Control of Stromal Keratitis by Inhibition of Neovascularization

Mei Zheng,* Margaret A. Schwarz,[†] Sujin Lee,*
Uday Kumaraguru,* and Barry T. Rouse*

From the Department of Microbiology,* University of Tennessee,
Knoxville, Tennessee; and the Departments of Pediatrics and
Surgery,[†] Children's Hospital of Los Angeles, University of
Southern California, Los Angeles, California

Stromal keratitis resulting from ocular infection with herpes simplex virus is a common cause of blindness. This report investigates the role of neovascularization in the pathogenesis of stromal keratitis by measuring the outcome of treatment with the potent anti-angiogenesis cytokine endothelial monocyte-activating polypeptide II (EMAP II). We show that systemic and topical administration of EMAP II from the outset of infection resulted in markedly diminished levels of herpes simplex virus-induced angiogenesis and significantly reduced the severity of stromal keratitis lesions. EMAP II treatment had no demonstrable pro-inflammatory or toxic effects and failed to express antiviral activity. The mechanism of action of EMAP II was shown to proceed by causing apoptosis in vascular endothelial cells. Our data document for the first time the essential role of angiogenesis in the pathogenesis of stromal keratitis and also indicate that the therapy of herpetic stromal keratitis could benefit by procedures that diminish angiogenesis. (*Am J Pathol* 2001, 159:1021–1029)

Infection of the eye with herpes simplex virus (HSV) may result in a blinding immunoinflammatory lesion in the cornea called herpetic stromal keratitis (HSK).¹ Studies in mouse models have shown that HSK is a multistep process that primarily is the consequence of an immunoinflammatory reaction orchestrated by CD4⁺ T cells.^{2,3} Although most studies on HSK have focused on the participation of different cell types, the cytokines and chemokines induced, and the issue of which antigens act as targets for CD4⁺ T-cell recognition,^{4–9} the principal event of the ingrowth of new blood vessels into the normally avascularized cornea has been virtually ignored. Although it remains unclear as to the likely multiple molecules responsible for HSV-induced angiogenesis, it seems likely that this event is a necessary step in HSK pathogenesis. Accordingly, we hypothesized that proce-

dures that minimize, or preferably abrogate angiogenesis, may diminish the severity of HSK.

In the present report, we have tested this idea choosing for study a cytokine endothelial monocyte-activating polypeptide II (EMAP II) shown previously to diminish neovascularization in a tumor system as well as in corneas implanted with the angiogenic factor basic fibroblast growth factor.¹⁰ The potential advantage of EMAP II is that this cytokine seems to inhibit growing blood-vessel endothelial cells.^{10–12} Thus EMAP II might act against a range of angiogenesis factors such as could be induced by HSV infection. Several effects of EMAP II on neovessels have been described. These include direct apoptosis of vascular endothelial cells by either activating caspase 3¹⁰ or Fas-associated death domain and down-regulation of Bcl-2¹¹ as well as indirect effects on these cells by up-regulated tumor necrosis factor receptors so making the endothelial cells subject to tumor necrosis factor- α -induced apoptosis.^{13,14} EMAP II may also damage vascular beds by causing up-regulation of tissue factor that in turn leads to local activation of clotting factors and hence small vessel occlusion.¹⁵ Besides effects on vascular endothelial cells, EMAP II has potential unwanted side effects because in some circumstances local or systemic pro-inflammatory effects have been described.^{15,16} These effects seem to be fleeting and considered as insignificant by some investigators.¹⁰ Thus, on balance EMAP II represents an attractive candidate anti-angiogenesis factor to test for inhibitory effects against herpetic ocular lesions.

Our results demonstrate that daily systemic and topical EMAP II administration to mice with HSV-infected eyes, led to diminished HSK lesions. The outcome seemed to result from the effect of EMAP II on new blood vessel development rather than from some direct or indirect antiviral activity. EMAP II was also shown to significantly reduce angiogenesis in the cornea caused by the potent angiogenic factor vascular endothelial growth factor (VEGF). This later effect seemed to result from the induction of apoptosis in developing blood vessel endothelial cells.

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Address reprint requests to Barry T. Rouse DVM, DSc, Dept. of Microbiology, M409 Walters Life Sciences Building, University of Tennessee, Knoxville, TN 37996-0845. E-mail: btr@utk.edu.

Materials and Methods

Mice

BALB/c mice (4 to 5 weeks old) obtained from Harlan Sprague-Dawley (Indianapolis, IN) were acclimated for 1 week before use in a specific pathogen-free animal colony accredited by the American Association for Accreditation of Laboratory Animal Care. All experimental procedures were conducted according to the Association for Research in Vision and Ophthalmology resolution on the use and care of laboratory animals.

Virus, Virus Corneal Infection, and Treatment Protocol

Wild-type RE HSV-1 was propagated and assayed on vero cells by a plaque assay. Mice were deeply anesthetized with methoxyflurane (Metofane; Pittman-Moore, Monroeville, IL). The corneal surfaces were scarified with a 27-gauge needle and 5×10^5 pfu of RE virus were applied in a 5- μ l volume and gently massaged into the eyes. One hour before virus infection, the mice were given rEMAP II,¹⁰ in the presence of mouse serum albumin at a concentration of 1 mg/ml (Sigma Chemical Co., St. Louis, MO), 1 μ g intraperitoneally and 1 μ g topically per animal. The treatment was continued for 20 days. Separate studies were conducted in which EMAP II was not applied until day 7 after infection, and treatment was continued for 2 weeks. The level of lipopolysaccharide in the recombinant EMAP II was <15pg/ml as measured with a limulus amoebocyte lysate (LAL) kit (BioWhittaker QCL-1000; BioWhittaker, Walkersville, MD). Signs of toxicity of EMAP II were looked for after systemic and local administration. Systemic signs would include failure to eat or drink, depression, and ruffled fur. Local effects in the eye would include ocular secretion and irritation, corneal edema, and cloudiness.

Eye Swab Viral Titration

At different time points after virus infection, eye swabs (four eyes at each time point) were taken using sterile swabs soaked in McCoy medium containing 100 UI/ml penicillin and 100 μ g/ml streptomycin (Life Technologies, Grand Island, NY). The swabs were then placed in tubes containing 500 μ l McCoy medium and stored at -80°C . To detect HSV in swabs, the samples were thawed and vortexed, and 100 μ l of each sample from individually marked mice was used for quantification of virus through recovery by standard PFU assay on vero cell cultures as described elsewhere.⁴

Clinical Scoring System

Mice were examined at different times after infection for the development of clinical lesions by slit-lamp biomicroscopy (Kowa Co., Nagoya, Japan), and the severity of stromal keratitis and angiogenesis were recorded as described elsewhere.^{4,17} The severity of the HSK lesions

and angiogenesis were also recorded by a stereomicroscope (Leica, Germany) and image system (Hamamatsu, Japan). Briefly, the clinical lesion score of HSK was described as 0, normal cornea; 1, mild haze; 2, moderate haze, iris visible; 3, severe haze, iris not visible; 4, severe haze and corneal ulcer; 5, corneal rupture. In reference to the angiogenic scoring system, the method relied on quantifying the degree of neovessel formation based on two primary parameters: 1) the circumferential extent of neovessels (as the angiogenic response is not uniformly circumferential in all cases); 2) the centripetal growth of the longest vessels in each quadrant of the circle; and 3) the longest neovessel in each quadrant was identified and graded between 0 (no neovessel) and 4 (neovessel in the corneal center) in increments of ~ 0.4 mm (radius of the cornea is ~ 1.5 mm). According to this system, a grade of 4 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 to 16) for each eye at a given time point. The extent of the neovessel ingrowth was also recorded by direct measurement using calipers (Symbol of Quality, Biomedical Research Instruments, Rockville, Maryland) under stereomicroscopy.

Corneal Lysate VEGF Enzyme-Linked Immunosorbent Assay (ELISA)

Corneas at different time points were isolated and put into RPMI 1640 without serum and stored at -80°C . The corneas were homogenized in an ice bath using a tissue homogenizer (PRO Scientific Inc., Monroe, CT) for 1 minute. The corneal lysates were collected and assayed for VEGF by sandwich ELISA. The two mVEGF monoclonal antibodies used were anti-mouse VEGF antibody and biotinylated anti-mouse VEGF antibody (R & D systems, Inc., Minneapolis, MN) and mVEGF164 was used as a standard (R & D systems, Inc.).

Histopathological and Immunohistochemical Staining

At various times after infection, whole eyes were fixed in 10% buffered neutral formalin, embedded in paraffin, and tissue sections were stained with hematoxylin and eosin as described previously.⁴ Sections were observed for thickness of the cornea, the presence of inflammatory infiltrates, neovascularization, and corneal perforation. For immunohistochemistry, eyes were removed and snap-frozen in OCT compound (Miles, Elkhart, IN). Six- μ m sections were cut, air-dried, and fixed in cold acetone for 5 minutes. The sections were then blocked with 3% bovine serum albumin and stained with biotinylated anti-pan-endothelial antigen (PharMingen, San Diego, CA). Sections were then treated with horseradish peroxidase-conjugated streptavidin (1:1000) and 3,3'-diaminobenzidine (Vector, Burlingame, CA) and counterstained with hematoxylin.

Corneal Micropocket Assay

The murine corneal neovascularization model followed the general protocol of Kenyon and colleagues.¹⁸ Pellets for insertion into the cornea were made by combining rhVEGF (40 μ g, R&D system) and sulcralfate (10 mg, Bulch Meditec, Vaerlose, Denmark) and hydron polymer in ethanol (120 mg/1 ml ethanol; Interferon Sciences, Brunswick, NJ) and applying the mixture to a 15 $\times$ 15 mm² piece of synthetic mesh (Sefar America, Inc., Kansas City, MO). The mixture was allowed to air-dry and fibers of the mesh were pulled apart, yielding pellets containing 90 ng of VEGF. Pellets containing VEGF were inserted into a corneal pocket created 1 mm from the limbus at the lateral canthus of the eye under stereomicroscope. Mice were then treated with vehicle or EMAP II (2 μ g/day, 1 μ g intraperitoneally and 1 μ g topically) for the following 4 days. Then the eyes were evaluated for corneal neovascularization according to that previously described.¹⁰ Briefly the number of vessels originating from the limbus was counted over the entire orbit, and the area of angiogenesis was calculated according to the formula for an ellipse. $A = [(\text{clock hours}) \times 0.4 \times (\text{vessel length in mm}) \times \pi]/2$. Each clock hour is equal to 30° at the circumference.

Cell Culture and Cell Death Detection

Human endothelial cells were isolated from umbilical cord vein by collagenase treatment as described elsewhere¹⁹ and used at passage 1 to 4. Human umbilical vein endothelial cells (HUVECs) were plated on two-well chamber slides and allowed to rest overnight, after which the cells were starved for 6 hours in RPMI 1640 without serum. The medium was replaced with either 10 ng/ml rhVEGF (R & D Systems) or 10 ng/ml rhVEGF plus 0, 10, 100, and 1000 ng/ml EMAP II, respectively. Briefly, the cells were incubated at 37°C for 24 hours. HUVEC apoptosis and corneal cell (corneal sections) apoptosis from both EMAP II- and vehicle-treated groups were monitored by using the *in situ* cell death detection kit, alkaline phosphatase (AP) (Boehringer Mannheim). Briefly, the cells on the chamber slides or the corneal sections were air-dried and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) solution for 1 hour at room temperature. The samples were then permeabilized in 0.1% Triton X-100, 0.1% sodium citrate for 2 minutes on ice. The slides were then washed with PBS and 50 μ l of terminal deoxynucleotidyl transferase [TdT]-mediated deoxyuridinetriphosphate [dUTP] nick end labeling (TUNEL) reaction mixture was added and incubated in a humidified chamber for 60 minutes at 37°C. The HUVEC samples were then analyzed under fluorescence microscope (Leica, Germany) at this stage and the images were taken by imaging system. The corneal sections were processed further for converter-AP and substrate nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Roche Diagnostics GmbH, Mannheim, Germany) and analyzed by light microscopy.

Myeloperoxidase Assay

Myeloperoxidase, a marker for neutrophils—mainly existed in polymorphonuclear and macrophage—was detected according to the method of Bradley and colleagues²⁰ as previously described. Briefly, myeloperoxidase was extracted from the homogenized single cornea by suspending the tested corneas in 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical Co.) in 50 mmol/L potassium phosphate buffer, pH 6.0, before homogenization in an ice bath for 1 minute. The specimens were freeze-thawed three times, after which homogenization was repeated. Suspensions were then centrifuged at 10,000 $\times$ g for 15 minutes and the resulted supernatants were assayed. Myeloperoxidase was assayed spectrophotometrically: A total of 200 μ l of serially diluted corneal lysate samples and standard (myeloperoxidase, Sigma Chemical Co.) were added in duplicate and twofold diluted in 0.5% hexadecyltrimethylammonium bromide and then mixed with 100 μ l of 50 mmol/L phosphate buffer, pH 6.0 containing 0.167 mg/ml *O*-dianisidine dihydrochloride (Sigma Chemical Co.) and 0.0005% hydrogen peroxidase. The color reaction was measured by an ELISA reader (SpectraMAX 340; Molecular Devices, Sunnyvale, CA) at 460 nm. Quantification was performed with Spectramax ELISA reader software version 1.2.

Statistics

Statistical analysis was performed using Student's *t*-test. $P < 0.05$ was considered significant.

Results

HSV Infection of the Cornea Results in VEGF Production, Angiogenesis, and Chronic Stromal Inflammation

Using a well-defined model of ocular infection with HSV, susceptible BALB/c mice developed a chronic immunoinflammatory response in the corneal stroma termed herpetic stromal keratitis (HSK).^{2,21} Evident clinically ~8 days after infection, the lesion progresses in severity to peak levels ~15 to 21 days after infection.^{4,7} Initially after infection, a vigorous neutrophil response is evident histologically in the cornea but this response declines by 4 to 5 days after infection, as does the presence of demonstrable infectious virus in corneal swabs (Figure 1, A and B). Usually neither infectious virus, nor viral antigen can be demonstrated at the time HSK lesions become clinically overt (Figure 1C). The undamaged cornea is normally avascular, but after HSV infection, new blood vessel development invades from the corneal limbus (Figure 2A). The angiogenesis increases and at the time of severe HSK may reach the central corneal region (Figure 2C). At ~5 to 7 days after infection, when virus is no longer detectable and inflammatory lesions are inapparent clinically, angiogenesis up to 0.6 to 0.8 mm beyond the limbus is usually present (Figure 2B and Figure 1D).

Figure 1. HSK pathogenesis. **A:** Neutrophil infiltration in HSV corneal infection. HSV-1 RE (5×10^5) was applied on the scarified cornea. At indicated time points after infection, the mice were sacrificed and the corneas were enucleated. Routine paraffin sections were made and the infiltrating neutrophils and central corneal section were counted under microscopic examination. The number was derived from four eyes. **B:** Eye swab viral titration showed virus was cleared from the infected cornea at approximately day 5 after infection. HSV-1 RE (5×10^5) was inoculated on the scarified cornea, and at the indicated time points the presence of infectious virus in eye swabs was determined by the agarose overlay method and the number of plaque forming units (pfu) per milliliter were estimated. At each time point, eye swabs were collected from four mice ($n = 4$), and viral titers were determined. **C** and **D:** Clinical lesion and angiogenesis scores measured as described in Materials and Methods.

The means by which HSV infection results in angiogenesis has not been defined. However, as shown in Figure 3, HSV infection results in induction of the potent angiogenesis factor VEGF. In corneal lysates made with mouse corneas at various times after infection and tested with a capture ELISA assay, VEGF levels in infected eyes were significantly above those present in trauma control eyes at 48 hours after infection and beyond. Application of UV-inactivated HSV to eyes failed to induce VEGF levels above those observed in the trauma controls (data not shown). No significant differences in VEGF values were observed between EMAP II- and vehicle-treated groups at days 2 and 4 after infection with the values of 31.5 ± 5.0 and 35.2 ± 6.9 pg/ml in the vehicle-treated and 34.6 ± 7.5 and 33.2 ± 7.6 pg/ml in EMAP II-treated mice. On the contrary, significant differences in VEGF values at days 10 and 15 after infection (clinical phase) was noted

Figure 2. Angiogenic response to HSV infection. **A:** The initial neovessel formation at day 2 after infection (original magnification, $\times 20$). **B:** At day 5 after infection, angiogenesis was progressing but the cornea remains relatively clear. Original magnification, $\times 25$. **C:** To show the HSK lesion clearly, FITC-dextran (Sigma Chemical Co.) was applied topically on the corneal surface and the cornea was viewed under GFP filter by stereomicroscopy. It shows that at day 16 after infection, the cornea was covered by vigorous angiogenesis and was damaged. Original magnification, $\times 20$. The pictures were taken by stereomicroscopic imaging system.

Figure 3. Corneal lysate mVEGF164 ELISA showed that VEGF was induced in HSV-infected corneas. At different time points after infection, the infected corneas were treated with vehicle or EMAP II and trauma control corneas ($n = 4$) were isolated and stored at -80°C until use. The corneas were homogenized, the lysates were collected, and mVEGF164 was assayed by ELISA. Detectable levels of VEGF in control corneas could not be detected beyond 48 hours. VEGF levels in UV-inactivated HSV-infected corneas at all time points were the same as trauma controls (data not shown).

between the two groups with values of 81.3 ± 8.5 and 87.8 ± 9.0 pg/ml in the vehicle-treated and 53.4 ± 7.4 and 50.2 ± 8.5 pg/ml in EMAP II-treated mice.

Effect of EMAP II on Angiogenesis and HSK

Because angiogenesis may represent an essential step in the pathogenesis of HSK, the effect of the angiogenesis-blocking cytokine EMAP II on lesion severity was investigated. Initially, the effect of systemic and topical administration of various doses of EMAP II was tested in a corneal micropocket assay for anti-angiogenic effects against an optimal dose of VEGF incorporated into slow release pellets. VEGF was chosen for study because this molecule is a potent angiogenic factor^{22,23} and was induced in the eye after HSV infection. In this model, we established that a daily dose of EMAP II, $1 \mu\text{g}$ intraperitoneally and $1 \mu\text{g}$ topically for 4 days, inhibited angiogenesis (Figure 4B) by up to 65% (Figure 4C) when compared to vehicle-treated animals (Figure 4A). In addition, EMAP II was shown to cause DNA fragmentation by the TUNEL assay in cultured HUVECs stimulated with rhVEGF (Figure 5A). Evaluation of 10 random high-power fields of cultured HUVECs on chamber slides, revealed that there was a 14.5-fold increase in the number of apoptotic cells/high-power fields in 1000 ng/ml EMAP II-treated cells (29 ± 9.3) as compared to control (2.1 ± 2.0) ($P \leq 0.001$). Whereas 10 ng/ml EMAP II treatment had no significant difference in the number of apoptotic cells induced compared to the control cells ($P \geq 0.05$). This indicated that despite the presence of VEGF, EMAP II is a potent inducer of apoptosis.

Furthermore, at days 2, 6, 11, and 15 after infection, before and during clinical HSK, corneal frozen sections were stained for TUNEL-positive cells. Corneal neovascular endothelial cells showed TUNEL positivity to various

Figure 4. Corneal angiogenesis model showed the effect of rEMAP II on rhVEGF-induced neovascularization. **A** and **B**: Hydron pellets (asterisks) containing VEGF (90 ng) were implanted into the corneal pockets. Mice were treated with either vehicle or rEMAP II (1 μ g intraperitoneally and 1 μ g topically every 24 hours for 4 days), and the corneal neovascular response was assessed. **C**: The total number of neovessels originating in the limbus was counted and the area of neovascularization was calculated. Data shown are the results of eight eyes and the experiment was repeated twice.

degrees in the EMAP II-treated group at all of the time points observed (Figure 5B: day 6 after infection cornea is a representative example). However TUNEL-positive cells were not observed in the neovessels of vehicle-treated groups. Some corneal epithelial cells were also positive (Figure 5B) for TUNEL staining but few if any corneal endothelial cells showed signs of DNA fragmentation.

To measure the effects of EMAP II on the severity of lesions in HSK, groups of mice were ocularly infected with HSV and treated daily with either EMAP II (as described above) or vehicle alone. In infected mice the extent of neutrophil infiltration in corneas was measured by a myeloperoxidase assay. Infected plus vehicle and infected plus EMAP II 24-hour samples had values of 121.5 ± 15.3 and 97.5 ± 9.2 μ U, respectively, and 48-hour samples had values of 198.6 ± 14.6 and 211.4 ± 13.0 μ U, respectively. These differences were not significantly different ($P > 0.05$). Similarly, viral titers were

Figure 5. EMAP II-induced vascular endothelial cells undergo apoptosis. **A**: HUVECs were incubated with 10 ng/ml rhVEGF together with 0, 10, 100, and 1000 ng/ml EMAP II, respectively, in serum-free media for 24 hours. The apoptotic cells were shown by TUNEL assay for DNA fragmentation. Each number of the apoptotic cells was derived from 10 random high-power fields (original magnification, $\times 400$). **B**: Corneal frozen sections showed TUNEL-positive neovascular endothelial cells (arrow) in the limbal region (right) as well as in some corneal epithelial cells (original magnification, $\times 200$).

measured daily in EMAP II-treated and vehicle-treated mice. In both groups, virus was present until days 5 to 6 after infection and titers at all time periods were approximately the same.

To test the possible pro-inflammatory effect of EMAP II, neutrophil infiltration was measured at 24 hours after infection by the myeloperoxidase assay in control mice (scratched corneas only) as well as in test mice treated with EMAP II. Values of 24.6 ± 2.3 and 34.4 ± 7.2 μ U, respectively, were recorded (these values were not significantly different).

The effects of EMAP II on HSV-induced lesions, as well as angiogenesis, is shown in Figures 6 and 7. As is readily apparent, mice given EMAP II had significantly diminished HSK lesions at all phases of clinical disease (Figure 6, B and D) in comparison to controls (Figure 6, A and C). Lesions in EMAP II-treated animal were approximately twofold reduced in severity (Figure 6G), but the effect was quite variable. Thus 1 out of 24 treated eyes still showed severe lesions (score of 5) yet others had no apparent lesions ≤ 2.0 (Figure 6F). In control animals, examined at the same time points, only 2 out of 24 eyes

Figure 6. Effect of EMAP II on HSK elicited by HSV-1 infection. One hour before infection, the animal received 1 μ g of rEMAP II intraperitoneally and 1 μ g topically per animal and treatment was repeated daily for 20 days. Control animals were given 1 mg/ml of bovine serum albumin (vehicle). The eyes were examined daily by slitlamp microscopy to judge the severity of the inflammatory response. The scores were derived from 24 eyes (in two separate experiments). **A–D:** Images were taken by stereomicroscopic imaging system at days 8 and 16 after infection (original magnification, $\times 30$). The histological photos **E** and **F** showed the difference in corneal pathology in control and EMAP II-treated cornea at day 16 after infection (original magnifications, $\times 200$). Signs of systemic or local ocular toxicity were not observed (see Materials and Methods for criteria used). If EMAP II treatment was commenced at day 10 and continued for a further 6 days, no significant measurable differences were observed in either the severity of HSK or extent of angiogenesis between treated and untreated animals.

showed a lesion score ≤ 1.0 and the rest of them were ≥ 3.0 (Figure 6E). The average score was 2.0 ± 0.3 in the

Figure 7. Effect of EMAP II on HSV-induced angiogenesis. Experiment design was as described in Figure 6. **G:** Angiogenesis scores in HSK treated with EMAP II and vehicle. The scores were derived from 24 eyes by slitlamp microscopical examination. **A–F:** Immunohistochemical staining for new blood vessels in the corneas by pan-endothelial antigen staining (four eyes/each time point). The corneal structure was located by two white lines. i, iris; c, cornea. Reduced neovascularization in EMAP II-treated group was evident compared to the vehicle-treated group at days 3, 9, and 16 after infection (**A**, **C**, and **E** represent the control and **B**, **D**, and **F** the EMAP II-treated group). Original magnifications, $\times 100$.

EMAP II-treated group versus 3.9 ± 0.4 in the control group at day 16 after infection ($P < 0.05$) (Figure 6G).

In addition to measuring the effect of EMAP II on the clinical severity of HSK, the effects on HSV-induced neovessel formation from the limbus was also recorded. As shown in Figure 7, throughout a 20-day observation period, the angiogenesis score derived from both the circumferential extent of angiogenesis and the distance from the limbus was markedly reduced in EMAP II-treated

animals at all time points observed (Figure 7G). At day 8 after infection the neovascularization in EMAP II-treated eyes extended 0.3 ± 0.2 mm from the limbus (Figure 6B), whereas in vehicle-treated eyes extensive neovessels were evident (0.8 ± 0.2 mm from the limbus) (Figure 6A). After day 16 after infection, in the control group, abundant neovessels were evident, some already reaching the central region of the cornea (1.3 ± 0.4 mm from the limbus) (Figure 6C). In contrast, in the EMAP II-treated group at 16 days after infection, the average centripetal length of the neovessels was only 0.6 ± 0.3 mm from the limbus (Figure 6D). As measured by an anti-angiogenesis scoring method (see Materials and Methods), at 16 days after infection, the treated animal had a value of 8.5 ± 1.3 , compared to the control value of 15.3 ± 1.5 ($P < 0.05$) (Figure 7G). Immunohistochemical staining for neovessels using pan-endothelial antigen also was used to show the extent of the corneal neovessel ingrowth into the stroma at different time points after HSV-1 infection (Figure 7, A to F). By this analysis, the number of the neovessels existing in the EMAP II-treated group at all time points was significantly less than that of the vehicle-treated group ($P < 0.05$). At days 3 and 16 after infection, the number of positively stained neovessels was 1.6 ± 0.9 and 38 ± 5.0 /corneal section, respectively, in EMAP II-treated group versus 6.0 ± 1.9 and 80 ± 9.3 /corneal section, respectively, in the control group ($P < 0.05$). Whereas EMAP II treatment from the onset of infection diminished both angiogenesis and HSK, delaying treatment until day 10, had no significant measurable effects either on the severity of subsequent HSK or the extent of angiogenesis compared to the control vehicle-treated animal (see footnote Figure 6).

Discussion

HSK is a chronic immunoinflammatory lesion in the corneal stroma that results from ocular infection with HSV.^{1,3} The pathogenesis of HSK is complex and involves the participation of numerous cell types and chemical mediators.¹⁻⁹ In the present report, we demonstrate for the first time, the critical role of neovascularization for HSK lesion development. Accordingly, we showed that treatment of HSV-infected mice with EMAP II, a cytokine-like molecule with robust anti-angiogenesis properties,^{10-13,24} markedly diminished the severity of HSV lesions. Mice treated with EMAP II showed significantly diminished corneal angiogenic sprouting both to HSV infection as well as to the potent angiogenesis factor VEGF. Although the numerous factors likely responsible for HSV-induced angiogenesis remain ill-defined, they do include VEGF. EMAP II, however, seems able to diminish angiogenesis by VEGF and presumably other HSV-induced angiogenesis factors likely because it induced apoptosis in endothelial cells involved in neovascularization.

Ocular infection by HSV is the most common infectious cause of blindness in North America.²⁵ Approximately 20% of HSV infections result in HSK, a lesion that requires prolonged management with anti-inflammatory drugs and oftentimes corneal transplantation.²⁶ The pathogen-

esis of HSK, as studied in a mouse model, involves a complex of events with stromal keratitis mainly the consequence of a CD4⁺ T-cell-orchestrated inflammatory process.^{2-4,21} Such CD4⁺ T cells are present in corneal tissues from ~8 days after infection, a time when replicating virus has usually disappeared.^{8,26} At this stage, neovascularization of the normally avascular cornea is prominent. We assume that this is a necessary event for CD4⁺ T cells to gain access to the central cornea, a concept supported by the present data. Thus mice treated with EMAP II had markedly diminished HSK lesions. As well documented elsewhere, EMAP II may express potent anti-angiogenesis activity.¹⁰ Such effects were demonstrated against pathological angiogenesis in tumor models^{10,11,24} as well as against physiological angiogenesis occurring during organ development.^{12,27,28} EMAP II may also be a pro-inflammatory molecule under some circumstances,^{15,16} although such effects were not observed in the eyes of uninfected or recently infected mice treated with the cytokine in the present study.

Using a corneal micropocket assay, the eye acts as a convenient site to measure the activity of molecules that induce angiogenesis.¹⁸ One of the most potent angiogenesis factors (AFs) is the several isoforms of VEGF.^{22,23,29} In our studies, HSV-1 ocular infection was shown to induce VEGF production. However, it is not clear how HSV infection results in VEGF production, or if VEGF is the only, or even the predominant AF induced after HSV infection. Most likely, in fact, multiple AFs are involved, many of which could derive from inflammatory cells such as neutrophils that promptly invade the stroma of HSV-infected eyes.^{7,8} Because the corneal tissue thickens during HSK, this may cause hypoxia, also a known stimulus for angiogenesis.^{30,31} Identification of additional candidate AFs involved in HSV-induced angiogenesis, as well as their cellular source, requires further investigation. Some other herpesviruses can be involved in aberrant angiogenesis but this may result from the virus encoding molecular mimics of known angiogenesis factors.³²⁻³⁴ The absence of any reported AF molecular homologues with HSV-1 virion proteins makes this mechanism seem unlikely to account for HSV angiogenesis. Moreover HSV infection of cells results in the rapid shut-down of most host-cell protein synthesis.^{35,36} Accordingly, HSV-infected cells themselves represent unlikely primary sources of AF production. Indeed, preliminary data were providing evidence that VEGF production in tissues after HSV infection is predominantly a paracrine event (Zheng and Rouse, submitted).

Although multiple AFs could mediate HSV-induced angiogenesis, interestingly exogenous treatment with EMAP II seemed to limit the activity of all factors involved. Furthermore, EMAP II minimized angiogenesis induced by the potent molecule VEGF as well as another well-characterized AF, basic fibroblast growth factor.²³ Accordingly, the mechanism by which EMAP II achieves anti-angiogenesis might be global. In fact, the present results, as well as some previous reports,^{10,11} indicate that EMAP II causes apoptosis in stimulated vascular endothelial cells. In addition, as shown in this report, EMAP II also caused apoptosis of corneal neovessels in HSV-infected

eyes. Such an effect likely served to minimize the extent of new vessel growth. Nevertheless, as observed both in the VEGF-induced micropocket assay and after HSV infection, EMAP II treatment failed to abrogate angiogenesis. Moreover, once vessels were fully established we failed to observe any effect of EMAP II treatment. Conceivable, concomitant treatment with additional anti-angiogenesis molecules, especially those with a different mechanism of action, could further limit the extent of HSV angiogenesis and maybe even prevent HSK lesion expression. Experiments of this design are currently underway in our laboratory. Finally, targeting angiogenesis with appropriate inhibitors merits investigation as a management approach for human HSK.

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