Effect of the Synthetic NC-1059 Peptide on Diffusion of Riboflavin across an Intact Corneal Epithelium

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PURPOSE. To investigate the effect of the peptide NC-1059 on riboflavin (RF) diffusion across an intact corneal epithelium into the stroma.

METHODS. NC-1059 peptide was synthesized by solid-phase synthesis with 9-fluorenylmethoxycarbonyl chemistry, characterized by reversed-phase HPLC, and matrix-assisted laser desorption ionization time-of-flight mass spectroscopy. The diffusion of RF across embryonic day 18 chick corneal epithelium ex vivo was monitored using confocal microscopy. The depth distributions of RF in the corneal stroma were calculated using a group of linear equations based on the relationship between RF fluorescence intensity and concentration.

RESULTS. Data presented in this study demonstrate that the NC-1059 peptide can transiently open the intact epithelial barrier to allow the permeation of RF into the stroma. The effect of NC-1059 peptide on RF diffusion across the corneal epithelium was concentration and time dependent. The amount of RF reaching a 50- μ m depth of chick corneal stroma increased dramatically after exposure to NC-1059 for 10 minutes, reaching a plateau by 30 minutes. The concentrations of RF in the presence of NC-1059 at corneal stromal depths of 50, 100, and 150 μ m were significantly higher than in the absence of the peptide, and almost as high as in corneas in which the epithelium first had been physically removed. In addition, a cell viability assay indicated that the NC-1059 peptide did not kill corneal epithelial cells.

CONCLUSIONS. NC-1059 peptide significantly enhances the diffusion of RF across intact corneal epithelium into the stroma. (*Invest Ophthalmol Vis Sci.* 2012;53:2620–2629) DOI:10.1167/iops.12-9537

Keratoconus is a vision disorder of unknown molecular etiology, but with some genetic basis. It occurs when the normally spherical cornea begins to bulge outward acutely. This abnormal shape usually occurs where the central stromal

region becomes thinner, preventing the light entering the eye from being focused correctly on the retina and causing distortion of vision.¹ Keratoconus may progress for 10–20 years and then slow in its progression. Each eye may be affected differently. Keratoconus affects 1 in 2000 people.² Treatment options include conservative approaches aimed at maintaining visual acuity, such as rigid contact lenses placed to correct corneal aberrations.³ Alternatively, more invasive methods are applied using intrastromal corneal implants,⁴ performing anterior lamellar keratoplasty, and penetrating keratoplasty in extreme cases.^{5,6} However, all of these techniques only correct the refractive errors of keratoconus but do not treat the cause underlying the corneal ectasia and, therefore, do not block the progression of keratoconus.

Recently, a new technique of corneal cross-linking was devised that immediately increases the biomechanical rigidity of the corneal stroma and stops further degradation of the cornea.⁷ This approach consists of irradiation with ultraviolet-A in the presence of stromal riboflavin, the photosensitizing chromophore and UVA blocking agent, to stop the progression of the keratoconus syndrome. The basic principle of corneal cross-linking is thought to be that in the presence of UVA at 370 nm, riboflavin in the corneal stroma is excited into its triplet state, generating singlet oxygen species that can react further with various molecules, inducing formation of covalent bonds bridging amino and carbonyl groups of collagen fibrils,^{8,9} as well as proteoglycan core proteins.¹¹ During this process, sufficient concentrations of exogenously applied RF in the stroma are required for corneal cross-linking as well as for absorbing the UVA irradiation, thereby preventing damage to the cells of deeper ocular tissues, such as the lens and retina.¹²

In the standard clinical protocol of corneal cross-linking, the central 7 mm of the corneal epithelium is removed surgically to allow immediate diffusion of riboflavin into the de-epithelialized stroma. Before irradiation with UVA, a 0.1% riboflavin solution (10 mg riboflavin-5-phosphate in 10 mL of a 20% dextran solution) is applied to the debrided central cornea every 5 minutes for 30 minutes. Then, as the application of riboflavin continues, UVA irradiation at 370 nm with irradiance of 3 mW/cm² (5.4 J/cm²) is applied at a 1-cm distance for an additional 30 minutes.^{13–17} Unfortunately, the need to remove the epithelium causes mild to severe postoperative pain for the first 3 to 4 days and is associated with an increased risk of corneal infections.^{18–21} Thus, there are valid reasons for seeking to modify the standard protocol and to perform the treatment with epithelial retention (“epi-ON” protocols).^{22–24} Early attempts to accomplish this have included the use of multiple applications of the topical anesthetic, tetracaine 1%,^{25,26} 20% ethanol solution,²⁷ 0.02% benzalkonium chloride (BAC),²⁸ 0.44% NaCl in combination with 0.01% BAC,²⁹ or by removing the epithelium in a grid pattern rather than complete debridement²⁷ to assist the diffusion of RF into the stroma before beginning the irradiation with UVA.

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Supported by the National Institutes of Health R01EY000952 to GWC, the Research Career Development Core (Brychta) in the Division of Biology at Kansas State University G0B0000657 to GWC, NIH-R01GM074096 to JMT, and by NIH-P20-RR017686 to the Center of Biomedical Research Excellence Core B in the Veterinary School at Kansas State University.

Submitted for publication January 19, 2012; revised February 22, 2012; accepted March 13, 2012.

Disclosure: **Y. Zhang**, None; **P. Sukthankar**, None; **J.M. Tomich**, None; **G.W. Conrad**, None

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FIGURE 1. Schematic diagram of chicken cornea incubation ex vivo and monitoring the diffusion of RF with confocal microscopy.

Corneal epithelium is a significant biological barrier (e.g., preventing drug permeation into the eye).^{30–32} The corneal epithelium is composed of five to six layers of columnar epithelial cells; a basal layer of columnar cells; two to three layers of wing cells; and one to two outermost layers of squamous, polygonal-shaped, superficial cells.³¹ In the normal corneal epithelium, intercellular tight junctions (zonula occludens) completely surround the most superficial cells that restrict the passage of foreign molecules across the cornea and also act as a barrier to ion diffusion.^{33–35} Therefore, drug absorption into the corneal stroma relies on transcellular passage or strategies that can modulate (e.g., transiently open) the epithelial cell tight junctions.^{36–40} Interestingly, previous studies have demonstrated that NC-1059, a synthetic nonselective ion channel-forming peptide, enhances drug permeation across corneal epithelial cell monolayers incubated in vitro by transiently opening the paracellular pathways.^{41–43} These properties suggest that this peptide could assist the diffusion of RF into the stroma before the irradiation with UVA. In this study, the potential ability of NC-1059 peptide to facilitate RF diffusion across an intact corneal epithelium ex vivo into the stroma has been investigated.

MATERIALS AND METHODS

Materials

Riboflavin 5'-monophosphate sodium salt was purchased from Sigma (St. Louis, MO). Pico-Green (PicoGreen dsDNA Quantitation Kit) and LIVE/DEAD Viability/Cytotoxicity Kit were purchased from the Invitrogen Corporation (Carlsbad, CA).

Peptide Synthesis

The peptides used in this study were generated by solid-phase synthesis with 9-fluorenylmethoxycarbonyl chemistry, as described in detail previously.⁴⁴ The peptide was designed so that the transmembrane segment had an N-terminal tetra-lysine domain and so that a central leucine was flanked by a palindrome that is based on the N-terminal portion, and was designed so that the transmembrane segment had an N-terminal tetra-lysine domain and with a central leucine defining a palindrome based on the N-terminal portion of second transmembrane segment of the alpha subunit of the spinal cord glycine receptor M2GlyR and was designated NC-1059 (KKKKAARVGLGITTTLVLTIGLVRAA).⁴⁵ The peptides were purified and characterized by reversed-phase HPLC and matrix-assisted laser desorption ionization time-of-flight mass spectroscopy.

Cornea Incubation Ex Vivo

All animals were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Fertilized White Leghorn chick eggs were purchased from Nelson Poultry Farms, Inc. (Manhattan, KS). These freshly fertilized eggs were incubated at 38°C and 45% humidity from embryonic day of incubation zero, the day when the eggs were put into the incubator. At embryonic day 18, the entire anterior half of the eyeball, including the cornea and surrounding limbus and conjunctiva (but with lens and iris removed), was used as a model system to investigate the effect of the NC-1059 peptide on RF diffusion across an intact corneal epithelium into the stroma. Immediately after harvest, such E18 anterior half eyeballs (hereafter simply referred to as "corneas"), were rinsed in a balanced salt solution (BSS) (122 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 1.57 mM Na₂HPO₄, 25 mM NaHCO₃, 5 mM dextrose, 0.3 mM glutathione disulfide; pH approximately 7.4). Subsequently, each fresh cornea was incubated with 0.1% RF in BSS with/without NC-1059 peptide at 37°C in a glass-bottom dish incubator and examined with confocal microscopy (Zeiss LSM 510; Carl Zeiss, Oberkochen, Germany) (Fig. 1).

Monitoring the Diffusion of RF

The diffusion of RF across the corneal epithelium and into the stroma was monitored using confocal microscopy (Carl Zeiss) every 5 minutes for 60 minutes. The parameters set were: HFT 488 nm, NFT 545 nm, and BP 505–530 nm emission filter (RF). Z-series through corneas were collected with a Plan-Neofluar 40×/1.3 differential interference contrast objective.

Quantification of RF Concentrations in the Stroma

The relationship of fluorescence intensity and molar concentration of RF was established by determining the intensities of standard RF solution 25-μL drops on glass slides without a glass cover slip.⁴⁶ A group of linear equations (shown in Fig. 5A) was derived and used to calculate the molar concentration of RF at different standard corneal depths of 50 μm, 100 μm, and 150 μm from the surface of the cornea, while corneas were incubated with NC-1059 peptide in BSS at 37°C for 30 minutes.

Imaging Nuclei of Corneal Epithelial Cells

The corneas from E18 chick embryos were dissected and rinsed in BSS. Subsequently, the tissues were incubated with 200 μM NC-1059 peptide, 5 mM EDTA, 0.02% BAC, or BSS at 37°C for 30 minutes. Freshly dissected E18 chick corneas were used as normal positive controls. All samples were stained in the dark using Pico-Green

FIGURE 2. The absorption spectrum and fluorescence emission spectrum of 0.1% riboflavin in BSS. **(A)** From 300 to 600 nm of wavelength, absorption spectra of riboflavin demonstrated absorption peaks at 371 and 442 nm. **(B)** Scanning the excitation wavelength range from 490 to 800 nm, an emission maximum was observed at 533 nm for the fluorescence emission spectrum of riboflavin in BSS when exciting at wavelengths of 442, 458, and 488 nm, respectively.

(Invitrogen).⁴⁷ An aqueous working solution of the PicoGreen reagent was made by a 200-fold dilution of the concentrated DMSO solution in TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH, 7.5): PicoGreen commercial stock solution (100 μ L) was added to 19.9 mL of the working solution to produce a 1:200 dilution of the dye. The fresh anterior halves of chick eyes were incubated in the dye solution for 30 minutes at room temperature, rinsed twice with Hank's buffered salt solution for 5 minutes, and then viewed by confocal microscopy (Carl Zeiss) as shown in Figure 1. The parameters set were: HFT 488 nm, NFT 545 nm, and BP 505–530 nm emission filter (PicoGreen). Z-series through corneas were collected with a Plan-Neofluar 40 $\times$ /1.3 DIC objective. The nuclei of both live and dead cells were stained by PicoGreen.

Live/Dead Epithelial Cell Assay

A LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen) also was used to discriminate between live and dead epithelial cells. A staining solution containing both 2- μ M calcein AM and 4- μ M ethidium homodimer-1 was made by adding both 5 μ L of 4-mM calcein AM stock solution and 20 μ L of 2-mM ethidium homodimer-1 stock solution to 10 mL of Dulbecco's phosphate buffered saline (2.68 mM KCl, 1.47 mM KH_2PO_4 , 136.75 mM NaCl, 8.06 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; pH 7.4). The corneas from E18 chick embryos were dissected and rinsed in BSS. Subsequently, the tissues were incubated with BSS containing 200 μ M NC-1059 peptide, 5 mM EDTA, or 0.02% BAC at 37°C for 30 min, separately. Freshly dissected E18 chick corneas stained directly (subjected to no

incubation *in vitro*) were used as live-cell controls. For the dead-cell control, all cells were killed by incubating freshly dissected E18 chick corneas in 70% (v/v) methanol at room temperature for 30 minutes. After incubating, all samples were rinsed three times with D-PBS, each for 1 minute, and then stained with 2- μ M calcein AM and 4- μ M ethidium homodimer-1 working solution at room temperature for 10 minutes, rinsed three times with D-PBS for 1 minute each, and then imaged by confocal microscopy (Carl Zeiss). The parameters used were: a 488-nm laser line from an argon ion laser, a 543-nm laser line from a HeNe laser, HFT 488/543 nm, NFT 545 nm, BP 505–530 nm emission filter (calcein AM), and LP 585-nm emission filter (ethidium homodimer-1). Z-series images through corneas were collected with a Plan-Neofluar 40 $\times$ /1.3 DIC objective. By this assay, the polyanionic dye calcein is well retained within live epithelial cells, producing an intense uniform green fluorescence. In contrast, ethidium homodimer-1 enters only cells with damaged membranes and undergoes an enhancement of fluorescence upon binding to DNA, thereby producing a bright-red fluorescence only in the nuclei of dead cells.

Statistical Analysis

Data are presented as means $\pm$ standard deviation (SD) from three separate experiments. Statistical analyses were performed using Student's *t*-tests to compare readings from the concentrations of RF detected at different depths in the stroma of corneas, assuming equal variances. A *P* value less than 0.05 was considered significant.

FIGURE 3. The features of epithelium, stroma, endothelium, and epithelial cells of E18 chick corneas. **(A)** Histological cryostat section of E18 chick cornea stained with hematoxylin eosin. **(B)** Bright field face-on image of the epithelial cells of fresh E18 chick cornea. Scale bars = 50 μ m.

FIGURE 4. Effects of NC-1059 peptide modulating the diffusion of riboflavin across the corneal epithelium into the stroma. All corneas below were incubated in the solution described for 30 minutes at 37°C before confocal examination. (A) E18 cornea incubated with BSS alone. (B) Effect of epithelial removal from an E18 cornea, followed by incubation with 0.1% RF in BSS. (C) Effect of epithelium retention in an E18 cornea, followed by incubation with 0.1% RF in BSS. Bright layer at the top is the RF solution, dark layer below it is the epithelium, which has effectively excluded most RF from entering the stroma. Red arrow diagrammatically represents the diffusion of RF across the epithelium and into the stroma. (D) Effect of epithelium retention in an E18 cornea with 0.1% RF and 200 μ M NC-1059 peptide in BSS. The graphs of (E), (F), (G), and (H) corresponding to each (A), (B), (C), and (D) image, correlate the fluorescence intensity of RF with corneal depth. Scale bar = 50 μ m.

RESULTS

Fluorescence Determination of Riboflavin

Riboflavin is poorly soluble in water, hence riboflavin-5'-phosphate (abbreviated here as RF) was used in this study (unless stated otherwise), consistent with its use in clinical protocols.^{12,14,17} The absorption spectrum and fluorescence emission spectrum of 0.1% riboflavin in BSS are presented in Figure 2. Over the wavelength range of 300–600 nm, the absorption spectrum of riboflavin demonstrated absorption peaks at 371 and 442 nm (Fig. 2A). Scanning the excitation wavelength range from 490 to 800 nm showed an emission maximum at 533 nm for the fluorescence emission spectrum of riboflavin when excited at 442, 458, and 488 nm (Fig. 2B). The highest fluorescence emission intensity of 0.1% RF in BSS was obtained with excitation at 488 nm. Thus, the 488-nm laser line from the argon laser and BP 505–530 nm emission filter was chosen for monitoring the diffusion of RF into the stroma of E18 chick corneas using confocal microscopy (Carl Zeiss).

Effect of NC-1059 Peptide on the Diffusion of Riboflavin

The features of epithelium, stroma, endothelium, and epithelial superficial cells of E18 chick corneas are shown in Figures 3A and 3B, suggesting that chick corneas of E18 embryos are a suitable animal model for investigating the effect of NC-1059 on the diffusion of RF into the cornea as used previously. Figure 4 shows the effects of NC-1059, modulating the diffusion of RF across the intact corneal epithelium into the stroma. For the negative control, no endogenous fluorescence was detected in E18 cornea incubated in BSS without RF (Fig. 4A). As a positive control, diffusion of RF into stroma was seen when the epithelium was surgically removed (Fig. 4B), whereas only weak fluorescence intensity of RF appeared in the stroma when the epithelium remained intact (Fig. 4C), demonstrating the barrier function of intact chick E18 corneal epithelium. In contrast, strong fluorescence intensity of RF was detected in the stroma after exposure of the cornea with an

intact epithelium to 200 μ M NC-1059 in 0.1% RF BSS for 30 minutes at 37°C (Fig. 4D), suggesting that the epithelial intercellular tight junctions were opened by NC-1059, allowing diffusion of RF into the stroma. As expected of a simple diffusion gradient formed across the epithelium, the concentration of RF was highest in the most anterior stroma and progressively lower toward the posterior stroma (Figs. 4C, 4D).

Data presented in Figure 5 demonstrate that the effect of the NC-1059 peptide on the diffusion of RF across the epithelial layer into the stroma is concentration and time dependent. The fluorescence intensity of RF detected in the stroma significantly increased with the concentrations of NC-1059 from 50 μ M to 200 μ M, as well as with incubation times from 10 to 50 minutes. However, fluorescence intensity of RF in the stroma did not appear to increase with incubation time when corneas were treated with 5 mM EDTA (Fig. 5E) or with 0.02% BAC (Fig. 5F), indicating that the mechanisms by which EDTA and BAC open the epithelium to RF diffusion are different from those induced by the peptide.

Quantification of the Permeation of RF into the Stroma

Results presented in Figure 6A indicate that the effect of 200 μ M NC-1059 on diffusion of RF into corneal stroma was time dependent and that the fluorescence intensity of RF at the 50 μ m depth of the stroma significantly increased, compared with that seen in the absence of the NC-1059. The concentration of RF increased approximately linearly with time during the first 25 minutes of exposure to NC-1059, reached a maximum level by 30 minutes, and then remained stable at that high concentration during the next 20 minutes (50 minutes total incubation time). In the absence of NC-1059, a much lower level of RF diffused into the stroma; but, as in the presence of peptide, a stable RF concentration was also reached by approximately 30 minutes, and then maintained for the next 20 minutes (50 minutes total incubation time). Determining the fluorescence of riboflavin drops of a series of standardized concentrations allowed the construction of a standard curve, and the derivation of a group of linear equations relating RF

FIGURE 5. NC-1059 peptide causes a concentration-, and time-dependent effect on the diffusion of RF across the E18 corneal epithelial barrier into the stroma. (A) The absence of NC-1059 peptide in 0.1% RF (B) 50 μ M NC-1059 peptide in 0.1% RF (C) 100 μ M NC-1059 peptide in 0.1% RF (D) 200 μ M NC-1059 peptide in 0.1% RF (E) 5 mM EDTA in 0.1% RF (F) 0.02% BAC in 0.1% RF. From left to right, each single image corresponding to the time of the cornea culture ex vivo at 10, 20, 30, 40, and 50 minutes. Scale bar = 50 μ m.

fluorescence intensity with concentration at depths of 50, 100, and 150 μ m from the surface of epithelium was achieved (Fig. 6B). For E18 chick corneas treated with NC-1059, statistical analysis revealed that the concentrations of RF detected at stromal depths of 50, 100, and 150 μ m were all significantly higher than those detected in the absence of NC-1059. As expected, the surgical removal of the epithelium allowed maximal diffusion of RF into the stroma, but only slightly more than the concentrations attained in the presence of an intact epithelium treated with NC-1059 (Fig. 6C).

Live/Dead Epithelial Cell Assay

Figure 7 shows the morphologies of PicoGreen-stained nuclei of E18 chick corneal epithelial after incubation in alternative solutions. The patterns of the corneal epithelial nuclei treated with NC-1059 (Fig. 7C) do not differ from those of the BSS group (Fig. 7B) or from fresh cornea alone (Fig. 7A). However, exposure of corneas to 5 mM EDTA or 0.02% BAC resulted in a distinct aggregation of nuclei of E18 chick corneal cells (Figs. 7D, 7E). Representative images of live and dead epithelial cells of E18 chick corneas after incubation in alternative solutions are shown in Figure 8. There was no significant difference in the incidence of dead epithelial cells (red) observed between

BSS with 200 μ M NC-1059 versus BSS alone versus fresh corneas, whereas almost all epithelial cells were dead after incubation in 0.02% BAC. In addition, many epithelial cells were dead after incubation in 5 mM EDTA. Thus, these results suggest that NC-1059 at 200 μ M for 30 minutes greatly increases the paracellular permeability of the corneal epithelium cell layer to RF and its effects do not appear to cause toxicity in those cells.

DISCUSSION

In this study, chicken corneas at E18 were chosen as an animal model to investigate the ability of NC-1059 peptide to facilitate RF diffusion across an intact corneal epithelium. During development of the chicken corneal epithelium, the superficial cells with villi and apical junctions begin to appear on the ninth day.⁴⁸ Between E10 and E13, superficial cells begin to peel off and the three-cell-layer epithelia become visible.⁴⁹ The gap junctions and sealing junctions near the free surface of the epithelium appear during this time period. On E18 (stage 44), the corneal epithelium, stroma, and endothelium are close to maturity.⁴⁹ Importantly, a previous study demonstrated that the epithelial junctions effectively act as a barrier to ruthenium red

FIGURE 6. Effect of NC-1059 peptide on RF diffusion across an intact corneal epithelium into the stroma. (A) NC-1059 peptide (200 μM) causes a time-dependent increase in RF diffusion into the stroma. (B) The linear equations derived that correlate fluorescence intensity and RF concentrations at three different depths, 50, 100, and 150 μm in the stroma in E18 chick corneas. (C) Concentrations of RF detected at three standard depths in the stroma of corneas incubated for 30 minutes without peptide and with epithelium intact, versus with peptide NC-1059 and with epithelium intact ("Epi-ON+NC-1059"), without peptide and with epithelium surgically removed ("Epi-Removed").

penetration into chick corneal stroma from E12 (stage 38) to hatching at E21 (stage 45), suggesting that the outer cell layer of the chick embryonic corneal epithelium is the site of functioning tight junctions, and acts as an effective barrier, preventing large molecules from crossing the epithelium.⁵⁰ Thus, the chick corneas of E18 embryos are a suitable animal model for investigating the effect of NC-1059 on the diffusion of riboflavin into the corneal stroma.

Keratoconus is a noninflammatory, progressive thinning process of the cornea. It is a relatively common disorder of unknown etiology that can involve each layer of the cornea and often leads to high myopia and astigmatism.²⁴ Riboflavin-catalyzed ultraviolet light-activated cross-linking has received a significant amount of attention in recent years. It is currently approved in Europe as a treatment for keratoconus and is also being used for other corneal disorders.^{51,17} During corneal cross-linking treatment, riboflavin is a key component as it is excited by UVA into its triplet state, generating singlet oxygen that can react further with various molecules,⁹ inducing chemical covalent bonds bridging amino groups of collagen fibrils,¹⁰ and other components of the corneal stroma extracellular matrix.¹¹ In addition, concentrations of RF in the stroma adequate to catalyze crosslinking also increase the absorption of UVA, and thus protect the deeper ocular tissues from UVA damage.¹² Many recent publications on corneal cross-linking for keratoconus have advocated the complete removal of the epithelium to allow RF penetration into the corneal stroma.^{7,13–17} To overcome the postoperative pain and risk of corneal infections caused by epithelial removal, the E18 chick cornea were used as an ex vivo model system to investigate the effects of NC-1059 peptide on RF diffusion across an intact corneal epithelium into the stroma. Study data demonstrate that NC-1059 can open the epithelial intercellular tight junctions to allow the diffusion of RF into the stroma and can do so without killing corneal epithelial cells.

A synthetic peptide, NC-1059, exhibits the barrel and stave mechanism to produce ion channels.^{42,43} The peptide undergoes supramolecular assembly as parallel helices to form oligomeric pores that show opening and closing on the millisecond time scale. The palindromic sequence, NC-1059, forms a nonselective ion channel that reversibly alters epithelial barrier function. This modulation occurs through the reorganization of epithelial tight junctions in Madin-Darby canine kidney epithelial cell monolayers.⁴² Moreover, a recent study has demonstrated that NC-1059 can modulate the barrier function of corneal epithelium to enhance drug permeation across cultured corneal epithelial cell monolayers by transiently affecting the paracellular pathway.⁴¹ Study data presented in Figure 4 show that NC-1059 can affect intact, living corneal epithelium in such a manner that the cells remain alive, but RF diffuses across the cell layer, which suggests that the paracellular pathway of E18 chick corneal epithelium may be transiently opened by NC-1059, by a mechanism yet to be characterized. These results are consistent with the findings from corneal epithelial cell monolayers in vitro⁴¹ and suggest a possible nonselective ion channel mechanism of NC-1059.⁴² In addition, in this study corneas were incubated ex vivo less than 60 minutes. All E18 chick corneas were freshly collected in the lab just before treating with RF and NC-1059 peptide. During this short time, the corneas may not degenerate, as indicated by the results of live/dead epithelial cell assay: There were no dead epithelial cells observed in the BSS treatment group in Figure 8.

Data presented in Figure 5 demonstrate that NC-1059 induced a concentration-dependent increase in RF across the corneal epithelium. Compared with the absence of NC-1059, an effective concentration of NC-1059 was observed as low as 50 μM at about 10 minutes after NC-1059 exposure. With increasing concentrations of NC-1059, significant increase in RF across the corneal epithelial barrier was observed with 100 μM and 200 μM of NC-1059, respectively. It is important to note that there was no obvious difference in RF fluorescence intensity in corneal stroma with 400 μM of NC-1059 as compared with that of 200 μM of NC-1059 (data not shown); hence higher concentrations were not tested. As can be seen from Figures 5 and 6, the effect of NC-1059 on diffusion of RF

FIGURE 7. Nuclear morphologies of E18 chick corneal epithelial cells stained with PicoGreen. (A) Fresh cornea alone. (B) Fresh cornea incubated with BSS for 30 minutes. (C) Fresh cornea incubated in 200 μ M NC-1059 in BSS for 30 minutes. (D) Fresh cornea incubated in 5 mM EDTA in BSS for 30 minutes. (E) Fresh cornea incubated in 0.02% BAC in BSS for 30 minutes. Scale bar = 50 μ m. PicoGreen, as used here, stains the nuclei of both live and dead cells.

into corneal stroma exhibited a time-dependence. The concentration of RF at 50- μ m depth of chick corneal stroma increased distinctly after exposure to NC-1059 for 10 minutes, reaching a much higher, stable level by 30 minutes. These results were consistent with previous studies showing that NC-1059 exposure increased dextran permeation when compared with basal permeation,⁴¹ an effect considered to be due to an opening of the paracellular pathway, as well as due to the introduction of an ion channel into the apical membrane.⁴² Permeability was dependent on the size of the diffusing molecule, a size selectivity that was maintained after NC-1059 exposure, but not after exposure to EDTA.⁴² EDTA is a $\text{Ca}^{2+}/\text{Mg}^{2+}$ chelator that has been reported to cause a pronounced disruption of the junctional proteins in cornea⁵² and has been used to improve the delivery of drugs across the corneal epithelium. BAC is used in many ophthalmic drop solutions because in addition to its bacteriostatic activity, it can enhance corneal epithelial permeability by loosening the tight junctions.^{53,54} In this study, both EDTA and BAC increased the permeation of RF, but both of these reagents also killed significant numbers of epithelial cells.^{55,56} It has been reported that the BAC damages the corneal epithelium and results in the loss of the protective barrier,⁵⁷ as observed here as well. Interestingly, the epithelial barrier completely recovers within 90 minutes after NC-1059 exposure,⁴² suggesting that the effect of NC-1059 on corneal epithelium is very gentle. The depths to which RF diffuses in E18 chick corneal stroma were illustrated in Figure 6C.

Using a group of linear equations that correlated fluorescence intensity with RF concentrations (Fig. 6B), the RF concentrations in the E18 chick corneas at three standard depths of 50, 100, and 200 μ m were calculated (Fig. 6C). Study data demonstrate that the effect of NC-1059 on the corneal epithelium allows the generation of significant concentrations

of RF in the anterior stroma (50- μ m depth), as well as in the middle stroma (100- μ m depth) and posterior stroma (150- μ m depth) of E18 chick corneas, even after the same relatively short time of 30 minutes, used for current clinical RF+UVA treatments. Confocal fluorescence microscopy and other kind fluorescence spectrometer such as two-photon fluorescence axial scanning measurements are commonly used for quantifying concentrations of fluorophores, proteins, and ions at defined depths and for localizations in tissue *in vivo* and *in vitro*.^{46,58–60} This method allows rapid nondestructive optical sectioning of the specimen at high resolution. In this study, confocal microscopy was successfully used to monitor and quantify the concentrations of RF at various depths in E18 chick corneal stroma. Study data on the concentrations of RF in E18 chick cornea without epithelium were consistent with previous measurements that used infrared-excited two-photon microscopy to determine the RF concentrations in the corneas after epithelial abrasion,^{60–63} suggesting that the quantification method established here is reliable and practicable.

Moreover, data presented in Figures 7 and 8 demonstrate that 30-minute incubations with 200 μ M NC-1059 do not cause deleterious effects to the corneal epithelium. As can be seen from Figure 7, there was no distinct difference observed in the staining pattern of nuclei of E18 chick corneas in the absence (BSS alone) or presence of NC-1059, as compared with fresh cornea. Use of vital dyes to stain and distinguish live cells from dead has proven to be a useful assay to determine the cytotoxicity of topical ophthalmic medications.^{64,65} In the LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen) used here, the polyanionic dye calcein is retained within live epithelial cells, producing an intense uniform green fluorescence in live cells. In contrast, ethidium homodimer-1 only enters cells with damaged membranes and undergoes an enhancement of fluorescence upon binding to DNA, thereby producing a

FIGURE 8. Representative images of E18 chick corneal epithelial cells after treatment with 70% methanol, 200 μ M NC-1059 peptide, 5 mM EDTA, 0.02% BAC, or BSS alone, and then stained with calcein-AM and ethidium homodimer-1 to show live cells (green) and dead cells (red). **Live:** Patterns of the live epithelial cells. **Dead:** Features of the dead epithelial cells. **Live + Dead:** Fused images (green and red) showing both live and dead epithelial cells. Scale bar = 50 μ m.

bright red fluorescence in dead cells. As can be seen from the images presented Figure 8, the patterns of epithelial cells of E18 chick corneas after exposure to 200 μ M NC-1059 for 30 minutes appear similar to those of the fresh cornea or BSS alone. In contrast, almost all epithelial cells were dead after the 0.02% BAC treatment. In addition, many dead epithelial cells were visible after the 5 mM EDTA treatment. It is worth noting that the results of E18 chick cornea treated with 0.02% BAC are in agreement with previous findings that the BAC has significant in vitro cytotoxicity to cultured ocular epithelial cells,^{64,66,67} suggesting that the BAC should not be used to modify the epithelial permeability for corneal cross-linking.

Taken all together, the work presented here represents a novel approach for studying the effect of NC-1059 peptide on RF diffusion across an intact corneal epithelium. Study data demonstrate that the NC-1059 peptide enhances the diffusion of RF into the corneal stroma, perhaps by transiently opening the epithelial intercellular tight junctions (the paracellular

pathway). Moreover, the live/dead epithelial cell assay in combination with the imaging of nuclei reveals that the NC-1059 peptide does not cause any measurable cytotoxicity to corneal epithelial cells, whereas both EDTA and BAC killed epithelial cells. Study data suggest that the NC-1059 peptide may be useful in preparing human corneas with intact corneal epithelium for various RF+UVA treatments.

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