Suppression of Transcription Factor Early Growth Response 1 Reduces Herpes Simplex Virus Type I-induced Corneal Disease in Mice

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Running head: Egr-1 aggravates HSV-1-induced corneal disease

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ABSTRACT

Herpes simplex virus type 1 replication initiates angiogenesis and inflammation in the cornea. This can result in herpetic stromal keratitis (HSK), which is a leading cause of infection-induced corneal blindness. Host cellular factors mediate the progression of HSK, but little is known about these cellular factors and their mechanisms of action. We show here that the expression of the cellular transcription factor early growth response 1 (Egr-1) in HSV-1-infected mouse corneas was enhanced. Enhanced Egr-1 expression aggravated HSK by increasing viral replication and subsequent neovascularization with high levels of potent angiogenic factors, fibroblast growth factor 2 and vascular endothelial growth factor. Furthermore, Egr-1 deficiency due to a targeted disruption of the gene or knockdown of Egr-1 expression topically using a DNA-based enzyme significantly reduced HSK by decreasing both viral replication and the angiogenic response. The present study provides the first evidence that endogenous Egr-1 aggravates HSK and that blocking Egr-1 reduces corneal damage.
INTRODUCTION

Herpes simplex virus type 1 (HSV-1) infects about 80% of adults worldwide and can induce devastating diseases (34). For example, HSV-induced stromal keratitis (HSK) can lead to corneal blindness. Indeed, HSK is the leading cause of infection-induced vision impairment in the western world (5, 26). In the United States of America alone, more than 400,000 persons are affected with 20,000 new cases per year (31).

In the early stage of HSK, viral replication in the cornea initiates angiogenesis and inflammation (5, 40, 47). Viral replication is eventually terminated by the host immune response. However, neovascularization and inflammation may intensify, in part because neovessels bring in more inflammatory infiltrates. Currently, a combination of antiherpetic drugs and anti-inflammatory agents are used to treat HSK (16, 22, 29, 32, 33). Unfortunately, some patients fail to respond to this regimen or develop virus with resistance to antiherpetic drugs (3, 13, 14), so additional alternative therapies are needed.

Studies using the murine model show that HSV infection of the cornea induces neovascularization by enhancing the expression of potent angiogenic factors, such as fibroblast growth factor 2 (FGF-2; also known as basic fibroblast growth factor) and vascular endothelial growth factor-A (5, 47). Furthermore, suppression of VEGF-A improves HSK in mice, so inhibition of angiogenesis has been proposed as a potential therapy for HSK patients (20, 37). More studies are needed to elucidate how HSV infection induces FGF-2 and VEGF-A, because blocking of factors inducing FGF-2 and VEGF-A might be a very effective treatment for HSK.

We previously found that HSV-1 infection increased the expression of a cellular transcription factor, early growth response 1 (Egr-1) (10), which is known to
enhance FGF-2 and VEGF-A expression by binding and activating their promoters (19, 23, 38, 43). We also showed that Egr-1 could activate the promoter of HSV-1 gene, infected cell protein 4 (ICP4), which is recently reported to activate the VEGF-A promoter (10, 44). The induced FGF-2 and VEGF-A can in turn augment Egr-1 expression (27, 35). Moreover, Egr-1 mediates the angiogenic response of VEGF-A and FGF-2 by up-regulating VEGF receptor 1 and enzymes needed for angiogenesis (17, 42). Egr-1 has also been shown to intensify inflammation in the ischemic mouse lung by enhancing the expression of chemokines, such as IFN-γ-inducible protein 10 (IP-10) and macrophage inflammatory protein-2 (MIP-2) (45), which are reported to aggravate HSK by recruiting leukocytes (7, 39, 46). Although Egr-1 may potentially aggravate HSK by enhancing viral replication (10), angiogenesis, and inflammatory responses, there are no reports on the induction and role of Egr-1 in HSK. Since Egr-1 could be a potential target to treat HSK, the present study was undertaken. We used mice deficient in Egr-1 due to a targeted disruption of the gene or a topically applied specific inhibitor to block Egr-1 expression to address the role of Egr-1 in HSK.
MATERIALS AND METHODS

Viruses and cells. African green monkey kidney (Vero) cells were maintained and propagated according to the instructions of the American Type Culture Collection. Wild-type HSV-1 strain RE and strain KOS-derived mutant, ΔLTRZ1 (12) were propagated and titrated on Vero cell monolayers.

Opacity and angiogenesis scoring systems. All mouse experiment protocols were approved by the Laboratory Animal Committee of National Cheng Kung University. Five- to six-week-old male and female C57BL/6 mice and C57BL/6-derived mice deficient in Egr-1 due to a targeted disruption of the gene (9, 24, 25, 30) were used for study. Egr-1 deficiency does not result in growth abnormality or defects except that female mice lacking Egr-1 are infertile (9, 24, 25, 30). Mice were anesthetized and infected with 5 × 10⁵ PFU of HSV-1 strain RE or mock-infected with lysates of uninfected Vero cells topically on the right eye following scarification of the cornea with a needle 20 times. Mouse eyes were examined weekly after infection to monitor the corneal opacity and angiogenesis for one month. The corneal opacity was scored as previously described (28). Briefly, HSK was graded on a scale of 0 to 5 as follows: 0, normal cornea; 1, mild corneal haze; 2, moderate corneal opacity or scarring; 3, severe corneal opacity, iris visible; 4, opaque cornea, iris invisible; 5, necrotizing stromal keratitis, often with perforation in corneas. The corneal angiogenesis was scored by measuring the length of neovessels as previously described (11, 47). Briefly, the cornea was divided into 4 quadrants. The length of the longest neovessel in each quadrant was graded between 0 (no neovessel) and 4 (neovessel in the corneal center) in increments of about 0.4 mm (the radius of the cornea is about 1.5 mm). A grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.2 to 1.5 mm.
toward the corneal center. The angiogenesis score for each cornea (ranging 0 to 16) was the sum of four quadrants. In separate experiments, infected mice were sacrificed, and their eyes were harvested to determine viral titers using plaque assay or for staining.

**Western blot analysis.** Mouse corneas were harvested, and total proteins were extracted using ice-cold RIPA buffer containing 0.1% SDS and protease inhibitor cocktail (Sigma-Aldrich). The amounts of total proteins in samples were determined by protein assay dye reagent concentrate (Bio-Rad Laboratories) according to the manufacturer’s instructions. Total protein (150 μg/lane) was separated by 8% SDS-PAGE and transferred to PVDF membranes. Membranes were probed with antibodies against mouse Egr-1 (Santa Cruz Biotechnology, Inc.) or mouse β-actin (clone C4; Millipore) followed by appropriate horseradish peroxidase-conjugated secondary antibodies. The protein bands were developed using an enhanced chemiluminescence kit (Millipore).

**Histological and immunohistochemical staining.** Mouse eyes were harvested for detection of HSV antigens, Egr-1, or FGF-2 or for histological examination. Briefly, samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. Sections (6 μm) were deparaffinized and stained with hematoxylin and eosin. In addition, deparaffinized sections were treated with 1% fetal bovine serum to block non-specific binding before incubating with antibodies against HSV-1 (Dako), mouse Egr-1, or mouse FGF-2 (Santa Cruz Biotechnology) or isotype-matched control antibodies overnight at 4°C. Subsequently, sections were treated with peroxidase-conjugated streptavidin (LSAB+ kit; Dako) followed by 3-amino-9-ethylcarbazole (AEC kit; Zymed Laboratories) before counterstaining with hematoxylin. Mouse eyes were also embedded in OCT medium (Thermo Fisher Scientific), snap-frozen in liquid nitrogen, and sectioned. Sections (6 μm)
were air-dried, fixed in cold acetone, and incubated with antibody against mouse CD31 (clone MEC13.3; BD Biosciences) or isotype-matched control antibody overnight at 4°C. The resulting sections were treated with horseradish peroxidase-labeled donkey anti-rat immunoglobulin G (Jackson ImmunoResearch Laboratories) and 3-amino-9-ethylcarbazole before counterstaining with hematoxylin. Antibodies against mouse Egr-1, FGF-2, CD31, or HSV antigens detected specific signals, whereas isotype-matched control antibodies failed to detect signals.

**Measurement of FGF-2, VEGF-A, IP-10, and MIP-2.** Four corneas were pooled in 500 μl of ice-cold lysis buffer containing phosphate-buffered saline (pH 7.4), 0.5% Tween-20, 1 mM EDTA, and protease inhibitor cocktail (Sigma-Aldrich), grinded, and centrifuged. The resulting supernatants were collected to measure FGF-2 (Signosis), VEGF-A (R&D Systems), IP-10 (R&D Systems), and MIP-2 (R&D Systems) using ELISA kits according to the manufacturer’s instructions.

**DNAzyme treatment.** The scarified corneas of C57BL/6 mice were treated with 5 μl of solution containing 7.5-30 μg of ED5SCR or DNAzyme ED5 (TriLink BioTechnologies) (15), MgCl₂ (25 mM), and 3 μl of FuGENE 6 (Roche Applied Science) 15 min before infection or 1 day post-infection once daily through day 7 post-infection as well as once every two days from days 9-15 post-infection or once daily from 9 to 28 days after infection with 5 × 10⁶ PFU of HSV-1 strain RE. In addition, the scarified corneas of ICR mice were treated with ED5SCR or ED5 15 min before infection and once daily from days 1-3 after infection with 5 × 10⁶ PFU of tkLTRZ1 (12)

**Statistical analyses.** For statistical comparison, Egr-1, FGF-2, VEGF-A, IP-10, and MIP-2 levels were analyzed by the Student’s t test. Corneal opacity scores, angiogenesis scores, and viral loads were analyzed by the Mann-Whitney U test.
HSK incidences were analyzed by the Fisher’s exact test. All $P$ values are for two-tailed significant tests. A $P$ value of $<0.05$ is considered statistically significant.
RESULTS

HSV-1 infection enhances Egr-1 expression in the cornea. We first investigated whether HSV-1 infection could induce Egr-1 expression in the cornea. C57BL/6 mice were inoculated with 5 \times 10^5 PFU of HSV-1 strain RE topically on the scarified cornea, which did not induce death. Infected mice developed HSK progressively with moderate to severe corneal opacity and visible irises (an opacity score of 2-3) by day 7 post-infection (p.i.), opaque corneas and invisible irises (an opacity score of 4) by day 14 p.i., and necrotizing stromal keratitis with perforation in 65-75% of corneas (an opacity score of 5) by days 21-28 p.i. Mouse corneas were harvested after infection to detect Egr-1 protein by Western blotting. The Egr-1 expression in the cornea mock-infected with lysates of uninfected Vero cells was very minimal (Fig. 1A). In the infected cornea, Egr-1 expression was enhanced at day 1 p.i. with a level 1.7-fold greater than that in the mock-infected cornea, and then Egr-1 levels remained constant from days 1-14 p.i. (Fig. 1A and data not shown). Egr-1 was most abundant in the infected cornea at day 28 p.i. at a 2.5-fold greater level than that in the mock-infected cornea (P < 0.05, Student’s t test).

We also performed immunohistochemical staining to detect the corneal region, which expresses viral antigens and Egr-1. Viral antigen was not detected in the mock-infected cornea (Fig. 1B). Of the infected cornea, abundant viral antigen was detected around the lesion in the epithelium, and little viral antigen was detected in the stroma at day 1 p.i. At day 28 p.i., the stroma became very thick with profound edema and inflammatory infiltrate, but very little viral antigen was detected in both the epithelium and stroma. Our findings of abundant viral antigen at day 1 p.i. and little viral antigen at day 28 p.i. in the cornea are consistent with most reports showing that in the mouse eye, viral titers normally reach a peak at 1-3 days p.i. and
become undetectable by 7-9 days p.i. before the development of severe and permanent HSK 14-28 days p.i. (2, 40, 41).

Egr-1 expression in the mock-infected cornea was very minimal (Fig. 1B). Of the infected cornea, Egr-1 expression was clearly enhanced in the epithelium and to a lesser degree in the stroma at day 1 p.i. Notably, Egr-1 expression in the stroma with severe lesions was greatly enhanced, while Egr-1 expression in the epithelium was below detection at day 28 p.i.

**Absence of Egr-1 improves HSK.** We determined the role of Egr-1 in HSK, as Egr-1 is abundantly expressed in the infected cornea. C57BL/6 mice and C57BL/6-derived mice with a targeted disruption of the gene encoding Egr-1 (9, 24, 25, 30) were infected with 5 × 10^5 PFU of HSV-1 strain RE and monitored for corneal opacity and angiogenesis, two important parameters of HSK. With this dose, all infected wild-type mice and Egr-1 gene knock-out (Egr-1^-^-) mice survived. Most infected wild-type mice developed HSK progressively with very severe corneal opacity with mean scores of > 4 from days 14-28 p.i. (Fig. 2A). However, most infected Egr-1^-^- mice displayed only mild corneal haze with visible irises with mean scores of 1 from days 7-28 p.i. The differences in corneal opacity scores between wild-type and Egr-1^-^- mouse groups from days 21-28 p.i. are statistically significant (P < 0.001, Mann-Whitney U test). About 90% of wild-type mice displayed severe HSK with an opacity score of ≥ 4 by day 21 p.i. (Fig. 2B). In contrast, only 17% of Egr-1^-^- mice displayed severe HSK which was significantly lower than that of wild-type mice (P < 0.001, Fisher’s exact test). Abundant and extended neovessels were found in the infected cornea of wild-type mice with an angiogenesis score significantly higher than that in the infected cornea of Egr-1^-^- mice (Fig. 2C, P < 0.01, Mann-Whitney U test).

To further assess corneal opacity and neovascularization, we performed...
histochemical staining. Mouse eyes were harvested 28 days p.i., sectioned, and stained with hematoxylin and eosin. Histologically, the mock-infected corneas of wild-type and Egr-1−/− mice were similar in morphology (Fig. 2D). However, the infected corneas of wild-type mice were much thicker with profound edema, inflammatory infiltrate, and vascularization with erythrocyte-filled blood vessels, especially in the stroma relative to those seen in the infected corneas of Egr-1−/− mice. The results of hematoxylin and eosin staining confirm opacity score results. Endothelial cells constituting the newly formed blood vessels express CD31 (4).

To further assess neovascularization in the cornea, we performed immunohistochemical staining to detect CD31 in mouse eyes harvested 28 days p.i. Abundant CD31 was detected in the corneal stroma of infected wild-type mice, but not in the corneas of infected Egr-1−/− mice or mock-infected wild-type and Egr-1−/− mice (Fig. 2E and data not shown). The CD31 staining result is consistent with the results of angiogenesis scores. Collectively, endogenous Egr-1 aggravates HSK with increased opacity and neovascularization.

Absence of Egr-1 decreases the eye viral load and corneal FGF-2 and VEGF-A levels. To determine how Egr-1 exacerbates HSK, we compared the viral loads in eyes of wild-type and Egr-1−/− mice, because our previous report showed that Egr-1 increased HSV-1 replication (10). The mean viral titers in the eyes of wild-type mice were all higher than those of Egr-1−/− mice from days 1-7 p.i. by 5- to 3-fold from days 1-3 p.i. (Fig. 3). The difference in viral titers between wild-type and Egr-1−/− mouse groups at day 1 p.i. is significant (P < 0.05, Mann-Whitney U test).

We next measured angiogenic factors, FGF-2 and VEGF-A in the cornea, because Egr-1 can activate both FGF-2 and VEGF-A promoters (19, 23, 38, 43). ELISA results showed a low level (47.9 ± 8.4 pg/cornea) of FGF-2 in the
mock-infected cornea of wild-type mice (Fig. 4A). In wild-type mice, HSV-1 infection enhanced corneal FGF-2 expression, particularly from days 14-28 p.i. with a level (339.5 ± 59.0 pg/cornea) significantly higher than that of the mock-infected cornea at day 28 p.i. (P < 0.05, Student’s t test). Of infected Egr-1-/- mice, corneal FGF-2 levels were low and steady from days 14-28 p.i. with a level (90.0 ± 19.4 pg/cornea) significantly lower than that of infected wild-type mice at day 28 p.i. (P < 0.01, Student’s t test). Few studies detect the corneal region, which expresses abundant FGF-2 in the corneal stroma of infected wild-type mice, but little FGF-2 in the corneas of infected Egr-1-/- mice and mock-infected wild-type and Egr-1-/- mice at day 28 p.i. (Fig. 4B and data not shown).

VEGF-A results were very similar to FGF-2 results (Fig. 4C). In wild-type mice, HSV-1 infection enhanced corneal VEGF-A expression, particularly from days 7-14 p.i. with levels significantly higher than that of the mock-infected cornea at days 7 and 28 p.i. (P < 0.05, Student’s t test). Of infected Egr-1-/- mice, corneal VEGF-A levels were low and steady from days 14-28 p.i. with a level (7.9 ± 2.2 pg/cornea) significantly lower than that of infected wild-type mice (23.6 ± 2.9 pg/cornea) at day 28 p.i. (P < 0.01, Student’s t test). A previous report using immunohistochemical staining detected VEGF-A at sites with neovascularization in the corneal stroma of wild-type mice infected with HSV-1 for 15 days (47).

We found a positive correlation in levels of corneal Egr-1 and inflammatory infiltrate (Fig. 2D). Egr-1 has been shown to augment inflammation in the ischemic mouse lung by enhancing the expression of chemokines, IP-10 and MIP-2, which are reported to aggravate HSK by recruiting leukocytes (7, 39, 45, 46). Accordingly, we measured IP-10 and MIP-2 in corneas of wild-type and Egr-1-/- mice. ELISA results showed that absence of Egr-1 did not significantly reduce IP-10 and MIP-2
levels in infected mouse corneas (Fig. 5). Thus, Egr-1 may not markedly affect the leukocyte infiltrate in HSK via these two chemokines.

Suppression of Egr-1 expression topically on the cornea using a DNA-based enzyme, ED5 improves HSK by reducing viral replication and angiogenic response. A DNA-based enzyme (ED5), which suppresses Egr-1 protein expression by cleaving mRNA, has been shown to block neovascularization in the rat cornea implanted with VEGF-A and tumor growth in mice mediated by FGF-2-dependent angiogenesis (15, 36). ED5 treatment given both topically on the mouse cornea infected with HSV-1 and systemically by intravenous injection is required to significantly reduce the lethality of mice with encephalitis (10). Here we tested whether ED5 given topically on the cornea could diminish HSK. C57BL/6 wild-type mice were treated with or without ED5 once daily right before infection through 7 days p.i. and then once every two days from days 9-15 days p.i. Several doses of ED5 (7.5-30 μg/eye) were tested, and ED5 reduced the corneal opacity in a manner dependent on the dose (Fig. 6). ED5 at the dose of 30 μg/eye significantly decreased the corneal opacity when compared with no treatment (\(P < 0.05\), Mann-Whitney U test). This dose was therefore chosen for further studies. C57BL/6 wild-type mice were given 30 μg/eye of ED5 or a scramble oligomer (ED5SCR) (15, 36). Western blotting analysis showed that ED5 reduced Egr-1 protein level by > 60% in the infected cornea harvested at day 1 p.i. when compared with ED5SCR (see Fig. S1 in the supplemental material). ED5 reduced the mean corneal opacity scores from days 7-28 p.i. (Fig. 7A) when compared with ED5SCR with significant differences found from days 21-28 p.i. (\(P < 0.05\), Mann-Whitney U test). Hematoxylin and eosin staining analysis showed that ED5 dramatically decreased edema and inflammatory infiltrate in the mouse cornea, when compared with ED5SCR at day 28 p.i (Fig. 7B). Moreover, immunohistochemical staining
analysis showed that ED5 markedly suppressed the expression of CD31, a marker of endothelial cells in the newly formed blood vessels, when compared with ED5SCR (Fig. 7B).

We harvested mouse eyes to measure viral titers, FGF-2, and VEGF-A. ED5 reduced the mean viral titers in mouse eyes from days 1-5 p.i. when compared with ED5SCR (Fig. 7C) with a significant difference found at day 5 p.i. ($P < 0.05,$ Mann-Whitney U test). ED5 also significantly reduced the mean corneal levels of FGF-2 and VEGF-A at day 28 p.i. when compared with ED5SCR (Fig. 7D and 7E; $P < 0.05,$ Student’s $t$ test).

We also tested the effect of delaying treatment. ED5 given to mice 1-15 days p.i. still improved HSK when compared with ED5SCR (see Fig. S2A in the supplemental material), but the mean corneal opacity scores were not statistically significant between ED5- and ED5SCR-treated groups (Fig. S2A). In addition, ED5 given to mice 9-28 days p.i., when viral replication was below detection, only delayed HSK development, but failed to reduce the final disease score when compared with ED5SCR (see Fig. S2B in the supplemental material).

**ED5 reduces the growth of acyclovir-resistant HSV-1 in the eye.** Anti-HSV drugs, acyclovir and related nucleoside analogs are used to treat HSK in patients, but acyclovir treatment is hindered by the emergence of drug-resistant viruses, mostly containing mutations in the viral $tk$ gene (3, 13, 14). We tested the efficacy of suppressing Egr-1 to reduce the replication of an acyclovir-resistant HSV-1, $tk$LTRZ1 derived from HSV-1 strain KOS without TK activity due to an insertion within the $tk$ gene (12). ICR mice were treated with 30 $\mu$g/eye of ED5 or ED5SCR topically on the cornea once daily right before infection through 3 days after infection with $tk$LTRZ1. ED5 reduced the mean viral titers in mouse eyes from days 1-3 p.i. by 20- to 3-fold when compared with ED5SCR (see Fig. S3 in the
supplemental material) with a significant difference found at day 1 p.i. \( (P < 0.05, \) Mann-Whitney U test). This result also shows that suppression of Egr-1 to reduce corneal HSV replication is not a phenomenon specific to a particular virus or mouse strain. We did not monitor HSK, because tkLTRZ1 cannot induce the disease in mice.
DISCUSSION

Our study is the first to reveal that HSV infection of the mouse cornea increases the expression of Egr-1 to exacerbate HSK. Furthermore, suppression of Egr-1 improves HSK. These findings enhance our understanding of HSK pathogenesis, and also identify a therapeutic target to treat HSK.

Our present and previous studies show enhanced Egr-1 expression in various mouse tissues (corneas and brains) and a human cell line infected with HSV (10). We previously investigated how HSV infection enhanced Egr-1 expression and found that HSV replication was required for Egr-1 induction (10). HSV-1 protein(s) may activate the Egr-1 promoter directly as indicated by the finding that the Zta protein of another herpesvirus, Epstein-Barr virus is reported to bind and activate the Egr-1 promoter (8). Additionally, HSV-1 infection may increase Egr-1 expression indirectly through cellular factors. We previously revealed that the 5’ end of Egr-1 promoter containing a cAMP response element and binding sites for several transcription factors, including SP1, AP1, and Egr-1 itself, was required for HSV infection to increase Egr-1 expression (1, 10). HSV infection increases the expression or the activities of cAMP, SP1, and AP1 (6, 18, 21), and may enhance Egr-1 expression in infected cells through these mediators. Moreover, our present and previous studies observed that some cells in the mouse cornea or brain expressing Egr-1 are not positive for viral antigen (10). These indicate that some uninfected cells may express Egr-1 as a response to paracrine effects or the factors induced by HSV-1 infection, such as FGF-2 and VEGF-A, well-known inducers of Egr-1 (27, 35). This is particularly evident in the infected mouse cornea which shows abundant FGF-2 and Egr-1, but only little viral antigen, at day 28 p. i.

Egr-1 may exacerbate HSK mainly by increasing viral replication. This notion
is supported by the evidence that ED5 treatment reduced HSK severity only when
the treatment was given to mice before viral replication or from 1 day p.i. (on the
peak of viral replication), but not from 9 days p.i. (after viral replication). In
addition, wild-type mice infected with a lower inoculum \((5 \times 10^4 \text{ PFU})\) displayed
less severe HSK with a corneal opacity score of 2.2 significantly lower than mice
infected with \(5 \times 10^5 \text{ PFU}\) of virus with a corneal opacity of 4.4 by days 21-28 p.i.
Egr-1 may also contribute to the angiogenesis response by increasing VEGF-A and
FGF-2 expression through activating \(VEGF-A\), \(FGF-2\), and \(ICP4\) promoters \((10, 19, 23, 38, 43)\). Subsequently, Egr-1 and FGF-2 or VEGF-A further amplify each other
to promote neovascularization. This view is based on the findings that VEGF-A,
FGF-2, and Egr-1 levels reach peaks in similar time frames \((14-28 \text{ days p.i.)}\).
Withdrawal of ED5 treatment 15 days p.i. increases HSK severity \((\text{Fig. 7 and Fig.}
S2A)\). In addition, ED5 treatment given to mice 9-28 days p.i. (after viral replication)
delayed HSK development.

HSV infection is known to enhance FGF-2 and VEGF-A expression to promote
angiogenesis during HSK \((5, 47)\), but how HSV infection enhances these two
angiogenic factors has not been clear until recently. The HSV transcription factor
(ICP4) is reported to activate the \(VEGF-A\) promoter \((44)\). Here we show that the
cellular transcription factor, Egr-1 capable of activating \(ICP4\), \(FGF-2\), and \(VEGF-A\)
promoters, is also involved. During acute infection, Egr-1 can enhance FGF-2 and
VEGF-A expression directly and indirectly through ICP4. After acute infection \((\text{in}
the absence of ICP4)\), Egr-1 and FGF-2 or VEGF-A can amplify each other. Egr-1 is
very likely to serves as a master switch coordinating upregulation of angiogenic
factors during HSK. Few studies characterize FGF-2 expression in the
HSV-1-infected mouse cornea. To our knowledge, our present study might be the
first one to show corneal FGF-2 levels and kinetic as well as the expression of
FGF-2 in the mouse corneal stroma with severe HSK. HSV-1 infects the majority of the world’s population (34), but only induces HSK in some individuals. Here we show that Egr-1 promotes HSK in mice. In the future, it will be of interest to investigate the influence of Egr-1 expression on human susceptibility to HSK. Combined treatment of acyclovir and steroids is currently used to treat HSK. The extensive use of acyclovir leads to the emergence of resistant virus (3, 13, 14), so more alternative therapies are needed. Although Egr-1 participates in many cellular activities, Egr-1 deficiency in mice does not result in obvious defects except that female mice lacking Egr-1 are infertile (24, 25, 30). Additionally, suppression of Egr-1 using ED5 does not influence the body weight, wound healing, blood coagulation, or other hematological parameters of mice (15). In the present study, blocking Egr-1 expression using ED5 improves HSK and reduces viral loads in the mouse eye infected with acyclovir-resistant HSV-1. These results confirm the significance of Egr-1 in HSK and most importantly, show the potential of reducing HSK by suppressing Egr-1. In human cells, knockdown of Egr-1 using antisense oligonucleotide and DNAzyme has also been shown to reduce HSV-1 replication and angiogenesis (10, 15). Therefore, in the future, it is worth testing whether blocking Egr-1, which is unlikely to mutate, could be a potential therapy to reduce HSK caused by drug-resistant mutants in patients.
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REFERENCES


FIGURE LEGENDS

FIG 1  HSV-1 infection enhances Egr-1 expression in the cornea. Corneas (A) or eyes (B) of C57BL/6 mice mock-infected or infected with HSV-1 were harvested at the indicated days post-infection (days p.i.). (A) Mouse corneas were assayed by Western blotting to detect Egr-1 and β-actin (left panel). For each sample, the intensity of the Egr-1 band was normalized to that of β-actin band. Data show mean plus SE values (error bars) of ≥ 3 samples per group with 8 corneas per sample. The value of mock-infected cornea samples was set as 100%, and those of infected corneas were calculated accordingly (right panel). *, P < 0.05, via the Student’s t test. (B) Mouse eyes were sectioned and stained with antibodies against HSV (top panel) or mouse Egr-1 (bottom panel). The reddish brown color denotes a positive reaction. Data are representative of at least 3 samples from 2-3 independent experiments. Scale bars: 100 μm. The dashed box in the bottom right panel is enlarged and shown in the top right corner.

FIG 2  Absence of Egr-1 improves HSK. Wild-type (C57BL/6) mice (n = 19) and Egr-1−/− mice (n = 12) were infected with HSV-1. (A) The corneal opacity scores of infected mice at the indicated times post-infection are shown. Data show mean ± SE values (error bars). ***, P < 0.001, via the Mann-Whitney U test, when compared with Egr-1−/− mice. (B) The incidences of HSK lesions scored ≥ 4 in infected mice 21 days post-infection are shown. Data show mean plus SE values (error bars). ***, P < 0.001, via the Fisher’s exact test. (C) The corneal angiogenesis scores of infected mice 21 days post-infection are shown. **, P < 0.01, via the Mann-Whitney U test. The eyes of wild-type and Egr-1−/− mice mock-infected or infected with HSV-1 were harvested 28 days post-infection, sectioned, and stained with (D) hematoxylin and eosin (H&E) or (E) antibody against mouse CD31. The corneal
portion is shown. Data are representative of at least 3 samples from 2-3 independent experiments. The reddish brown color in the panel E denotes a positive reaction.

Scale bars: 100 μm.

**FIG 3** Absence of Egr-1 decreases viral titers in the eye. The eyes of wild-type (C57BL/6) mice and Egr-1−/− mice were harvested at the indicated times post-infection to determine viral titers. Data show mean ± SE values (error bars) of ≥ 3 samples per data point with one eye per sample. *, P < 0.05, via the Mann-Whitney U test, when compared with Egr-1−/− mice.

**FIG 4** Absence of Egr-1 reduces the expression of angiogenic factors. The corneas of wild-type (C57BL/6) mice and Egr-1−/− mice mock-infected or infected with HSV-1 were harvested at the indicated times post-infection to measure FGF-2 (A) and VEGF-A (C) by ELISA. Data show mean ± SE values (error bars) of ≥ 3 samples per data point with 4 corneas per sample. *, P < 0.05, via the Student’s t test, when compared with the mock-infected corneas of wild-type mice or the infected corneas of Egr-1−/− mice. (B) The eyes of wild-type and Egr-1−/− mice mock-infected or infected with HSV-1 were harvested 28 days post-infection, sectioned, and stained with antibody against mouse FGF-2. The corneal portion is shown. Data are representative of at least 3 samples from 2-3 independent experiments. The reddish brown color denotes a positive reaction. Scale bar: 100 μm.

**FIG 5** The effect of Egr-1 on IP-10 and MIP-2 levels in the infected mouse cornea. The corneas of wild-type mice and Egr-1−/− mice mock-infected (Mock) or infected with HSV-1 (Infected) were harvested 2 days post-infection to measure IP-10 and MIP-2 using ELISA. Data show mean plus SE values (error bars) of 3 samples per
group with 4 corneas per sample. B.D.: below detection.

**FIG 6** Knockdown of Egr-1 by ED5 treatment reduces HSK in a manner dependent on the ED5 dose. The corneal opacity scores of infected C57BL/6 mice treated with ED5 at the doses of 0 μg \((n = 3)\), 7.5 μg \((n = 3)\), 15 μg \((n = 3)\), or 30 μg \((n = 9)\) 28 days post-infection are shown. Data show the mean plus SE values (error bars). *, \(P < 0.05\), via the Mann-Whitney U test.

**FIG 7** ED5 treatment improves HSK with reduced levels of viral titer and angiogenic factors. (A) The corneal opacity scores of infected C57BL/6 mice treated with 30 μg of ED5 \((n = 9)\) or ED5SCR \((n = 7)\) at the indicated times post-infection are shown. Data show mean ± SE values (error bars). *, \(P < 0.05\), via the Mann-Whitney U test, when compared with ED5. (B) The eyes of mock-infected mice without DNAzyme treatment or infected mice treated with ED5 or ED5SCR were harvested 28 days post-infection, sectioned, and stained with hematoxylin and eosin (H&E; top panel) or antibody against mouse CD31 (bottom panel). The reddish brown color in the bottom panel denotes a positive reaction. Data are representative of at least 3 samples from 2-3 independent experiments. Scale bars: 100 μm. (C) Infected mouse eyes treated with ED5SCR or ED5 were harvested at the indicated times post-infection to determine viral titers. Data show mean ± SE values (error bars) of ≥ 6 samples per data point with one eye per sample. *, \(P < 0.05\), via the Mann-Whitney U test, when compared with ED5. Infected mouse corneas treated with ED5 or ED5SCR were harvested 28 days post-infection to measure FGF-2 (D) and VEGF-A (E) by ELISA. Data show mean plus SE values (error bars) of ≥ 3 samples per group with 4 corneas per sample. *, \(P < 0.05\), via the Student’s t test.
FIG 1

A

<table>
<thead>
<tr>
<th>HSV-1</th>
<th>Mock</th>
<th>1</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egr-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relative Egr-1 expression (%)

B

Mock | Infected
---|--------
1 | 28 days p.i.

Anti-HSV

Anti-Egr-1
D

Wild-type

Mock

Infected

Egr-1−/−

E

Mock

Infected

Wild-type

Wild-type

Egr-1−/−

Anti-CD31

100 μm
FIG 3

![Graph showing viral yield (PFU/eye) over days post-infection.](image-url)
FIG 4

A

FGF-2

Days post-infection

Mock wild-type
Infected wild-type
Infected Egr-1

B

Mock
Wild-type
Infected wild-type
Infected Egr-1

Anti-FGF-2

C

VEGF-A

Days post-infection

Mock wild-type
Infected wild-type
Infected Egr-1
FIG 6

![Graph showing corneal opacity levels with ED5 (μg/eye) in the x-axis and opacity levels in the y-axis. There is a significant difference indicated by an asterisk (*) between the groups.](http://jvi.asm.org/)
FIG 7

A

Duration of treatment

Conjunctival opacity

Days post-infection

B

Mock

Infected

No treatment

ED5SCR

ED5

H&E

Anti-CD31

C

Viral yield (PFU/eye)

Days post-infection

D

FGF-2 protein (pg/cornea)

E

VEGF-A protein (pg/cornea)