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*J Immunol* 2011; 186:3653-3665; Prepublished online 16 February 2011; doi: 10.4049/jimmunol.1003239

http://www.jimmunol.org/content/186/6/3653

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[http://www.jimmunol.org/content/suppl/2011/02/14/jimmunol.1003239.DC1](http://www.jimmunol.org/content/suppl/2011/02/14/jimmunol.1003239.DC1)

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Ocular Neovascularization Caused by Herpes Simplex Virus Type 1 Infection Results from Breakdown of Binding between Vascular Endothelial Growth Factor A and Its Soluble Receptor

Amol Suryawanshi,*1 Sachin Mulik,*1 Shalini Sharma,* Pradeep B. J. Reddy,* Sharvan Sehrawat,† and Barry T. Rouse*

The normal cornea is transparent, which is essential for normal vision, and although the angiogenic factor vascular endothelial growth factor A (VEGF-A) is present in the cornea, its angiogenic activity is impeded by being bound to a soluble form of the VEGF receptor-1 (sVR-1). This report investigates the effect on the balance between VEGF-A and sVR-1 that occurs after ocular infection with H SV, which causes prominent neovascularization, an essential step in the pathogenesis of the vision-impairing lesion, stromal keratitis. We demonstrate that H SV-1 infection causes increased production of VEGF-A but reduces sVR-1 levels, resulting in an imbalance of VEGF-A and sVR-1 levels in ocular tissues. Moreover, the sVR-1 protein made was degraded by the metalloproteinase (MMP) enzymes MMP-2, -7, and -9 produced by infiltrating inflammatory cells that were principally neutrophils. Inhibition of neutrophils, inhibition of sVR-1 breakdown with the MMP inhibitor marimastat, and the provision of exogenous recombinant sVR-1 protein all resulted in reduced angiogenesis. Our results make the novel observation that ocular neovascularization resulting from H SV infection involves a change in the balance between VEGF-A and its soluble inhibitory receptor. Future therapies aimed to increase the production and activity of sVR-1 protein could benefit the management of stromal keratitis, an important cause of human blindness. The Journal of Immunology, 2011, 186: 3653–3665.

N ormal ocular function requires that photons can pass through ocular tissues anterior to the retina with minimal impedance. In consequence, tissues anterior to the retina are transparent and numerous strategies are used by the normal eye to maintain transparency. These include mechanisms to suppress inflammatory and immune reactions, and means to suppress ocular vascularization, both enemies of normal vision (1, 2). Nevertheless, in some circumstances, damage to the eye can result in chronic inflammatory reactions and/or pathological neovascularization, and these events may seriously impair vision. This is often the outcome of ocular infection with HSV-1 (3, 4). Invariably, HSV-1 infection is followed by lifelong viral persistence, and this source of virus can create subsequent infections in the cornea (5, 6). Repeated recurrences often result in significant vision-impairing lesions that occur mainly in the corneal stroma (5, 6). Such stromal keratitis (SK) lesions have a complex pathogenesis but result mainly from T cell-orchestrated immunopathological reactions to viral or perhaps unmasked ocular Ags (5, 7). Characteristically, SK also includes pathological neovascularization that can be extensive, especially in animal models of SK (8).

The therapeutic management of SK usually targets the inflammatory reaction, but controlling pathological angiogenesis also represents a useful approach, as has been demonstrated in experimental situations (9, 10). Although several molecules may be involved in pathological neovascularization, the vascular endothelial growth factor (VEGF) family of proteins, especially VEGF-A signaling through the VEGF receptor 2 (VEGFR-2), is likely to be the most consequential (11, 12). In support of this, inhibiting VEGF-A, or interfering with its receptor function, were shown to be effective therapeutic strategies in mice models of SK (8–10).

One unsolved issue with regard to VEGF-A and pathological angiogenesis in SK is the source of the VEGF-A that induces neovascularization. Initial studies indicated that VEGF-A was synthesized de novo in epithelial cells as a consequence of the infection, although it is not clear whether the primary source of VEGF-A is virus-infected cells themselves or nearby cells exposed to products released from infected and dying cells (13, 14). Another VEGF-A source, especially after the initial event of SK pathogenesis, may be infiltrating inflammatory cells, especially neutrophils that are prominent cells in SK lesions (8). More recently, it was realized that VEGF-A is continuously produced in biologically functional amounts by corneal epithelial cells. However, this source of VEGF-A fails to mediate angiogenesis because it is bound to a soluble form of the VEGF receptor 1 (sVR-1) (15). This so-called VEGF-A trap may be the primary reason the un-damaged cornea is not vascularized (15). Curiously, angiogenesis can be elicited in the normal eye if the bond between VEGF-A and sVR-1 is disrupted in some way (15). Of interest, some species fail
to express sVR-1, and in one genetic disease of humans, synthesis of the sVR-1 molecule is defective (16). Both situations result in corneal vascularization.

In light of the existence of the VEGF-A trap in normal eyes, pathological angiogenesis, as, for example, can occur after HSV-1 infection, could also be influenced by the balance between VEGF-A and the sVR-1 molecules. We analyze this possibility in this article. Our results confirm the previous report that VEGF-A is present and bound to sVR-1 in normal ocular tissue (15). We further show that one consequence of HSV-1 infection is the occurrence of an imbalance in the levels of VEGF-A and sVR-1 mRNA and proteins. Our results could mean that therapies designed to increase the presence, or retard the breakdown, of sVR-1 may be useful approaches to help manage the severity of SK, an important cause of blindness.

Materials and Methods

Mice, virus, and cell lines

Female 6- to 8-wk-old C57BL/6 mice were purchased from Harlan Sprague Dawley (Indianapolis, IN). Animals were housed in the animal facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care at The University of Tennessee, and all experimental procedures were in complete agreement with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. HSV-1 RE Tumpey and HSV-KOS viruses were grown in Vero Cell monolayers (ATCC no. CCL81). The virus was concentrated, titrated, and stored in aliquots at −80˚C until use. MK/T-1 cell line (immortalized keratocytes from C57/B6 mouse corneal stroma) was kindly gifted by Dr. Reza Dana (Scripps Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, MA).

Corneal HSV-1 infection and clinical scoring

Corneal infections of C57BL/6 mice were conducted under deep anesthesia induced by i.p. injection of trichloremethanol (Avertin) as previously described (8). Mice were scarified on their corneas with 27-gauge needle, and a 3-μl drop containing 1 × 10^4 PFU virus was applied to the eye. The eyes were examined on different days postinfection (p.i.) for the development and progression of clinical lesion by slit-lamp biomicroscope (Kowa Company, Nagoya, Japan). The progression of SK lesion severity and angiogenesis of individually scored mice were recorded. The scoring system was as follows: 0, normal cornea; +1, mild corneal haze; +2, moderate corneal opacity or scarring; +3, severe corneal opacity but iris visible; +4, opaque cornea and corneal ulcer; +5, corneal rupture and necrotizing keratitis. The severity of angiogenesis was recorded as described previously (17). According to this system, a grade of 4 for a given quadrant of the circle represents a centripetal growth of 1.5 cm toward the corneal center. The score of the four quadrants of the eye were then summed of the circle represents a centripetal growth of 1.5 cm toward the corneal center. The score of the four quadrants of the eye were then summed.

Subconjunctival injection

Subconjunctival injections of different drugs were performed as described previously (18). In brief, subconjunctival injections were done using a 2-cc, 32-gauge needle and syringe (Hamilton, Reno, NV) to penetrate the perivascular region of conjunctiva, and the required dose of sVR-1 or metalloproteinase inhibitor (MMPI) was delivered into subconjunctival space. Control mice received isotype fusion protein or solvent used for MMPI.

Treatment of mice with recombinant sVR-1 and MMPI

Six- to 8-wk-old C57BL/6 mice were ocularly infected under deep anesthesia with 1 × 10^4 PFU HSV-1 RE Tumpey and divided randomly into groups. One group of mice was administered with 5 μg recombinant sVR-1 (rsVR-1; R&D Systems, Minneapolis, MN) in 7 μl PBS subconjunctivally on 36 h p.i. and then every alternate day from day 5 until day 13 postinfection. In some experiments, rsVR-1 was injected subconjunctivally every alternate day starting from day 7 until day 13 postinfection. Animals in the control group were given rhlgG1Fc protein (R&D Systems) following either of the regimens for respective experiment. The MMPI, marimastat (200 μg/mouse; Tocris Cookson, Ellisville, MO) was administered subconjunctivally either starting from day 2 or 5 p.i. followed by every alternate day until day 13 p.i. Mice in the control group were given the mock injections. Mice were observed for the development and progression of angiogenesis and SK lesion severity from day 7 until day 15 p.i. In some experiments, MMPI treatment was started from day 2 and continued every alternate day until day 6 p.i. Mice were sacrificed on day 7 p.i. and corneas were collected for further analysis. All the experiments were repeated at least two times.

Neutrophil depletion with mAb

Six- to 8-wk-old C57BL6 mice were ocularly infected under deep anesthesia with 1 × 10^4 PFU HSV-1 RE Tumpey and divided randomly into groups of mice. One group of animals was administered with 100 μg anti-Gr-1 mAb (RB6-8C5; BioXcell, West Lebanon, NH) i.p. on days 7 and 9 p.i. Experiments were terminated on day 11 p.i. and corneal samples were collected for further analysis. Ab treatment was given every alternate day starting from day 7 until day 13 p.i. to study the effect of neutrophil depletion on HSV-1–mediated corneal angiogenesis. Mice were observed for the development and progression of angiogenesis and SK lesions until day 15 p.i. Animals in control group were given isotype control (IgG2b) Ab (LT/E-2; BioXcell) following the same regimen. All experiments were repeated at least two times.

Immunofluorescence staining

For immunofluorescence staining, the eyes from naive uninfected mice were enucleated and snap frozen in OCT compound. Six-micrometer-thick sections were cut, air-dried, and fixed in acetone/methanol (1:1) at room −20˚C for 10 min. Sections were blocked with 10% goat serum containing 0.05% Tween 20 and 1:200 dilution of Fc block (Clone 2.4G2, BD Biosciences-Pharmingen, San Diego, CA). Rat anti–sVR-1 (141515; R&D Systems) was diluted in 1% BSA containing 0.1% Triton X-100 and incubated at room temperature for 1 h. After incubations, sections were washed several times with PBS Tween-20 and then stained with rabbit anti-rat Alexa 488 for 45 min. The corneal sections were repeatedly washed with PBS Tween-20 and mounted with ProLong gold antifade mounting media containing DAPI as a nuclear stain (Molecular Probes, Invitrogen) and visualized under an immunofluorescence microscope. For whole-mount corneal staining, corneas from rsVR-1 and isotype-treated mice were dissected under stereomicroscope. Corneal whole mounts were rinsed in PBS for 30 min and flattened on a glass slide under stereomicroscope. Corneal flat mounts were dried and fixed in 100% acetone for 10 min at −20˚C. Nonspecific binding was blocked with 5% BSA containing 1:200 dilution of Fc block for 2 h at 37˚C. CD31-PE (MEC13.3; BD Biosciences-Pharmingen) Ab was used at a concentration of 1:250 in 1% BSA overnight followed by subsequent washes in PBS. Corneas were mounted with ProLong Gold antifade and visualized with fluorescence microscope.

Purification of neutrophils

Ly6G+ neutrophils were purified from the pooled Liberases (Roche Diagnostics, Indianapolis, IN) digested corneal single-cell suspension obtained from HSV-infected mice using anti-Ly6G+ microbeads (Miltenyi Biotec, Auburn, CA). The purity was achieved at the extent of 90%. Purified neutrophils were further analyzed by RT-PCR and Western blot (WB) for the expression of different metalloproteinases (MMPs).

Flow cytometry

Corneas were excised, pooled groupwise, and digested with 60 U/ml Liberases for 35 min at 37˚C in a humified atmosphere of 5% CO2. After incubation, the corneas were disrupted by grinding with a syringe plunger on a cell strainer, and a single-cell suspension was made in complete RPMI 1640 medium. The single-cell suspensions obtained from corneas were stained with different cell surface markers for FACS. All steps were performed at 4˚C. In brief, cell suspension was first blocked with an unjugated anti-CD3/CD16 mAb for 30 min in FACS buffer. After washing with FACS buffer, samples were incubated with CD45-allophy- cocyanin (30-F11), CD11b-PerCP (M1/79), Ly6G-PE (1A8), CD4-allophycocyanin (RM4.5) (all from BD Biosciences-Pharmingen) for 30 min on ice. Finally, the cells were washed three times and resuspended in 1× paraformaldehyde. The stained samples were acquired with a FACScalibur (BD Biosciences), and the data were analyzed using the FlowJo software.

qPCR

Corneal cells were lysed and total mRNA was extracted using TRIzol LS reagent (Invitrogen). Total RNA was made with 1 μg RNA using oligo(dT) primer. Quantitative PCR (qPCR) was performed using SYBR Green PCR.
Master Mix (Applied Biosystem, Foster City, CA) with iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA). The expression levels of different molecules were normalized to β-actin using ΔCT calculation. Relative expression between control and experimental groups was calculated using the $2^{-\Delta\Delta CT}$ formula. The PCR primers used are included as Supplemental Table I.

**RT-PCR**

RT-PCR was performed to detect the presence of mRNA transcripts of various molecules (VEGF-A, sVR-1, β-actin, and different MMPs) according to manufacturer’s protocol (Promega, Madison, WI). In brief, cDNA was prepared from total mRNA extracted from different samples, cDNAs were amplified by PCR and were resolved on 1% agarose gel. The PCR primers used are shown in Supplemental Table II.

**Western blot analysis**

The supernatants from lysed corneal cells were quantified using bicinchoninic acid protein assay kit (Thermo Scientific, Waltham, MA) using BSA as a standard, and samples with equal protein concentrations were denatured by boiling in Laemmli buffer. Polypeptides were resolved by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with 5% BSA in Tris-buffered saline with Tween 20 (20 mM Tris [pH 7.4], 137 mM NaCl, and 0.1% Tween 20) overnight at 4°C and probed with specific primary and secondary Abs. Proteins were detected using chemiluminescent HRP substrate (Millipore, Billerica, MA). The membrane was kept in stripping buffer for 10 min and reprobed using anti–β-actin Ab. The Abs used were rat anti–sVR-1 (141515; R&D Systems), mouse anti–VEGF-A (EE02; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti–MMP-2 (8B4; Santa Cruz Biotechnology), goat anti-mouse MMP-8 (M-20; Santa Cruz Biotechnology), goat anti-mouse MMP-9 (C-20; Santa Cruz Biotechnology), goat anti-mouse MMP-12 (M-19; Santa Cruz Biotechnology), mouse anti–VEGF-A (AC74; Sigma-Aldrich, St. Louis MO), goat anti-rat IgG-HRP (R&D Systems), goat anti-mouse IgG-HRP (Santa Cruz Biotechnology), and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology).

**Coimmunoprecipitation**

Coimmunoprecipitation was performed using Pierce Coimmunoprecipitation kit according to manufacturer’s protocol (Thermo Scientific). In brief, 50 μg anti–sVR-1 Ab was immobilized to Ab coupling resin by incubating the Ab to resin containing column for 2 h at room temperature. Care was taken to keep resin slurry in suspension during incubation. The 200-μl supernatant from lysed corneal cells (800 μg total protein) was incubated for 2 h at 4°C with the earlier mentioned resin immobilized Ab. Finally, 50 μl of the elution buffer was added, and immunoprecipitate was eluted out and analyzed by WB.

**ELISA**

The pooled corneal samples were homogenized using a tissue homogenizer, and supernatant was used for analysis. The concentrations of VEGF-A and sVR-1 were measured using Quantikine sandwich ELISA kits (R&D Systems) according to the manufacturer’s protocol.

**Quantification of total number of molecules for sVR-1 and VEGF-A**

The total number of sVR-1 and VEGF-A molecules from naive and different day p.i. samples were calculated using following formula: total number of molecules = quantity of protein × molecular size × Avogadro’s number.

**In vitro assay for VEGF-A and sVR-1 production**

A mouse stromal fibroblast cell line (MK/T-1) was used to study differential effect of HSV-1 on production of VEGF-A and sVR-1. The cells were cultured and plated onto six-well tissue culture plates in 10% DMEM. The cells around >90% confluent were infected with HSV-KOS at 3 multiplity of infection (adsorption for 1 h). Uninfected cells were used as control. Cells were harvested at different time points, and total mRNA was extracted and used to quantify the mRNA levels of the VEGF-A and sVR-1.

**Degradation assays**

Degradation assays were performed as previously described (19). In brief, 100 ng sVR-1 (R&D Systems) was incubated with active mouse MMP-2, -7, -8, -9, and -12 (R&D Systems), at 1:1 substrate/ enzyme molar ratio at 37°C for 24 h. Pro-MMPs were activated by p-aminophenylmercuric acetate (Sigma-Aldrich) before use according to manufacturer’s protocol. To study the degradation of mouse native sVR-1 from cornea, the supernatant from lysed corneal cells was collected and incubated with different activated MMPs (1:1 substrate enzyme molar ratios) at 37°C for 24 h. MMP activity was inhibited using 20 mM EDTA. The samples were then analyzed by WB.

**Statistics**

Student t test was performed to determine statistical significance, and data are expressed as mean ± SEM.

**FIGURE 1.** Normal cornea expresses VEGF-A bound to sVR-1. Wild-type (WT) mice were sacrificed, and four to six corneas were collected and pooled for WB, coimmunoprecipitation, and mRNA analysis by RT-PCR. Data are representative of two independent experiments. A, Reducing WB analysis for sVR-1 and VEGF-A reveals presence of sVR-1 at 62 kDa and two isoforms of VEGF-A at 22 and 16 kDa in normal cornea. B, Nonreducing WB for VEGF-A shows the presence of a much larger band between molecular size of 100 to 150 kDa consistent with its being the bound form. C, sVR-1 was immunoprecipitated from normal corneal extracts, with anti–sVR-1 mAb, and the complex was analyzed for the coimmunoprecipitation of VEGF-A by WB. Normal cornea shows the presence of VEGF-A bound to sVR-1. D, Agarose gel analysis of VEGF-A (214 bp; lane 3) and sVR-1 (358 bp; lane 5) cDNA from normal uninfected cornea. Lane M, marker; lane 1, β-actin (92 bp); lane 2, 4, and 6, reverse transcriptase negative controls for β-actin, VEGF-A, and sVR-1. E, Representative immunofluorescence staining of corneal section for sVR-1 (left panel); nuclei (middle panel) reveals the presence of sVR-1 mainly in the corneal epithelial layer and, to a lesser extent, in the stroma (Merge). Nuclei were stained with DAPI. Image is representative of two independent experiments. Original magnification ×10.
FIGURE 2. Corneal HSV-1 infection causes an imbalance between VEGF-A and sVR-1. WT mice corneas were scarified and infected with 10^4 PFU HSV in PBS or mock infected with only PBS (naive control mice). Corneas were harvested from infected mice at indicated time points p.i. and from naive control mice at 24 h p.i. At each time point, six corneas were collected and pooled for mRNA extraction. The expression levels of VEGF-A molecule were normalized to $\beta$-actin using $\Delta$Ct calculation. Relative expression between control and infected groups was calculated using the $2^{-\Delta\Delta C_t}$ formula. Kinetic analysis for the expression of VEGF-A mRNA by qPCR at different days p.i. The expression of VEGF-A shows a biphasic upregulation pattern, early on day 2 p.i. and then again on day 11 p.i. as compared with naive cornea. Figure is a summary of two independent experiments, and each experiment represents a group of six corneas.

B, At each time point, six corneas were harvested from infected or naive mice and the levels of VEGF-A were determined by ELISA. Quantification of VEGF-A protein levels in the normal and infected corneas at different days p.i. reveals a similar pattern as that of gene expression. Figure is a summary of at least two independent experiments, and each experiment represents a group of six corneas.

C, At each indicated time point, three to four corneas were harvested, and total protein concentrations were measured by bicinchoninic acid protein assay. Samples with equal protein concentration were subjected to SDS-PAGE followed by WB analysis. VEGF-A expression was analyzed by WB analysis and showed an increase in the VEGF-A protein levels at different days p.i. compared with naive control mice. D, Three to four corneas were harvested from day 3 p.i. or naive control mice at 24 h p.i. Samples with equal protein concentration were subjected to native PAGE followed by WB analysis. Left panel shows a band of molecular size between 100 to 150 kDa in naive corneal sample. Right panel is from day 3 infected corneas. It shows the large 100- to 150-kDa band, as well as the 62-kDa band.
Results

Presence of VEGF-A in ocular tissues of normal mice

Recently, it was demonstrated that VEGF-A is present in normal corneal tissues, but it fails to induce angiogenesis because it is bound to sVR-1 (15). Both VEGF-A and sVR-1 are produced continuously by normal ocular tissues. As a prelude to measuring the influence of HSV-1 ocular infection on the balance between VEGF-A and sVR-1, we repeated some of the normal mice experiments previously described (15). We were able to confirm many of their observations. Accordingly, as shown in Fig. 1, corneal extracts from naive mice analyzed by WB under reducing conditions revealed a band of 62 kDa for sVR-1 and two bands for VEGF-A at molecular sizes of 22 and 14 kDa (Fig. 1A). These bands correspond to two isoforms of VEGF-A. However, under nonreducing conditions, blots revealed only a single band that was far larger than those observed in reducing gels and had an apparent size of between 100 and 150 kDa (Fig. 1B). This would be consistent with VEGF-A being bound to another molecule, presumably sVR-1. Support for this was obtained performing coimmunoprecipitation that revealed the interaction of VEGF-A and sVR-1 (Fig. 1C).

The fact that normal ocular tissue expressed mRNA for both the VEGF-A and the sVR-1 proteins was also demonstrated by RTPCR (Fig. 1D). The sVR-1 molecule was also readily detectable in epithelial and stromal layers of corneal sections by immunohistochemistry (Fig. 1E). Our findings are consistent with and confirm those reported by the Ambati group, and demonstrate that VEGF-A is present in cornea and is mostly bound to sVR-1, forming a so-called VEGF-A trap (15, 20, 21).

Changes in VEGF-A and sVR-1 as a consequence of HSV-1 infection

The principal objective of our study was to evaluate whether the VEGF-A trap affected the outcome of pathological angiogenesis caused by ocular infection with HSV-1. To do this, mice were ocularly infected with virus, and the expression levels of both VEGF-A and sVR-1 mRNA and proteins were measured and compared with controls at various times p.i. We focused on initial time points postinfection when replicating virus is readily detectable in the cornea (up to 5 d p.i.), as well as at the stage of clinical SK (day 8 p.i.) when replicating virus is no longer demonstrable and the stroma is heavily infiltrated by inflammatory cells, particularly neutrophils (4, 22). At each time point, six corneas were collected and pooled for analysis from infected and control animals by qPCR. The data expressed in Fig. 2, A and B, shows a significant decrease in the expression of sVR-1 mRNA at different days p.i. Relative change in the expression of sVR-1 mRNA by qPCR at different days p.i. as compared with the uninfected mice revealed a sharp decrease in the sVR-1 expression from day 3 p.i., which reached above normal around day 11 p.i. and back to normal levels at day 14 p.i. The figure is a summary of two independent experiments, and each experiment represents a group of six corneas. F, sVR-1 protein levels analyzed by ELISA from the normal and infected corneas at different days p.i. shows an early decrease after HSV-1 infection that reaches to level found in normal controls at day 14 p.i. The figure is a summary of at least two independent experiments, and each experiment represents a group of six corneas. *p ≤ 0.05, **p = 0.0023, ***p ≤ 0.0001. G, Quantification of total number of sVR-1 molecules per VEGF-A molecule from naive and infected corneas at different days p.i. using protein quantities obtained by ELISA (B, F) shows the significant reduction of total number of sVR-1 molecules per VEGF-A molecule on day 1 and from day 7 to day 14 p.i. The total number of molecules for sVR-1 and VEGF-A were calculated by multiplying the total quantity of the protein by its m.w. and Avogadro’s number. As another at 50 kDa indicating the presence of both bound and free VEGF-A. E, Kinetic analysis for the expression of sVR-1 mRNA by qPCR at different days p.i. Relative change in the expression of sVR-1 mRNA at different days p.i. as compared with the uninfected mice revealed a sharp decrease in the sVR-1 expression from day 3 p.i., which reached above normal around day 11 p.i. and back to normal levels at day 14 p.i. The figure is a summary of two independent experiments, and each experiment represents a group of six corneas. F, sVR-1 protein levels analyzed by ELISA from the normal and infected corneas at different days p.i. shows an early decrease after HSV-1 infection that reaches to level found in normal controls at day 14 p.i. The figure is a summary of at least two independent experiments, and each experiment represents a group of six corneas. *p ≤ 0.05, **p = 0.0023, ***p ≤ 0.0001. G, Quantification of total number of sVR-1 molecules per VEGF-A molecule from naive and infected corneas at different days p.i. using protein quantities obtained by ELISA (B, F) shows the significant reduction of total number of sVR-1 molecules per VEGF-A molecule on day 1 and from day 7 to day 14 p.i. The total number of molecules for sVR-1 and VEGF-A were calculated by multiplying the total quantity of the protein by its m.w. and Avogadro’s number. The figure is a summary of values obtained from B and F. *p = 0.0216, **p = 0.0032, ***p ≤ 0.0002. H, Reducing WB analysis for sVR-1 from uninfected and infected corneas at different time p.i. shows degradation of sVR-1 after HSV-1 infection. Degraded products are indicated by black arrowheads.
FIGURE 3. rsVR-1 administration hinders angiogenic activity of VEGF-A and inhibits corneal angiogenesis post HSV-1 infection. WT mice were ocularly infected by corneal scarification with 10^4 PFU HSV. A, rsVR-1 or isotype Fc protein was administered subconjunctivally at 5 μg/eye as shown. The extent of angiogenesis and SK lesion severity in the eyes of HSV-infected mice was quantified in a blinded manner using a scale as described in Materials and Methods. The progression of angiogenesis and SK lesion severity was significantly reduced in the group of mice treated with rsVR-1 as compared with isotype-treated mice. Data are representative of two independent experiments (n = 10–12 mice/group). **p ≤ 0.0007. B, Representative whole-mount corneas stained for CD31 (lower panel) at day 15 p.i. shows reduced angiogenic response in rsVR-1–treated mouse. Scale bars, 100 μm. C, Representative eye photos show reduced SK lesion severity, as well as angiogenesis, from rsVR-1–treated mouse compared with isotype-treated mouse. D, rsVR-1– or isotype Fc protein-treated mice were sacrificed on day 15 p.i., and corneas were collected for surface staining of eye-infiltrating cells. Total cell numbers per
The Journal of Immunology

points p.i. (Fig. 2H). In this study, it was evident in corneal extracts from uninfected animals that sVR-1 protein was represented almost entirely as a single band at ~60 kDa size. However, by day 3 p.i., there was notable fragmentation of the sVR-1, and this situation was also evident in samples collected at other time points postinfection. The degradation of sVR-1 could explain why we observed minimal sVR-1 protein at day 11 time point even though sVR-1 mRNA levels peaked at this time. These observations led us to conclude that HSV infection of the cornea diminished the expression of sVR-1, and that the intact protein was degraded, at least partially, at different phases of virus infection. Both effects would serve to diminish the inhibitory activity of sVR-1 on VEGF-A–induced angiogenesis.

An additional series of experiments supported the notion that the activity of sVR-1 has a constraining effect on the angiogenic activity of VEGF-A. We anticipated that the provision of an additional exogenous source of sVR-1 protein to HSV-1–infected mice might act to diminish the extent of angiogenesis that occurs subsequent to infection. To evaluate this possibility, we obtained mouse rsVR-1 fusion protein that had previously been used by others to counteract VEGF-A activity in a VEGF-A–induced micro-pocket assay for angiogenesis (15). In our pathological system, we chose to administer a higher dose rsVR-1, or an isotype fusion protein (isotype). To evaluate the possible VEGF-A inhibitory effects in vivo, animals were given either rsVR-1 or isotype subconjunctivally to HSV-1–infected animals, either starting at day 1 (Fig. 3A) or day 7 p.i. (Fig. 3E). Animals were followed until day 15 p.i. to measure the extent of angiogenesis. Results shown in Fig. 3A–C and Supplemental Fig. 2A indicated that rsVR-1 recipients had significantly diminished angiogenic responses, as well as SK lesion severity, compared with the isotype group. Corneal cell preparations collected at the end of the experiment revealed diminished frequency and numbers of CD31+ (a marker for blood vessel endothelial cells), neutrophils, as well as CD45+ T cells (Fig. 3D, Supplemental Fig. 2B). It is of particular interest to note that even when rsVR-1 administration was delayed until day 7 p.i. (Fig. 3E), a significant level of SK severity and angiogenesis inhibition was still evident. These results further support the concept that sVR-1 may limit the angiogenic response to VEGF-A in pathological angiogenesis.

Metalloproteinases could be responsible for sVR-1 degradation

The observation that sVR-1 protein may be degraded in HSV-1–infected corneas raised the question of how the breakdown was accomplished. Likely enzyme candidates include MMPs, some of which were shown to be present in damaged eyes in other systems (1). Previous reports suggest that MMP-9 could induce neovascularization in vivo and inhibition of MMP-9 reduces angiogenesis (27). Moreover, MMP-9 protein is present in the cornea after HSV-1 infection, as we have reported previously (28). Experiments were performed to measure the expression profile by qPCR of several MMP mRNAs at various time points after HSV-1 infection. The results shown in Fig. 4A demonstrate that compared with uninfected controls, expression levels of several MMPs were increased after the infection. Peak levels that varied between experiments differed between the MMPs tested, but always occurred between days 5 and 11 p.i. Of the multiple MMP mRNAs examined, expression levels of MMP-9 were the most elevated, with its peak evident in day 7 p.i. samples (average 249-fold). Another MMP of interest was MMP-7 because this enzyme was shown recently to be uniquely capable degrading human sVR-1 (19). Levels of MMP-7 were at their peak on day 11 p.i. Additional experiments were done in vitro with the activated form of selected MMPs (MMP-2, -7, -8, -9, and -12) to measure their ability to degrade rsVR-1 in vitro as detected by WB. As is evident in Fig. 4B, MMP-7 was most effective at degrading rsVR-1 with MMP-9 also active, but less so. In these assays, MMP-8 and MMP-12 were without activity, and MMP-2 had a minimal effect. In additional experiments, MMP-2, -7, and -9 were shown to degrade sVR-1 present in naïve corneal extracts (Fig. 4C).

Experiments were also done to determine whether inhibiting the function of MMP enzymes in vivo affected sVR-1 degradation, as well as the outcome on the extent of angiogenesis and SK severity. To accomplish MMP inhibition, we administered marimastat (29–31) subconjunctivally at different times p.i. and compared the results with infected mice given subconjunctival injections of mock. In an experiment in which animals received marimastat on days 2, 4, and 6 p.i., corneal extracts examined by WB on day 7 p.i. revealed a markedly reduced level of sVR-1 degradation compared with controls (Fig. 5A). In another experiment, two marimastat injections given on days 2 and 4 also revealed inhibited sVR-1 degradation in samples examined on day 5 (data not shown).

When the outcome of angiogenesis and SK severity was compared in treated and control mice, the marimastat recipients had significantly reduced levels of angiogenesis and SK severity, at least at day 15 p.i. time point, when experiments were terminated (Fig. 5B). Corneal cell preparations collected at the end of the experiment revealed diminished frequency and numbers of CD31+ endothelial cells, neutrophils, and CD45+ T cells (Fig. 5C, Supplemental Fig. 3A). Furthermore, in an experiment in which marimastat treatment was begun on day 5 p.i. and given additional injections on days 7, 9, and 11, there was also a significant reduction of angiogenesis and lesion scores at day 15 p.i. (Fig. 5D). Corneal cell preparations collected at the end of the experiment revealed diminished frequency and numbers of CD31+ endothelial cells, neutrophils, and CD45+ T cells (Fig. 5E, Supplemental Fig. 3B). Taken together, our results support the concept that MMPs, particularly MMP-2, -7, and -9, are upregulated after HSV-1 infection, and these enzymes are responsible for degrading the sVR-1 protein that, in turn, constrains the angiogenic activity of VEGF-A.

Neutrophils play multiple roles in ocular angiogenesis

A prominent cell type in the inflammatory response to ocular HSV-1 infection is the neutrophil (8, 13, 22). To investigate the role played by neutrophils in HSV-1–mediated corneal angiogenesis, we performed kinetic studies for neutrophil infiltration in the cornea after HSV infection. As shown in Fig. 6A, neutrophils constitute about 30–80% of total infiltrated leukocytes at all tested time points and infiltrate the cornea in a biphasic manner. An initial wave of infiltration peaked around day 2 p.i. followed by cornea for CD31+ cells, neutrophils (CD11b+, Ly6G+ cells) gated on total CD45+ cells, and CD45+ T cells showed the significant reduction in total numbers of the respective cell population from sVR-1–treated mice as compared with the isotype-treated mice. Data are representative of two independent experiments (n = 3–5/group). *p = 0.012; **p = 0.0015. E: Therapeutic administration of the rsVR-1 was started from day 7 p.i. when SK becomes visibly evident. Therapeutic administration of rsVR-1 also showed significant reduction in angiogenesis, as well as lesion severity, further confirming the inhibitory effect of sVR-1 on the angiogenic activity of VEGF-A. Data are representative of two independent experiments (n = 8 mice/group). *p ≤ 0.012.
FIGURE 4. Metalloproteinases are upregulated after HSV-1 infection, and MMP-2, -7, and -9 degrade rsVR-1 and sVR-1. A, WT mice corneas were scarified and infected with 10^6 PFU HSV in PBS or mock infected with only PBS (naive control mice). Corneas were harvested from infected mice at indicated time points p.i. and from naive control mice at 24 h. At each time point, six corneas were collected and pooled for mRNA extraction. The expression levels of different MMP molecule were normalized to β-actin using ΔΔCt calculation. Relative expression between control and infected groups was calculated using the 2^-ΔΔCt formula. Kinetic analysis for the relative fold change in the expression levels of MMP-2, -7, -8, -9, and -12 mRNAs at different days p.i. The mRNA expression of all the tested MMPs was upregulated in biphasic manner after HSV-1 infection, with the first peak expression at day 1 p.i. and maximum expression between days 7 and 11 p.i. B, Mouse rsVR-1 was incubated with activated form of MMP-2, -7, -8, -9, and -12 for 12 h at 37˚C and analyzed by WB. MMP-7 (lane 3) showed maximum degradation of rsVR-1 followed by MMP-9 (lane 5) and MMP-2 (lane 2). Addition of the MMPi EDTA inhibited the degradation of rsVR-1 by MMP-7 (lane 7) and MMP-9 (lane 8). Figure is a representative of three independent experiments. C, Corneal lysates from four to six pooled corneas of uninfected WT mice were incubated with activated form of MMP-2, -7, -8, -9, and -12 either for 6 or 12 h at 37˚C. The digested corneal lysates were analyzed by WB for sVR-1. MMP-7 showed degradation of corneal sVR-1 even after 6 h of incubation (upper panel, lane 2), with complete degradation after 12 h of incubation (lower panel, lane 2). MMP-2 (lower panel, lane 1) and MMP-9 (lower panel, lane 4) also showed degradation of corneal sVR-1 after 12 h of incubation. Addition of MMPi EDTA with MMP-7 (lane 6) showed inhibition of sVR-1 degradation. Figure is a representative of three independent experiments.

Discussion

In this report, we investigate mechanisms by which HSV-1 infection of the eye results in neovascularization, an essential step in pathological angiogenesis, we did experiments to compare the extent of angiogenesis and differences in expression levels of VEGF-A and sVR-1 in mice depleted of neutrophils compared with controls. Depletion (with anti-Gr1 mAb) was commenced on day 7 p.i. As shown in Fig. 6I, whereas the extent of angiogenesis and SK lesion severity continued to increase in magnitude in isotype Ab-treated animals, by day 15 p.i., when the experiment was terminated, the average angiogenic response and SK severity were significantly decreased in animals treated with neutrophil-depleting Ab. Although cell types in inflammatory reaction other than neutrophils can express Gr1, neutrophils are the predominant Gr1+ population in lesion during clinical phase of SK. We interpret Gr-1 depletion experiment where the VEGF-A levels were decreased to mean that neutrophils represent a major source of the VEGF-A that is involved in HSV-1–induced angiogenesis even though a minority of other cell types in inflammatory reaction other than neutrophils can express Gr1. Taken together, these results demonstrate that neutrophils contribute to the severity of the pathological angiogenic response caused by HSV-1 infection. This occurs because neutrophils can act as one of the sources of VEGF-A, as well as the MMP enzymes that degrade sVR-1, the molecule that binds to VEGF-A and inhibits its angiogenic activity (Fig. 7).

To show the influence of neutrophils during HSV-1–induced pathological angiogenesis, we did experiments to compare the decline and second wave starting from day 8 that peaked around day 15 p.i. (Fig. 6B). Because neutrophils studied in other systems are known to be a source of different MMPs, as well as VEGF-A (28, 32–34), we anticipated that neutrophils could be a source of MMP enzymes that act to increase angiogenesis by breaking down and releasing bioactive VEGF-A from its bond to sVR-1. In support of this, we purified neutrophils from the day 2 infected corneas, and WB analysis was conducted to check the presence of protein for MMP-2, -8, -9, and -12. Fig. 6C shows the presence of MMP-2, -8, -9, and -12. In addition, depleting neutrophils from day 7 p.i. (Fig. 6D) in infected mice led to significantly diminished VEGF-A protein levels and restored levels of sVR-1 protein in day 11 samples (Fig. 6E). QPCR analysis also revealed the decreased expression of VEGF-A and MMP-9 in the depleted mice as compared with isotype Ab-treated mice (Fig. 6F). To further implicate neutrophils as a source of VEGF-A during the clinical phase of SK, we purified neutrophils from pooled corneas at day 11 p.i., and carried out RT-PCR and WB to check for the presence of VEGF-A mRNA message, as well as protein. Both VEGF-A mRNA (Fig. 6G) and protein (Fig. 6H) could be demonstrated, supporting a role for neutrophils in contributing to angiogenesis during the clinical phase of SK when replicating virus is no longer present in the cornea.
FIGURE 5. Blocking MMPs activity in vivo rescues sVR-1 degradation and diminishes angiogenesis and SK severity. WT mice corneas were scarified and infected with 10^4 PFU HSV or mock infected with only PBS (naive control mice). Marimastat, a broad-spectrum MMPi, was administered subconjunctivally at 200 μg/mouse as shown. Mice in control group received HSV infection with mock treatment. A, WB analysis of the corneal lysates collected from naive (N), control-treated (C), and MMPi-treated mice at day 7 p.i. shows that MMPi rescues degradation of sVR-1 after HSV-1 infection. The figure is a representative of two independent experiments. B, Preventive treatment of MMPi in mice infected with HSV-1 was started from day 2 p.i. as shown. MMPi treatment showed a significant reduction of angiogenesis, as well as SK lesion severity, at day 15 p.i. Data are representative of two independent experiments (n = 9–12 mice/group). *p ≤ 0.04, **p = 0.0065. C, MMPi-treated or control mice were sacrificed on day 15 p.i., and corneas were collected for surface staining of eye-infiltrating cells. Analysis for total cell numbers per cornea for CD31+ cells, neutrophils (CD45+CD11b+Ly6G+ cells), and CD4+ T cells showed significant reduction in frequencies, as well as total numbers of the respective cell population in the MMPi-treated mice. Data are representative of two independent experiments (n = 7/group). *p = 0.0115, **p = 0.0078, ***p = 0.0002. D, Therapeutic administration of the MMPi (started from day 5 p.i.) also showed reduced angiogenesis, as well as SK lesion severity, at day 15 p.i. Data are a summary of two independent experiments (n = 6 mice/group). *p ≤ 0.021. E, Analysis of total cell numbers per cornea for CD31+ cells, neutrophils (CD45+CD11b+Ly6G+ cells), and CD4+ T cells also showed the significant reduction in the total cell numbers per cornea after preventive MMPi treatment. Data are representative of two independent experiments (n = 5–6/group). **p ≤ 0.0094.
FIGURE 6. Neutrophil, the principal cell in cornea after HSV-1 infection, is the source of MMPs and VEGF-A. Wild-type (WT) mice corneas were scarified and infected with 10^5 PFU HSV or mock infected with only PBS (naive control mice). At each indicated day p.i., two mice were sacrificed, corneas were harvested, and single-cell suspensions were analyzed for surface staining by flow cytometry. A. Representative FACS plot from each time point shows the percentage of neutrophils (CD11b+Ly6G+) gated on total CD45+ cells. B. Total number of neutrophils per cornea at each indicated time point after HSV-1 infection. Data are expressed as mean ± SEM. Figure is a summary of two independent experiments, and each experiment represents a group of two corneas. C. Ly6G+ neutrophils were purified from day 2 p.i. corneas using anti-Ly6G beads. Cell suspension from six to eight pooled corneas was used for neutrophil purification. Western blot analysis for MMP-2, -8, -9, and -12 from purified neutrophils. Data are from a single experiment and represent each sample represents neutrophils from six to eight corneas. 

FIGURE 7. Scheme illustrating the mechanism for HSV-1-mediated corneal angiogenesis. Normal uninfected cornea constitutively secretes large amount of sVR-1 and small amounts of VEGF-A. sVR-1 constrains the physiological effect of VEGF-A by binding it with very high affinity (left panel). Early after corneal HSV-1 infection, there is sudden increase in the levels of VEGF-A primarily being produced by infected or nearby uninfected corneal epithelial cells. However, levels of sVR-1 decline mainly because of decreased production of sVR-1 by corneal epithelial cells and also because of degradation by MMP-2, -7, and -9. This, in turn, leads to more physiologically active VEGF-A, which is now free from inhibitory effect of sVR-1. This active form of VEGF-A drives the initial angiogenic sprouting early after HSV infection (middle panel). During chronic phase of HSK, when HSV-1 is no longer detected in the cornea, inflammatory cells, particularly neutrophils, act as a source of VEGF-A and different MMPs. This second wave of VEGF-A and MMPs further maintains the angiogenic response by continuous supply of active form of VEGF-A and MMPs, which degrades sVR-1. The development of new leaky blood vessels leads to the release of more plasma and inflammatory cells in the cornea, thus setting the stage for chronic SK and blindness (right panel).
cells, to exert more effective angiogenic activity. Procedures that decrease the breakdown of sVR-1, such as the administration of the MMPi marimastat or the provision of exogenous rsVR-1 protein, resulted in lessened angiogenesis. Our results support the notion that the binding of sVR-1 to VEGF-A, a so-called VEGF-A trap, acts to limit the extent of pathological angiogenesis such as that caused by ocular infection by HSV-1. It is also conceivable that future therapies to control SK could benefit by procedures that influence the production and activity of the sVR-1. Our overall results are summarized in Fig. 7, showing how HSV-1 infection leads to corneal angiogenesis.

The pathogenesis of SK involves multiple events that include neovascularization of the normally transparent tissue anterior to the retina. Thus, any new blood vessel development in the anterior tissues damages vision by diffracting light, and because new vessels are leaky (4, 11), they readily permit escape of inflammatory cells that further contribute to the loss of transparency. Accordingly, preventing, and ideally reversing, neovascularization is an important objective to retain optimal vision. Numerous mechanisms have been suggested to explain how HSV infection could cause neovascularization (8, 28, 36), but perhaps the most important is the induction of VEGF family members, especially VEGF-A, which stimulates corneal angiogenesis by engaging the receptor VEGFR-2 (8, 10). Supporting this, prior studies have shown that counteracting the VEGF-A made or inhibiting its receptor can significantly reduce HSV-induced angiogenesis (8, 10).

Others, as well as ourselves, have already reported that HSV infection of the corneal epithelium in the early stages of keratitis results in the expression of VEGF-A protein either in infected or nearby cells (13, 14). What we had not appreciated, until the observations of the Ambati group (15) appeared, is that VEGF-A is continually produced by uninfected normal eyes but fails to cause corneal vascularization because the VEGF-A is bound to a soluble form of one of its receptors (15). The important aspect of our study is our observations that the binding of sVR-1 to VEGF-A made during a pathological disease process may also act to modulate VEGF-A activity and influence the magnitude of angiogenesis. A series of observations led to this conclusion.

First, in the early stages after HSV infection, much of the VEGF-A detected by WB under native conditions was of a molecular size consistent to its being bound to another protein that was shown to be sVR-1. Of interest, soon postinfection, one consequence of HSV-1 was to exert a differential effect on gene expression of VEGF-A and sVR-1 proteins. Accordingly, whereas infection caused an increased expression of VEGF-A, the production of the sVR-1 was inhibited. In addition, that infection of cells could enhance VEGF-A, but suppress sVR-1, gene expression could also be shown by in vitro studies. Thus, the initial angiogenesis observed postinfection could be the consequence of enhanced VEGF-A activity arising both from increased production and diminished inhibition. In support of the latter mechanism, if an additional exogenous source of sVR-1 was provided during the early stages of the disease process, the outcome was diminished angiogenesis. These observations taken together support the idea that a VEGF-A trap operates to limit the extent of pathological angiogenesis at least early postinfection.

It is characteristic of HSV-1 ocular infection in the mouse model that the virus is cleared within a few days, but the extent of angiogenesis continues to advance, as does the chronic inflammatory process in the corneal stroma that is indicative of SK (4, 8). Our studies show that VEGF-A may also be responsible for the additional angiogenesis, but the principal cell source of the VEGF-A is no longer the epithelium (that is no longer infected), but instead derives from invading inflammatory cells, particularly neutrophils.

We could show that depleting Gr1+ cells with specific mAb on day 7 p.i. diminished the extent of the neovascular response compared with nondepleted controls, indicating their role in VEGF-A production. Depleted animals also had less VEGF-A protein in corneal extracts when quantified by ELISA. However, we anticipated that at the later phases of the disease process, the activity of VEGF-A was less likely to be impeded by its binding to the sVR-1 protein. Thus, in addition to being a source of VEGF-A, the invading inflammatory cells are well-known to produce many different MMP enzymes (28, 37), some of which could degrade proteins that bind to VEGF-A and limit its activity (34). For example, in some cancer systems where VEGF-A is shown to be bound to a matrix protein, MMP-9 may degrade the matrix protein, thus releasing VEGF-A to exert angiogenic activity (38). Moreover, VEGF-A processed by different MMPs retains biological activity (39). Also of relevance, MMP-7 was shown recently to degrade human sVR-1, resulting in increased angiogenic activity in an in vitro tube formation assay using HUVECs (19). In this study, we could demonstrate that the inflammatory exudate cells could express several MMPs, and that at least two of them (MMP-7 and -9) were highly effective in vitro at degrading the mouse sVR-1 protein, which had not previously been reported. We could show the effect using an rsVR-1 protein, as well as with sVR-1 preparations isolated from normal mouse corneas. However, of special relevance to our viewpoint as the constraining role of sVR-1 on VEGF-A activity, we could clearly demonstrate that if we treated animals with marimastat, an inhibitor of MMP enzyme activity, then the extent of the neovascular response was significantly diminished compared with untreated animals. Furthermore, WB revealed that sVR-1 protein concentrations were greater in marimastat-treated corneal samples.

Taken together, we contend that our studies have provided novel insight on mechanistic aspects of SK pathogenesis and have revealed a previously unrecognized event that could be targeted for therapeutic manipulation. Accordingly, we show that the important event of ocular angiogenesis mediated by VEGF-A is markedly influenced by the presence and activity of the sVR-1 protein. When bound to sVR-1, as occurs with all of the VEGF-A made by the normal cornea, VEGF-A is unable to mediate angiogenesis. In a pathological situation, this VEGF-A trap is also occurring, but it breaks down when levels of MMPs arrive that can degrade the sVR-1 protein (Fig. 7). It would seem logical to develop therapies to manage SK that either increase the concentration or retard the breakdown of the sVR-1 protein. Further studies are under way to investigate this issue.

Acknowledgments
We thank Dr. John Dunlop for assistance on confocal microscopy. We also thank Naveen Rajasagi, Tamara Veiga Parga, and Greg Spencer for invaluable assistance during manuscript preparation in many ways.

Disclosures
The authors have no financial conflicts of interest.

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