VIP and Growth Factors in the Infected Cornea

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PURPOSE. Vasoactive intestinal peptide (VIP) is an anti-inflammatory neuropeptide that downregulates proinflammatory cytokines and promotes healing in a susceptible model of *P. aeruginosa* keratitis. Growth factors also play a role in corneal healing and restoration of tissue homeostasis after wounding. However, whether VIP treatment modulates growth factors to promote healing in the infected cornea remains untested and is the purpose of this study.

METHODS. C57BL/6 (B6) mice were injected with VIP and mRNA and protein levels, and immunostaining for EGF, FGF, HGF, and VEGF-A were done. Exogenous treatment with a mixture of the growth factors also was tested and levels of cytokines, defensins, and bacterial counts were determined.

RESULTS. Real-time RT-PCR, immunostaining, and ELISA data demonstrated that treatment with VIP enhanced levels of EGF, FGF, and HGF during disease, and that VEGF-A, and associated angiogenic molecules also were increased by VIP. Moreover, immunohistochemical studies confirmed that both epithelial and stromal cells participated in growth factor production. Most notably, treatment with a mixture of EGF, FGF, and HGF after disease onset, prevented corneal perforation when compared with controls. This outcome was associated with downregulation of proinflammatory cytokines such as macrophage inflammatory protein-2 (MIP-2), upregulation of anti-inflammatory cytokines such as TGF- β , and antimicrobials β -defensins 2 and 3, as well as decreased plate counts at 1 day postinfection (p.i.) ($P = 0.0001$).

CONCLUSIONS. Collectively, the data provide evidence that VIP treatment modulates growth factors, angiogenic molecules, and defensins in the infected cornea and that this in turn promotes healing and restoration of tissue homeostasis. (*Invest Ophthalmol Vis Sci.* 2011;52:6154–6161) DOI:10.1167/iovs.10-6943

An opportunistic, gram-negative pathogen, *Pseudomonas aeruginosa* is one of the most virulent organisms associated with microbial keratitis and is often associated with contact lens usage.¹ Both bacterial (e.g., lipopolysaccharide) and host factors released from infiltrating cells during infection are thought to contribute to a rapidly progressing liquefactive stromal necrosis.^{2–5} Experimental murine models of the disease have been established: Th1 responder mouse strains (e.g.,

C57BL/6, B6) are susceptible (cornea perforates), whereas Th2 responder strains (e.g., BALB/c) are resistant (cornea heals).⁶

In previous studies using these murine models, we provided evidence that an anti-inflammatory neuropeptide, vasoactive intestinal peptide (VIP) given exogenously, promotes resistance against *P. aeruginosa* corneal infection by regulation of cytokine production and subsequent alteration of the host inflammatory cell response.⁷

Neuropeptides, such as VIP are small protein-like molecules used by neurons to bidirectionally communicate with the cells of the immune system.⁸ In the eye, VIP can be detected in corneal nerves and the aqueous humor in both mice and humans.⁹ Others also have shown previously that VIP reduces pro-inflammatory cytokine production, limits cell-mediated immunity, and inhibits macrophage and T cell proliferation⁹; and by downregulation of IL-6 and TNF- α , the neuropeptide showed a protective effect in models of endotoxemia.¹⁰ Despite these anti-inflammatory effects which have been reported in diverse systems and which clearly modulate disease outcome, the role of VIP in regulation of corneal healing, specifically, control of growth factor production, has not been examined.

Classically, growth factors, which also may be considered as cytokines, bind to their receptors and regulate cell growth, proliferation, migration, and differentiation.¹¹ Thus, the current studies investigated the potential regulation by VIP of growth factors in *P. aeruginosa*-infected corneas in B6 (susceptible) mice that exhibit lower levels of VIP when compared with resistant (BALB/c) mice.⁷ Exogenous VIP treatment was given to B6 mice to determine whether VIP modulates growth factor production and thereby, favors resolution of disease. Data from these studies provide evidence that VIP treatment leads to upregulation of growth factor production in the cornea after *P. aeruginosa* infection, and that both epithelial and stromal cells are participatory. Furthermore, evidence is provided to show that treatment with a mixture of growth factors, given topically after infection, prevents perforation of the cornea. Mechanistically this is achieved by downregulation of proinflammatory cytokines, upregulation of anti-inflammatory cytokines, antimicrobials β -defensins 2 and 3, and decreased bacterial plate counts. The data suggest that VIP modulates growth factor production in the infected cornea and that this in turn, contributes to better disease outcome.

MATERIALS AND METHODS

Infection

Eight-week-old female C57BL/6 (B6) mice (The Jackson Laboratory, Bar Harbor, ME) were anesthetized with ethyl ether and placed beneath a stereoscopic microscope at $\times 40$ magnification. The cornea of the left eye was wounded¹² and a 5- μ L aliquot containing 1.0×10^6 CFU/ μ L of *P. aeruginosa* (American Type Culture Collection, strain 19,660, Manassas, VA) was topically delivered. Animals were treated humanely and in compliance with the Association for Research in Vision and

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Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research.

Ocular Response to Bacterial Infection

Corneal disease was graded¹³: 0 = clear or slight opacity, partially or fully covering the pupil; +1 = slight opacity, fully covering the anterior segment; +2 = dense opacity, partially or fully covering the pupil; +3 = dense opacity, covering the entire anterior segment; and +4 = corneal perforation or phthisis. After infection, a clinical score was recorded (days 1, 3, 5, and/or 7) for each mouse ($n = 5$ per group per treatment for two experiments = 20 mice).

VIP Treatment

In vivo treatment with synthetic VIP (Bachem, Torrance, CA) has been described previously.⁷ In brief, B6 mice received daily IP injections of VIP (5 nM in 100 μ L sterile phosphate buffered saline, PBS) at 1 day before infection (Day = -1) through a maximum of 7 days p.i. Control mice were injected similarly with sterile PBS (100 μ L).

Real-time RT-PCR

Total RNA was isolated from an individual cornea of PBS, VIP, or growth factor-treated mice ($n = 5$ per group per time per treatment for two experiments = 140 mice) using RNA-Stat 60 (TelTest, Friendswood, TX), per the manufacturer's recommendations and quantitated spectrophotometrically (260 nM). One microgram of total RNA was reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase. The 20- μ L reaction mixture contained 200 U of MMLV-reverse transcriptase, 10 U of RNasin, 500 ng of oligo (dT) primers, 10 mM dNTPs, 100 mM DTT, and MMLV reaction buffer (Invitrogen, Carlsbad, CA). After, cDNA was amplified using SYBR Green Master Mix (SABiosciences, Frederick, MD), per the manufacturer's recommendation. Briefly, the 20- μ L reaction system contained 10 μ L of SYBR Green PCR Master Mix, 1.0 μ L of 10- μ M primer mix, 2 μ L of cDNA (diluted 1/10), and diethyl pyrocarbonate water. All primers were purchased from SABiosciences, except for β -actin which was designed using PrimerQuest (Integrated DNA Technologies, Cambridge, MA) whose forward primer is 5'-GAT TAC TGC TCT GGC TCC TAG C-3'; the reverse primer is 5'-GAC TCA TCG TAC TCC TGC TTG C-3'. The primer sequences for MIP-2, TNF- α , TGF- β ,⁷ and β -defensins-2^{14,15}) were provided previously.

Quantitative real-time RT-PCR reactions were performed (MyiQ single color real-time RT-PCR detection system; Bio-Rad, Hercules, CA). Optimal conditions for PCR amplification of cDNA were established using routine methods.¹⁶ Relative mRNA levels were calculated after normalization to β -actin.

Immunofluorescent Staining

Normal, uninfected, and infected eyes were enucleated from VIP, PBS, and growth factor-treated B6 mice ($n = 5$ per group per time for two experiments = 60 mice) at 1 and 5 days p.i. Tissue was immersed in 1X Dulbecco's PBS (Mediatech, Inc., Herndon, VA), embedded (Tissue-Tek OCT compound; Miles, Elkhart, IN), and frozen in liquid nitrogen. Ten-micrometer thick sections were cut, mounted to polylysine-coated glass slides, and incubated in a moist chamber at 37°C overnight. After a 2-minute fixation in acetone, nonspecific staining was blocked with 10 mM sodium phosphate buffer containing 2.5% bovine serum albumin and donkey IgG (1:100) for 30 minutes at room temperature. For immunostaining, sections were incubated for 1 hour each with goat anti-mouse VEGF-A, EGF and/or HGF (R&D, Minneapolis, MN), goat anti-human FGF-7 (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-mouse β -defensin 2 (1:50, Santa Cruz) or rabbit anti-mouse β -defensin 3 (1:50, Santa Cruz), followed by a secondary Ab, Alexa Fluor 594 conjugated donkey anti-goat (1:1500, Invitrogen, Eugene, OR) or Alexa Fluor 594 conjugated donkey anti-rabbit Ab (1:1500, Invitrogen; both = red color). Sections were then incubated for 2 minutes with nuclear acid stain (1:20,000, SYTOX Green; Lonza, Walkersville, MD). Controls

were similarly treated, but the primary Ab was replaced with the same host IgG. Finally, sections were visualized and digital images captured with a confocal laser scanning microscope (Leica TCS SP2; Leica Microsystems, Bannockburn, IL).

ELISA

Growth factor protein levels were tested using ELISA kits (R&D). Corneas from PBS and VIP-treated B6 mice were individually collected ($n = 5$ per group per time per treatment for two experiments = 100 mice) at 1, 5, and 7 days p.i. Corneas were homogenized in 0.5 mL of PBS with 0.1% Tween 20. All samples were centrifuged at 13,000 rpm for 5 minutes and an aliquot of each supernatant was assayed in duplicate for EGF, KGF/FGF-7 (FGF), HGF, and VEGF-A per the manufacturer's instruction. ELISA also was used to test for receptor protein levels for VEGFR1 and VEGFR2. The reported sensitivity of these assays is <0.95 pg/mL for EGF, <156.25 pg/mL for HGF, 15.62 pg/mL for KGF/FGF-7 (FGF), <3.0 pg/mL for VEGF-A, <9.8 pg/mL for VEGFR1, and <27 ng/mL for VEGFR2.

Growth Factor Treatment

Recombinant mouse EGF, KGF/FGF-7 (FGF) and HGF (all from R&D) were dissolved in PBS to a final concentration of either 40 μ g/mL (EGF and HGF) or 20 μ g/mL (FGF). After mice were infected, 5 μ L of a mixture of the three growth factors (2 μ L EGF, 0.4 μ L FGF, and 2 μ L HGF in 0.6 μ L PBS) was delivered topically, once on the day of infection, twice at 1 day p.i. and once daily through 4 days p.i. Concentrations for the growth factors were selected and modified after review of the literature.¹⁷⁻¹⁹ Controls received PBS similarly.

Bacterial Plate Counts

Corneas from PBS or growth factor-treated B6 mice were collected ($n = 5$ per group per time for two experiments = 60 mice) at 1, 3, and 5 days p.i. and the number of viable bacteria were quantitated. Individual corneas were homogenized in sterile water containing 0.85% (wt/vol) NaCl containing 0.25% BSA. Serial 10-fold dilutions of the samples were plated on *Pseudomonas* isolation agar (Difco Laboratories, Sparks, MD) in triplicate and plates were incubated overnight at 37°C and bacteria counted. Results are reported as log₁₀ number of CFU per cornea $\pm$ SEM.

Statistics

The difference in clinical score between the two groups at each time point was tested by the Mann-Whitney *U* test. An unpaired, two-tailed Student's *t*-test was used to determine significance of all other data. For each test, differences were considered significant at $P < 0.05$ and represented as mean $\pm$ SD. Each experiment was performed twice for reproducibility and representative data from a typical experiment are shown.

RESULTS

mRNA Expression of Growth Factors

Whether VIP modulated mRNA expression patterns for selected growth factors was first assessed. The mRNA expression patterns of EGF, FGF, HGF, and EGF receptor (EGFR) are shown in Figures 1A, 1C, 1E, and 1G. Compared with PBS, treatment with VIP significantly increased EGF (Fig. 1A) mRNA expression in normal and infected cornea at 3 days p.i. ($P < 0.05$ and $P < 0.001$). FGF mRNA expression (Fig. 1C) was not significantly different between groups at all times tested. HGF mRNA expression (Fig. 1E) was significantly upregulated by VIP in normal, uninfected, and infected corneas at 1 and 3 days p.i. ($P < 0.01$, $P < 0.05$, and $P < 0.01$). EGFR was significantly increased at days 5 and 7 p.i. ($P < 0.01$ for both). There were no significant differences between the two groups for any of the growth factors at other time points tested (Figs. 1A, 1C, 1E, 1G).

FIGURE 1. mRNA and protein expression patterns after VIP. EGF (A) mRNA levels were significantly increased in VIP- versus PBS-treated B6 mice in the normal, uninfected cornea and at 3 days p.i. mRNA levels of FGF (C) were unchanged between the two groups. HGF mRNA expression levels (E) were significantly increased by VIP in normal, uninfected cornea and at 1 and 3 days p.i. EGFR mRNA levels (G) were increased after VIP treatment at 5 and 7 days p.i. ($n = 5$ per group per time per treatment for two experiments = 80 mice). ELISA analysis: When compared with PBS, VIP significantly increased EGF protein expression (B) at 5 and 7 days p.i., but earlier in the normal cornea and at 1 day p.i., EGF protein levels were lower after VIP treatment. For FGF (D), VIP increased protein levels only at 7 days p.i. For HGF (F), VIP significantly increased protein levels at 1 and 5 days p.i. No significant changes were seen at other times tested for all assays ($n = 5$ per group per time per treatment for two experiments = 60 mice). Results for mRNA and protein data are mean $\pm$ SD.

ELISA

Protein expression patterns for the three growth factors were assessed after examination of mRNA expression patterns. Protein expression for EGF, FGF, and HGF also is shown in Figures 1B, 1D, and 1F. When compared with PBS, VIP treatment upregulated EGF protein expression (Fig. 1B) later in disease (5 and 7 days p.i.; $P < 0.01$ and $P < 0.01$). Before infection and at 1 day p.i., levels were higher in the PBS treated group ($P < 0.001$ and $P < 0.001$). VIP treatment enhanced protein levels of FGF (Fig. 1D) only at 7 days p.i. ($P < 0.05$). For HGF (Fig. 1F), VIP significantly increased protein expression at 1 and 5 days p.i. ($P < 0.05$ and $P < 0.01$) and remained elevated at 7 days p.i., but was not significant.

mRNA Expression of Angiogenic Molecules

Whether mRNA expression of angiogenic molecules were changed after VIP also was assessed. For VEGF-A (Fig. 2A), treatment with VIP versus PBS significantly increased mRNA levels only in the normal, uninfected cornea ($P < 0.001$); but no difference between the two groups was seen at either 1 or 5 days p.i. For VEGFR1 (Fig. 2C), VIP increased mRNA expres-

FIGURE 2. VEGF mRNA, protein, and photography with a slit lamp to document VIP regulation. VIP versus PBS modestly elevated mRNA expression of VEGF-A (A) only in the normal, uninfected cornea. VEGFR1 (C) mRNA levels were increased by VIP in the normal, uninfected cornea, but levels decreased at 5 days p.i. VIP treatment increased VEGFR2 (E) in the normal cornea. No significant changes were seen at other times tested for all assays ($n = 5$ per group per time per treatment for two experiments = 40 mice). ELISA analysis: For VEGF-A (B), VIP versus PBS increased protein levels at 7 days p.i. For VEGFR1 (D), levels were unchanged at all times tested. For VEGFR2 (F), protein levels were increased only at 7 days p.i. Photography using a slit lamp showed that VIP treatment decreased opacity and increased peripheral corneal vascularization when compared with PBS (D) treatment (magnification, $\times 15$; $n = 5$ per group per time per treatment for two experiments = 40 mice). Results for mRNA and protein data are mean $\pm$ SD. (G, H) Photographs reprinted with permission from Szlitter EA, Lighvani S, Barrett RP, Hazlett LD. Vasoactive intestinal peptide balances pro- and anti-inflammatory cytokines in the *Pseudomonas aeruginosa*-infected cornea and protects against corneal perforation. *J Immunol.* 2007;178:1105-1114. Copyright 2007. The American Association of Immunologists, Inc.

sion in normal, uninfected cornea ($P < 0.001$), did not differ between groups at 1 day, but decreased levels at 5 ($P < 0.01$) days p.i. VIP increased VEGFR2 mRNA expression (Fig. 2E) in normal, uninfected ($P < 0.05$) cornea but no differences were seen between groups at 1 or 5 days p.i.

ELISA Analysis

Protein expression patterns for angiogenic molecules were assessed after VIP treatment and compared with controls. VIP versus PBS significantly increased VEGF-A (Fig. 2B) protein expression in the infected cornea at 7 days p.i. ($P < 0.05$). VEGFR1 (Fig. 2D) was not significantly different between groups at all times tested. VEGFR2 protein levels (Fig. 2F) were significantly increased (more than two-fold) by VIP versus PBS at 7 days p.i. ($P < 0.001$). No differences in protein levels were detected between the two groups at the other times tested (Figs. 2B, 2D, 2F). Consistent with these data (Figs. 2G, 2H), a photograph taken with a slit lamp at 7 days p.i., shows greater vascularity in the peripheral cornea of VIP- (Fig. 2H) versus PBS- (Fig. 2G) treated mice.

FIGURE 3. Immunostaining at 1 day p.i. for growth factors and VEGF-A. EGF immunostaining was slightly increased in PBS- (A) versus VIP- (B) treated mice (stromal as shown in the insets and epithelial). For FGF, epithelial staining was seen and similar between groups (C, D). For HGF VIP enhanced stromal immunostaining, while more epithelial staining was seen in the PBS group (E, F). For VEGF-A, staining was similar between groups in both the epithelium and stroma (G, H, and insets). An example of a negative control (primary antibody substituted with species-specific IgG) shows lack of positive immunostaining (I, J) seen with each of the factors tested. *Green*: Sytox green nuclear label; *red*: Alexa Fluor 594 secondary antibody staining; $n = 5$ per group per treatment for two experiments = 20 mice).

FIGURE 4. Immunostaining at 5 days p.i. for growth factor and VEGF-A. At this time, EGF, FGF, HGF, and VEGF-A epithelial and stromal staining was more intense in VIP- (B, D, F, H) versus PBS- (A, C, E, G) treated corneas. An example of a negative control (primary antibody substituted with species-specific IgG) shows lack of positive immunostaining (I, J) seen with each of the factors tested. *Green*: Sytox green nuclear label; *red*: Alexa Fluor 594 secondary antibody staining. Magnification, $\times 100$; $n = 5$ per group per treatment for two experiments = 20 mice.

Immunostaining

Immunohistochemistry was used to allow us to spatially localize EGF, FGF, HGF, and VEGF-A in the corneas of VIP- versus PBS-treated B6 mice at 1 (Figs. 3A–J) and 5 days p.i. (Figs. 4A–J). At 1 day p.i. slightly increased immunostaining was seen for EGF after PBS versus VIP treatment (Figs. 4A, 4B, and insets) in the epithelium and stroma. For FGF, staining was similar between groups and in the epithelium (Figs. 4C, 4D). For HGF VIP-enhanced stromal immunostaining, while more epithelial staining was seen in the PBS group (Figs. 4E, 4F), VEGF-A staining was similar between groups and seen in both the epithelium and stroma (Figs. 4G, 4H, and insets). Negative controls (primary antibody substituted with species-specific IgG) showed no positive immunostaining for each factor tested. An example of these negative controls shows lack of positive immunostaining (Figs. 4I, 4J) seen with each of the factors tested.

At 5 days p.i., EGF (Figs. 4A, 4B), FGF (Figs. 4C, 4D), HGF (Figs. 4E, 4F), and VEGF-A (Figs. 4G, 4H) staining of increased intensity was detected in the epithelium and stroma of VIP- versus PBS-treated mice. Negative controls (primary antibody substituted with species-specific IgG) showed no positive immunostaining

for EGF, FGF, or VEGF-A staining at either time point tested for either treatment. An example of these negative controls shows typical lack of positive immunostaining seen with each of the factors tested (Figs. 4I, 4J).

Growth Factor Treatment

Because results suggested that VIP exerted some of its healing effects in the infected cornea via modulation (enhancing) of growth factor production, the next study was performed to test that hypothesis. To assess whether the healing effects of VIP was through growth factor modulation, a mixture of EGF, FGF, and HGF was topically applied after infection. Clinical score data (Figure 5A) and photography with a slit lamp (Figs. 5B, 5C) revealed significantly less disease in the growth factor-treated mice at 5 days p.i. ($P < 0.05$ for clinical score). Although most corneas of growth factor-treated mice exhibited a +3 opacity, (only one perforated) all corneas of PBS-treated mice had perforated at 5 days p.i. In addition, RT-PCR analysis of growth factor-versus PBS-treated corneas showed that mRNA levels of the pro-inflammatory cytokine, MIP-2 (Fig. 5D), were significantly decreased ($P < 0.05$), while TGF- β (Fig. 5F; $P < 0.001$), VEGF-A (Fig. 5G; $P < 0.001$), and β -defensins 2 (Fig. 5H; $P < 0.05$) and 3 (Fig. 5I; $P < 0.001$) all were upregulated. There was no difference between the two treatment groups for TNF- α (Fig. 5E), or for VEGFR1 or VEGFR2 (data not shown).

Immunostaining

To begin to determine the mechanism by which growth factors given topically contributed to corneal defense, we used immunohistochemistry to examine infected corneas for antimicrobial molecules which had been shown by this laboratory to be protective in the infected cornea.^{14,15} Staining for antimicrobial β -defensins in the cornea is shown in Figures 6A–F. After growth factor versus PBS treatment, increased immunostaining for mBD2 was detected in both the epithelium and stroma at 5 days p.i. (Figs. 6A, 6B). However, negligible staining for mBD3 was observed, with no difference between groups (Figs. 6C, 6D). Controls (primary Ab substituted with species-specific IgG) showed no positive immunostaining for mBD2 or mBD3. These negative controls showed lack of positive immunostaining and appeared similar to staining with nuclear label (SYTOX Green; Lonza; Figs. 6E, 6F).

Bacterial Counts

To determine whether increased β -defensin production correlated with decreased bacterial number in the cornea, bacterial plate counts (Fig. 6G) were used to detect viable bacteria in the infected cornea of mice treated with growth factors versus PBS at 1, 3, and 5 days p.i. When compared with controls, growth factor treatment led to decreased bacterial plate counts at 1 ($P = 0.0001$), 3 ($P = 0.35$), and 5 days p.i. ($P = 0.09$), but was not significant at either of the latter times.

DISCUSSION

Previously, this laboratory has provided evidence that VIP, a 28-amino acid, anti-inflammatory neuropeptide, regulates cytokine production in *P. aeruginosa*-induced keratitis, leading to decreased stromal destruction in a B6 mouse model in which the infected cornea normally perforates.⁷ In this regard, VIP is involved in the regulation of a wide variety of immune reactions and among its numerous anti-inflammatory functions, participates in the maintenance of immune homeostasis.⁹ In addition, VIP has been considered in itself to be a growth factor,²⁰ whereas other studies have suggested that it does not function of itself in this, but that it may regulate other more classic growth factors such as VEGF²¹ and nerve growth factor (NGF).²² These growth factors can facilitate noninfectious

wound healing and may also function as anti-inflammatory cytokines.

The present study indicates that VIP promotes production of EGF, FGF, HGF, and VEGF-A and participates in regulating angiogenesis, all of which contribute to resistance to *P. aeruginosa* keratitis. It is highly likely that these intriguing effects may be indirect, through facilitating wound healing, and/or epithelial barrier function, for example. It also is possible that the effect of VIP and growth factors may modulate inflammation, balancing its required contribution to the healing process, with its downregulation which is permissive to healing. In this regard, the present study has shown that both epithelial and stromal cells participate in growth factor production and are

FIGURE 5. Topical growth factor treatment and assessing disease response by clinical evaluation and RT-PCR. Clinical scores (A) indicated statistically significant differences at 5 ($P < 0.05$) days p.i. between groups. Photographs taken with a slit lamp at 5 days p.i. showed a worsened disease response (perforation) when comparing control (B) versus growth factor (C) treatment. Magnification, $\times 6$ ($n = 5$ per group per treatment for two experiments = 20 mice). mRNA levels (D–I) showed a significant decrease in MIP-2 (D), no change in TNF- α (E), and increased TGF- β (F), VEGF-A (G), and β -defensins 2 (H) and 3 (I) in growth factor versus control treated eyes ($n = 5$ per group per treatment for two experiments = 20 mice). Results are shown as mean $\pm$ SD.

FIGURE 6. Immunostaining and bacterial counts after topical growth factor treatment. At 5 days p.i., β -defensin 2 epithelial and stromal staining was more intense in growth factor- (B and inset) versus PBS- (A) treated corneas. At this time, negligible staining for β -defensin 3 was seen after either growth factor (D) or PBS (C) treatment. Controls, in which the primary Ab was replaced by IgG, were negative for immunostaining (E, F) for both defensins. Green: Sytox green nuclear label; red: Alexa Fluor 594 secondary antibody staining ($n = 5$ per group per treatment for two experiments = 20 mice). (G) Bacterial counts: Growth factor versus PBS treatment reduced bacterial plate counts at 1, 3, and 5 days p.i., but were significant only at 1 day p.i. ($n = 5$ per group per treatment for two experiments = 60 mice). Results are shown as mean $\pm$ SD.

modulated by VIP treatment. Unfortunately, there is a dearth of other studies concerning the role of VIP regulating many of the classic growth factors which have been studied herein; thus, the effects of VIP on EGF, HGF, and FGF production are initially characterized in the present study. Despite this, previous investigators have shown that VIP can directly activate the EGF receptor (EGFR)²³ which is confirmed at the mRNA level in the present study described herein. In addition, we found that VIP increases EGF expression at the mRNA and protein levels and by immunostaining at mid-to-later times p.i. How-

ever, early after infection, EGF mRNA levels were similar between the two groups, but unexpectedly, protein and immunostaining was slightly increased in the PBS- versus VIP-treated group initially, suggesting that VIP has a complex regulatory function for this growth factor. In addition, investigators reported that either EGF or VIP inhibited the secretion of several proinflammatory cytokines, such as IL-1 and IL-8, and also reduced adhesion of neutrophilic leukocytes to bronchial epithelial cells in vitro.²⁴ We reported previously that VIP significantly reduced proinflammatory cytokine production in microbial keratitis, including IL-1 and MIP-2 (murine homolog of IL-8) levels,⁷ but the present study shows that this effect may have been enhanced indirectly through VIP modulation of EGF. In fact, in another study it was reported that during infection with an enteropathogenic strain of *Escherichia coli* oral administration of EGF significantly reduced *E. coli* colonization.²⁵ These findings are consistent with a past study which has shown that VIP reduces *P. aeruginosa* corneal bacterial load and with the present study which suggests that this effect may have been at least in part, due to upregulation of EGF at later times p.i. via VIP administration.

Another growth factor, KGF/FGF-7 also participates in disease resolution through reduction of bacterial load. In this regard, clearance of *P. aeruginosa* was increased by intratracheal treatment with KGF in an induced lung injury bacterial infection model.²⁶ KGF also increased antimicrobials β -defensin 2 and cathelicidin (LL-37) mRNA levels which were associated with reduced bacterial load in *P. aeruginosa* infection in a skin model.²⁷ These data are consistent with the present study, in that VIP increased FGF (KGF/FGF-7) expression and when used in a growth factor cocktail, applied to the cornea after infection, increased β -defensins 2 (mRNA and protein) and 3 (mRNA). These data compliment prior work from this laboratory in which we showed that in the cornea, both defensins are protective in a murine (BALB/c, resistant) model of *P. aeruginosa*-induced microbial keratitis^{14,15}; this inbred strain also was shown to have increased levels of VIP when compared with B6 mice after infection.⁷

VIP also increased HGF expression at both mRNA and protein levels (ELISA and immunostaining). This growth factor has anti-inflammatory properties,²⁸ in that it can target vascular endothelial cells and disrupt nuclear factor-kappa B (NF- κ B) signaling,²⁹ critical to regulating inflammation. We also have reported that VIP treatment of susceptible B6 mice, decreased proinflammatory cytokines, including but not limited to IFN- γ , IL-1 β , TNF- α , and MIP-2 and enhanced protective cytokines such as TGF- β and IL-10.⁷ TGF- β , in addition to its regulation of immune responses, is also profibrotic,³⁰ which may reflect increased wound healing in the VIP- and/or growth factor-treated mice in which stromal destruction is diminished. Because the present study has shown that HGF levels are enhanced after VIP treatment in B6 mice, it is not inappropriate to suggest that the observed protective effects of VIP could be affected by HGF enhancement. Immunostaining for VEGF-A was observed in both epithelium and stroma at 5 days p.i., confirming the ELISA data and providing information regarding protein localization. High expression of VEGF-A mRNA in the newly formed epidermis after wounding³¹ is somewhat consistent with our data, which shows a more modest effect. Elevated expression of VEGF-A may be indicative of its exerting its growth factor functions to induce healing, cell division, migration, cell survival, and proliferation.³² In addition to its growth factor properties, VEGF-A can also regulate angiogenesis.^{33,34} Data from the present study show that VIP upregulates VEGFR2 protein levels more than two-fold at 5 days p.i. These data indicate that VIP, via upregulation of VEGFR2 has an angiogenic effect that is beneficial to the avascular cornea for disease resolution, (confirmed by photography using a slit

lamp). The data are also consistent with the tenet that the cornea must balance its usually high threshold for resisting vascular ingrowth (corneal hemangiogenesis) with an ability to react to sight-threatening injuries (e.g., *P. aeruginosa*), with a robust and rapid angiogenic response, required to enhance immune defenses.^{35,36} We hypothesize that inflammation is reduced through the actions of VIP on proinflammatory cytokines and chemokines, but also, with increased VEGF-A and R2 protein levels, as reported herein, immune cells are better able to enter the cornea, more rapidly participate in bacterial disposal, thus limiting bacterial-induced stromal damage; this is followed by healing through the role of VIP in promoting anti-inflammatory cytokines,⁷ and modulating growth factor production as shown herein. These data also are consistent with other studies using a rat sponge model for quantitative assessment of angiogenesis which have shown that VIP treatment improved angiogenesis in sponge sections.³⁷

In vitro studies have shown that exogenous application of EGF can stimulate corneal epithelial cell migration³⁸ which may have a positive effect in microbial keratitis, contributing to restoration of epithelial barrier function.

In addition, other studies have shown that topical application of EGF and FGF can promote wound healing in several types of corneal wound models,^{17–19} consistent with the lack of perforation in VIP-treated mice in which these growth factors are elevated. In this regard, topical treatment with a growth factor cocktail containing EGF, FGF, and HGF provided evidence that at least some of the effects seen with VIP may be evoked through these growth factors. For example, in growth factor-treated eyes, only one of five versus all 5 corneas of PBS-treated eyes perforated; MIP-2, a chemokine that attracts neutrophils (PMN) into the cornea² mRNA was reduced; while TGF- β , an anti-inflammatory cytokine, was upregulated as shown before with VIP treatment.⁷ Growth factor treatment also significantly increased β -defensins 2 and 3 mRNA expression at 5 days p.i. and β -defensin 2 staining was enhanced at 5 days p.i. in both epithelium and stroma. These data are consistent with a previous study showing that KGF increased β -defensin 2 expression levels in a skin model expressing KGF/FGF-7.²⁷ Most importantly, after growth factor treatment, bacterial counts also were reduced at 1, 3, and 5 days p.i., but were significant only at 1 day p.i. These data suggest that there is a delay in bacterial growth in the growth factor-treated cornea which may reflect less stromal damage which could be permissive to bacterial growth.

Overall, this study provides evidence that VIP modulates growth factors, angiogenic molecules (both epithelium and stroma), β -defensin production, and bacterial counts and that many of the effects observed with VIP treatment can be compensated for through exogenous growth factor delivery.

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