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Disruption of CFTR-Dependent Lipid Rafts Reduces Bacterial Levels and Corneal Disease in a Murine Model of *Pseudomonas aeruginosa* Keratitis

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Abstract

Purpose—*Pseudomonas aeruginosa* enters corneal epithelial cells in vitro via membrane microdomains or lipid rafts. Bacterial entry, mediated by the cystic fibrosis transmembrane conductance regulator (CFTR), promotes infection and disease. This study was conducted to determine whether *P. aeruginosa* and CFTR are colocalized to rafts in isogenic corneal cells expressing wild-type (WT) or mutant $\Delta F508$ -CFTR and whether disruption of the rafts both in vitro and in vivo affects the bacterial levels and the course of the disease.

Methods—Transformed human corneal epithelial cells from a patient homozygous for $\Delta F508$ -CFTR, and the same cells corrected with WT-CFTR, were exposed to six isolates of *P. aeruginosa*—three invasive and three cytotoxic strains—in the presence of β -cyclodextrin (CD), which disrupts rafts. Association and cellular uptake of the invasive strains were measured, as was lactate dehydrogenase release induced by the cytotoxic strains. Scratch-injured mouse eyes were infected with the six *P. aeruginosa* strains, and the effect of prophylactic or therapeutic administration of CD on bacterial levels and disease was evaluated.

Results—*P. aeruginosa* and CFTR were colocalized with lipid rafts in cells with WT-CFTR, and CD treatment of these cells disrupted bacterial association, internalization, and cytotoxic effects. Cells expressing $\Delta F508$ -CFTR were marginally affected by CD. Prophylactic and therapeutic topical application of CD ameliorated corneal disease and reduced the bacterial count in the eye.

Conclusions—*P. aeruginosa* enters human corneal epithelial cells via lipid rafts containing CFTR, and disruption of raft-mediated uptake of this organism by CD protects against disease and reduces bacterial levels in the mouse model of keratitis.

It is now well appreciated that cholesterol-rich membrane microdomains or lipid rafts are locations on the plasma membranes of eukaryotic cells often used by bacterial,¹ viral,² and protozoal parasites³ to gain access to the cell's interior. This process is important in the pathogenesis of diverse organisms such as *Neisseria gonorrhoeae*, *Escherichia coli*, and *Pseudomonas aeruginosa*,¹ human immunodeficiency and herpes simplex viruses,² and *Plasmodium spp.*³ The broad use of such host cell features among diverse pathogens suggests that therapies aimed at disrupting the ability of parasites to use lipid rafts could be effective in preventing or treating infections. Although no general approach has yet been devised, it is of note that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, broadly known as statin drugs, which reduce cholesterol levels and thus the ability of cells to form cholesterol-rich membrane microdomains, have been suggested by observational studies to improve outcomes in sepsis and other types of infection.^{4,5} However, since these drugs have multiple

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biological effects,⁶ any benefit attributable to statins in the context of sepsis has not been associated with any specific mechanism of action of these drugs. Nonetheless, disruption of cholesterol-rich membrane microdomains and reduction of microbial uptake into host cells when this interaction promotes pathogenesis is a plausible, if unproven, effect of statins in modulating infection.

One important infectious process shown to involve lipid-raft uptake of a pathogen and subsequent disease is microbial keratitis,^{7,8} a sight-threatening infection often associated with both the extended wear of contact lenses^{9–12} and the use of soft daily-wear, frequent-replacement lenses.¹³ Keratitis also occurs in the setting of eye trauma, ocular disease, and even systemic disease.^{14,15} A prominent pathogen in these settings is *Pseudomonas aeruginosa*,^{11,16} which presents a particularly difficult therapeutic challenge owing to the rapid progression of infection, the need to deliver antibiotics locally via instillation or injection into the eye, and the antibiotic resistance that commonly occurs in strains of this pathogen. Therefore, new adjunctive therapies, derived from an understanding of the basic aspects of pathogenesis of the microbe–host interaction, could markedly augment the clinical treatment of this serious eye disease.

Several recent studies have shown that *P. aeruginosa* enters epithelial cells of the lung^{17–20} and cornea^{7,8} through cholesterol-rich membrane microdomains. Uptake of *P. aeruginosa* into corneal cells promotes pathogenesis and bacterial survival.^{21–23} The bacterial ligand has been identified as the outer-core oligosaccharide of the bacterial lipopolysaccharide (LPS),²² and the main epithelial cell receptor implicated in binding of this bacterium is the cystic fibrosis transmembrane conductance regulator (CFTR).^{23–25} Disruption of the bacteria–CFTR interaction reduces disease severity in a murine model of scratch-injury keratitis,^{22,24} and transgenic CF mice are markedly resistant to *P. aeruginosa* keratitis.²⁴ One recent report has disputed that CFTR is the *P. aeruginosa* receptor for corneal epithelial and conjunctival cells²⁶; however, the lack of CFTR-negative animals and cells as comparators raises questions as to whether the conclusions dismissing CFTR as the corneal epithelial cell receptor for *P. aeruginosa* have been adequately justified by the experimental data.

Disruption of lipid rafts by extraction of cholesterol from the plasma membrane of epithelial cells by agents such as β -cyclodextrin (CD) usually reduces the amount of bacteria that can enter the epithelial cells.^{17,27,28} However, neither the potential prophylactic use of such an agent to prevent eye infections in a setting where there is increased risk of infection (i.e., trauma, surgery, or otokeratology) nor therapeutic use of this modality in the setting of established *P. aeruginosa* infection has been tried. To extend further the understanding of the interaction of *P. aeruginosa* and corneal epithelial cells and evaluate whether an intervention that disrupts this interaction has therapeutic potential, we used confocal laser microscopy to visualize the binding of *P. aeruginosa* to CFTR in lipid rafts of corneal epithelial cells expressing wild-type (WT)-CFTR, as well as the organism's interaction with isogenic cells carrying only mutant $\Delta F508$ CFTR alleles. We further evaluated the ability of CD to inhibit epithelial cell association with and uptake of invasive strains of *P. aeruginosa* and its effect on the cytotoxicity of exotoxin U-positive (ExoU⁺) *P. aeruginosa*, by using these CFTR-isogenic cells. We also determined whether prophylactic or therapeutic topical administration of CD to the scratch-injured eye of mice infected with *P. aeruginosa* ameliorated the pathologic effects of infection and modulated bacterial levels within the cornea.

Materials and Methods

Bacterial Strains

The bacterial strains used in these assays included three invasive (i.e., ExoU⁺) strains—PAO1V, 6294, and 6354—the first a laboratory strain with a determined genome

sequence²⁹ and the latter two clinical isolates from *P. aeruginosa* keratitis²¹; and three cytotoxic (ExoU⁺) strains—PAO1 ExoU⁺, 6077, and 6206. PAO1 ExoU⁺ is strain PAO1 into which we cloned and expressed the gene and related chaperone for ExoU,³⁰ and the other two cytotoxic strains are clinical isolates from patients with keratitis who have been shown to carry and express the ExoU gene and protein.^{31,32} Strain PAO1V-expressing green-fluorescent protein (GFP) was produced as described.³³

Cell Culture and In Vitro Infection of Cells

Isolation, transformation, and the initial propagation of isogenic human corneal epithelial cells expressing either a cloned WT-*CFTR* gene or endogenous, homozygous genes for the mutant Δ F508 allele of *CFTR* have been described.²⁵ Cells were obtained from frozen stocks and passaged fewer than three times in vitro for experimental studies. The cells were grown in keratinocyte-serum-free (SF) medium supplemented with 1 ng epidermal growth factor/mL, 25 μ g bovine pituitary extract/mL, and 5% fetal bovine serum.

The epithelial cells were grown in 96-well tissue culture plates as described²⁴ and incubated with 0, 0.5, 2, or 5 mM CD for 30 minutes, then infected for 3 hours with various *P. aeruginosa* strains at a multiplicity of infection of ~10:1. In brief, approximately 10⁶ CFU of *P. aeruginosa* was added to monolayers of 10⁵ cultured corneal cells. Infected cells were placed in 5% CO₂ for 3 hours at 37°C. Nonadherent bacteria were washed away until <10 CFU of *P. aeruginosa*/0.1 mL was present in the wash solutions, as determined from prior assays,³⁴ and the total bacteria remaining were determined by dilution and plating after lysis of the cells with 0.5% Triton X-100. To determine the internalized colony-forming units, gentamicin-exclusion assays were used as described elsewhere.^{21,24,35–37} Three-hundred micrograms gentamicin per milliliter was added to the infected epithelial cell cultures to kill extracellular bacteria. After 1 hour of exposure to the antibiotic, the cells were washed, and intracellular bacteria were released from them by lysis with 0.5% Triton X-100, diluted, and plated for bacterial enumeration.

To determine the cytotoxic effect of ExoU⁺ *P. aeruginosa* strains, human corneal epithelial cells expressing either WT or mutant Δ F508 alleles of *CFTR* were incubated with 0 mM, 0.001, 0.01, 0.1, or 1 mM CD for 30 minutes and then infected for 30 minutes with *P. aeruginosa* strains 6206, 6077, or PAO1 ExoU⁺. The release of lactate dehydrogenase (LDH) as a marker of cytotoxicity was determined (LDH TOX-7 kit; Sigma-Aldrich, St. Louis, MO). The percentage of LDH released was calculated by subtracting the background OD readings obtained with uninfected cells and then dividing the mean OD reading from experimental cells by the maximum LDH activity measured from uninfected cells lysed with Triton X-100 and multiplying by 100.

Growth of Primary Human Corneal Cells and siRNA Treatment to Reduce CFTR Expression

To determine whether invasion of human primary corneal epithelial (HPCE) cells by *P. aeruginosa* in SF conditions is dependent on the expression of CFTR, HPCE cells, obtained from human corneal rings removed at surgery and cultured as described previously,²⁴ were either left untreated or transfected with a short-interfering (si)RNA specific for CFTR (CFTRsi) or a control siRNA (CTRLsi) as described for transfection of siRNA to the major vault protein.³⁸ The tissue was obtained and used in accordance with the provisions of the Declaration of Helsinki for research involving human tissue. Bases 2336 to 2354 (5'-GGC UGU CCU UAG UAC CAG A dTdT-3') in the open reading frame of human CFTR mRNA were found to mediate optimal inhibition of CFTR protein production by the siRNA.³⁹ The CTRLsi RNA was siRNA 1 (Dharmacon, Lafayette, CO), which lacks any specific mammalian gene target. The cells were used 68 to 72 hours after transfection, based on initial results showing that this duration was optimal for minimizing CFTR levels in the siRNA-transfected cells.

Confirmation of CFTR inhibition by siRNA was made by Western blot of cell lysates with the CFTR-specific monoclonal antibody L12B4 (Chemicon, Temecula, CA). Lysates were incubated at 37°C for 30 minutes before loading onto SDS-polyacrylamide gels. Gentamicin exclusion assays to determine *P. aeruginosa* invasion were as described earlier, except that no serum was present during the invasion assay. Invasion levels (colony-forming units *P. aeruginosa* internalized/10⁵ cells) in the nontransfected cultures were set to 100%, and the relative internalization levels in siRNA-treated cultures were determined.

Confocal Microscopy

Analysis of the expression of lipid rafts by WT and Δ F508 homozygous *CFTR* corneal cells was performed as described previously for lung epithelial cells^{17,38} by visualizing the binding of the lipid raft marker GM1 to labeled cholera toxin. Briefly, corneal epithelial cells were grown in dishes containing a glass coverslip and infected with strain PAO1V expressing GFP³³ for 60 minutes. The cells were rinsed twice with PBS, fixed with 1% paraformaldehyde, and stained for 1 hour at room temperature with AlexaFluor 594-conjugated cholera toxin (1:2000 dilution; Invitrogen-Molecular Probes, Eugene, OR) diluted in 2% normal goat serum and 0.2% BSA, to visualize GM1 in lipid rafts. The cells were rinsed five times for 5 minutes in PBS and then reacted overnight at 4°C with rabbit polyclonal antibody to CFTR (PA1-935; Affinity Bioreagents, Golden, CO). The cells were washed five times for 5 minutes each in PBS and then reacted with an AlexaFluor 647-conjugated secondary antibody to rabbit IgG (1:500 dilution; Invitrogen-Molecular Probes) to visualize CFTR. The cells were rinsed five times for 5 minutes per rinse, and the stained cells were viewed with a confocal system (MRC 1024 Multi-photon system equipped with a krypton/argon laser and a Spectra Physics Tsunami Multi-Photon Laser with 63× objective; Bio-Rad, Hercules, CA). Samples were excited at wavelengths of 596, 488, and 760 nm (collection at HQ598/40, HQ515/30, and HQ485/30, respectively), and the images collected in the *x-y* plane at these wavelengths were obtained individually as well as overlaid to form a merged image. Sections in the *x-z* and *y-z* planes were also obtained to document colocalization of *P. aeruginosa*, CFTR, and GM1-stained by cholera toxin as a marker of lipid rafts.

Animal Model of Corneal Infection

Scratch-injured eyes of anesthetized mice were challenged in vivo with the *P. aeruginosa* strains as described.⁴⁰ The infecting inoculum was delivered in a 5- μ L volume and varied depending on the strain and its associated virulence. In an initial series of experiments mice with scratch-injured but uninfected eyes were treated with 5 mM CD three times a day for 3 days, to assess corneal disease resulting from this treatment. No change from time 0 or in comparison to an uninjured, uninfected, control contralateral eye was ever observed (Zaidi T, unpublished observation, 2006).

For prophylactic treatment with CD, 5 mM was applied topically to the infected eye in 10- μ L volumes 30 minutes after infection and continued for five more treatments at 8- to 12-hour intervals for two more days. Control eyes were given the PBS vehicle in an identical fashion. The severity of corneal disease was scored by a masked observer on a scale of 0 to 4: 0, eye macroscopically identical with the uninfected contralateral control eye; 1, faint opacity partially covering the pupil; 2, dense opacity covering the pupil; 3, dense opacity covering the entire anterior segment; 4, perforation of the cornea, phthisis bulbi (shrinkage of the globe after inflammatory disease), or both. The disease severity scores observed at 48 hours after infection were determined, the mice were euthanatized by CO₂ narcosis, the corneas were removed, and the extracellular and internalized colony-forming units of *P. aeruginosa* were determined. The excised corneas were placed in DMEM with 1% fetal bovine serum (FBS) and vortexed for 3 minutes. The extracellular bacterial counts were determined from the solution obtained after vortexing. The cornea was removed from the solution and placed in a solution of DMEM with

300 μ g gentamicin/mL and 1% FBS and incubated for 1 hour at 37°C, to kill any remaining extracellular bacteria. After exposure to gentamicin, the cornea was removed from the gentamicin solution and washed three times in 5-mL solutions of DMEM-1% FBS. Next, the cornea was homogenized in 0.5% Triton X-100 in trypticase soy broth, serial dilutions were made in DMEM-1% FBS, and bacteria were plated for enumeration.

For therapeutic treatment, mouse eyes were scratch injured and infected as just described, and then 5 mM CD was topically applied to the treatment groups in a 10- μ L volume starting 8 hours after infection and then for two more days, once or twice daily. PBS vehicle was applied to control eyes on an identical schedule. Treatment was continued for 48 hours after infection, and the mice were followed for 7 days and the corneal disease scores determined at 24-hour intervals, as described.⁴⁰ However, no changes in disease severity scores after 72 hours of infection and treatment were noted, so that all the results were plotted at this time interval. Survival curves were plotted as the proportion of mice with disease scores <3, and the times needed to achieve this score between CD-treated and control PBS-treated mice compared by Kaplan-Meier survival curves. All animal studies were approved by the Harvard Medical Area Institutional Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Statistical Analysis

Uptake of *P. aeruginosa* by transformed epithelial and HPCE cells was analyzed by ANOVA and the Dunnett test for multiple pair-wise comparisons. Differences in bacterial levels in mouse corneas were compared by Mann-Whitney U test, and the time to development of corneal disease of severity ≥ 3 was determined by Kaplan-Meier survival analysis and a log rank test (Prism 4; GraphPad, San Diego, CA).

Results

Colocalization of *P. aeruginosa*, CFTR, and Lipid Raft Marker GM1

To show that *P. aeruginosa* entered corneal epithelial cells in association with both CFTR and lipid rafts, which could be visualized by binding of raft-associated GM1 to labeled cholera toxin, isogenic cells expressing either WT-CFTR or Δ F508 CFTR were exposed to *P. aeruginosa* PAO1V for 60 minutes, fixed, and stained, and the nonpermeabilized cells were visualized by confocal microscopy. As shown in Figure 1, in cells with WT-CFTR infected with GFP-*P. aeruginosa*, bacteria were readily seen bound to the surface membrane at locations where lipid rafts and CFTR were also present. In contrast, in cells homozygous for the Δ F508 CFTR allele, few lipid rafts were even seen in the plasma membrane, and GFP-*P. aeruginosa* were rarely associated with the cells at any point. No CFTR staining was detected in the Δ F508-CFTR cells and no association of *P. aeruginosa* and lipid rafts was observed in the three-channel overlay.

siRNA Treatment of Primary Human Corneal Epithelial Cells

It has recently been questioned whether *P. aeruginosa* enters human corneal epithelial cells under serum-free conditions via CFTR.²⁶ We therefore analyzed *P. aeruginosa* uptake by HPCE cells, which are free of confounding from transformation and selection for optimal in vitro growth and long-term propagation, that were treated with either a control siRNA or siRNA that reduces CFTR levels. Prior studies to show CFTR specificity for *P. aeruginosa* entry into cells used peptides specific to amino acids 108 to 117 of CFTR to inhibit bacterial binding,^{24,37} but Yamamoto et al.²⁶ could not confirm this peptide inhibited *P. aeruginosa* interactions with transformed human corneal epithelial cells. As siRNA knockdown of CFTR is more specific and provides for experimental conditions where identical cell cultures either do or do not express CFTR, we identified a 21-nucleotide siRNA that reduced CFTR expression

by HPCE cells, whereas a control siRNA had no effect on CFTR expression (Fig. 2). Reduction of CFTR expression by the HPCE cells also reduced *P. aeruginosa* internalization by >60%, whereas bacterial uptake by the cells treated with the control siRNA was no different from that with untreated cells (Fig. 2). This finding establishes CFTR expression as essential for full uptake of *P. aeruginosa* by HPCE cells, a process previously shown to be essential for the full virulence of *P. aeruginosa* to be manifest in the murine scratch-injured-eye model of keratitis.²⁴

Effect of Disruption of Lipid Rafts by CD on Association and Internalization of Invasive Strains of *P. aeruginosa*

We next used the isogenic WT-CFTR and Δ F508-CFTR cell lines to analyze the role of lipid rafts in association (total colony-forming units of bacteria present after extensive washing) and internalization of three invasive strains of *P. aeruginosa*. As shown in Figure 3, CD in a dose-response manner inhibited the association of *P. aeruginosa* strains PAO1V, 6294, and 6354 with cells expressing WT-CFTR. In contrast, isogenic corneal epithelial cells that could only synthesize Δ F508 CFTR bound much lower levels of the three *P. aeruginosa* strains, and with only one strain, PAO1V, at the highest dose of CD used (5 mM) was any inhibition of the low level of association of *P. aeruginosa* observed. Thus, in the presence of CFTR, membrane microdomains that can be disrupted by CD are critical for maximum association of *P. aeruginosa* with corneal epithelial cells, whereas in the absence of CFTR, membrane microdomains are generally not involved in the low level of association of invasive strains of *P. aeruginosa* with corneal epithelial cells.

Along similar lines, internalization of the three invasive *P. aeruginosa* strains in the absence of CD (0 mM CD) was markedly higher in the cells with WT-CFTR than in the cells with Δ F508 CFTR (Fig. 4). Note the different y-axis used for the cells with WT-CFTR compared with that for Δ F508-CFTR corneal cells. For the former cells, CD inhibited bacterial uptake in a dose-dependent manner, whereas only at the highest concentration of CD used, 5 mM, was there any inhibition of the low-level ingestion of *P. aeruginosa* by the Δ F508-CFTR cells.

Role of Lipid Rafts in the Manifestation of Cytotoxic Effects of ExoU⁺ *P. aeruginosa*

Strains of *P. aeruginosa* expressing the phospholipase cytotoxin ExoU comprise a notable proportion of isolates from corneal infections.^{31,32} These strains generally rapidly kill target cells within 1 to 2 hours, due to injection of the ExoU toxin via the type III secretion system into target cells^{41–43} after binding of the bacterium to the eukaryotic cell. To evaluate the role of lipid rafts in ExoU-mediated cytotoxicity of corneal epithelial cells, we determined the effect of CD treatment on cytotoxicity during 30 minutes of infection using cells with either WT-CFTR or Δ F508 CFTR. As with the invasive strains, CD treatment inhibited LDH release induced by infection with all three cytotoxic *P. aeruginosa* strains (Fig. 5) when cells with WT-CFTR were used. However, the cells expressing Δ F508 CFTR had less than half the release of LDH compared to the cells with WT-CFTR, probably because of the reduced ability of *P. aeruginosa* to associate with the cells, and there was no effect of CD on this lower level of cytotoxicity. CFTR-dependent lipid raft-mediated binding of these cytotoxic strains of *P. aeruginosa* to the corneal epithelial cell membrane appears to be needed for the maximum activity of cytotoxic type III secretion factors of *P. aeruginosa*.

Effect of Prophylactic Treatment with CD on Disease and Bacterial Levels in Infected Mouse Eyes

Prophylactic topical application of an adjuvant therapy such as CD in situations of potential high risk for *P. aeruginosa* infection could be beneficial in preventing infection or reducing the consequences of infection. To evaluate this possibility, we treated scratch-injured mouse eyes with CD, starting within 30 minutes of infection with the three noncytotoxic strains of *P.*

aeruginosa (infectious dose $2\text{--}5 \times 10^6$ CFU/eye) and continuing for 2 days with topical application of 5 mM CD in 10 μ L of PBS. This treatment reduced the corneal disease severity score at 48 hours after infection (Fig. 6), although the difference with strain 6354 was of borderline significance ($P = 0.065$). However, for the other two invasive *P. aeruginosa* strains, PAO1 and 6294, CD treatment significantly reduced the corneal disease from infection. For all three strains of *P. aeruginosa*, CD treatment reduced the total levels of *P. aeruginosa* in the eye and the levels inside corneal cells as determined by resistance to gentamicin. The less successful treatment of disease from strain 6354 may have been due to higher levels of bacterial cells of this strain in the eye compared with the other two strains.

For the ExoU⁺ cytotoxic strains (infectious dose 1×10^5 CFU/eye for all three strains), prophylactic CD treatment was highly effective in reducing disease severity, total levels of *P. aeruginosa* in the eye, and internalized levels of *P. aeruginosa* within the corneal cells (Fig. 7). This finding indicates that lipid rafts have a central role in the pathogenesis of corneal infection due to ExoU⁺ *P. aeruginosa*. The greater effectiveness of CD against the more virulent cytotoxic strains may be related to the lower dose of these strains needed to achieve corneal disease comparable to that of the invasive strains, which require 10- to 50-fold higher inocula for achieving disease scores ≥ 3 in most mice.

Effect of Therapeutic Treatment with CD on Disease in Infected Mouse Eyes

Another potential use for adjuvant therapy to disrupt lipid rafts and reduce the consequences of *P. aeruginosa* infection could be topical application of an agent such as CD, which clinically would be used with other standard treatments. However, to investigate whether CD alone could provide a therapeutic effect, we waited 8 hours after infection to begin treatment and continued treatment for two more days, and then followed the progress of the disease for seven more days, a time at which there were no residual bacteria in the eye. We first compared once or twice daily treatment with CD on eyes of mice infected with 1×10^6 CFU/eye of PAO1V (Fig. 8). Both treatments were effective in reducing disease severity. Of note, although a slightly more significant difference was seen between the control and the once-daily-treated animals in the corneal pathology score than between the control and the twice-daily-treated animals (see probabilities in Fig. 8), this result was due to a couple of control mice in the twice-daily treatment group that unexpectedly had disease scores of +1, whereas all other controls in both groups had the expected scores of +3. However, the twice-daily treatment resulted in more mouse eyes with lower overall disease scores ($P = 0.01$, Mann-Whitney U test comparing disease scores of mice treated twice daily versus once daily with CD) and, thus, the twice-daily regimen was chosen for further study. Twice-daily therapeutic CD treatment of mouse eyes infected with invasive strains 6294 (2×10^6 CFU/eye) and 6354 (5×10^5 CFU/eye) significantly reduced corneal disease at 72 hours (Fig. 9). Similarly, therapeutic treatment of mouse eyes infected with the three cytotoxic strains of *P. aeruginosa* ($7.5 \times 10^5\text{--}2.5 \times 10^6$ CFU/eye) was also highly successful at reducing corneal disease severity (Fig. 10).

Discussion

A major goal of modern biomedical research is to use basic insights gained from analysis of the pathogenesis of disease to develop and assess potential therapies and interventions. For many microbial pathogens, it appears that use of membrane microdomains on cell surfaces is critical for their interaction with the host, and in many instances this interaction is essential for the development and progress of disease. When common mechanisms of pathogenesis are identified, the molecular and cellular factors needed for disease to ensue become tempting targets for pharmacologic interventions, because of the potential for such therapies to be broadly applicable in numerous clinical settings. To determine whether disruption of lipid rafts formed by corneal cells and used by *P. aeruginosa* to enter the cells and then cause disease

represents a possible target for therapeutic intervention, we defined the cellular factors needed for optimal entry of this pathogen into these epithelial cells, or optimal cytotoxic effects for ExoU⁺ *P. aeruginosa* strains, and then proceeded to test whether disruption of lipid raft formation had any effect on the outcome of *P. aeruginosa* corneal infection. Overall, we found that both CFTR and lipid rafts were needed for maximum *P. aeruginosa* entry or cytotoxicity and that disruption of rafts by CD, either prophylactically or therapeutically, had a marked positive effect on corneal disease and on bacterial levels in the eye.

Two studies by Yamamoto et al.^{7,8} initially identified lipid rafts as points of entry of *P. aeruginosa* into corneal cells. Their finding prompted us to use the keratitis model to assess the value of CD treatment in *P. aeruginosa* keratitis. This model is particularly amenable for testing this intervention, as the eye is easily accessible for topical treatment. Whether CD has any potential for use against infections in a confined tissue setting such as the lung or gut, or systemically, is not known, but different types of CDs have been used in formulating drugs for optical, oral, and parenteral delivery.⁴⁴ However, as *P. aeruginosa* keratitis is also a clinically significant disease with serious consequences for vision loss, new therapies leading to better outcomes from this infection are also urgently needed.

The use of CFTR by *P. aeruginosa* to promote infection in the scratch-injured eye is different from the consequences of this interaction in the lung, where absence of CFTR leads to hypersusceptibility to chronic *P. aeruginosa* infection.⁴⁵ Indeed, lack of CFTR in the eye makes mice essentially totally resistant to experimental *P. aeruginosa* keratitis.²⁴ The basic reason for this difference is that the corneal epithelium in the eye is five to six layers thick, and the scratch injury, like trauma, allows the bacterial cells to travel down to the anatomic location at which the epithelial cells border on the stroma, where the microbes spread out, enter the epithelial cells in the lowest layer of the corneal epithelium and are trapped there to multiply, spread, activate, and prolong inflammation, which ultimately damages the cornea.²¹ In the lung, the one-layer-thick epithelium responds to *P. aeruginosa* with activation of protective innate immunity, where binding to CFTR mediates IL-1 release, NF- κ B nuclear translocation, cytokine secretion, and apoptosis, which lead to the resolution of infection.^{46–48} A similar process probably occurs on the surface of the intact corneal epithelium, wherein entry of *P. aeruginosa* into these surface cells is a manifestation of effective innate immunity that is compromised when the *P. aeruginosa*-laden epithelial cells are trapped beneath a contact lens, thus prolonging the infection in the eye. It is encouraging that the CD treatment was effective even with the bacteria buried within the corneal epithelium, suggesting that CD concentrations remain high enough while diffusing through the eye to disrupt the lipid rafts effectively.

In another more recent study, Yamamoto et al.²⁶ found that CFTR in lipid rafts did not mediate *P. aeruginosa* entry into rabbit corneal conjunctival epithelium after contact lens wear or into transformed human corneal epithelial cells in serum-free conditions. However, the conclusions of that study are uncertain, because of the lack of use of any CFTR-negative rabbits, ocular tissues, or human corneal epithelial cells in the study to validate the specificity of the antibody reagents. Of note, one of the monoclonal antibodies they used to detect CFTR in rabbit corneal and conjunctival tissues, CF3, has been reported by the investigators who produced this reagent to react with a protein of 170 kDa that is not CFTR.⁴⁹ In addition, visualization of bacterial cells in the rabbit surface corneal epithelium after contact lens wear and infection was nonspecific when a red nucleic acid stain that binds to DNA (Cyto 59; Invitrogen-Molecular Probes) was used to detect bacteria. However, that stain could also detect extracellular DNA appearing to resemble bacterial cells. Also, it was not clear that their transformed human corneal epithelial cells expressed CFTR in the plasma membrane as a functional chloride channel. Overall, there are significant uncertainties in this challenge²⁶ to the evidence showing that *P. aeruginosa* binds to CFTR in the corneal epithelium, primarily because of the lack of

sufficient CFTR-negative controls to validate the specificity of the reagents used and because they did not demonstrate the presence of functional WT-CFTR in their human cell line.

In summary, our cell culture data and in vivo efficacy data support the concept that CFTR in lipid rafts of corneal cells mediates *P. aeruginosa* uptake by these cells, providing a protected niche for the microbe to survive host defenses, multiply, and activate and prolong the inflammatory response that damages the cornea. We saw no gross toxic effect on the corneas of mice treated with CD, a drug commonly used at high concentrations (up to 45%) in ophthalmic drug delivery systems,⁴⁴ suggesting that a good safety profile for this treatment has already been obtained. However, further development of this treatment as a therapeutic modality for *P. aeruginosa* eye infections will require extensive safety testing to ensure that no untoward interactions between CD and infected corneal tissues ensue. Nonetheless, if there is acceptable toxicity associated with CD application to the infected human eye, this simple and inexpensive compound could be used as an additional therapy and perhaps reduce the serious consequences of *P. aeruginosa* keratitis and possibly keratitis induced by other microbes.

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Figure 1.

Confocal microscopic imaging of GFP-*P. aeruginosa* strain PAO1V interacting with transformed human corneal epithelial cells expressing either wild-type (WT) or $\Delta F508$ -CFTR. Two separate images for the WT CFTR (top) and $\Delta F508$ CFTR (bottom) corneal epithelial cells are shown. Edges of the overlay panel in the top left image depict the $x-z$ -plane (top slice) and $y-z$ plane (right slice) showing GFP-*P. aeruginosa* associated with the plasma membrane at the top of the cell and closely associated with lipid rafts and CFTR. Yellow arrows: colocalization of *P. aeruginosa*, lipid rafts, and CFTR. GFP, GFP-*P. aeruginosa* PAO1V; LR, lipid rafts visualized with Alexa 594-conjugated cholera toxin; CFTR, CFTR stained with polyclonal rabbit antibody PA1-395 to CFTR and secondary anti-rabbit-IgG conjugated to Alexa 647. Overlay: green, red, and blue.

Figure 2.

Expression of CFTR by HPCE cells after transfection with control or CFTR-specific siRNA and effect on internalization of *P. aeruginosa* PAO1V. Nontreated cells and cells transfected with control siRNA had readily detectable CFTR, whereas the cells treated with CFTR-specific siRNA had markedly reduced protein expression. The loss of CFTR expression reduced uptake of *P. aeruginosa* PAO1V by >60%. * $P < 0.01$, ANOVA and the Dunnett multiple comparison test comparing the nontreated and control siRNA-treated HPCE cells with the CFTR siRNA-treated cells.

Figure 3.

Effect of CD on association of three invasive strains of *P. aeruginosa* with transformed human corneal epithelial cells expressing either wild-type (WT) or Δ F508-CFTR after 3 hours of interaction. *,**Comparison to 0 mM CD, by ANOVA and by the Dunnett multiple comparison test.

Figure 4.

Effect of CD on internalization of three invasive strains of *P. aeruginosa* by transformed human corneal epithelial cells expressing either wild-type (WT) or ΔF508-CFTR after 3 hours of interaction. * $P < 0.01$, by ANOVA and comparison to 0 mM CD by the Dunnett multiple comparison test. Note the different y-axis scales for cells with WT-CFTR versus ΔF508-CFTR.

Figure 5. Effect of CD on LDH release induced by three cytotoxic strains of *P. aeruginosa* after 30 minutes of infection of transformed human corneal epithelial cells expressing either wild-type CFTR (WT-CFTR) or Δ F508-CFTR. * P < 0.01 when compared with 0 mM CD by the Dunnett multiple comparison test. Note the different y-axis scales for cells with WT-CFTR versus Δ F508-CFTR.

Figure 6.

Efficacy of prophylactic treatment with CD on disease severity, bacterial levels in the eye, and bacterial CFU internalized on scratch-injured mouse eyes infected with three invasive strains of *P. aeruginosa*. Treatment was initiated 30 minutes after infection and continued twice daily at 8- to 12-hour intervals. Probabilities were determined by Mann-Whitney U test. Bacterial inocula (CFU/eye): PAO1 = 5×10^6 ; 6354 and 6294 = 2×10^6 .

Figure 7.

Efficacy of prophylactic treatment with CD on disease, bacterial levels in the eye, and bacterial colony-forming units internalized on scratch-injured mouse eyes infected with three cytotoxic strains of *P. aeruginosa*. Treatment was initiated 30 minutes after infection and continued twice daily at 8- to 12-hour intervals. Probabilities were determined by Mann-Whitney U test. Inocula for all three strains were 1×10^5 CFU/eye.

Figure 8.

Effect of therapeutic treatment, with CD given once (*top*) or twice (*bottom*) a day starting 8 hours after infection, on disease developing on scratch-injured mouse eyes infected with 1×10^6 CFU/eye of *P. aeruginosa* strain PAO1V. Probabilities for the survival curves determined by log-rank test. Probabilities for the disease score at 72 hours determined by Mann-Whitney U test.

Figure 9.

Effect of therapeutic treatment with CD, given twice daily starting 8 hours after infection, on disease achieved in scratch-injured mouse eyes infected with two invasive strains of *P. aeruginosa*. Probabilities for the survival curves determined by log rank test. Significance of the disease score at 72 hours determined by Mann-Whitney U test. Inocula (CFU/eye): strain 6294 = 2×10^6 ; strain 6354 = 5×10^5 .

Figure 10.

Effect of therapeutic treatment with CD, given twice daily starting 8 hours after infection, on disease achieved in scratch-injured mouse eyes infected with three cytotoxic strains of *P. aeruginosa*. Probabilities for the survival curves determined by log rank test. Probabilities for the disease score at 72 hours determined by Mann-Whitney U test. Inocula (CFU/eye): PAO1V ExoU⁺ = 1×10^6 ; 6206 = 7.5×10^5 ; 6077 = 2.5×10^6 .