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Activation of Endothelial Roundabout Receptor 4 Reduces the Severity of Virus-Induced Keratitis

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Antiangiogenic molecules exert a feedback control to restrain pathological angiogenesis, which includes physical binding or inhibition of angiogenic signaling in blood vessel endothelial cells. The latter is the case in which Slit2 ligand-dependent activation of the blood vessel endothelial cell receptor roundabout 4 (Robo4) occurs. In this study, we demonstrate that Robo4 receptors are upregulated following HSV infection of the eye on the majority of the new blood vessel endothelial cells that occur in the corneal stroma. However, expression levels of the ligand for Robo4 receptors, Slit2, was not significantly increased during the disease process, and the knockdown of Slit2 gene expression using lentiviral short hairpin RNAs had no effect on the extent of pathological angiogenesis. In contrast, providing additional Slit2 protein by subconjunctival administration resulted in significantly reduced angiogenesis. The Slit2 binding to Robo4 was shown to block the downstream vascular endothelial growth factor signaling molecules Arf 6 and Rac 1 and reduce the antiapoptotic molecule Bcl-xL in blood vessel endothelial cells. Our results indicate that augmenting the host Robo4/Slit2 system could provide a useful therapeutic approach to control pathological angiogenesis associated with HSV induced stromal keratitis. The Journal of Immunology, 2011, 186: 000–000.

Ocular HSV infection may result in a chronic inflammatory lesion in the corneal stroma that often results in blindness (1–4). Understanding the pathogenesis of stromal keratitis (SK) lesions has mainly come from animal model studies, particularly using the mouse (5). Such studies have revealed that neovascularization of the normally avascular cornea is a key event in SK pathogenesis (6–8). In consequence, understanding how to control pathological angiogenesis, which is also a critical event in human SK, is relevant because it could result in improved therapy.

Many molecules induced in the eye in response to infection can contribute to neovascularization with vascular endothelial growth factor (VEGF) family molecules, particularly VEGF-A signaling via VEGF receptor-2 receptors, being the major stimulus for angiogenesis (9–11). Curiously, VEGF-A is produced within the normal cornea, but it fails to drive blood vessel development because it is bound to an excess of the soluble form of one of its receptors (12–14). Virus infection results in the increased synthesis of VEGF-A as well as some other angiogenic molecules (15, 16) and, in addition, causes the breakdown of the VEGF-A soluble receptor bond (17). Interference with VEGF levels or blocking the binding or signaling of receptors are the major approaches so far evaluated to control pathological neovascularization (6, 7, 9, 18). However, the host itself also has one or more means of shifting down VEGF-induced angiogenesis (13). For example, the roundabout 4 (Robo4) receptor that is expressed by endothelial cells in newly formed blood vessels can transduce signals to the cell that will negate or counteract the positive stimulus caused by VEGF binding to its receptors (19, 20). Robo4 receptors can be activated by the host ligand, Slit2, that is produced in some pathological lesions by vascular endothelial cells (19, 21). Accordingly, the balance between the positive signals produced by VEGF may become modulated and perhaps terminated by Slit2-induced Robo4 receptor activation. In some circumstances, such as in laser-induced retinopathy, Robo4 knockout mice revealed an accelerated pathological angiogenesis process (19). Additionally, Slit2/Robo4 signaling reduced vessel permeability in the lung and other organs and protected mice from a pathogen-induced cytokine storm (22).

Currently, no reports have evaluated the relevance of the Slit2/Robo4 regulatory system in an infectious disease that involves pathological angiogenesis. This issue is addressed in this study using ocular infection with HSV that causes neovascularization of the cornea and the blinding lesion SK. Our results show that following HSV infection, Robo4 transcripts were significantly upregulated in corneal tissues, with the majority of endothelial cells of the newly developed blood vessels expressing Robo4 receptors. However, the Robo4 ligand, Slit2 transcripts, and protein were not significantly increased after HSV ocular infection. This could mean that the amount of Slit2 available for binding to Robo4 receptors was limited and that the Slit2/Robo4 host regulatory system was not contributing to effectively control angiogenesis during SK. In support of this notion, blocking Slit2 gene expression using lentiviral short hairpin RNA (shRNA) vectors had no effect on the extent of angiogenesis, but in contrast, the provision of additional Slit2 protein by subconjunctival injection significantly reduced neovascularization. The results of these studies have therapeutic implications for the control of HSV-induced vision loss.

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Abbreviations used in this article: HSK, herpetic stromal keratitis; p.i., postinfection; QPCR, quantitative PCR; Robo4, roundabout 4; shRNA, short hairpin RNA; SK, stromal keratitis; VEGF, vascular endothelial growth factor; WB, Western blot.

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Materials and Methods

**Mice**

Female 6-8-wk-old C57 BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). All mice were housed conventionally in an animal facility at the University of Tennessee. All investigations followed the guidelines of the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

**Virus**

HSV-1 strain RE Tumpey was propagated in Vero cell monolayers (ATCC number CCL81; American Type Culture Collection). Infected Vero cells were harvested, titrated, and stored in aliquots at −80˚C until used.

**Corneal HSV-1 infection and scoring**

Corneal infections of mice were performed under deep anesthesia. The mice were lightly scotched on their corneas with a 27-gauge needle, and a 3-µl drop containing 108 PFU HSV-1 RE Tumpey was gently applied to one eye. The development of SK lesion severity and angiogenesis in the eyes of mice was examined by slit-lamp biomicroscopy (Kowa, Nagoya, Japan). The scoring system used was as follows: 0, normal cornea; 1, mild corneal haze; 2, moderate corneal opacity; 3, severe corneal opacity; 4, opaque cornea and ulcer; and 5, corneal rupture. The severity of angiogenesis was recorded as described previously (23). According to this system, a grade of 0 indicates no discernable neovascularization of the cornea, while 1 indicates a focal neovessel, 2 indicates small vessels, 3 indicates small vessels growing perpendicularly toward the corneal center, 4 indicates a centripetal growth of vessels in each quadrant of the cornea, and 5 represents a centripetally growing neovessel in each quadrant of the eye. The score of the four quadrants of the eye for a given quadrant of the circle represents a centripetal growth of 1.5 mm toward the corneal center. The score of the four quadrants of the eye were then summed to derive the neovessel index (range 0–16) for each eye at a given time point.

**Subconjunctival injections**

Subconjunctival injections were performed as reported previously (23). Briefly, these injections were carried out using a 2-µl, 32-gauge needle and syringe (Hamilton, Reno, NV) to penetrate the perivascular region of conjunctiva, and an appropriate amount of Slit2 protein (R&D Systems, Minneapolis, MN) was administered into subconjunctival space. Slit2 protein used in this study is a Gln26-Gln900 amino terminal fragment, which is expressed in Chinese hamster ovary cells. Mock treatment was given in control mice.

**Mice treatment with recombinant Slit2 protein**

The mice oculoarly infected with HSV-1 RE Tumpey were separated into two groups. The Slit2 protein (1 µg) was given to one group in 10 µl PBS subconjunctivally at 2 d postinfection (p.i.) with additional doses on alternate days until day 12 p.i. The Slit2 treatment was begun at day 7 in another experiment with additional doses on alternate days until day 13 p.i. The control group received mock drug subconjunctivally with the same regimen for respective experiments. These animals were carefully followed for the progression of angiogenesis and SK development. All experiments were repeated at least two times.

**Purification of endothelial cells**

The excised corneas were pooled and digested with 60 U/ml Liberase for 35 min at 37˚C in a humidified atmosphere of 5% CO2. The single-cell suspension was prepared and stained with anti-CD31-FITC for 30 min on ice. The FITC+ endothelial cells (CD31+) were sorted using a FACS sorter. The purity at the extent of 90–90% was achieved. These sorted endothelial cells were used for pulldown assays, detection of Bcl-xL, cyclin D1, and caspase-3 proteins, and confocal microscopy.

**Confocal microscopy**

For confocal microscopy, the purified endothelial cells were taken on a slide using cytospin and fixed in 4% paraformaldehyde at room temperature for 10 min. The cells were blocked with 10% goat serum containing 0.05% Tween 20 and 1:200 dilution of Fc block (clone 2.42G2; BD Pharmingen, San Diego, CA). Rat anti-Bo-roβ4-PE (R&D Systems, Minneapolis, MN) and rat anti–CD31-FITC (clone 390; BD Pharmingen) were diluted in 1% BSA containing 0.1% Triton X-100 and incubated at room temperature for 1 h. After incubations, slides were washed several times with PBS with Tween 20, mounted with Prolong gold antifade mounting media (Invitrogen), and visualized under a confocal microscope.

**Flow cytometry**

The corneal single-cell suspensions were prepared following Liberase digestion of corneas. These corneal cell suspensions were stained for different cell-surface molecules for FACS. Shortlly, cell suspensions were incubated with CD45-allophycocyanin (30-F11), CD11b-PerCP (M179), Gr1-PE (1A8), CD4-allophycocyanin (RM4.5), CD31-FITC (all from BD Pharmingen), and Robo4-PE (R&D Systems) for 30 min on ice. Thereafter, cells were washed three times and resuspended in 1% paraformaldehyde. The stained samples were acquired with a FACS Calibur (BD Biosciences), and the data were analyzed using the FlowJo software (Tree Star).

**Quantitative PCR**

Total mRNA was isolated from corneal cells using Trizol LS reagent (Invitrogen). The cDNA prepared using 1 µg RNA was used for subsequent analysis. Quantitative PCR (QPCR) was done using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) with iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). The expression levels of Slit2 and Robo4 were normalized to β-actin with the Δ threshold cycle method, and relative quantification between control and infected mice was performed using the 2−DDCT formula. The primers used were as follows: β-actin forward, 5′-CTACCTCATGAGATCTGGAC-3′; β-actin reverse, 5′-CTTCTAGAACAATAGACAC-3′; Slit2 forward, 5′-CCCTGTCGTATGCTCTC-3′; Slit2 reverse, 5′-GTAGTATGAGTTGTCCTGGG-3′; Robo4 forward, 5′-GACCTATATGTGTATGGCCAC-3′; Robo4 reverse, 5′-CTTCTAGTGTTCCTTGTGGTCC-3′; Cxcl2 forward, 5′-CCAAAAGAGGCCTACGAG-3′; Cxcl2 reverse, 5′-CTTCAAGCGTCAAGAGCACC-3′; Nos2 forward, 5′-TGTTTGGTCCTCACACCA-3′; and Nos2 reverse, 5′-CCTCAATCTGTCCTACCC-3′. The primers for β-actin, Ng, Ib6, and Cxcl1 were all identified in the laboratory (24).

**RT-PCR**

RT-PCR for the presence of Slit2 and Robo4 transcripts was done according to the manufacturer’s protocol (Promega, Madison, WI). The amplified products were resolved on 1% agarose gel. Primers for RT-PCR were as follows: β-actin forward, 5′-CTACCTCATGAGATCTGGAC-3′; β-actin reverse, 5′-GGCATAGGTCTTGCTTACGAT-3′; Slit2 forward, 5′-GGGAAAGCAATGGTCATTGAGT-3′; Slit2 reverse, 5′-GTAGTATGAGTTGTCCTGGG-3′; Robo4 forward, 5′-GGTAGTGACCTAAGTCCTGGG-3′; and Robo4 reverse, 5′-CTAGTACGACCAACACAGAGT-3′.

**Western blot analysis**

The corneal cells were lysed, and total protein in the supernatants was quantified using a BCA protein assay kit (Thermo Scientific, Waltham, MA). Samples were denatured in Laemmli buffer and resolved by SDS-PAGE, and proteins were transferred onto a polyvinyldene difluoride membrane. The membrane was blocked with 5% BSA in TBS with Tween 20 or 4% nonfat dry milk overnight and subjected to incubation with specific primary and secondary Abs. Proteins bands were visualized using chemiluminescent HRP substrate (Millipore, Billerica, MA). After keeping in stripping buffer for 10 min, the membrane was reprobed using anti-β-actin Ab. The Abs used were as follows: goat anti-mouse Robo4 (C-20), goat anti-mouse Slit2 (D-16), anti-β-actin (C 4), mouse Bcl-xL (4), mouse cyclin D1 (72-13G), goat caspase-3 (T-20), goat anti-mouse IgG-HRP, donkey anti-goat IgG-HRP (all from Santa Cruz Biotechnology), mouse Arf 6 (26180D; Thermo Scientific), and mouse Rac 1 (89856D; Thermo Scientific).

**Arf 6 activation assay**

Arf 6 activation assays were performed as reported previously (20). These experiments were carried out using the Active Arf 6 pulldown and deactivation kit (Thermo Scientific) according to the manufacturer’s protocol. Briefly, reslin slurry was added to the spin cup in a collection tube, and 100 µg GST GGA-3-PBD fusion protein with 800 µg total protein (corneal cell lysate) was added to this slurry. This was incubated at 4˚C for 1 h, and finally, 50 µl reducing sample buffer was added, and GTP-Arf 6 was pulled down and subsequently analyzed by Western blot (WB). These experiments were repeated two times.

**Rac 1 activation assay**

The Rac 1 activation assays were carried out as described previously (20). The EZ-Detect Rac 1 activation kit (Thermo Scientific) was used to isolate active Rac 1 from corneal cell lysates. The immobilized glutathione disc was placed into a spin column, and 20 µg GST-human Pak1-PBD was added to it. Immediately, 700 µg total protein (corneal cell lysate) was added to the spin column, and it was incubated for 1 h at 4˚C without rocking. Finally, 50 µl × 5× SDS sample buffer containing 2-ME was added to the resin, and the mixture was heated at 100˚C for 5 min. Samples were analyzed by WB. These experiments were repeated two times.
Retrovirus-based RNA interference

Slit2 shRNA lentiviral particles and control lentiviral particles were procured from Santa Cruz Biotechnology. These lentiviral particles contain three to five expression constructs each encoding Slit2-specific shRNA, whereas control lentiviral particles encode scrambled sequences. The knockdown of Slit2 in MKT cells was performed using the manufacturer’s protocol. Briefly, cells were transfected when they were 50% confluent. Polybrene was added at a final concentration of 5 μg/ml, and Slit2 shRNA/ control lentiviral particles (ranging from 1 × 10^5–5 × 10^5) were added to cells. Medium was changed after 12 h, and cells were analyzed by QPCR for Slit2 knockdown over 2–4 d after transduction. For in vivo experiments in HSV-infected mice, 1 × 10^3 Slit2 shRNA/control lentiviral particles were inoculated subconjunctivally starting at day 1 with additional doses on alternate days until day 11, and mice were monitored for the progression of angiogenesis and SK.

Statistics

The statistical significance for SK lesion severity and angiogenesis between two groups was determined using an unpaired two-tailed Student t test. One-way ANOVA with Bonferroni’s post hoc test was used to calculate the level of significance for some experiments; *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001 were considered significant. All experiments were repeated at least two times, and results are expressed as means ± SEM. For all statistical analyses, GraphPad Prism software (GraphPad) was used.

Results

Expression of Robo4 and Slit2 after HSV infection

The pattern of expression of Robo4 and Slit2 gene and protein expression was measured at various time points after HSV infection, focusing on time points when neovascularization was prominent and SK lesions clinically apparent. Thus, our aim was to find evidence for a possible role of the Robo4/Slit2 interaction in constraining VEGF-driven angiogenesis and vascular permeability. Corneas were collected at different times post-HSV infection, dissected free of normal limbal blood vessels, and samples processed either to collect vascular endothelial cells for phenotyping or to prepare corneal RNA samples to quantify Robo4 and Slit2 mRNA levels by QPCR. As shown in Fig. 1A, naive avascular corneal samples do express Robo4 mRNA (likely produced by corneal epithelial cells), but Slit2 mRNA was barely detectable. The fact that naive corneas express Robo4 and Slit2 was also demonstrable by WB (Fig. 1B, 1C), and the positive controls for Robo4 and Slit2 were kept to the kidney and brain due to the expression pattern of these molecules reported previously (25, 26). Changes in the levels of Robo4 mRNAs (probably from blood vessel endothelial cells) occurred as early as day 2 after HSV infection, and levels peaked during the clinical phase (days 7–14), reflecting the appearance of neovascularization in the infected eye (Fig. 1D). However, Slit2 expression levels did not change significantly over the course of events (Fig. 1E).

By day 2, mRNA levels for Robo4 were increased by 28-fold but Slit2 only by 2.9-fold. At the time of overt neovascularization, on day 9 p.i., Robo4 mRNA was elevated by 51-fold, but Slit2 mRNA only 3.6-fold (Fig. 1D, 1E). Corneal extracts were also analyzed for Robo4 and Slit2 proteins by WB on day 9. Compared to controls, the elevated expression of Robo4 was evident with little change in Slit2 levels (Fig. 1F).

Cells isolated from infected corneas at different times p.i. were sorted for CD31+ cells (a marker for vascular endothelial cells), and Slit2 shRNA lentiviral particles and control lentiviral particles were processed either to collect vascular endothelial cells for phenotyping or to prepare corneal RNA samples to quantify Robo4 and Slit2 mRNA levels by QPCR. As shown in Fig. 1A, naive avascular corneal samples do express Robo4 mRNA (likely produced by corneal epithelial cells), but Slit2 mRNA was barely detectable. The fact that naive corneas express Robo4 and Slit2 was also demonstrable by WB (Fig. 1B, 1C), and the positive controls for Robo4 and Slit2 were kept to the kidney and brain due to the expression pattern of these molecules reported previously (25, 26). Changes in the levels of Robo4 mRNAs (probably from blood vessel endothelial cells) occurred as early as day 2 after HSV infection, and levels peaked during the clinical phase (days 7–14), reflecting the appearance of neovascularization in the infected eye (Fig. 1D). However, Slit2 expression levels did not change significantly over the course of events (Fig. 1E).

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FIGURE 1. Expression of Robo4 and Slit2 after HSV infection. Wild type mice were sacrificed, and six corneas were collected and pooled for mRNA analysis by RT-PCR and for protein analysis by WB. A, Agarose gel analysis of Robo4 (394 bp) (lane 4) and Slit2 (465 bp) (lane 6) transcripts from naive corneas (pooled n = 6 corneas). Lane M is marker; lane 2 is β-actin (325 bp). Lanes 1, 3, and 5 are reverse transcription negative control for β-actin, Robo4, and Slit2, respectively, whereas lane 7 is negative control (water). Data are representative of three independent experiments. Reducing WB analysis of Robo4 (B) and Slit2 (C) protein in naive corneas is shown. The kidney and brain were used as positive controls for Robo4 and Slit2, respectively. These experiments were repeated three times. Wild type mice were infected with HSV-1 RE Tumpey in one eye, and six corneas were collected and pooled for analysis either by QPCR or WB. D, Kinetic analysis of the expression of Robo4 mRNA levels shows peaks around days 7–14 p.i. Data are mean ± SEM (pooled n = 6 mice). E, Quantification of Slit2 mRNA at different time points post-HSV infection. Data are mean ± SEM (pooled n = 6 mice). F, Reducing WB analysis of corneal samples from naive uninfected and day 9 p.i. reveals increase in Robo4 protein in infected corneas, whereas only a slight change in Slit2 protein at day 9 p.i. (pooled n = 6 mice). These experiments were repeated three times.
and the expression of Robo4 was measured on such cells (Fig. 2A). First, we could show that the percentage of CD31+ cells that expressed Robo4 protein changed with time. Thus, at the earliest time point (day 1), 23.8% of cells were Robo4 positive, whereas at a later time (day 11), the expression increased to 59.2% average. Second, we also compared the numbers of Robo4+CD31+ cells at different time points. Maximal numbers of Robo4+CD31+ cells were evident in samples collected at days 11 and 14 p.i. (Fig. 2B).

At this time point, levels of Slit2 protein were minimal, and, as was already mentioned, mRNA levels were also low in comparison with Robo4. We also attempted to sort endothelial cells from naive uninfected and day 11 p.i. corneas (Fig. 2C, 2D). Accordingly, we could not sort any endothelial cells from naive corneas, but large numbers of endothelial cells were sorted from day 11 p.i. corneas (Fig. 2C, 2D). These sorted endothelial cells from day 11 p.i. corneas revealed the presence of Robo4 by WB (Fig. 2E). Additionally, the fact that blood vessel endothelial cells during SK expressed Robo4 was also demonstrable by confocal microscopy. Eyes taken from the day 11 time point and subjected to confocal microscopy revealed endothelial cells with Robo4 receptors (Fig. 2F–H). Thus, taken together, our results could mean that although Robo4 is abundantly expressed by the new blood vessels that result from HSV infection, there may be an inadequacy of the ligand Slit2 to trigger an effective antiangiogenic response.

**Endogenous Slit2 is dispensable during SK**

Additional experiments were performed to determine if the inhibition of Slit2 production in HSV-infected animals had any effect on the extent of the ocular angiogenesis. To inhibit Slit2 expression, a lentiviral vector was used that expressed Slit2 shRNAs. Initial experiments in vitro using an MKT cell line expressing Slit2 (Fig. 3A) were performed to assess the efficacy of the lentiviral knockdown system. The MKT cell line was transduced with lentiviruses expressing Slit2 shRNA/scrambled sequences. Fig. 3B demonstrates the average Slit2 gene expression values of two independent experiments. We could achieve ∼55% reduction in Slit2 mRNA transcripts when Slit2 shRNAs were introduced into MKT cells compared with controls. We did not observe any Slit2 knockdown using scrambled sequences, which confirmed in vitro knockdown of the Slit2 gene. In vivo experiments were done in HSV-infected animals to determine if the lentiviral vector expressing Slit2 shRNAs had any effect on the extent of angiogenesis. The subconjunctival delivery (27) of lentiviruses encoding Slit2 shRNAs was started from day 1 post-HSV infection with additional administrations on alternate days (Fig. 3C). Corneas collected at day 9 revealed diminished Slit2 mRNA and protein compared with controls (Fig. 3D, 3E), suggesting a reduction in Slit2 mRNA and protein in vivo after lentiviral Slit2 shRNA inhibition of HSV-1-induced corneal neovascularization.
administration. However, when lentiviruses expressing Slit2 shRNA treatment were further continued in another group of mice until day 11 and animals were observed at the end of the experiment (day 15) (Fig. 3F), no increase in angiogenesis or SK lesion severity was observed (Fig. 3G, 3H). These experiments were repeated three times, and the results suggest that endogenous Slit2 is dispensable during SK (due to low amounts).

Provision of recombinant Slit2 diminishes angiogenesis and herpetic SK

These experiments were carried out to observe the effect of subconjunctival Slit2 protein administration on HSV-1-induced angiogenesis. As shown in Fig. 4A, subconjunctival administration of Slit2 protein (starting from day 2 p.i. with additional doses on alternate days up to day 12) with different doses was done in mice, and animals were examined at the end time of the experiment (day 15). This resulted in a dose-dependent inhibition of angiogenesis, with peak reduction achieved at the maximum dose of 1 μg Slit2 protein when mice were examined at the day 15 time point (Fig. 4B). Thus, preventive administration of Slit2 resulted in reduction in SK and angiogenesis scores (Fig. 4C–E). The eyes from the Slit2-treated group revealed reduction in visible angiogenesis compared with the mock-treated group (Supplemental Fig. 1A–C). The frequency and numbers of CD31+ endothelial cells (Fig. 4F, 4G), CD4+ T cells (Fig. 4H, 4I), and neutrophils (Fig. 4J, 4K) were reduced in mice receiving Slit2 protein compared with mock-treated animals. Curiously, even though the treatment of mice with Slit2 protein was delayed up to day 7 post–HSV-1 infection (with additional doses on alternate days until day 13), this treatment modality also resulted in diminished angiogenesis and SK lesion severity (day 15) (Fig. 5A–C). Additionally, there was a reduction in the frequency and numbers of CD31+ endothelial cells (Fig. 5D, 5E), CD4+ T cells (Fig. 5F, 5G), and neutrophils (Fig. 5H, 5I) and in the Slit2-treated group. Additionally, when levels of various cytokines (IL-1β, IFN-γ, IL-6, and Nos2) and chemokines (Cxcl1 and Cxcl2) were examined in the Slit2/mock-treated group, a reduction in the levels of different cytokines and chemokines was evident in Slit2-treated mice compared with infected controls at both days 11 and 15 p.i. (Supplemental Fig. 2A–C).
Taken together, our results demonstrate that Slit2 protein-driven activation of endothelial Robo4 receptors can serve to modulate the extent of angiogenesis and subsequent immunopathology that occurs following HSV infection.

**Possible mechanism by which Slit2 exerts antiangiogenesis**

Blockade of Arf 6 and Rac 1 activity during herpetic SK by Slit2 treatment. Recent reports show that Slit2 inhibits angiogenesis by blockade of Arf 6 (20). GTP$^{\text{ac}}$ activation assays for Arf 6 and Rac 1 were performed to see whether exogenously delivered Slit2 protein causes similar effects in an infectious disease setting. Mice infected with ocular HSV-1 were provided with 1 mg Slit2 protein subconjunctively starting from day 2 with additional doses at days 4, 6, 8, and 10. Active Arf 6 and Rac 1 were pulled down from six corneas collected from each group at day 11 p.i. (Fig. 6A). These were subjected to WB to determine levels of active Arf 6 and Rac 1. As shown in Fig. 6B and 6C, total Arf 6 levels were similar in all groups, but there was an increase in GTP-Arf 6 in the case of mock-treated animals compared with uninfected controls. In contrast, a reduction in GTP-Arf 6 in the Slit2 treatment group was observed, indicating that Slit2 treatment resulted in the blockade of Arf 6 activity. Similar results were obtained in analyses from GTP-Rac 1 activation assays. There was an increase in GTP-Rac 1 in the mock treatment group over uninfected control animals, but levels of GTP-Rac 1 were undetectable in the Slit2-treated group (Fig. 6D, 6E). Total Rac 1 activity was similar in these groups. The above experiments were repeated two times. Additionally, the blood vessel endothelial cells (CD31$^+$ cells) were sorted from mock/Slit2-treated corneas at day 11 p.i. (purity up to the extent of 85–90% was achieved), and active Arf 6 and Rac 1 were pooled down from the endothelial cells of the respective groups. As shown in Supplemental Fig. 2A–E, levels of active Arf 6 and Rac 1 were lower in endothelial cells taken from Slit2-treated mice compared with mock, whereas total Arf 6 and Rac 1 levels were similar in both groups. These experiments were repeated two times. These results indicate that the provision of excess Slit2 to HSV-1–infected mice reduces angiogenic Arf 6 and Rac 1 activity in the corneas.

**Slit2 may reduce antiapoptotic and cell cycle signal molecules in blood vessel endothelial cells**

The observed reduced numbers and frequency of blood vessel endothelial cells in Slit2-treated animals prompted us to investigate...
whether Slit2 treatments induced apoptosis of endothelial cells in vivo. To evaluate the situation in HSV-infected ocular disease, infected mice were treated with 1 μg Slit2/mock starting from day 2 up to day 10 on every alternate day, and corneas collected at day 11 p.i were subjected to analysis (Fig. 7A). The blood vessel endothelial cells (CD31+) were sorted from mock/Slit2-treated corneas at day 11 (purity up to the extent of 90% was achieved) and analyzed for the expression of antiapoptotic Bcl-xL and cell cycle signal molecule cyclin D1 by WB. As shown in Fig. 7B–E, levels of Bcl-xL and cyclin D1 were downregulated in endothelial cells isolated from Slit2-treated mice compared with the mock-treated group. These experiments were repeated two times. Additionally, these sorted blood vessel endothelial from both groups were subjected to WB and analyzed for the expression of active caspase-3 (indicating apoptosis). As shown in Supplemental Fig. 3A–C, the level of caspase-3 was higher in endothelial cells taken from Slit2-treated mice (indicating more apoptosis) compared with the mock-treated group. The above experiments were repeated two times. The level of significance was determined by Student t test (unpaired). These experiments were repeated two times.

**Discussion**

HSV ocular infection results in neovascularization of the normally avascular cornea. This represents a key event in the pathogenesis of a chronic inflammatory reaction in the eye (5–7, 9). In consequence, understanding how to control pathological angiogenesis is relevant because it could result in improved therapy of a lesion, SK, that is an important cause of human blindness. In the present report, we have evaluated if a host feedback antiangiogenesis mechanism, namely the stimulation by Slit2 ligand of Robo4 receptors on vascular endothelial cells, is operative in an infectious disease situation and to determine if manipulating the feedback system could represent a useful approach to constrain lesion severity. We demonstrate that Robo4 receptors are upregulated following HSV infection and that the majority of new blood vessel endothelial cells express Robo4. However, the expression levels of the Robo4 ligand Slit2 were not significantly increased during the disease process. Moreover, the inhibition of Slit2 gene expression using lentiviral shRNAs had no effect on the extent of pathological angiogenesis. In contrast, providing additional Slit2 protein by subconjunctival administration resulted in significantly reduced angiogenesis. Mechanistically, the Slit2/Robo4 interaction was shown to block the downstream VEGF signaling molecules Arf 6 and Rac 1 and reduced the antiapoptotic molecule Bcl-xL in blood vessel endothelial cells. Our results indicate that augmenting the host Robo4/Slit2 antiangiogenesis system could provide a useful therapeutic approach to control pathological angiogenesis associated with HSV-induced SK.

SK remains a significant cause of vision impairment, and new forms of therapy are needed. Controlling the extent of neovascularization, a key event in SK pathogenesis, represents a logical target for therapy. In animal models of SK, we and others have shown that reducing angiogenesis, as can be achieved by inhibiting the expression of angiogenic factors, results in milder lesions (6,
Similarly, angiogenesis is damaging to the retina, and one treatment finding favor in the clinic is to use the anti-VEGF mAb bevacizumab (29, 30). Recently, it became apparent that the host itself has one or more systems that counteract the stimuli for new blood vessel development and malfunction. We chose to investigate the Slit2/Robo4 system because this antiangiogenic feedback mechanism was shown recently to influence vascular disease induced by trauma to the retina (19, 20). Moreover, both Slit2 and Robo4 can be artificially induced in the cornea by implants containing the angiogenic factor fibroblast growth factor (31). However, it was not known if the Slit2/Robo4 feedback mechanism had any regulatory effect in any natural infectious disease models in which typically, as in SK, multiple angiogenic factors are involved in driving the pathological angiogenesis.

Our results clearly show that the Robo4 upregulation becomes evident early p.i. We could show that the majority of the endothelial cells recovered from the neovascularized corneas expressed Robo4 receptors. Accordingly, the Robo4-mediated antiangiogenesis system was poised to perform its regulatory function. Nevertheless, we advocate that this potential regulatory event may be inadequate during SK lesion pathogenesis because the ligand for Robo4, Slit2, was in limited supply. The reasons for this were not established, but they could relate to an inhibitory effect of virus on Slit2 gene expression, which may be derived from corneal epithelial cells (31–

FIGURE 6. Blockade of Arf 6 and Rac 1 activity after Slit2 treatment. Wild type mice were infected with HSV-1 RE Tumpey in one eye, and six corneas were collected and pooled for analysis by GTP$^{\text{GTP}}$ activation assays. A, Wild type mice infected with HSV were treated with Slit2/mock as shown. The corneas collected at day 11 p.i. after Slit2/mock treatment were subjected to Arf 6 (B, C) and Rac 1 (D, E) pulldown followed by reducing WB analysis for Arf 6 and Rac 1 of the respective groups (pooled $n = 6$ mice/group). The results of two independent experiments are shown.

FIGURE 7. Slit2 may reduce antiapoptotic and cell cycle signal molecules in endothelial cells. Wild type mice were infected with HSV-1 RE Tumpey in one eye, and six corneas were collected and pooled for analysis by WB. The HSV-infected mice were treated with Slit2/mock as shown (A), and CD31$^+$ cells were sorted from mock- and Slit2-treated mice corneas. These sorted CD31$^+$ cells from respective groups were subjected to reducing WB for antiapoptotic molecule Bcl-xL (B, C) and cell cycle signal molecule cyclin D1 (D, E) (pooled $n = 6$ mice/group). The results of two independent experiments are shown.
34]. This issue is under investigation. Following infection, a modestly increased expression of Slit2 did occur, but this was considered insignificant. Moreover, when Slit2 gene expression was reduced in the cornea, as was achieved using a lentivirus system that expressed shRNA for Slit2, an anticipated increase in the extent of pathological angiogenesis [21] was not observed. Accordingly, we must conclude that during the pathogenesis of HSV ocular lesions, the host Slit2/Robo4 antiangiogenesis feedback mechanism fails to influence the outcome of events. Nevertheless, we could also show that the antiangiogenic system could be activated and play a modulatory effect on the virus-induced angiogenesis. This was done by providing an additional supply of Slit2 protein locally to the eye by subconjunctival injection. This procedure, which could be started as late as day 7 p.i., resulted in significantly reduced angiogenesis and SK lesion severity compared with untreated controls. Additionally, we could observe a reduction in levels of various cytokines and chemokines in the corneas of Slit2-treated mice, which is in line with recent findings of others [22]. Our results support the observations of Jones et al. [19, 20] using a noninfectious vascular disease model in the retina that intravitreal administration of additional Slit2 protein can diminish vascular disease.

Previous reports indicate that a number of biochemical events could explain the mechanisms by which Slit2 exerts its antiangiogenic effect [19, 20, 35]. These include activation of Robo4 receptors, which recruits paxillin and GIT1 that, in turn, block angiogenic Arf6 (VEGF downstream signaling) activity [19, 20]. Moreover, others suggest that Slit2 accelerates apoptosis in tumor systems by reducing the expression of the antiapoptotic molecule Bcl-2 as well as diminishing cell cycle signal molecules such as cyclin D1 [35]. Our results demonstrate that the Slit2 treatment in HSV-infected mice resulted in significant reduction in Arf6 and Rac1 activity in corneas, but the treatment modality also led to reduction in antiangiogenic Bcl-2 and cell cycle signal molecule cyclin D1, whereas it increased caspase-3 levels in blood vessel endothelial cells of Slit2-treated mice. Future studies are under investigation to determine whether blockade of downstream signaling molecules of VEGF such as Arf6 or increasing cell cycle inhibitors in blood vessel endothelial cells will lead to modulation of angiogenesis and SK.

In conclusion, we have demonstrated that enhancing natural Slit2/Robo4 antiangiogenic host feedback mechanism reduces virus-induced angiogenesis and subsequent immunopathology. In this study, we show that the majority of the blood vessel endothelial cells recovered from the neovascularized corneas during SK expressed the Robo4 receptors. Nevertheless, the ligand Slit2 was found to be minimal, leading to ineffectiveness of this natural feedback mechanism to control angiogenesis during this blinding immunopathology. Thus, the provision of additional amount of Slit2 protein led to significant reduction in angiogenesis and diminished immunopathology during SK. Accordingly, we advocate that manipulating the function of the host feedback mechanism holds promise as a potential approach for therapy of SK.

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Disclosures

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References


