VIP Promotes Resistance in the *Pseudomonas aeruginosa*-Infected Cornea by Modulating Adhesion Molecule Expression

Elizabeth A. Berger, Sharon A. McClellan, Ronald P. Barrett, and Linda D. Hazlett

PURPOSE. This study tested the hypothesis that the neuropeptide vasoactive intestinal peptide (VIP) regulates adhesion molecule expression, reduces inflammatory cell migration and infiltration into the *Pseudomonas aeruginosa*-infected cornea of susceptible B6 mice, and promotes corneal healing and resistance.

METHODS. B6 mice received daily intraperitoneal (IP) injections of VIP from –1 through 5 days after infection. Control mice were similarly injected with sterile phosphate-buffered saline (PBS). Transcript levels of adhesion molecules were determined by PCR array, then select molecules were tested individually by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and confirmed at the protein level by enzyme-linked immunosorbent assay (ELISA) or immunofluorescent staining with confocal laser scanning microscopy at various time points after infection to assess the effects of VIP treatment in the regulation of adhesion molecule expression.

RESULTS. Injection of B6 mice with VIP compared with PBS resulted in significant downregulation of intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, platelet-endothelial cell adhesion molecule-1, and P-selectin and L-selectin mRNA expression. Protein levels for ICAM-1 and VCAM-1, detected by ELISA, supported the mRNA data at similar time points. Immunofluorescence staining further confirmed the effects of VIP treatment, showing reduced corneal expression of ICAM-1/leukocyte function-associated antigen (LFA-1) and VCAM-1/very late antigen-4 (VLA-4) at select time points compared with PBS-treated animals.

CONCLUSIONS. VIP treatment downregulates the production of adhesion molecules integral to the transmigration process of host inflammatory cells (polymorphonuclear neutrophils, macrophages) into the infected cornea. This results directly in reduced cellular infiltration, less stromal destruction, and better disease outcome. (*Invest Ophthalmol Vis Sci.* 2010;51:5776–5782) DOI:10.1167/iovs.09-4917

The corneal epithelium, intermixed with Langerhans cells and sporadic dendritic melanocytes,¹ provides a physical barrier between the external environment and the inte-

rior of the eye. Attached to the basal cell layer of the epithelium is the epithelial basement membrane, which serves as an important physiological barrier between the epithelium and the stroma. The epithelial basement membrane consists primarily of collagens, proteoglycans, and noncollagenous proteins such as laminin.^{1,2} The posterior surface of the cornea is a monolayer of nonreplicating endothelial cells. The endothelium forms a leaky barrier between the aqueous humor and the stroma, allowing the passage of essential nutrients. The aforementioned barriers use adhesion molecules to maintain physical integrity and selectively permit the passage of molecules. During a state of inflammation, adhesion molecules are modified; as a result, these barriers are breached and permit the transmigration of inflammatory cells through the matrix into the injured or infected tissue site. The recruitment of inflammatory cells into infected or injured tissue is an essential component of inflammation and innate immunity. Transmigration and sequestration of leukocytes (monocytes, macrophages, polymorphonuclear neutrophils [PMNs]) are mediated by cell-adhesion molecules that are expressed on activated endothelia. It is a sequential, multistep cascade, whereby a number of adhesion molecules are upregulated on both endothelial cells and activated leukocytes to facilitate this process, resulting in further recruitment of large numbers of circulating immune cells into the site of infection. Macrophage-derived cytokines (e.g., tumor necrosis factor [TNF]- α) and lipopolysaccharide (LPS) activate endothelia to express molecules such as selectins, intercellular adhesion molecules (ICAMs), vascular cell adhesion molecule (VCAM)-1, and platelet-endothelial cell adhesion molecule (PECAM)-1. Counterreceptors, expressed on infiltrating inflammatory cells, typically include sulfated-sialyl-Lewis X moieties, LFA-1, VLA-4, and Mac-1.³

The extent of leukocyte recruitment is a major determinant of the intensity of the inflammatory response, and it may result in considerable tissue damage if not adequately regulated. Previous studies from our laboratory have provided evidence that vasoactive intestinal peptide (VIP) regulates cytokine/chemokine production and host inflammatory cell function to promote resistance against *Pseudomonas aeruginosa* corneal infection in normally susceptible C57BL/6 (B6) mice.⁴ In this respect, the present study sought to test the hypothesis that VIP also regulates adhesion molecule expression, thus reducing inflammatory cell migration and infiltration into the *P. aeruginosa*-infected corneas of B6 mice, promoting corneal healing and restoration of immune homeostasis.

MATERIALS AND METHODS

Experimental Animal Protocol

Eight-week-old female B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were housed in accordance with the

From the Department of Anatomy and Cell Biology, Wayne State University School of Medicine, Detroit, Michigan.

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Corresponding author: Elizabeth A. Berger, Department of Anatomy and Cell Biology, Wayne State University School of Medicine, 540 E. Canfield Avenue, Detroit, MI 48201; eberger@med.wayne.edu.

National Institutes of Health guidelines. Mice were anesthetized with ethyl ether, and the left cornea was wounded as previously described.⁵ A 5- μ L aliquot containing 1×10^6 CFU of *P. aeruginosa* American Type Culture Collection (Manassas, VA) strain 19660 was topically delivered to the ocular surface. At 24 hours postinfection (p.i.) and at additional time points mentioned below, eyes were examined to ensure that mice were similarly infected and to monitor disease. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all research was approved by the Wayne State University Animal Institutional Review Board.

VIP Treatment

B6 mice received daily intraperitoneal (IP) injections of VIP (5 nmol in 100 μ L; Bachem California, Inc., Torrance, CA) starting 1 day before infection (d - 1) and included the day of infection (d0) and d1 through d5 p.i. Control mice were similarly injected with sterile PBS.

Steroid Treatment

B6 mice were treated with prednisolone acetate (Prednisolone Acetate Ophthalmic Suspension USP 1%; Allergan, Inc., Irvine, CA), an adrenocortical steroid product prepared as a sterile ophthalmic suspension. Starting the day of infection (d0), a single 5- μ L aliquot of the steroid suspension was topically delivered to the corneal surface of the infected eye; mice were subsequently treated 3 $\times$ /d (5- μ L aliquots) from d1 through d5 p.i. Steroid treatment was used as a positive control, in conjunction with PBS as a negative control, for select experiments to allow for a more appropriate comparison of the immunosuppressive effects of VIP treatment.

Real-time RT-PCR

Total RNA was isolated from individual corneas using an extraction reagent (RNA-Stat 60; Tel-Test, Friendsville, TX) according to the manufacturer's recommendations and was quantitated by spectrophotometric determination (260 nm). One microgram of total RNA was reverse transcribed as previously described.⁴ All primer sets for the PCR reactions were purchased either as a 96-well plate (RT² Profiler PCR Array; SABiosciences Corporation, Frederick, MD) or as individual primer sets from SABiosciences Corporation. Quantitative real-time RT-PCR was processed (using MyiQ Single-Color Real-Time RT-PCR Detection System; Bio-Rad; Hercules, CA). PCR amplification conditions were set according to the recommendations of SABiosciences Corporation. Relative mRNA levels were calculated using the relative standard curve method that compares the amount of target normalized to an endogenous reference, β -actin. Briefly, the mean $\pm$ SD values of replicate samples were calculated. Samples were then normalized to β -actin. Results are expressed as the relative amount of mRNA between experimental test samples and normal control samples (all normalized to β -actin). Before using this method, a validation experiment was performed comparing the standard curve of the reference and the target to demonstrate that efficiencies were approximately equal. The correct size of the amplified products was verified by electrophoresis using an agarose gel.

ELISA

Protein levels for adhesion molecules were selectively tested using available ELISA kits (R&D Systems, Minneapolis, MN). Corneas from VIP-, PBS-, and steroid-treated B6 mice were individually collected ($n = 5$ /group/time) at 1, 3, and 5 days p.i. Corneas were homogenized in 250 μ L PBS with 0.1% Tween 20 and protease inhibitor cocktail tablets (containing protease inhibitors for serine, cysteine, and metalloproteases in bacterial, mammalian, yeast, and plant cell extracts; Roche, Mannheim, Germany). Samples were centrifuged at 5000g (10 minutes), and an aliquot of each supernatant was assayed in triplicate for soluble (s)ICAM-1 and sVCAM-1 protein according to the manufacturer's instruction. Assay sensitivity was 30 pg/mL for both sICAM-1 and

sVCAM-1. Results are expressed as average nanograms of sICAM-1/mL and picograms of sVCAM-1/mL $\pm$ SEM.

Immunofluorescence Staining

Corneal expression of ICAM-1 and VCAM-1 and their respective ligands, LFA-1 (CD11a/CD18) and VLA-4 (CD49), as well as L-selectin, P-selectin, and PECAM, was evaluated by immunofluorescent dual- or triple-label staining using confocal laser scanning microscopy of corneal tissue sections. Whole eyes were enucleated at 1 or 3 days p.i. (as indicated) from PBS- and VIP-treated B6 mice ($n = 3$ /group). For ICAM-1/LFA-1 staining, samples were fixed, dehydrated, and embedded in paraffin, as previously described,⁶ then stored at -20°C until used for analysis. Ten micron-thick sections were deparaffinized, then rehydrated through graded alcohols. For immunostaining of the remaining molecules to be tested, whole eyes were immersed in PBS, embedded in OCT compound (Tissue-Tek; Miles, Elkhart, IN), and frozen in liquid nitrogen. Frozen sections were cut (10- μ m thick), mounted to poly-L-lysine-coated glass slides, incubated at 37°C overnight, and fixed in acetone. All sections (paraffin and frozen) were incubated (30 minutes) with a blocking agent (goat IgG, 1:100; donkey IgG, 1:100; 2.5% BSA in 0.01 M PBS) and incubated for 1 hour with primary antibodies as follows: (1) primary goat anti-ICAM-1 (1:10; R&D Systems, Minneapolis, MN) and rat anti-LFA-1 (1:20; BD Biosciences, San Jose, CA); (2) goat anti-VCAM-1 (10 μ g/mL; R&D Systems) and rat anti-VLA-4 (CD49d) (2.5 μ g/mL; BD Biosciences); (3) rat anti-L-selectin (CD62L) (1:25; BD Biosciences); (4) rat anti-PECAM-1 (CD31) (1:25; BD Biosciences); and (5) rat anti-P-selectin (CD62P) (1:25; BD Biosciences) followed by PBS rinse. Secondary antibody AlexaFluor 546-conjugated donkey anti-goat (for ICAM-1 and VCAM-1), AlexaFluor 633-conjugated goat anti-rat (for LFA-1 and VLA-4), or AlexaFluor 594-conjugated donkey anti-rat IgG (for L-selectin, P-selectin, and PECAM-1) (1:1500; Molecular Probes, Inc., Eugene, OR) were applied to the sections for an additional hour. Slides were then incubated for 2 minutes with nuclear acid stain (SYTOX Green, 1:20,000; Fisher Scientific, Pittsburgh, PA) and mounted with mounting medium (Vectashield; Vector Laboratories, Inc., Burlingame, CA). All steps of the immunostaining procedure were carried out at room temperature.

Negative controls were similarly treated with species-specific IgG in lieu of the primary antibody. Sections were visualized, and digital images were captured with a confocal laser scanning microscope (TSC SP2; Leica Microsystems, Exton, PA). Positive controls (mouse spleen sections) were used where indicated.

Statistical Analysis

An unpaired Student's *t*-test was used to determine the significance of real-time RT-PCR and protein assays. Data were considered significant at $P < 0.05$. All experiments were performed at least twice, and combined data from the two experiments are shown unless otherwise indicated.

RESULTS

Adhesion Molecule mRNA Expression with VIP Treatment

To investigate the mechanisms by which VIP reduces inflammatory cell infiltration into the cornea during bacterial keratitis, mRNA levels of 84 genes important for cell-cell and cell-matrix interactions were profiled by 96-well plate (RT² Profiler PCR Array; SABiosciences Corporation). At 3 days p.i., VIP treatment resulted in differential expression (greater than threefold upregulation or downregulation) of 56 genes compared with PBS-treated controls (Fig. 1). Of these 56 genes, 12 genes were upregulated at the mRNA level; conversely, 46 genes were downregulated in corneas of VIP- compared with

FIGURE 1. VIP differentially regulates ECM and adhesion molecule mRNA production in the cornea after *P. aeruginosa* ocular infection, as depicted in this 3D profile generated by PCR array. Treatment with VIP resulted in twofold or greater mRNA expression levels for 12 ECM/adhesion molecules compared with PBS-treated controls. Conversely, 46 different genes were reduced by twofold or greater with VIP treatment. β -Actin served as the control for each sample. Each bar represents a single gene. Test, VIP; control, PBS.

PBS-treated mice, of which select molecules are shown in Table 1.

Of the adhesion molecules involved in the transmigration of inflammatory cells into and through the extracellular matrix (ECM), six of the most well-known of these molecules (L-selectin, P-selectin, ICAM-1, ICAM-2, PECAM-1, and VCAM-1) associated with the cornea and corneal vasculature were further analyzed through 5 days p.i. by real-time RT-PCR; the data are shown in Figures 2A to 2F, respectively. Both L-selectin and P-selectin were significantly reduced at 3 and 5 days p.i. (L-selectin, $P = 0.008$ and 0.003 ; P-selectin, $P < 0.001$ and 0.002) in VIP- compared with PBS-treated animals (Figs. 2A, 2B). In addition, treatment with VIP resulted in a significant decrease of mRNA transcripts at 1, 3, and 5 days p.i. for cellular adhesion molecules ICAM-1 ($P = 0.006$, <0.001 , and <0.001) and VCAM-1 ($P = 0.003$, 0.009 , and 0.004) compared with PBS-treated controls (Figs. 2C, 2F), whereas PECAM-1 was reduced only at 3 days p.i. ($P = 0.021$) and ICAM-2 showed no difference at any time point compared with controls (Figs. 2D, 2E, respectively). Treatment with VIP did not significantly change the normal, basal levels for these six adhesion molecules compared with PBS controls.

Effects of VIP Treatment on Adhesion Molecule Protein Expression

The influence of VIP treatment on ICAM-1/LFA-1 and VCAM-1/VLA-4 expression in the corneas of B6 mice was examined using triple-label fluorescent staining and confocal laser scanning microscopy. Figure 3 demonstrates the contrast between ICAM-1/LFA-1 coexpression in PBS- (Figs. 3A, 3C, 3E) and VIP-treated (Figs. 3B, 3D, 3F) corneas at 1 day p.i., depicted at both low ($340\times$) and high ($510\times$) magnification in Figs. 3A and 3B and in Figs. 3C and 3D, respectively. Regarding PBS-treated B6 mice, ICAM-1 (red) staining is evident throughout the corneal epithelium, stroma, and endothelium. Although LFA-1 (blue) was detected at low levels in the anterior third of the stroma and along the corneal endothelium, it appeared more intensely localized along the corneal epithelium. Nuclear acid stain revealed numerous infiltrating cells migrating through the posterior half of the corneal stroma and along the

epithelium, demonstrating the close interaction of infiltrating inflammatory cells (expressing LFA-1) with the corneal epithelium, endothelium, and throughout the stroma (expressing ICAM-1). Immunostaining for ICAM-1 and LFA-1 was dramatically less intense after VIP treatment than after PBS treatment at the same time point. This is supported by the reduced ICAM-1 expression throughout the corneal tissue and a marked decrease in infiltrating inflammatory cells as shown by the less intense LFA-1 and nuclear acid stain (SYTOX Green; Fisher Scientific) in VIP-treated sections. In addition, higher magnification illustrated more intact corneal epithelium after VIP treatment (Fig. 3D) than in PBS controls (Fig. 3C). Negative controls showed no detectable ICAM-1/LFA-1 immunostaining (Figs. 3E, 3F).

As demonstrated in Figure 4A, corneas of PBS-treated mice revealed positive staining for VCAM-1 (red) throughout the entire cornea; however, the most intense expression appeared along the epithelium and anterior half of the stroma. VLA-4 expression (blue) was detected adjacent to the epithelium, throughout the stroma, and along the endothelium. Nuclear acid stain revealed the interaction between resident cells of the cornea (coexpressing VCAM-1) with infiltrating inflammatory cells (coexpressing VLA-4) along the epithelium, endothelium, and throughout the stroma. VIP treatment (Fig. 4B) revealed prominently less positive staining for both VCAM-1 and its ligand, VLA-4, regarding the stroma and endothelium. VCAM-1 staining was similar in the corneal epithelium of both VIP- and PBS-treated animals. However, diffuse VLA-4 staining was detected throughout the epithelium with VIP treatment, whereas staining was localized to the borders of the corneal epithelium in PBS-treated controls. Further, nuclear acid stain was reduced overall after VIP treatment, indicating fewer infiltrating inflammatory cells, and revealed a more intact epithelium when compared with controls. Regarding L-selectin, staining was detected in spleen (used as a positive control); however, no positive staining was obtained in corneas of PBS- or VIP-treated animals when tested at 3 days p.i. (data not shown).

In addition to immunofluorescence microscopy, the expression of soluble (s)ICAM-1 and sVCAM-1 was further examined at the protein level by ELISA; results are provided in Figures 5A and 5B, respectively. Protein expression in the corneas of VIP-, steroid-, and PBS-treated animals was assessed at 1, 3, and 5 days p.i. Results at the protein level corroborated effects observed at the mRNA levels at the same time points and the results obtained through immunostaining. Both sICAM-1 and sVCAM-1 protein levels were significantly decreased as a result of VIP treatment under normal, uninfected conditions and at all time points p.i. when compared with PBS treatment (sICAM-1: $P = 0.012$, <0.001 , <0.001 , and <0.001 ; sVCAM-1: $P = 0.001$, <0.001 , 0.002 , and 0.003 , respectively). In addition, sICAM-1 and sVCAM-1 protein levels were significantly re-

TABLE 1. Select Molecules from RT² PCR Array

Gene	Fold Change VIP over PBS
<i>CD44</i>	8.08
<i>ICAM1</i>	-2.60
<i>ITGA1</i>	21.63
<i>ITGA2</i>	8.17
<i>ITGAL</i>	-6.87
<i>PECAM1</i>	-14.67
<i>LAMA1</i>	4.91
<i>L-SEL</i>	-3.45
<i>P-SEL</i>	-5.46
<i>SPG7</i>	7.86
<i>TGFB1</i>	6.61
<i>VCAM1</i>	-2.46

FIGURE 2. Adhesion molecule transcript levels as detected by real-time RT-PCR in VIP- and PBS-treated corneas after ocular infection. L-selectin (A) and P-selectin (B) mRNA levels were significantly decreased with VIP treatment at 3 and 5 days p.i. compared with PBS controls. mRNA expression for ICAM-1 (C) and VCAM-1 (F) was significantly reduced with VIP treatment at 1, 3, and 5 days p.i. over PBS-treated controls. VIP treatment significantly reduced PECAM-1 (E) at 3 days p.i. only, whereas ICAM-2 (D) mRNA expression showed no difference compared with controls. Normal levels remained similar in VIP- and PBS-treated mice.

duced after VIP treatment when compared with steroid treatment at 1, 3, and 5 days p.i. (sICAM-1: $P < 0.001$ for all time points; sVCAM-1: $P < 0.001$ for all time points).

Dual-staining fluorescence microscopy was used to examine the corneal expression of PECAM-1 and P-selectin at 3 days p.i. in VIP- and PBS-treated mice; results are presented in Figures 6 and 7, respectively. Pronounced PECAM-1 staining (red) was evident lining the lumen of stromal vessels, predominantly confined to the peripheral cornea (Fig. 6A). Similar results were obtained from corneas of VIP-treated animals (Fig. 6B). Negative controls (primary antibodies omitted) showed positive nuclear acid stain but no detectable PECAM-1 staining in the corneas of PBS- and VIP-treated mice (Figs. 6C, 6D, respectively).

Regarding P-selectin, positive staining was detected at low levels in the corneal stroma, potentially associated with activated endothelial cells of the stromal vasculature in both PBS- and VIP-treated mice (Figs. 7A, 7B, respectively). There were no visual differences in expression between the two groups at this time point. Negative controls (primary antibodies omitted) revealed positive nuclear acid stain but no detectable P-selectin immunostaining (Figs. 7C, 7D).

Although VIP appeared to downregulate L-selectin at the transcript level, results could not be corroborated at the protein level using immunohistochemistry. Although positive staining was observed using spleen sections as a control, there

were no signs of detectable staining in the corneas of either PBS- or VIP-treated animals (data not shown).

DISCUSSION

Included among the consequences of bacterial keratitis is the destruction of the ECM by proteolytic enzymes. Infiltrated immune cells and resident fibroblasts are thought to contribute to this degradation. We have shown previously that the neuropeptide VIP is able to balance proinflammatory and anti-inflammatory events in the *P. aeruginosa*-infected cornea and ultimately protect against corneal perforation.⁴ These studies delineated mechanisms by which VIP modulates inflammation, which include both cytokine/chemokine production and up-regulation of the VIP receptor VIPR1 on macrophages.⁴ This conclusion led to the present study, which demonstrated that VIP may be a pro-resolution molecule because it further balances the inflammatory response by decreasing inflammatory cell infiltration through the regulation of adhesion molecule expression, ultimately reducing destruction of the ECM and ameliorating disease pathogenesis.

ICAM-1 is an adhesion molecule potentially induced on and released by endothelial cells in response to TNF- α , and it mediates both firm adhesion of leukocytes and transmigration/infiltration into tissue sites. Previous studies from our labora-

FIGURE 3. Comparison of ICAM-1/LFA-1 immunostaining at 1 day p.i. in PBS- and VIP-treated B6 mice. Merged images of PBS-treated cornea stained positive for both ICAM-1 (red) and LFA-1 (blue) shown at low magnification (340 $\times$) (A), demonstrating the interaction (pink) between infiltrating inflammatory cells and corneal endothelium, stroma, and epithelium. ICAM-1 and LFA-1 adhesion molecules were also detected in the VIP-treated cornea (B), but staining was less intense than in the PBS control. High magnification (510 $\times$) revealed a mostly denuded epithelium in the PBS-treated control (C), whereas the epithelium appeared more intact after VIP treatment (D). Negative controls for (E) PBS- and (F) VIP-treated mice showed only nuclear acid stain (primary antibodies were omitted).

tory, using ICAM-1-deficient KO mice, demonstrated that ICAM-1 plays a significant role in the development of the severe pathology characteristic of *P. aeruginosa* eye infection through the recruitment of inflammatory cells (PMNs) into the infected eye.⁷ With regard to the chronically inflamed or infected human cornea, it has been shown that ICAM-1 was focally increased on keratocytes, endothelial cells, and vascular endothelial cells.⁸ In addition, VCAM-1 was found to be focally expressed on endothelial cells of the stromal vasculature and on infiltrated macrophages and monocytes.⁸ Results obtained from our murine model of infection are in accordance with this observation, whereby increased corneal expression of both ICAM-1 and VCAM-1 mRNA and protein were detected after *P. aeruginosa*-induced ocular infection. In addition to PBS, VIP treatment was compared with steroid treatment. Corticosteroid, a common immunosuppressant, is often used in the clinical setting to treat a number of inflammation-based conditions, including allergic conjunctivitis, superficial punctate keratitis, herpes zoster keratitis, and selected infective conjunctiv-

itides. Although the exact mechanism of steroid treatment remains unknown, it is generally accepted that it impedes the inflammatory response through a number of processes, such as inhibition of edema, fibrin deposition, capillary dilation, leukocyte migration, fibroblast proliferation, and collagen deposition. Results from our study, however, indicate that corticosteroid treatment does not modulate the expression of adhesion molecules. In fact, VCAM-1 expression was significantly up-regulated after steroid treatment compared with PBS at all time points. Because leukocyte migration is suppressed with corticosteroid treatment, it is plausible that the increase in adhesion molecule protein levels (above PBS levels) is a compensatory response to the decreased, yet required, migration of inflammatory cells into the infected cornea. Immunosuppression was further evidenced by a highly phthical eye (subsequently overrun by bacteria) and lack of mucopurulent exudate (typically present from PMN infiltration/persistence; data not shown). Overall, it supports the role of VIP as a pro-resolution immunomodulator, not merely an immunosuppressant, which functions to balance the immune response.

Most striking, however, were the results obtained by immunostaining for coexpression of ICAM-1/LFA-1 and VCAM-1/VLA-4, which visually confirmed both mRNA and protein data and further indicated that these adhesion molecules play a central role in the pathology of bacterial keratitis. After VIP treatment, expression of these four adhesion molecules is remarkably altered in corneal endothelium, stroma, and epithelium during bacterial infection, ultimately leading to a reduction in inflammatory cell infiltration and the preservation of corneal integrity. VIP has been shown to abrogate the inflam-

FIGURE 4. Comparison of VCAM-1/VLA-4 immunostaining at 3 days p.i. in PBS- and VIP-treated B6 mice. Merged image of PBS-treated cornea (A) showed positive staining throughout the entire cornea for VCAM-1 (red). Although VLA-4 (blue) was also demonstrated throughout the cornea, it was predominantly detected along the edges of the epithelium. VIP treatment (B) revealed less intense staining for VCAM-1 and VLA-4 throughout the stroma and endothelium. VCAM-1 staining remained similar along the epithelium; however, VLA-4 staining was more diffuse than in PBS controls. Negative controls for PBS- and VIP-treated mice (C, D, respectively) were positive for nuclear acid stain only.

FIGURE 5. Adhesion molecule protein levels as detected by ELISA in VIP- and PBS- and steroid-treated corneas after ocular infection. sICAM-1 (A) and sVCAM-1 (B) protein levels were significantly decreased with VIP treatment at 1, 3, and 5 days p.i. compared with both PBS and steroid controls. In addition, VIP treatment significantly decreased levels for both molecules under normal, uninfected conditions compared with PBS. * $P < 0.05$ for VIP compared with PBS; # $P < 0.05$ for VIP compared with steroid treatment.

matory lesion of infected bronchial epithelial cells by down-regulating ICAM-1 mRNA and NF- κ B activation.⁹ Martinez et al.¹⁰ have also demonstrated that VIP is involved in the inhibition of PMN infiltration by modulating sICAM-1 production and ICAM-1 and VCAM-1 expression. However, this study is the first to illustrate, using confocal laser scanning microscopy, the scope of influence VIP exerts on adhesion molecule expression during an active state of inflammation.

Contrary to results obtained for ICAM-1/LFA-1 and VCAM-1/VLA-4, the present study did not indicate a regulatory role of VIP overexpression of PECAM-1 and P-selectin. Although VIP appeared to significantly downregulate P-selectin (3 and 5 days p.i.) and PECAM-1 (1 day p.i.) at the transcript level, no visual differences were observed at the protein level when examined by immunofluorescence confocal laser scanning microscopy. These adhesion molecules are predominantly expressed on endothelial cells, though expression has been shown at lower levels on the surfaces of circulating platelets and/or leukocytes.^{11,12} Positive staining for both P-selectin and PECAM-1 reveal a close association with endothelial cells of the stromal vasculature located in the peripheral cornea. Although evidence denotes primarily anti-inflammatory actions, VIP has been described as an immunomodulator⁴ and, as such, has been shown to preserve proinflammatory activity under appropriate conditions. Selective expression of adhesion molecules along the vasculature may be such an example of immunomodulation whereby VIP does not abolish cell transmigration but pre-

FIGURE 6. Immunostaining of PECAM-1 in PBS- and VIP-treated B6 mice at 3 days p.i. Both (A) PBS- and (B) VIP-treated corneas stained positive (red) for PECAM-1 along the lumen of stromal vessels in the peripheral cornea. There were no visual differences between the two groups. Negative controls for (C) PBS- and (D) VIP-treated mice were positive for nuclear acid stain only.

serves the potential to allow for an influx of inflammatory cells if needed. These molecules are known to be constitutively expressed rather than upregulated during inflammation, and our results indicate that VIP maintains this expression. It should be noted that while VIP does not markedly alter the surface expres-

FIGURE 7. Immunostaining of P-selectin in PBS- and VIP-treated B6 mice at 3 days p.i. Although both groups stained positive for P-selectin, expression levels appeared comparable between (A) PBS- and (B) VIP-treated corneas. Negative controls for (C) PBS- and (D) VIP-treated mice revealed positive staining for nuclear acid stain only.

sion of PECAM-1, it is possible that the neuropeptide may regulate potential signal transduction events triggered by PECAM-1 engagement, leading to integrin activation and heterotypic cellular activation. This potential aspect of VIP immunoregulation remains to be determined.

Taken together, these studies demonstrate that VIP influences not only immune cells (as previously shown) but also the microenvironment, including corneal epithelium, stromal ECM, endothelium, and respective barriers, during an inflammatory response. We also further indicate that this neuropeptide is not strictly an "anti-inflammatory" molecule, in that it functions solely to deactivate inflammatory cells and associated molecules regardless of the circumstances. However, the observations that VIP modified selective adhesion molecules and that these modifications did not become apparent until 3 days p.i. support this molecule as a true immunomodulator, which permits inflammation for clearance of the infectious agent yet expeditiously transitions to the restoration of immune homeostasis.

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