of onset of AD (Corder et al., 1993; Itzhaki and Wozniak, 2006; Romas et al., 2002; Roses, 1996; Saunders et al., 1993; Tang et al., 1998). Patients who are homozygous for apoE4 (apoE4/4) have a ~70% chance of developing AD by the age of 85, while heterozygous individuals (apoE2/4 or apoE3/4) have a ~45% chance of developing AD by the age of 85

(Corder et al., 1993; Farrer et al., 1997). ApoE4 carriers also have an increased risk of developing recurrent herpes labialis and genital herpes (Itzhaki et al., 1997; Lin et al., 1998); however, the mechanism or mechanisms by which apoE4 acts as a risk factor for AD and HSV infections are unknown.

Apolipoprotein E (apoE) is a 299-residue monomeric protein. Structurally, apoE contains two functional domains; residues 1--191 are suggested to form the amino terminal "receptor binding domain" and residues 216--299 to form the carboxyl-terminal "lipid binding domain." Interactions between the N-terminal and C-terminal domains play a critical role in apoE function (Xu et al., 2004). In an AD model, the C-terminal truncated apoE4 is toxic both *in vitro* (Ljungberg et al., 2002) and *in vivo* (Harris et al., 2003), suggesting a role in the generation of neurofibrillary tangles. In addition, apoE4 is more susceptible to C-terminal truncation than apoE3 and has a greater capacity to induce cytoskeletal alterations (Harris et al., 2003).

Previous studies investigating the involvement of host genetics in HSK demonstrate differences in disease phenotype between different mouse strains and identified immune-related host genes involved in determining the outcome of HSV-1-induced keratitis (Deshpande et al., 2000; Tumpey et al., 1998; Zheng et al., 2001). Mouse ocular models using the corneal route of HSV-1 inoculation show that corneal opacity and neovascularization (angiogenesis) are the two cardinal features of mouse HSK. Furthermore, vascular endothelial growth factor (VEGF) is one of a group of angiogenic factors that are upregulated after HSV-1 infection (Zheng et al., 2001). VEGF is produced by both infected corneal epithelial cells and infiltrating inflammatory cells of the stroma in a paracrine nature (Zheng et al., 2001).

To evaluate if there is any apoE allele-specific role in ocular herpes, mice knocked in with human apoE3 or apoE4 and their parent C57Bl/6 mice were infected via corneal inoculation and the apoE isoform-dependent roles in ocular herpes were determined. We reasoned that if the apoE4 isoform was a factor in susceptibility to ocular HSV-1 infections, it could lead to the development of HSK. Therefore, we used these transgenic knock-in mice to investigate whether or not this disorder could be associated with a specific allele of apoE.

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. Age-matched female transgenic C57Bl/6 mice (10–14 weeks old) which were homozygous for human apoE3 (apoE3/3) or human apoE4 (apoE4/4) replacing the murine apoE gene (Taconic, Hudson, NY) and the parent C57Bl/6 mice (Jackson Lab, Bar Harbor, MA) were used. Females were chosen based on greater viral infectivity than males (Burgos et al., 2005). The apoE targeted replacement (TR) mouse model was developed by Piedrahita et al. (1992). These mice express the transgene under the regulation of the mouse apoE promoter. Levels of expression of apoE3 or apoE4 have been characterized and are not significantly different between the apoE3 TR and apoE4 TR mice (Brown et al., 2002; Knouff et al., 1999; Piedrahita et al., 1992; Sullivan et al., 1997; Sullivan et al., 1998).

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, penicillin G (100 U/ml), and streptomycin (100 mg/ml). A recombinant strain

of HSV-1 KOS that expresses GFP, KOS-GFP, was used for ocular infections (Foster et al., 1999).

Ocular inoculation

Before HSV-1 inoculation, mice were anesthetized by intraperitoneal administration of xylazine (6.6 mg/kg of body weight) and ketamine (100 mg/kg). Mouse corneas were scarified with a 2 by 2 cross-hatch pattern and inoculated with 2×10^5 PFU of virus (in a volume of 4 μ l) on each eye. Mock-infection is defined as application of 4 μ l of PBS in scarified eyes of apoE3, apoE4, or C57Bl/6 mice and selected groups of non-scarified eyes of apoE3 and apoE4 mice. Each mock-infection group comprised 3--5 mice. Following ocular infection, selected mice were sacrificed at different days P.I., and their cornea, TG, and brain were removed and processed for real-time PCR, reverse-transcription PCR, and Western blot analyses.

Determination of viral titer

On days 1, 3, and 6 P.I., tear film was collected with a sterile strip of dry filter paper (0.6 cm $\times$ 0.6 cm) placed on the lower cul-de-sac of mouse eyes and then placed in 1 ml of 10% EMEM. Standard plaque assays to quantify ocular infectious HSV-1 in the eye swabs were performed using CV-1 cells as indicator cells.

Slit-lamp examination and quantitation of corneal opacity and neovascularization

The progress and severity of HSK were determined by quantitation of corneal opacity and neovascularization. Following corneal HSV-1 infection, eyes were examined by slit-lamp microscopy (Eye Cap, Haag-Streit International, Mason, OH) in a masked fashion. Corneal opacity was graded as described by others (Keadle et al., 2005), such that 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. Figure 1a illustrates the stages of opacity. Neovascularization was scored as reported by others (Kim et al., 2006; Kim et al., 2004), such that in a given quadrant of the corneal circle, the longest centrifugal growth of a neovessel (1.5 mm) was graded as 4. The neovascularization index score of all four quadrants of the eye ranged from 0--16 (Figure 1b).

Quantitative real time PCR determination of HSV-1 DNA copy numbers in mouse tissues

Cornea, TG, and brain were collected and DNA was isolated (Sambrook et al., 1989). The number of HSV-1 copies per 100 ng of total DNA was determined using real-time PCR (Bhattacharjee et al., 2006; Kaufman et al., 2005; Marquart et al., 2003). Amplification of the HSV-1 polymerase gene was performed in triplicate using a Bio-Rad I-Cycler IQ (Hercules, CA). HSV-1 DNA copy numbers (Whelan et al., 2003) were calculated from a standard curve generated using the HSV-1 polymerase gene cloned as plasmid DNA. The forward and reverse primer sequences for HSV-1 polymerase used were 5'-CATCACCGACCCGGAGAGGGAC-3' and 5'-GGGCCAGGCGCTTGTTGGTGTA-3', respectively. The fluorescent probe sequence was 5'-6FAM-CCGCCGAAGTGAAGCAG-ACACCCGCGC-BHQ-1-3'. The purified plasmid DNA (generously provided by Dr. David C. Bloom, University of Florida, Gainesville, FL) was serially diluted as 10-fold dilutions of 10^6 to 10^0 . Positive and negative controls validated all PCR assays.

Extraction of RNA, reverse transcription, and PCR

Cornea samples of each genotype were harvested and placed immediately in RNA-later (Qiagen, Santa Clara, CA). Total cellular RNA was isolated using the RNeasy Mini Kit as specified by the manufacturer (Qiagen). One microgram of total RNA sample was reverse transcribed into cDNA using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). PCR for mouse β actin was performed on each of the test

samples. Two microliters of cDNA were added to 25 μ l of 2 $\times$ PCR reaction buffer (Applied Biosystems) and 50 ng of sense and antisense primers in a final reaction volume of 50 μ l. PCR was performed under the following conditions: 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 65°C for 1 min, and 72°C for 1 min, with one extension cycle at 72°C for 10 min. The primer sequences for mVEGF (murine VEGF) PCR were: 5'-GCGGGCTGCCTCGCAGTC-3' (sense) and 5' TCACCGCCTTGGCTTGTCAC-3' (antisense). The two mVEGF-specific primer sequences generated a 644 bp for mVEGF164 and a 512 bp for mVEGF120. The housekeeping gene, mouse β -actin, was used as a control. The primers used were β -actin (sense): 5'-AGCAGCCGTGGCCATCTCTTGCTCGAAGTC-3' and β -actin (anti-sense): 5'-AACCGCGAGAAGATGACCCAGATCATGTTT-3' which generated a 353 bp product.

Western blot analysis

Tissue homogenates were prepared with a commercial tissue protein extraction reagent, T-per (Pierce, Rockford, IL) containing a complete protease inhibitor cocktail (complete mini, Roche, Nutley, NJ). Following centrifugation, supernatants were collected. The protein concentrations of the supernatants were quantified with a BCA protein assay kit (Pierce), and all sample concentrations were diluted with the T-per reagent. To quantitate the apoE, 200 μ g/well of denatured protein samples were separated on 4%--20% gradient SDS-PAGE (Jule Inc, Milford, CT), electro-transferred to PVDF membranes (Bio-Rad), and blocked with 5% ECL blocking agent in TBS-T (0.05% Tween-20 in tris-buffered saline) for 2 h at room temperature. Blocking was followed by incubation of the membrane with a 1:5000 dilution of goat anti-human apoE (Calbiochem, San Diego, CA) or a column-purified polyclonal antibody against the C-terminal (272–299) fragment of apoE (generously provided by Dr. Y Huang, Gladstone Institute of Cardiovascular Disease, San Francisco, CA) overnight at 4°C. Following the primary antibody treatment, the membranes were washed three times for 10 min with TBS-T and incubated for 45 min at room temperature with rabbit anti-goat IgG conjugated with HRP (R&D, Minneapolis, MN). The unbound secondary antibody was removed by rinsing the membrane three times for 10 min in TBS-T. Immunodetection was performed using an ECL kit (Amersham, Piscataway, NJ).

Statistics

Results were reported as mean $\pm$ SEM. Significant differences between groups were evaluated using the Student's *t*-test, and a *p* value of < 0.05 was considered significant.

RESULTS

Quantification of infectious HSV-1 in the eye swabs of apoE4 mice results in higher titer than in apoE3 and C57Bl/6 mice

Ocular swabs were taken at 1, 3, and 6 days P.I. and assayed for infectious viral titers using CV-1 cells. In the apoE4 mice, infectious HSV-1 shedding was significantly higher ($p > 0.05$) as detected in ocular swabs at 6 days P.I., compared with apoE3 and C57Bl/6 mice (Figure 2).

The apoE4 mice have increased incidence and severity of corneal neovascularization following ocular HSV-1 infection

Ocular infection in the scarified corneas of transgenic mice induced angiogenic engorgement of limbal vessels, and centripetal extension of neovessels (sprouting) was calculated as described in the Materials and Methods. Starting on P.I. day 17 and through P.I. day 28, corneal neovascularization in apoE4 mice was significantly ($p < 0.05$) higher than in apoE3 mice (Figure 3a). At 28 days P.I., the incidence of neovascularization in apoE4 mice was 70%

compared with 10% in the apoE3 and 30% in the C57Bl/6 mice. No corneal neovascularization was observed (0/10) throughout the time course in any genotype of mock-infected mice.

Mice transgenic for apoE4 have an earlier onset of corneal opacity when compared with apoE3 mice

Corneal haze was detected in the eyes of infected apoE4 mice beginning at 9 days P.I., (Figure 3b). By day 11 P.I., we observed stromal opacity in 70% (7/10) of the apoE4 mice eyes while none (0/10) of the apoE3 mice exhibited any corneal opacity. While these differences in the time of onset between the two mouse genotypes were detected early in the infection, at 15 days P.I. and beyond, there was no significant difference in the incidence and severity of opacity between either group of the transgenic mice and the parent C57Bl/6. Between 15 and 28 days P.I., the incidence of corneal opacity in apoE3 mice was 80% and was 70% in C57Bl/6 mice eyes. No corneal neovascularization was observed (0/10) throughout the time course in any genotype of mock-infected mice.

At 28 days P.I., significantly higher copy numbers of HSV-1 DNA are found in TG and brain of apoE4 mice compared with apoE3 mice

Mice were sacrificed at 28 days P.I. and DNA was extracted from their corneas, TG, and brains. Infected mice of all genotypes had no significant differences in copy numbers of HSV-1 DNA in their corneas, indicating that HSV-1 DNA copy numbers in the cornea are independent of apoE isoform regulation (Figure 4, Table 1). At 28 days P.I., both apoE4 and C57Bl/6 mice had a significantly higher ($p = 0.001$) number of copies of HSV-1 DNA in TG compared with apoE3 mice (Figure 4, Table 1). ApoE4 mice also had a significantly higher ($p = 0.001$) number of copies of HSV-1 DNA in TG compared with C57Bl/6 mice (Figure 4). In brain, both apoE4 and C57Bl/6 mice had a significantly higher ($p \leq 0.03$) number of copies of HSV-1 DNA compared with apoE3 mice (Figure 4, Table 1). However, HSV-1 DNA copies in the brain of C57Bl/6 mice were not significantly different from apoE4 mice ($p = 0.1$). These data suggest that the apoE isoform E4 regulates the HSV-1 DNA load in the TG and brain.

mVEGF is up-regulated in the corneas of human apoE4 mice following ocular HSV-1 infection

To identify one possible factor in HSV-1-induced angiogenesis, we analyzed mVEGF gene expression, using RT-PCR, in the corneas of human apoE3 or human apoE4 knocked-in mice. No mVEGF expression was detected in the corneas of apoE3 mice at any time point. In the corneas of infected apoE4 mice, mVEGF expression was detected as early as 7 days P.I., and persisted at 14, 21, and 28 days P.I. (Figure 5, Table 2). The mVEGF expression was also seen in the corneas of C57Bl/6 mice at time points similar to apoE4 (Table 2). This finding suggests that human apoE4 is a risk factor for HSV-1-induced corneal neovascularization, in part, through expression of VEGF.

Human apoE3 and apoE4 concentrations are similar in cornea, TG, and brain of uninfected mice, and HSV-1 infection in the cornea of mice knocked-in for human apoE4 leads to apoE proteolysis

We assessed apoE expression in uninfected cornea, TG, and brain tissue and found no difference (Figure 6). The corneas of mice knocked-in for human apoE3 or apoE4 were analyzed by Western blot at 0, 7, 14, 21 and 28 days P.I. Using an antibody against full-length human apoE, we found increased apoE fragmentation in the cornea of human apoE4 knocked-in mice compared to none in human apoE3 knocked-in mice. The corneas of apoE3 mice did not display proteolysis (Figure 7A); however, the corneas of apoE4 mice displayed proteolysis at 14, 21, and 28 days P.I. (Figure 7B). The carboxyl-truncated apoE fragment (~29 kDa) was seen only in the corneas of apoE4 mice (Figure 7B). To verify that the ~29 kDa band was the C-terminal truncated apoE fragment of human apoE, we used a separate polyclonal antibody

(generously provided by Dr. Y Huang, Gladstone Institute of Cardiovascular Diseases, San Francisco, CA) against apoE C-terminal amino acids 272--299 and analyzed the pattern of apoE4 corneal homogenates using Western blot analysis (Huang et al., 2001). The antibody against C-terminal amino acids 272--299 failed to detect the ~29 kDa apoE protein fragment in HSV-1 infected apoE4 corneas, but recognized the full-length (34 kDa) apoE (Figure 7C). This result suggests that the ~29 kDa apoE fragment detected in the cornea of the HSV-1 infected human apoE4 knocked-in mice is the truncated form of apoE (minus the C-terminal, 272--299). Thus, HSV-1-induced ocular infection renders the apoE4 structure more susceptible to proteolysis than apoE3.

Discussion

Mouse apoE, like human apoE4, contains two arginines at positions 112 and 158 (Mahley and Rall, 2000). In a hematogenous route of HSV-1 inoculation, mouse apoE was analogous to the human apoE4 (Burgos et al., 2003; Burgos et al., 2006). In our ocular route of HSV-1 inoculation, we found similar results, i.e., both C57Bl/6 mice and apoE4 mice harbor significantly higher copy numbers of HSV-1 DNA compared with apoE3 mice. These results support our previous findings (Bhattacharjee et al., 2006) as well as those of others (Burgos et al., 2003; Burgos et al., 2006) that murine apoE is analogous to human apoE4 in terms of HSV-1 neuroinvasiveness.

There is controversy about the spontaneous reactivation of HSV in the mouse. Gebhardt and Halford (2005) reported that spontaneous reactivation of HSV-1 does not occur in mice. In their study, on days 10, 20, 30, 50, 70, and 100 after infection, both the ocular surface and the TG homogenates of latently infected mice failed to yield infectious virus. Other reports suggest that testing the mouse eye for infectious virus during post inoculation days ranging from 23 to 113 resulted in detection at a very low percentage. The rate of shedding was less than once per 100 days (Shimeld et al., 1990; Tullo et al., 1982; Willey et al., 1984). In most cases, only one eye shed infectious HSV-1 once on many continuous days of swabbing. Feldman et al. (2002) described abundant expression of select viral transcripts and proteins and noted viral DNA synthesis in about 1 neuron per 10 TGs at 37--47 days P.I. This process was termed "spontaneous molecular reactivation"; however, no evidence of infectious virus was reported in that study (Feldman et al., 2002). In a recent report, Margolis et al., 2007, reported that spontaneous reactivation of infectious HSV-1 indeed occurs in a limited percentage (~6%) of mouse TG at 37 days P.I. Thus, in our study, it is unknown whether the source of HSV-1 DNA at 28 days P.I. is due to latency or to a very low spontaneous reactivation of HSV-1.

There is also controversy related to viral contribution to the induction of HSK. Viral replication was considered necessary for the development of HSK since replication-defective HSV-1 mutants did not induce HSK (Babu et al., 1996). Viral proteins traveling by anterograde transport to the corneal nerve termini have been implicated in the immunopathology of HSK (Diefenbach et al., 2002; Holland et al., 1998; Holland et al., 1999). However, one recent report (Polcicova et al., 2005) claimed that such an anterograde spread of virus was not necessary. Using the mutant US9 deleted HSV-1, Polcicova et al. suggested that HSK can develop in the absence of the anterograde spread of HSV-1 to the cornea.

A continuous T-cell infiltration provides evidence of viral protein expression at the site of primary and secondary HSV-1 infection (Biswas and Rouse, 2005; Deshpande et al., 2001; Khanna et al., 2003). In a recurrent HSK model, shedding of small amounts of virus in the tears was not associated with the induction of HSK (Stumpf et al., 2001), suggesting that host genetics could be more important than viral genetic factors.

Our findings of apoE4 as a susceptibility gene for HSK apparently contradict a report by Lin et al. (1999) who found similar apoE4 allele frequencies in HSK and controls. The apoE2 frequency was higher in HSK, although the difference from controls was not statistically significant. The exact reasons are unknown. One possible factor may be due in part to the fact that in our study, mice expressing human apoE were homozygous, whereas, in human patients the subjects were predominantly heterozygous for apoE2, apoE3, or apoE4. In their study, only one out of 46 patients was homozygous for E4/E4 and one for E2/E2.

Although we did not include apoE null ($-/-$) mice in our current study, using a similar mouse ocular model, we have reported that murine apoE has no role in ocular viral shedding and acute corneal pathology (Bhattacharjee et al., 2006). We now report a comparative role of human apoE3 and apoE4 in ocular HSV-1 shedding. Our data suggest that apoE4 regulates ocular HSV-1 pathology by increased virus shedding from the eye. However, the exact mechanism underlying this regulation as it relates to HSK severity is unclear. One possibility is that carriage of the apoE4 allele in the presence of HSV-1 renders cells more vulnerable to lytic infection and possibly apoptosis. Enhanced immediate early gene expression and delayed or reduced levels of LAT expression in concert favor the lytic phase of the viral life cycle, during which replication and cell-cell spread occur. Thus, in the setting of apoE4, HSV-1 could replicate more extensively, and more rapidly gain access to the central nervous system (Miller and Federoff, 2008). Another possibility is that apoE influences isoform-dependent cellular susceptibility to HSV-1. Initial binding of HSV-1 to cells can be mediated by HSV-1 glycoprotein gB and gC (Melancon et al., 2005; Subramanian and Geraghty, 2007), and purified gB from HSV-1 can directly interact with apoE (Subramanian and Geraghty, 2007). Both apoE and HSV-1 can enter cells via a heparan sulphate proteoglycan (HSPG) binding site (Shieh et al., 1992), which results in the accumulation of both within the cell. The human apoE3 has been postulated to play a protective role by hindering the binding of HSV-1 to HSPG, while HSV-1 binding is increased in apoE4 (Itzhaki and Wozniak, 2006). Considering this, the outcome could result in enhanced uptake and increased neuronal transport of HSV-1 in apoE4 mice. Scarification of the corneas of transgenic mice in our current sterile keratitis model (absence of virus in mock-infected mice) did not result in the development of opacity or neovascularization. This finding suggests that mechanical stimulation is not sufficient to induce stromal inflammation. The paradox that apoE4 alone is not sufficient to cause disease (Poirier et al., 1993) is also true for the risk factor association of HSV-1 to AD (Ball, 1982; Jamieson et al., 1991; Poirier et al., 1993) and cold sores (herpes labialis) (Lin et al., 1995; 1998).

Human apoE has been reported to have allele-specific effects on reverse cholesterol transport, platelet aggregation, immune response, and oxidative processes that are likely to affect the overall pathological vascularization and wound healing potential ascribed to modulation of lipoprotein metabolism (Davignon et al., 1999; Mahley, 1988). The E4 allele that is associated with higher low-density lipoprotein cholesterol is considered pro-atherogenic (Davignon et al., 1999; Mahley, 1988; Song et al., 2004). The link between apoE4 and pathological vascularization has not been well elucidated; however, apoE4 is accepted as a risk factor for atherosclerosis (Altenburg et al., 2007; Knouff et al., 1999; Scuteri et al., 2005). In animal model studies, murine apoE is known to influence pathological vascularization (Couffignal et al., 1999; Pola et al., 2003). Evidence suggests that mice lacking apoE (ApoE $-/-$) have impaired angiogenesis, as well as a reduced capacity to upregulate VEGF, a prototypical angiogenic cytokine, in response to ischemic stimuli (Couffignal et al., 1999; Pola et al., 2003). We are aware of no reports confirming whether or not human apoE3 or apoE4 influences corneal neovascularization following ocular HSV-1 infection. We have observed that the human apoE4 isoform has a significantly higher potential for HSV-1-induced corneal neovascularization compared with that of the human apoE3 isoform. Vascularization can be related to wound healing (Folkman, 1995; Folkman, 2007). Human ApoE3 holoprotein or

mimetic peptides derived from the receptor binding region of apoE are critical in suppressing injury-mediated inflammation and promoting repair (Laskowitz et al., 2001; Laskowitz and Vitek, 2007). The presence of the human apoE4 gene, in contrast, is associated with an overactive pro-inflammatory immune phenotype (Brown et al., 2002; Colton et al., 2002; Guo et al., 2004; Lynch et al., 2001; Ophir et al., 2005). Our reverse-transcription PCR analysis has revealed the presence of a mVEGF-specific mRNA in the corneas of infected apoE4 mice but not in apoE3 mice. Furthermore, ocular HSV-1 infection of C57Bl/6 mice showed corneal mVEGF expression similar to apoE4 (unpublished results). These results suggest that human apoE4-induced upregulation of VEGF gene expression could play an important, positive endogenous role in HSV-1-induced corneal neovascularization. Because the transgenic mice used in this study had a targeted replacement of murine apoE with a homozygous insertion of human apoE (apoE3/3 or apoE4/4), we hypothesize that the human apoE4 gene carries specific angiogenic potential for HSK development. The lack of mVEGF gene expression in the corneas of infected apoE3 mice provides one possible explanation for the absence of neovascularization in the apoE3 mice. The human apoE4-induced angiogenesis has also been implicated as a possible risk factor for both diabetic retinopathy and age-related macular degeneration (Dorrell et al., 2007; Folkman, 2007).

The apoE3 and apoE4 mice used in this study were purchased from Taconic Farms (Germantown, NY). In these transgenic mice, the coding sequence for murine apoE has been replaced by the human apoE3 or apoE4 under the regulation of murine regulatory sequences. As a result, the mice express only the human apoE isoforms, with tissue distribution and levels very similar to those of endogenous mouse apoE (Knouff et al., 1999; Sullivan et al., 1997). We also measured apoE expression in uninfected cornea, TG, and brain tissue and found no significant differences. Transgenic mouse model studies of homozygous or heterozygous human apoE isoforms suggest that apoE4 is not only less neuroprotective but also acts as a dominant negative factor, retarding the beneficial effects of apoE3 (Buttini et al., 2002). The detrimental effect of apoE4 was associated with increased intracellular proteolysis (Harris et al., 2003; Huang et al., 2001). Full-length apoE has isoform-specific effects which promote cell survival (Demattos et al., 1998). Our results showed that at 14, 21, and 28 days P.I., apoE is expressed and proteolytically cleaved in the corneas of HSV-1 infected eyes of apoE4 mice; we, however, saw no proteolytic cleavage in apoE3 mice. In particular, a ~29 kDa C-terminal truncated apoE fragment was evident in the corneas of apoE4 mice but not in the apoE3 mice. The precise role of this C-terminal truncated fragment in the development of HSK is unknown; however, a similar ~29 kDa C-terminal truncated apoE4 fragment has been implicated as being toxic *in vitro* in neuronal cell culture as well as *in vivo* in AD patients (Huang et al., 2001). Further studies are needed to understand the relevance of this C-terminal truncated 29 kDa fragment in corneas of apoE4 mice in response to ocular HSV-1 infection.

HSK is a complex immunopathological disease. Research on HSK suffers from a lack of suitable animal models that mimic human HSK pathology. We have developed a mouse ocular model using transgenic mice that strongly links the apoE4 gene to ocular pathogenesis. This is the first report that establishes a link for a human gene as a risk factor for ocular HSV-1.

Acknowledgements

Supported in part by National Eye Institute Grants NEI R01 EY06311 (JMH), F32EY016316 (DMN), and P30EY002377 (LSU Eye Center Core Grant). Also supported in part by a Research to Prevent Blindness Senior Scientist Award (JMH), LSUHSC Translational Research Initiative Grants (PSB and DV), and an unrestricted departmental grant from Research to Prevent Blindness, New York, NY. We thank Maxine Simpson for assistance with tissue culture and swab analyses, Cheryl C. Vega for assistance with immunohistochemistry, and Dr. Manish Kumar for assistance with Western blot analysis. We also thank Dr. David C. Bloom for his generous gift of the HSV-1 polymerase plasmid and Dr. Yadong Huang for his generous provision of antibodies used in this study.

References

- Altenburg M, Johnson L, Wilder J, Maeda N. Apolipoprotein E4 in macrophages enhances atherogenesis in a low density lipoprotein receptor-dependent manner. *J Biol Chem* 2007;282:7817–7824. [PubMed: 17234631]
- Babu JS, Thomas J, Kanangat S, Morrison LA, Knipe DM, Rouse BT. Viral replication is required for induction of ocular immunopathology by herpes simplex virus. *J Virol* 1996;70:101–107. [PubMed: 8523513]
- Ball MJ. Limbic predilection in Alzheimer dementia: is reactivated herpesvirus involved? *Can J Neuro Sci* 1982;9:303–306. [PubMed: 7116237]
- Bhattacharjee PS, Neumann DM, Stark D, Thompson HW, Hill JM. Apolipoprotein E modulates establishment of HSV-1 latency and survival in a mouse ocular model. *Curr Eye Res* 2006;31:703–708. [PubMed: 16966142]
- Biswas PS, Rouse BT. Early events in HSV keratitis—setting the stage for a blinding disease. *Microbes Inf* 2005;7:799–810.
- Brown CM, Wright E, Colton CA, Sullivan PM, Laskowitz DT, Vitek MP. Apolipoprotein E isoform mediated regulation of nitric oxide release. *Free Radic Biol Med* 2002;32:1071–1075. [PubMed: 12031891]
- Burgos JS, Ramirez C, Sastre I, Alfaro JM, Valdivieso F. Herpes simplex virus type 1 Infection via the bloodstream with apolipoprotein E dependence in the gonads is influenced by gender. *J Virol* 2005;79:1605–1612. [PubMed: 15650186]
- Burgos JS, Ramirez C, Sastre I, Bullido MJ, Valdivieso F. ApoE4 is more efficient than E3 in brain access by herpes simplex virus type 1. *NeuroReport* 2003;14:1825–1827. [PubMed: 14534428]
- Burgos JS, Ramirez C, Sastre I, Valdivieso F. Effect of apolipoprotein E on the cerebral load of latent herpes simplex virus type 1 DNA. *J Virol* 2006;80:5383–5387. [PubMed: 16699018]
- Buttini M, Yu GQ, Shockley K, Huang Y, Jones B, Masliah E, Mallory M, Yeo T, Longo FM, Mucke L. Modulation of Alzheimer-like synaptic and cholinergic deficits in transgenic mice by human apolipoprotein E depends on isoform, aging, and overexpression of amyloid beta peptides but not on plaque formation. *J Neurosci* 2002;22:10539–10548. [PubMed: 12486146]
- Colton CA, Brown CM, Cook D, Needham LK, Xu Q, Czapiga M, Saunders AM, Schmechel DE, Rasheed K, Vitek MP. APOE and the regulation of microglial nitric oxide production: a link between genetic risk and oxidative stress. *Neurobiol Aging* 2002;23:777–785. [PubMed: 12392781]
- Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL, Pericak-Vance MA. Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* 1993;261:921–923. [PubMed: 8346443]
- Couffignal T, Silver M, Kearney M, Sullivan A, Wizenbichler BM, Magner M, Annex B, Peters K, Isner J. Impaired collateral vessel development associated with reduced expression of vascular endothelial growth factor in ApoE^{-/-} mice. *Circulation* 1999;99:3188–3198. [PubMed: 10377084]
- Davignon J, Cohn JS, Mabile L, Bernier L. Apolipoprotein E and atherosclerosis: insight from animal and human studies. *Clin Chim Acta* 1999;286:115–143. [PubMed: 10511288]
- Demattos RB, Curtiss LK, Williams DL. A minimally lipidated form of cell-derived apolipoprotein E exhibits isoform-specific stimulation of neurite outgrowth in the absence of exogenous lipids or lipoproteins. *J Biol Chem* 1998;273:4206–4212. [PubMed: 9461617]
- Deshpande S, Zheng M, Lee S, Banerjee K, Gangappa S, Kumaraguru U, Rouse BT. Bystander activation involving T lymphocytes in herpetic stromal keratitis. *J Immunol* 2001;167:2902–2910. [PubMed: 11509638]
- Deshpande SP, Kumaraguru U, Rouse BT. Dual role of B cells in mediating innate and acquired immunity to herpes simplex virus infections. *Cell Immunol* 2000;202:79–87. [PubMed: 10896767]
- Diefenbach RJ, Miranda-Saksena M, Diefenbach E, Holland DJ, Boadle RA, Armati PJ, Cunningham AL. Herpes simplex virus tegument protein US11 interacts with conventional kinesin heavy chain. *J Virol* 2002;76:3282–3291. [PubMed: 11884553]
- Dorrell MI, Aguilar E, Schepke L, Barnett FH, Friedlander M. Combination angiostatic therapy completely inhibits ocular and tumor angiogenesis. *Proc Natl Acad Sci U S A* 2007;104:967–972. [PubMed: 17210921]

- Farrer LA, Cupples LA, Haines JL, Hyman B, Kukull WA, Mayeux R, Myers RH, Pericak-Vance M, Risch N, van Duijn C. Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer's disease. A meta analysis. JAMA 1997;278:1349–1356. [PubMed: 9343467]
- Feldman LT, Ellison AR, Voytek CC, Yang L, Krause P, Margolis TP. Spontaneous molecular reactivation of herpes simplex virus type 1 latency in mice. Proc Natl Acad Sci U S A 2002;99:978–983. [PubMed: 11773630]
- Folkman J. Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 2007;6:273–286. [PubMed: 17396134]
- Folkman J. Seminars in medicine of the Beth Israel Hospital, Boston. Clinical applications of research on angiogenesis. N Engl J Med 1995;333:1757–1763. [PubMed: 7491141]
- Foster TP, Chouljenko VN, Kousoulas KG. Functional characterization of the HveA homolog specified by African green monkey kidney cells with a herpes simplex virus expressing the green fluorescence protein. Virology 1999;258:365–374. [PubMed: 10366573]
- Gebhardt BM, Halford W. Evidence that spontaneous reactivation of herpes virus does not occur in mice. Virology Journal 2005;2:67. [PubMed: 16109179]
- Guo L, LaDu MJ, Van Eldik LJ. A dual role for apolipoprotein e in neuroinflammation: anti- and pro-inflammatory activity. J Mol Neurosci 2004;23:205–212. [PubMed: 15181248]
- Harris FM, Brecht WJ, Xu Q, Tesseur I, Kekoni L, Wyss-Coray T, Fish JD, Masliah E, Hopkins PC, Scarce-Levie K, Weisgraber KH, Mucke L, Mahley RW, Huang Y. Carboxyl-terminal-truncated apolipoprotein E4 causes Alzheimer's disease-like neurodegeneration and behavioral deficits in transgenic mice. Proc Natl Acad Sci U S A 2003;100:10966–10971. [PubMed: 12939405]
- Hill JM, Bhattacharjee PS, Neumann DM. Apolipoprotein E alleles can contribute to the pathogenesis of numerous clinical conditions including HSV-1 corneal disease. Exp Eye Res 2007;84:801–811. [PubMed: 17007837]
- Holland DJ, Cunningham AL, Boadle RA. The axonal transmission of *Herpes simplex* virus to epidermal cells: a novel use of the freeze substitution technique applied to explant cultures retained on cover slips. J Microscopy 1998;192:69–72.
- Holland DJ, Miranda-Saksena M, Boadle RA, Armati P, Cunningham AL. Anterograde transport of herpes simplex virus proteins in axons of peripheral human fetal neurons: an immunoelectron microscopy study. J Virol 1999;73:8503–8511. [PubMed: 10482603]
- Huang Y, Liu XQ, Wyss-Coray T, Brecht WJ, Sanan DA, Mahley RW. Apolipoprotein E fragments present in Alzheimer's disease brains induce neurofibrillary tangle-like intracellular inclusions in neurons. Proc Natl Acad Sci U S A 2001;98:8838–8843. [PubMed: 11447277]
- Itzhaki RF, Lin WR, Shang D, Wilcock GK, Faragher B, Jamieson GA. Herpes simplex virus type 1 in brain and risk of Alzheimer's disease. Lancet 1997;349:241–244. [PubMed: 9014911]
- Itzhaki RF, Wozniak MA. Herpes simplex virus type 1, apolipoprotein E, and cholesterol: a dangerous liaison in Alzheimer's disease and other disorders. Prog Lipid Res 2006;45:73–90. [PubMed: 16406033]
- Jamieson GA, Maitland NJ, Wilcock GK, Craske J, Itzhaki RF. Latent herpes simplex virus type 1 in normal and Alzheimer's disease brains. J Med Virol 1991;33:224–227. [PubMed: 1649907]
- Kaufman HE, Azcuy AM, Varnell ED, Sloop GD, Thompson HW, Hill JM. HSV-1 DNA in tears and saliva of normal adults. Invest Ophthalmol Vis Sci 2005;46:241–247. [PubMed: 15623779]
- Keadle TL, Morris JL, Stuart PM. The effects of aminoguanidine on primary and recurrent ocular herpes simplex virus infection. Nitric Oxide Biology and Chemistry 2005;13:247–253.
- Khanna KM, Bonneau RH, Kinchington PR, Hendricks RL. Herpes simplex virus-specific memory CD8 + T cells are selectively activated and retained in latently infected sensory ganglia. Immunity 2003;18:593–603. [PubMed: 12753737]
- Kim B, Suvas S, Sarangi PP, Lee S, Reisfeld RA, Rouse BT. Vascular endothelial growth factor receptor 2-based DNA immunization delays development of herpetic stromal keratitis by antiangiogenic effects. J Immunol 2006;177:4122–4131. [PubMed: 16951377]
- Kim B, Tang Q, Biswas PS, Xu J, Schiffelers RM, Xie FY, Ansari AM, Scaria PV, Woodle MC, Lu P, Rouse BT. Inhibition of Ocular Angiogenesis by siRNA Targeting Vascular Endothelial Growth

- Factor Pathway Genes: Therapeutic Strategy for Herpetic Stromal Keratitis. *Am J Pathol* 2004;165:2177–2185. [PubMed: 15579459]
- Knouff C, Hinsdale ME, Mezdour H, Altenburg MK, Watanabe M, Quarfordt SH, Sullivan PM, Maeda N. Apo E structure determines VLDL clearance and atherosclerosis risk in mice. *J Clin Invest* 1999;103:1579–1586. [PubMed: 10359567]
- Laskowitz DT, Thekdi AD, Thekdi SD, Han SK, Myers JK, Pizzo SV, Bennett ER. Downregulation of microglial activation by apolipoprotein E and apoE-mimetic peptides. *Exp Neurol* 2001;167:74–85. [PubMed: 11161595]
- Laskowitz DT, Vitek MP. Apolipoprotein E and neurological disease: therapeutic potential and pharmacogenomic interactions. *Pharmacogenomics* 2007;8:959–969. [PubMed: 17716229]
- Lin WR, Graham J, MacGowan SM, Wilcock GK, Itzhaki R. Alzheimer's disease, herpes virus in brain, apolipoprotein E4 and herpes labialis. *Alzheimer's Rep* 1998;1:173–178.
- Lin WR, Shang D, Wilcock GK, Itzhaki RF. Alzheimer's disease, herpes simplex virus type 1, cold sores and apolipoprotein E4. *Biochem Soc Trans* 1995;23:594S. [PubMed: 8654779]
- Lin WR, Tullo AB, Itzhaki RF. Apolipoprotein E and herpes virus diseases: herpes simplex keratitis. *Eur J Hum Genet* 1999;7:401–403. [PubMed: 10234519]
- Ljungberg MC, Dayanandan R, Asuni A, Rupniak TH, Anderton BH, Lovestone S. Truncated apoE forms tangle-like structures in a neuronal cell line. *Neuroreport* 2002;13:867–870. [PubMed: 11997703]
- Lynch JR, Morgan D, Mance J, Matthew WD, Laskowitz DT. Apolipoprotein E modulates glial activation and the endogenous central nervous system inflammatory response. *J Neuroimmunol* 2001;114(1–2):107–113. [PubMed: 11240021]
- Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 1988;240:622–630. [PubMed: 3283935]
- Mahley RW, Rall SC. Apolipoprotein E: far more than a lipid transport protein. *Annu Rev Genomics Hum Genet* 2000;1:507–537. [PubMed: 11701639]
- Mahley RW, Weisgraber KH, Huang Y. Inaugural Article: Apolipoprotein E4: A causative factor and therapeutic target in neuropathology, including Alzheimer's disease. *Proc Natl Acad Sci U S A* 2006;103:5644–5651. [PubMed: 16567625]
- Margolis TP, Elfman FL, Leib D, Pakpour N, Apakupakul K, Imai Y, Voytek C. Spontaneous reactivation of herpes simplex virus type 1 in latently infected murine sensory ganglia. *J Virol* 2007;81:11069–11074. [PubMed: 17686862]
- Marquart ME, Bhattacharjee PS, Zheng X, Kaufman HE, Thompson HW, Varnell ED, Hill JM. Ocular reactivation phenotype of HSV-1 strain F(MP)E, a corticosteroid-sensitive strain. *Curr Eye Res* 2003;26:205–209. [PubMed: 12815548]
- Melancon JM, Luna RE, Foster TP, Kousoulas KG. Herpes simplex virus type 1 gK is required for gB-mediated virus-induced cell fusion, while neither gB and gK nor gB and UL20p function redundantly in virion de-envelopment. *J Virol* 2005;79:299–313. [PubMed: 15596825]
- Miller RM, Federoff HJ. Isoform-specific effects of ApoE on HSV immediate early gene expression and establishment of latency. *Neurobiol Aging* 2008;29:71–77. [PubMed: 17101197]
- Ophir G, Amariglio N, Jacob-Hirsch J, Elkon R, Rechavi G, Michaelson DM. Apolipoprotein E4 enhances brain inflammation by modulation of the NF- κ B signaling cascade. *Neurobiol Dis* 2005;20:709–718. [PubMed: 15979312]
- Pepose JS, Keadle TL, Morrison LA. Ocular herpes simplex: changing epidemiology, emerging disease patterns, and the potential of vaccine prevention and therapy. *Am J Ophthalmol* 2006;141:547. [PubMed: 16490506]
- Piedrahita JA, Zhang SH, Hagaman JR, Oliver PM, Maeda N. Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. *Proc Natl Acad Sci U S A* 1992;89:4471–4475. [PubMed: 1584779]
- Poirier J, Davignon J, Bouthillier D, Kogan S, Bertrand P, Gauthier S. Apolipoprotein E polymorphism and Alzheimer's disease. *Lancet* 1993;342:697–699. [PubMed: 8103819]
- Pola R, Gaetani E, Flex A, Aprahamian T, Proia AS, Bosch-Marce M, Smith RC, Pola P. Peripheral nerve ischemia: apolipoprotein E deficiency results in impaired functional recovery and reduction of associated intraneural angiogenic response. *Exp Neurol* 2003;184:264–273. [PubMed: 14637097]

- Polcicova K, Biswas PS, Banerjee K, Wisner TW, Rouse BT, Johnson DC. Herpes keratitis in the absence of anterograde transport of virus from sensory ganglia to the cornea. *Proc Natl Acad Sci USA* 2005;102:11462–11467. [PubMed: 16055558]
- Romas SN, Santana V, Williamson J, Ciappa A, Lee JH, Rondon HZ, Estevez P, Lantigua R, Medrano M, Torres M, Stern Y, Tycko B, Mayeux R. Familial Alzheimer disease among Caribbean Hispanics: a reexamination of its association with APOE. *Arch Neurol* 2002;59:87–91. [PubMed: 11790235]
- Roses AD. Apolipoprotein E alleles as risk factors in Alzheimer's disease. *Annu Rev Med* 1996;47:387–400. [PubMed: 8712790]
- Sambrook, J.; Fritsch, EF.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory; 1989.
- Saunders AM, Strittmatter WJ, Schmechel D, George-Hyslop PH, Pericak-Vance MA, Joo SH, Rosi BL, Gusella JF, Crapper-MacLachlan DR, Alberts MJ. Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease. *Neurology* 1993;43:1467–1472. [PubMed: 8350998]
- Scuteri A, Najjar SS, Muller D, Andres R, Morrell CH, Zonderman AB, Lakatta EG. ApoE4 allele and the natural history of cardiovascular risk factors. *Am J Physiol Endocrinol Metab* 2005;289:E322–E327.
- Shieh M-T, WuDunn D, Montgomery RI, Esko JD, Spear PG. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J Cell Biol* 1992;116:1273–1281. [PubMed: 1310996]
- Shimeld C, Hill TJ, Blyth WA, Easty DL. Reactivation of latent infection and induction of recurrent herpetic eye disease in mice. *J Gen Virol* 1990;71:397–404. [PubMed: 2155293]
- Song Y, Stampfer MJ, Liu S. Meta-analysis: apolipoprotein E genotypes and risk for coronary heart disease. *Ann Intern Med* 2004;141:137–147. [PubMed: 15262670]
- Stumpf TH, Shimeld C, Easty DL, Hill TJ. Cytokine production in a murine model of recurrent herpetic stromal keratitis. *Invest Ophthalmol Vis Sci* 2001;42:372–378. [PubMed: 11157869]
- Subramanian RP, Geraghty RJ. Herpes simplex virus type 1 mediates fusion through a hemifusion intermediate by sequential activity of glycoproteins D, H, L, and B. *Proc Natl Acad Sci U S A* 2007;104:2903–2908. [PubMed: 17299053]
- Sullivan PM, Mezdour H, Aratani Y, Knouff C, Najib J, Reddick RL, Quarfordt SH, Maeda N. Targeted replacement of the mouse apolipoprotein E gene with the common human APOE3 allele enhances diet-induced hypercholesterolemia and atherosclerosis. *J Biol Chem* 1997;272:17972–17980. [PubMed: 9218423]
- Sullivan PM, Mezdour H, Quarfordt SH, Maeda N. Type III hyperlipoproteinemia and spontaneous atherosclerosis in mice resulting from gene replacement of mouse Apoe with human Apoe*2. *J Clin Invest* 1998;102:130–135. [PubMed: 9649566]
- Tang MX, Stern Y, Marder K, Bell K, Gurland B, Lantigua R, Andrews H, Feng L, Tycko B, Mayeux R. The APOE-epsilon4 allele and the risk of Alzheimer disease among African Americans, whites, and Hispanics. *JAMA* 1998;279:751–755. [PubMed: 9508150]
- Tullo AB, Shimeld C, Blyth WA, Hill TJ, Easty DL. Spread of virus and distribution of latent infection following ocular herpes simplex in the non-immune and immune mouse. *J Gen Virol* 1982;63:95–101. [PubMed: 6294236]
- Tumpey TM, Cheng H, Cook DN, Smithies O, Oakes JE, Lausch RN. Absence of macrophage inflammatory protein-1alpha prevents the development of blinding herpes stromal keratitis. *J Virol* 1998;72:3705–3710. [PubMed: 9557652]
- Weisgraber KH, Roses AD, Strittmatter WJ. The role of apolipoprotein E in the nervous system. *Curr Opin Lipidol* 1994;5:110–116. [PubMed: 8044413]
- Whelan JA, Russell NB, Whelan MA. A method for the absolute quantification of cDNA using real-time PCR. *J Immunol Methods* 2003;278(1–2):261–269. [PubMed: 12957413]
- Wiley DE, Trousdale MD, Nesburn AB. Reactivation of murine latent HSV infection by epinephrine iontophoresis. *Invest Ophthalmol Vis Sci* 1984;25:945–950. [PubMed: 6086546]
- Xu F, Sternberg MR, Kottiri BJ, McQuillan GM, Lee FK, Nahmias AJ, Berman SM, Markowitz LE. Trends in herpes simplex virus type 1 and type 2 seroprevalence in the United States. *JAMA* 2006;296:964–973. [PubMed: 16926356]

- Xu Q, Brecht WJ, Weisgraber KH, Mahley RW, Huang Y. Apolipoprotein E4 domain interaction occurs in living neuronal cells as determined by fluorescence resonance energy transfer. *J Biol Chem* 2004;279:25511–25516. [PubMed: 15054100]
- Zheng M, Deshpande S, Lee S, Ferrara N, Rouse BT. Contribution of vascular endothelial growth factor in the neovascularization process during the pathogenesis of herpetic stromal keratitis. *J Virol* 2001;75:9828–9835. [PubMed: 11559816]

Figure 1. Representative photographs of ocular lesions following HSV-1 infection. (a) Representative photomicrographs of corneal opacity with scores (X 100). (b) Representative photomicrographs of corneal neovascularization with scores (X 100).

Figure 2.

Quantification of infectious HSV-1 in tear fluid. Swabs were plaque-assayed in CV-1 cells and these data are representative of two experiments with a total of 10 eyes at each time point. At 6 days P.I., infectious HSV-1 titer in the eyes of apoE4 mice was significantly higher ($p < 0.05$) than apoE3 and C57Bl/6 mice. * = $p < 0.05$

Figure 3.

Figure 3a. Corneal neovascularization. On days 17, 20, 23, 25, and 28 P.I., the corneas of HSV-1 infected apoE4 mice exhibited a progression of the corneal neovascularization that was significantly ($p < 0.05$) higher than apoE3 mice. The incidence of corneal neovascularization in apoE4 mice was 70% (7/10) compared to 10% in the apoE3 and 30% in C57Bl/6 mice group. * = $p < 0.05$

Figure 3b. Corneal opacity. Following ocular infection with HSV-1 KOS-GFP, corneal opacity was first detected in apoE4 mice eyes at 9 days P.I., and continued to be detected at days 10, 11, 15, 17, 20, 23, 25, and 28 P.I. Corneal opacity was not visible in the apoE3 mice until day 15 P.I., but then increased gradually through 28 days P.I. The results on P.I. days 9, 10, and 11 were significantly different (* = $p < 0.05$) between apoE3 and apoE4 mice. By day 11 P.I., 70% (7/10) of the apoE4 mice eyes were affected, but up to this point, none (0/10) of apoE3 mice had exhibited any corneal opacity. At 15 days P.I. and beyond, there was no significant difference in the incidence and severity of opacity in the transgenic mice and their parent C57Bl/6 mice. The incidence of corneal opacity in apoE3 mice was 80% and was 70% in both C57Bl/6 and apoE4 mice, respectively.

Figure 4.

Real-time PCR analysis of HSV-1 DNA copies. At 28 days P.I., infected mice of all genotypes had no significant differences in copy numbers of HSV-1 DNA in their corneas, indicating that HSV-1 DNA copy numbers in cornea are independent of apoE isoform regulation. At 28 days P.I., both apoE4 and C57Bl/6 mice had significantly higher ($p = 0.001$) numbers of copies of HSV-1 DNA in TG compared with apoE3. ApoE4 mice also had significantly higher ($p = 0.001$) numbers of copies of HSV-1 DNA in their TG compared with C57Bl/6 mice. In brain, both apoE4 and C57Bl/6 mice had significantly higher ($p \leq 0.03$) copy numbers of HSV-1 DNA compared with apoE3 mice. However, the HSV-1 DNA copy number in the brain of C57Bl/6 mice was not significantly different from apoE4 mice ($p = 0.1$). * = $p < 0.05$

Figure 5.

Reverse transcription PCR analysis of mVEGF gene expression in apoE4 mice. Representative gel picture of mVEGF and β -actin. Increased neovascularization of apoE4 mice was associated with upregulated expression of mVEGF-specific mRNA in the cornea of apoE4 mice. The mVEGF expression was detected in cornea of the infected apoE4 mice as early as 7 days P.I. and was detected at 14, 21, and 28 days P.I. The mVEGF expression was also seen in the corneas of C57Bl/6 mice at time points similar to apoE4 mice. No mVEGF expression was detected in the cornea of apoE3 mice at any time point assayed.

Figure 6. Western blot analysis of human apoE expression in the cornea, TG, and brain of apoE3 and apoE4 mice. In uninfected mice, human apoE expression was similar in both apoE3 and apoE4 mice. Immunoblotting was done using a polyclonal antibody against full-length human apoE

Figure 7.

Western blot analysis of corneal protein extracts of humanized mice. Corneal protein extracts of human apoE3 knocked-in or human apoE4 knocked-in mice at set time points were analyzed by Western blot. A 4%--20% gradient gel was used. Immunoblotting was done using either a polyclonal antibody against full-length human apoE or a polyclonal antibody against C-terminal peptide 272--299 of human apoE. No apoE proteolysis was seen in apoE3 mice (Figure 7a). However, at 14 days P.I., proteolytic cleavage products of apoE were seen in the cornea of apoE4 mice. In particular, at 14, 21, and 28 days P.I., a ~29 kDa apoE fragment was detected only in the cornea of apoE4 mice (Figure 7b), but never in apoE3 mice (Figure 7a). Using a polyclonal antibody against C-terminal 272--299 amino acids, the full-length 34 kDa apoE was virtually the only protein detected in human apoE4 knocked-in mice corneas (Figure 7c).

Table 1

HSV Copy Numbers in Cornea, TG, and Brain

DNA copy numbers	Cornea			Trigeminal Ganglia			Brain	
	C57Bl/6	apoE3	apoE4	C57Bl/6	apoE3	apoE4	C57Bl/6	apoE3
number positive/total number of samples	1 0	15 ± 6 5/10	10 ± 5 5/10	528 ± 106 10/10	41 ± 10 10/10	3893 ± 796 10/10	403 ± 172 10/10	12 ± 2 10/10

Table 2

RT-PCR Analysis of Corneal VEGF Expression

Mouse	VEGF expression at days post infection			
	0	7	14	21
•apofE3	—	—	—	—
•apofE4	—	+	+	+
•C57Bl/6	—	+	+	+