Regulation of *Pseudomonas aeruginosa* Internalization after Contact Lens Wear In Vivo and in Serum-Free Culture by Ocular Surface Cells

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**PURPOSE.** To determine the effects of contact lenses (CLs) and *Pseudomonas aeruginosa* (PA) infection on localization of cystic fibrosis transmembrane conductance regulator (CFTR) on corneal surface epithelial cells and the association between lipid raft formation and CFTR in mediating PA binding and internalization in ocular surface epithelium.

**METHODS.** CFTR immunolocalization was evaluated in vivo in rabbit corneal–conjunctival epithelium (with/without CL wear) before and after PA exposure and in serum-free human corneal epithelial cell culture (hTCEpi). Lipid raft formation was visualized with Alexa555-conjugated cholera toxin β-subunit. Lipid raft involvement in PA internalization was assayed in vivo by gentamicin survival assays after topical filipin pretreatment. Involvement of CFTR in PA binding and internalization was evaluated by blockade with CFTR peptides or LPS.

**RESULTS.** CL wear in vivo enhanced anti-CFTR staining, but CFTR localization did not correlate with the PA binding by ocular surface cells. Conjunctival epithelial cells stained for CFTR but did not bind or internalize PA. Corneal epithelial cells in vivo did not stain for CFTR unless challenged by contact lens-induced hypoxia. PA internalization by hTCEpi was significantly inhibited by LPS (*P* < 0.01), but not by CFTR peptides. Remarkably, normal conjunctival epithelial cells showed lipid raft formation and CFTR staining but did not bind PA. Inhibition of raft formation by filipin blocked PA internalization in vivo after CL wear.

**CONCLUSIONS.** CFTR is not the predominant receptor for ocular surface PA infection, and after hypoxic CL challenge, neither lipid rafts nor CFTR localization alone predicts PA binding; however, lipid rafts are critical to CL-mediated PA internalization.

The pathogen *Pseudomonas aeruginosa* is a Gram-negative bacterium most commonly involved in corneal infections associated with extended wear of contact lenses (CLs).1,2 Previous studies have demonstrated that many clinical isolates of PA can bind, invade, and replicate within corneal epithelial cells in animal models of corneal infection.3 Numerous PA and host factors contribute to the pathogenesis of PA keratitis, including actin cytoskeleton and protein tyrosine kinase.4–5 Although the outer-core polysaccharide portion of the lipopolysaccharide (LPS) in the outer membrane of PA is commonly considered to be the bacterial ligand for adherence to corneal epithelium and bacterial internalization,6 the binding mechanism has not been firmly established. Some reports have implicated gangliotetraosylceramide (asialo-GM1) on the cellular membrane surface as a receptor for PA/LPS,7,8 whereas others have shown that clinical corneal isolates of PA fail to bind to asialo-GM1.9 Recently, cystic fibrosis transmembrane conductance regulator (CFTR), a fairly ubiquitous chloride channel protein found apically in many surface epithelia, has been reported to serve as a receptor for PA internalization in cultured mammalian nasal, tracheal, lung, and corneal epithelial cells.10,11 A portion of the first predicted extracellular loop of CFTR, composed of amino acids 108–117, recognizes the conserved outer-core oligosaccharide of LPS in the outer membrane of PA. Mutations in CFTR, LPS, or an associated flagellum assembly apparatus have been shown to reduce PA internalization.6,12–15 Additional studies have further suggested that the specific phospholipids, phosphatidylserine (PS) and phosphatidylcholine (PI), present in mucus or on the surface of the corneal epithelium also function as PA receptors and contribute to selective bacterium–host interactions.16 Taken together, all these findings suggest that PA binding and internalization in corneal epithelium is a complex process, and that the pathogenesis of contact lens (CL)-mediated PA keratitis in vivo remains to be fully elucidated.

Lipid rafts are glycosphingolipid- and cholesterol-enriched domains in the plasma membrane that move within the fluid lipid bilayer,17 and are distinct from the remaining areas of the plasma membrane, which predominantly consist of phospholipids. Lipid rafts can undergo macromolecular reorganization and form large membrane platforms, which have been implicated in various cellular functions, including membrane trafficking17; signal transduction,18,19 particularly as defined for T cells and other leukocytes20,21, and the regulation of integrin function.22 Recent studies have also revealed that lipid rafts appear to be involved in the internalization of pathogenic microorganisms in host cells.23,24 Using a rabbit CL-wearing model and hTERT immortalized human corneal epithelial cells (hTCEpi), we recently reported that lipid rafts are necessary for PA internalization by corneal epithelial cells in vivo, and after CL wear.25 In addition, in more recent studies, we have demonstrated that polymethylmethacrylate (PMMA) con-
tact lens- or eyelid closure-induced hypoxia initiates the formation of lipid rafts on occasional corneal surface epithelial cells in vivo. Subsequent exposure to PA resulted in preferential PA binding to lipid raft-forming cells, and PA binding to these corneal surface cells triggered a dynamic process of lipid raft aggregation, leading to PA clustering and subsequent internalization. Of interest, we further observed that normal rabbit conjunctival epithelial cells exhibit robust lipid raft expression detected by β-CT staining, but do not bind or internalize PA. This result suggests that the presence of membrane lipid rafts alone is insufficient to trigger the process of PA binding and subsequent PA internalization.

In addition, a recent confocal microscopy and immunocytochemistry study with Madin-Darby canine kidney (MDCK) cells transfected with green fluorescent protein (GFP)-CFTR and cultured in serum-containing medium has shown that CFTR protein translocates in lipid rafts in response to PA infection and may act coordinately with lipid rafts to promote PA internalization. However, the functional association between CFTR and lipid rafts as a requirement for PA infection in ocular surface cells in vivo has not been determined.

In this study, we observed the immunolocalization of lipid rafts and CFTR on normal rabbit ocular surface epithelial cells in vivo (cornea, conjunctiva) and also examined the effects of exposure to three infectious strains of PA on CFTR, lipid raft association, and bacterial uptake, using sucrose gradient ultracentrifugation, confocal microscopy and a gentamicin survival assay in the rabbit CL-wearing model and in human corneal epithelial (hTCEpi) cells in serum-free culture.

Materials and Methods

Bacteria

Three PA strains—ATCC27853, -6294, and -6487—were used for this study. Strain ATCC27853 (American Type Culture Collection, Manassas, VA) is a nonconical isolate that has been shown to be fully infectious in the cornea and has been used as a standard test organism in experimental corneal studies with rabbits and humans. In addition, ATCC27853 has been used in bacterial adherence and PA internalization and lipid raft studies in many cell and tissue systems. Two infectious corneal isolates, strains 6294 and 6487, were kindly provided by Suzanne M. Fleisz (University of California, Berkeley) and have been characterized as invasive. Bacteria were cultured on Mueller Hinton II agar (BD Biosciences, Sparks, MD) for 17 hours at 37°C before use. Individual bacterial strains were then suspended in phosphate-buffered saline (PBS). The bacterial concentration was adjusted to 1 × 10^9 CFU/mL with a spectrophotometer. The bacterial suspension was then diluted with minimum essential medium (MEM; Invitrogen Corp., Carlsbad, CA) to a concentration appropriate for each experiment as described in the following sections.

Animals

Thirty-one New Zealand White rabbits (body weight, 2.5–3.5 kg) were used in this study. All rabbits were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. To facilitate contact lens retention, the nictitating membrane of both eyes was surgically excised under anesthesia (50 mg/kg ketamine and 20 mg/kg xylazine), and animals were allowed to recover for at least 1 week before contact lens wear.

Lens Fitting

As previously reported, 25 O2-nontransmissible polymethylmethacrylate (PMMA) rigid contact lenses (Dk = 0) specially designed for the rabbit model of CL wear (diameter, 14.0 mm; thickness, 0.15 mm) were used to induce the maximal CL-associated hypoxic challenge to both the corneal and paralimbal conjunctival epithelium. The best-fitting base curve was selected, after trial fitting, with radii of 7.60, 7.80, 8.00, and 8.20 mm by using fluorescein and blue light. PMMA contact lenses were fitted on the right eye of each rabbit. The left eye served as the control. After 24 hours of CL wear, animals were anesthetized, the CLs were removed, and the rabbits were killed humanely with intravenous pentobarbital (120 mg/kg). Both eyes were then enucleated immediately and used for subsequent studies.

Human Corneal Epithelial Cell Culture

Human corneal epithelial cells immortalized with hTERT (hTCEpi) were prepared and characterized as described previously. Cells were cloned and maintained in serum-free keratinocyte growth medium (KGM)-2 (Clonetics; BioWhittaker, Inc., Walkersville, MD) containing 0.15 mM Ca^2+ and routinely passaged at 50% to 60% confluence.

For immunofluorescence staining, 5 × 10^5 cells were grown on glass coverslips (12 mm diameter) coated with collagen (Vitrogen; Cohesion Technologies Inc., Palo Alto, CA) in 24-well culture plates (Corning Inc., Corning, NY) with serum-free KGM-2 containing 0.15 mM Ca^2+. Thereafter, cells were incubated at 37°C in 5% CO2 for 2 days before experiments. For quantification of bacterial internalization, 1 × 10^5 cells were seeded into 12-well culture plates (2.5 × 10^5 cells/cm^2; Corning Inc.) with serum-free KGM-2 and incubated at 37°C in 5% CO2 for 2 days.

PA Infection in Rabbit Cornea

For rabbit corneas, normal and CL-wearing eyes were enucleated and placed, corneal surface facing upward, into 12-well culture plates. As reported previously, a silastic tube was placed on the eye that exposed the entire cornea and the paralimbal conjunctival epithelium. Test strains of PA (0.5 mL, 1 × 10^8 CFU/mL) were then layered onto the ocular surface, and the culture plates were incubated at 37°C for 30 minutes. Eyes were then washed with MEM three times, followed by incubation at 37°C for an additional 30 minutes, to allow bacterial internalization. In some experiments, to disrupt lipid rafts, ex vivo enucleated eyes were pretreated with a cholesterol metabolism inhibitor, filipin (2 μg/mL) at 37°C for 30 minutes, prepared in PBS and diluted with MEM (Invitrogen Corp.) to final concentration, and then infected with PA in the presence of the same inhibitor.

Immunofluorescence Staining for CFTR and Lipid Rafts

Uninfected (control) and infected eyes or cells were fixed with 1% paraformaldehyde (PFA) in MEM (pH 7.2) on ice for 30 minutes, and the whole cornea and paralimbal conjunctiva were excised. For cryostat sectioning, tissues were embedded in tissue embedding medium (Leica Instruments, GmbH, Heidelberg, Germany), frozen in liquid nitrogen, and then sectioned by a cryostat (CM 3050S; Leica Microsystems Nussloch GmbH, Nussloch, Germany). hTCEpi cells were fixed with 1% PFA-MEM (pH 7.2) for 30 minutes before and after PA exposure.

For CFTR staining, cryostat sections or wholemounts of cornea and paralimbal conjunctiva, or cells were washed with PBS, and blocked with 10% goat serum. Samples were then incubated with mouse anti-CFTR monoclonal antibody, clone CF313 or clone 24-1 (R&D Systems, Minneapolis, MN) for 1 hour at 37°C, followed by FITC-conjugated anti-mouse IgM or anti-mouse IgG for 1 hour at 37°C. Samples were then counterstained with propidium iodide (5 μg/mL), to visualize cell nuclei and PA, and were examined with a laser scanning confocal microscope (SP2; Leica, Nussloch GmbH). Irrelevant IgM or IgG were used as negative controls of primary antibodies. Lipid raft formation was assessed with 555-conjugated cholera toxin β-subunit (β-CT; Invitrogen, Eugene, OR), followed by counterstaining with syto59 (Invitrogen).

Quantification of Bacterial Internalization

After eyes or cells were infected with PA test strains, bacteria internalization was quantified by gentamicin survival assay (GSA), as previously
Inhibition of PA Internalization with CFTR Peptides or LPS

For analysis of the role of CFTR in PA internalization, we used amino-acid peptides corresponding to the first extracellular domain of CFTR (amino acids 103-117) or 108-117). These peptides have been reported to inhibit PA internalization in serum-containing cultures of human and rabbit corneal cells (in vitro) and in scratch-wounded murine eyes (in vivo). A peptide composed of amino acids 103-117 was purchased from Abcam Inc. (Cambridge, MA), and a peptide of amino acids 108-117 was synthesized in the Protein Chemistry Technology Center of the University of Texas Southwestern Medical Center at Dallas.

Briefly, various concentrations of the CFTR peptides, along with a 10^5 CFU/mL of PA, were added to monolayers of 10^5 cells. Cells were then incubated at 37°C for 3 hours. Thereafter, bacterial internalization of PA was assayed by GSA as described earlier.

To evaluate the role of LPS in mediating PA internalization in vivo, 10^5 cells were preincubated with various concentrations of LPS from PA (Sigma-Aldrich, St. Louis, MO) for 1 hour at 37°C before PA infection. Thereafter, cells were infected with 5 x 10^5 CFU of PA at 37°C for 3 hours in the presence of LPS, and PA internalization was then quantified by GSA.

LPS Toxicity Studies

The cytotoxicity of LPS was determined using a standard viability assay (Live/Dead Viability/Cytotoxicity Kit; Invitrogen). Cells were incubated with 0, 25, or 100 μg/mL of LPS at 37°C in 5% CO2 for 4 hours. After the inhibitors were removed, cells were washed with MEM, stained with 2 μM calcein AM and 4 μM ethidium homodimer (EthD-1) for 30 minutes at room temperature and then immediately examined by fluorescence microscopy. To quantify cellular viability, we counted and averaged the number of live and dead cells in four randomly selected fields of each coverslip.

The effects of LPS on PA growth were also investigated. PA (5 x 10^5 CFU/mL, strain 6294) was incubated with 0 or 100 μg/mL of LPS at 37°C for 4 hours. Bacterial growth was then assayed by serial dilution of suspensions and culture on agar plates.

Isolation of Lipid Rafts and Western Blot Analysis for CFTR and Flotillin in hTCEpi Cells

Lipid rafts were isolated by discontinuous sucrose gradient centrifugation, as previously reported. Briefly, subconfluent hTCEpi cells grown in four 100-mm dishes were exposed to PA for 1 hour at 37°C. The cells were then washed with ice-cold PBS three times, lysed with 1% Triton X-100 in TNE buffer (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA; Sigma-Aldrich) at 4°C for 30 minutes in the presence of protease inhibitors (10 μg aprotinin, 10 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF) and passed five times through a syringe and 21-gauge needle. The 2 mL of pooled lysate was added to 2 mL of 80% sucrose (final concentration), placed in an ultracentrifuge tube, and overlaid with 6 mL of 30% sucrose, followed by an additional 2 mL of 5% sucrose in TNE buffer to form a discontinuous gradient. Samples were centrifuged overnight at 57,000 rpm, 4°C for 20 hours (SW41Ti rotor; Beckman, Inc., Fullerton, CA), and 1-mL fractions were collected from the top of the gradient. The Triton-insoluble lipid rafts were found to float in the interface of the 5% to 30% sucrose layers (fractions 2–4).

For electrophoresis, fractions were precipitated as described by Kowalski and Pier. Briefly, 10 μL of 5% deoxycholic acid was added to 500 μL of various gradient fractions to solubilize the proteins, and the samples were incubated on ice for 10 minutes. Four hundred microliters of methanol and 700 μL of chloroform were then added to solubilized samples and mixed well; samples were centrifuged at 4°C for 15 minutes at 15,000 rpm in a refrigerated centrifuge (Legend T/RT unit; Sorval, Asheville, NC). The top (aqueous) layer was removed without disturbing the interface. Seven hundred microliters of methanol was then added to the remaining lower (organic) phase and interface and mixed well, and proteins were pelleted at maximum speed for 30 minutes at 4°C. Pelleted proteins were resuspended in lysis buffer including 1% SDS (50 μL for fractions 1–8; 100 μL for fractions 9–12). All samples were incubated at 37°C for 30 minutes to avoid CFTR aggregation. Identical sample fraction volumes (20 μL) were run on 7.5% SDS-PAGE gel and then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Billerica, MA). Blots were blocked in 5% skim milk in 0.05% Tween 20PBS (TPBS) and incubated overnight with primary antibodies at 4°C. Blots were then washed with TPBS and incubated with horseradish (HRP)-conjugated secondary antibodies for 1 hour, followed by visualization using Western blot detection reagents (ELC Plus; GE Healthcare; Piscataway, NJ). Mouse anti-CFTR monoclonal IgG antibody, clone CF3 (Alexis Biochemicals, San Diego, CA), and mouse anti-flotillin monoclonal IgG antibody (BD Biosciences) were used as primary antibodies.

Statistics

Differences between treatment and control groups were evaluated with paired Student’s t-test and one-way ANOVA, on computer (Sigmastat for Windows; SPSS Inc., Chicago, IL).

RESULTS

CFTR Expression in CL-Wearing Rabbit Corneas In Vivo

The reactivity of the two anti-human CFTR antibodies used in this study with rabbit CFTR was confirmed by Western blot assay (data not shown).

CFTR staining of rabbit corneal cross-sections of the whole-thickness epithelial cell layer is shown in Figure 1. Before PA infection, expression of CFTR was confined to occasional superficial cells (Fig. 1A, arrow), and increased in underlying cells after 24 hours of CL wear (Fig. 1B). Bacterial infection with each of the three test PA strains appeared to increase CFTR staining slightly in control eyes (data shown for PA 6294; Fig. 1C), while not increasing CFTR staining after CL wear (compare Figs 1D and 1B). Similar staining patterns were obtained with both anti-CFTR antibodies. Staining with irrelevant IgG showed no CFTR localization in CL-wearing eyes even after PA infection (Fig 1E).

By contrast, corneal wholemounts from normal rabbit eyes showed uniform CFTR staining on scattered surface epithelial cells in conjunctiva (Fig. 2B) but not in central corneal epithelial cells before CL wear (Fig. 2A). This finding is consistent with a previous study using the same anti-human CFTR antibody that showed that CFTR protein is detectable in the surface membrane of normal rabbit conjunctival epithelium. Hypoxic PMMA lens wear induced CFTR protein localization on central corneal surface epithelial cells (Fig. 2C), whereas surface conjunctival epithelial cells remained occasionally stained (Fig. 2D). The former finding is also in good agreement with a previous report that found that hypoxia increases...
CFTR expression in organ-cultured human corneal epithelium, associated with increased PA internalization.13

PA 6294 exposure to normal rabbit eyes did not appear to affect CFTR protein staining on corneal (Fig. 3A) or conjunctival epithelium surface cells in wholemount preparations (Fig. 3B). Of note, normal eyes also failed to show any binding or internalization of PA to either the corneal or conjunctival surface epithelial cells after PA exposure alone, even though CFTR remained uniformly detectable on occasional conjunctival cells (Fig. 3B). By contrast, PA 6294 exposure in CL-wearing eyes resulted in the binding of numerous PA (small red bacteria, arrowheads) to some CFTR-expressing cells in the central corneal surface (Fig. 3C) but not to others; however, conjunctival epithelium failed to bind PA (Figs. 3D, 3F). It should also be noted that PA binding was not exclusive to CFTR-positive cells and that some surface cells that did not stain for CFTR showed significant binding of PA (Fig. 3E; arrow). Similar staining patterns were obtained with the three test invasive PA strains, ATCC27853, 6294, and -6487 (data not shown for ATCC27853 and -6487).

FIGURE 1. CFTR expression in rabbit corneas after CL wear, shown in a cross section. Before PA exposure, expression of CFTR (green) was confined to occasional superficial cells (A, arrow), but increased in underlying cells after CL wear (B). Bacterial infection with each of the three test strains consistently appeared to induce slight CFTR expression in control eyes (C), while not affecting CFTR expression in CL wearing eyes (D). Data are shown for the PA 6294 strain, but was similar for all test strains. Irrelevant IgG showed no CFTR staining in CL-wearing eyes even after PA infection (E). Propidium iodide nuclear staining (red). Bar, 20 μm.

FIGURE 2. CFTR expression in rabbit eye after CL wear, shown in a wholemount. Normal eyes showed CFTR expression (green) in occasional surface conjunctival epithelial cells (B), but not in central corneal surface epithelial cells (A). PMMA lens wear induced CFTR expression in central cornea (C), but not in conjunctival cells (D). Results were similar for both CFTR antibodies tested. Propidium iodide nuclear staining (red). Bar, 40 μm.

FIGURE 3. CFTR expression induced by PA exposure after CL wear, shown in a wholemount. After PA exposure, normal eyes failed to bind PA on either surface corneal (A) or conjunctival epithelial cells (B), even though CFTR (green) was uniformly expressed on occasional surface conjunctival cells. PA exposure to PMMA lens-wearing eyes resulted in the binding of numerous PA (small red bacteria, arrowheads) to some CFTR-expressing surface cells in central cornea (C); however, conjunctival epithelium failed to bind PA (D, F). Note cells (red) binding PA (E, arrow) but not expressing CFTR in corneal cells. Results were similar for all three test strains. Propidium iodide nuclear staining (red). Bar, 20 μm.
Costaining of CFTR and lipid rafts in PA-infected rabbit eyes after CL wear indicated that CFTR and lipid rafts were present in different cell populations and also had different membrane localization patterns on surface conjunctival epithelium (Fig. 4). Of note, even those conjunctival limbal epithelial cells that manifested both CFTR and lipid rafts in normal eyes did not bind PA (Fig. 4D, arrows). It is interesting that lipid raft–positive cells appeared larger than CFTR-positive cells. This may reflect differences in staining of the surface (larger) cells versus the underlying sublayer of (smaller) cells in the normal two-layered conjunctival epithelium. Results are shown for PA 6294 but were similar for all three test strains. In addition, not all surface corneal epithelial cells exhibiting lipid rafts and binding PA 6294 expressed CFTR (Fig. 5aC). Some corneal epithelial cells however, expressed both CFTR and lipid rafts and bound PA (Fig. 5bD). Results were similar for all three PA strains tested. Bar: 40 μm.

Inhibition of PA Internalization with CFTR Peptides or LPS In Vitro

To assess further the role of CFTR in PA internalization, PA binding was blocked with CFTR peptides or LPS from PA in corneal isolate test strains 6294 and 6487. CFTR peptides 103-117 and 108-117 correspond to the first extracellular domain, and have been reported to inhibit PA internalization by deeper corneal epithelial cells in the in vivo scratch-infection murine model and in vitro, serum-containing cultures of rabbit and human corneal epithelial cells. Cultures of hTCEpi cells in serum-free conditions were first treated with the same concentration of these CFTR peptides and infected with PA at the same cell–bacteria ratio (multiplicity of infection [MOI] = 10) as previously reported with human or rabbit cultured corneal epithelial cells; however, under serum-free experimental conditions, none of the CFTR peptides significantly reduced PA internalization (data not shown). Because previous murine studies have shown that sensitivity of PA internalization in vivo to these peptides depends on the challenge dose of PA, hTCEpi cells were next infected with one-fifth times as much PA as in the challenge in the previous in vitro study. Further, much higher concentrations of each peptide than previously used were also added to the cells (25 times for
peptide 103-117, 100 times for peptide 108-117); however, even under these test conditions, neither CFTR peptide significantly inhibited PA internalization of either test strain (Fig. 6). CFTR expression in hTCEpi was confirmed by Western blot of cell lysate (data not shown) and hTCEpi cells are clearly able to internalize PA25; thus, CFTR appears not to be a major receptor for PA internalization by the human corneal cell line used in this study under serum-free culture conditions.

By contrast, LPS from PA significantly decreased PA internalization by hTCEpi in a dose-dependent manner for both test strains (Fig. 7). LPS blocked bacterial internalization of PA strain 6294 by 59% \((P < 0.01,\) one-way ANOVA) and strain 6487 by 69% at \((P < 0.01) 100 \mu g/mL.\)

**LPS Effects on Cell and PA Viability**

LPS did not produce cytotoxicity for hTCEpi cells cultured in serum-free conditions over the concentration range studied \((0 – 100 \mu g/mL).\) Average cell viability assays revealed 96.62% \(\pm 1.14\%\) for control cultures \((0 \text{ mg/mL}), 97.68\% \pm 1.14\%\) at LPS 25 \(\mu g/mL,\) and 96.27\% \(\pm 1.47\%\) at LPS 100 \(\mu g/mL,\) \((P > 0.05,\) nonsignificant; Fig 8). No effect on PA growth of either test strain was observed (data not shown).

**Inhibition of Bacterial Internalization in Rabbit Corneas by Filipin In Vivo**

After 24-hour PMMA lens wear, bacterial internalization was assayed with or without filipin treatment \((2 \mu g/mL).\) We have reported that fillipin and other cholesterol metabolism inhibitors significantly decreased PA internalization with the same three test strains in serum-free cultured hTCEpi cells in vitro compared with control cells.\(^{25}\) In this in vivo corneal whole-mount study, pretreatment with filipin abrogated the increase in PA ATCC27853 internalization induced by PMMA lens wear \((Fig. 10, \ P = 0.034,\) paired Student’s \(t\)-test). These results demonstrate that lipid raft formation is involved in PA internalization in the in vivo rabbit CL-wearing model. Taken together, previous in vitro findings\(^{28}\) and the current results support our ongoing hypothesis that PMMA lens wear enhances the number of surface corneal epithelial cells that form lipid rafts and subsequently internalize PA through lipid raft aggregation.

**CFTR Flotilin Localization in Sucrose Density Isolated Rafts**

To investigate whether CFTR translocates to lipid rafts after PA infection, pooled lysates of PA-infected hTCEpi cells were fractionated by discontinuous sucrose gradient centrifugation.

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**Figure 6.** Effects of synthetic CFTR peptide on PA internalization in vitro. Neither of two CFTR peptides (amino acid 103-117 A; or 108-117 B) corresponding to the first extracellular domain of CFTR altered PA internalization of stains 6294 and 6487 in hTCEpi in serum-free cultures.

**Figure 7.** LPS Effects on PA Internalization by hTCEpi. LPS significantly decreased PA internalization by hTCEpi in a dose-dependent manner for both test strains. LPS blocked bacterial internalization of PA strain 6294 by 59% \((P < 0.01,\) one-way ANOVA) and strain 6487 by 69% at \((P < 0.01) 100 \mu g/mL.\)

**Figure 8.** LPS Effects on Cell Viability. LPS did not produce cytotoxicity for hTCEpi cells cultured in serum-free conditions over the concentration range studied \((0 – 100 \mu g/mL).\) Average cell viability assays revealed 96.62% \(\pm 1.14\%\) for control cultures \((0 \text{ mg/mL}), 97.68\% \pm 1.14\%\) at LPS 25 \(\mu g/mL,\) and 96.27\% \(\pm 1.47\%\) at LPS 100 \(\mu g/mL,\) \((P > 0.05,\) nonsignificant; Fig 8). No effect on PA growth of either test strain was observed (data not shown).

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Flotilin was used as a marker for lipid raft fractions. As expected, flotilin localized in Triton-insoluble fractions which contained lipid rafts at the 5% to 30% sucrose gradient interface (fractions 2-4) and in Triton-soluble fractions (fractions 8-10); however, CFTR was detected only in the soluble (nonraft) fractions (Fig. 11).

DISCUSSION

CL wear has been and currently remains the most frequent cause of corneal infection, and PA remains the most frequent microbial pathogen. Therefore, mechanistic understanding of how PA is internalized by surface corneal epithelial cells is of critical importance to understanding the pathogenesis of CL-related infectious keratitis.

One of the most remarkable features of membrane lipid organization in mammalian cells is the high concentration of sphingolipid and cholesterol in the late Golgi, plasma membrane, and endosomes. These complex lipid bilayer structures demonstrate a rich functional repertoire of shape-transition behavior, including vesicle fission and budding, associated with transmembrane transport. Membranes formed from multiple lipid components can partition into coexisting liquid phases or organized domains. Areas rich in cholesterol and sphingomyelin can separate from neighboring membrane regions containing dioleoylphosphatidylcholine. These latter areas are in a relatively fluid liquid-disordered state whereas other areas rich in cholesterol and sphingomyelin partition into a more ordered compartment (liquid-ordered state) known as lipid rafts. The ganglioside GM1 partitions preferentially into raft domains where it can be detected by the fluorescently labeled β subunit of cholera toxin. It is now also recognized that lipid can be sorted into vesicles in the absence of any membrane proteins suggesting that lipids can function alone in vesicle formation or budding processes. Thus, simple sphingolipid metabolites, such as ceramide and sphingolipid-1-phosphate, have been found to be important mediators in signaling cascades of apoptosis, proliferation, and stress responses.

In addition, sphingolipid-based membrane microdomains, or rafts, have been proposed to sort membrane proteins along the cellular pathways of membrane transport. Currently, however, it remains unknown what molecular properties determine the specific interaction between these proteins and different intraplasma membrane lipid domains. In a complex phenomenon such as raft-mediated PA internalization, the critical question is whether specific membrane proteins that appear to colocalize with rafts spatially and temporally are required for bacterial internalization, or whether they are fellow travelers mediating immunomodulatory or other related cell-signaling pathway(s) downstream within the cell.

Recently, CFTR has been reported to serve as a protein receptor for PA internalization in serum-containing, organ-cultured human corneas and in serum-containing cultures of mammalian nasal, tracheal, lung, and corneal epithelial cells; and CFTR colocalization with lipid rafts has been demonstrated in cultured MDCK epithelial cells. We have examined the

![Figure 7](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932940/) Inhibition of PA internalization by LPS in vitro. LPS from PA significantly blocked bacterial internalization of corneal isolate PA strains 6294 and 6487 by hTCEpi in a dose-dependent manner. **P < 0.01, one-way ANOVA.

![Figure 8](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932940/) Effects of LPS on corneal cell viability. Cell viability assays showed that LPS did not affect cultured cell viability at any concentrations used in this study.
effect of hypoxic CL wear and after infection with three invasive PA strains on the localization of CFTR in vivo in the ocular surface epithelium of the rabbit (cornea, conjunctiva) and determined the relationship between CFTR, lipid rafts, and PA colocalization and internalization. Parallel studies were also performed in serum-free cultures of hTEPi cells. To date, CFTR expression of corneal and conjunctival tissue has been identified by the measurement of chloride current on the living murine ocular surface,43 RT-PCR of corneal and conjunctival tissue, and by immunocytochemical studies of rabbit and porcine conjunctival epithelia.33 Similarly, we have reported the presence of diffuse β-cholera toxin, staining identifying the abundant presence of lipid raft component GM1 in normal rabbit conjunctival epithelial cells.25 In the present study, however, when challenged with exposure to three invasive PA strains, conjunctival epithelial cells failed to bind or internalize PA despite the presence of β-CT25 and CFTR staining. Exposure to PA alone did not appear to induce significant localization of CFTR to the surface epithelial cells in the normal rabbit cornea in vivo and also did not induce lipid raft formation.25 In contrast to these findings, hypoxic PMMA lens wear induced CFTR localization on rabbit corneal epithelial cells, and PA exposure with all test strains then led to binding of numerous PA to occasional but not all CFTR-positive cells in the central cornea. An important finding was that PA was able to bind to and internalize into CFTR-negative corneal epithelial cells; however, conjunctival epithelial cells were not capable of binding PA despite positive CFTR staining, even after contact lens wear. Collectively, these findings indicate that CFTR localization alone is insufficient to explain all PA binding to or internalization by corneal or conjunctival epithelial surface cells. Thus, receptor(s) other than CFTR appear to play a predominant role in mediating PA binding and subsequent PA uptake, and these as yet unidentified receptor(s) could be absent or masked in conjunctival surface cells in vivo.

In further support of this view, the attempt to interrupt the binding of CFTR with PA in serum-free cultures of hTCEpi cells using two synthetic CFTR peptides resulted in no significant reduction in bacterial internalization; whereas, LPS from PA significantly blocked PA internalization of the two invasive strains tested. By contrast, an essential requirement for lipid rafts in mediating PA internalization of the three infectious PA strains tested has been shown by inhibition with cholesterol metabolism inhibitors in the in vitro serum-free cultures of human corneal epithelial cells (hTCEpi)25; and, in the present study, the cholesterol-binding inhibitor filipin also signifi-

![Figure 9](Image)

**FIGURE 9.** Colocalization of PA, CFTR, and lipid rafts in in vivo cell culture of hTEpi cells. (A) Nuclei and PA (cyan, syto 59 staining); (B) lipid rafts (red); (C) CFTR (green); (D) merged image. Compare with Figures 5A and 5B for in vivo wholemounts. After 60 minutes of infection, PA 6294 appeared to bind to and aggregate on corneal epithelial cells (A, arrows). Lipid rafts (B) and CFTR (C) also aggregated at the sites of cell-PA interaction. Merged confocal image (D) showed colocalization of PA, CFTR, and lipid rafts (arrows) only in some corneal cells. Controls with irrelevant antibodies showed no staining of CFTR (E), but the merged image indicated colocalization of lipid rafts and PA as expected (F, arrow). Data show only for PA 6294 but was similar for the other two test strains. Bar: 10 µm.

![Figure 10](Image)

**FIGURE 10.** Inhibition of bacterial internalization in rabbit corneas by filipin in vivo using strain ATCC27853. After 24-hour PMMA lens wear, bacterial internalization was assayed with or without filipin treatment (2 µg/mL) in corneal wholemounts. Pretreatment with filipin abrogated the increase of PA uptake induced by PMMA lens wear (P = 0.034, paired Student’s t-test). These results suggest lipid raft formation is required for PA internalization in the in vivo rabbit contact lens wearing model.
cantly blocked bacterial internalization in the in vivo rabbit model of contact lens wear. Because membrane lipid rafts aggregate in response to PA binding, resulting in the formation of large platforms, lipid rafts appear to play the central role in PA internalization in the cornea, but may also concurrently coordinate downstream signaling responses to PA infection by concentrating other membrane-associated proteins, such as CFTR, specific binding of PA to lung or corneal epithelial rafts has been established by flow cytometry.

Direct isolation of lipid membrane rafts by sucrose density ultracentrifugation also failed to show detectable CFTR protein in Triton-insoluble, raft-rich fractions of PA-exposed hCTEpi cell lysates in Western blot analysis. As expected however, the raft-specific membrane protein flotilin was easily identified. These data are further consistent with the view that CFTR is not a major component of corneal epithelial membrane rafts induced by exposure to PA.

The inability to establish or to confirm a primary role for CFTR in inhibiting uptake of either of the invasive PA test strains by corneal epithelial cells in serum-free cultures with synthetic CFTR/LPS sequence peptides is different from a previous report in which corneal epithelial cells (rabbit, human) were used in cultures containing serum. hTCEpi cells used in our studies were originally cloned and have been maintained thereafter in serum-free conditions. These cells form a multi-layered, differentiated epithelium under 7-day air-lifted conditions. By contrast, primary outgrowth cultures of human eye bank and rabbit corneal cells (Pel-Freeze, Rogers, AR) grown in serum were used in previously reported studies. Such cultures are not known to form a multilayered, differentiated epithelium. Furthermore, the irreversible effects of serum in culture in shifting the behavior of cells from a normal differentiated state in situ to a wound-healing proliferative phenotype has recently been established for keratocytes. Collectively, these results indicate that interpretations of in vitro corneal cell culture experiments in medium containing serum should be approached with caution.

Similar caution should also be exercised in assessing the requirement for membrane lipid raft integrity in mediating PA uptake in ocular surface cells under in vitro culture conditions. In agreement with our studies, Grassme et al. have recently shown that serum-free cultures of human nasal epithelial cells and murine tracheal cells (in vitro and in vivo infection) require intact lipid rafts and raft aggregation for PA internalization. The same authors also report however, that cultures of Chang conjunctival epithelial cells containing serum exhibit GM1/ cholera-toxin-stained rafts that bind and internalize PA in vitro; however, our current studies, demonstrated the presence of detectable membrane rafts on normal rabbit surface conjunctival epithelium in vivo, but no PA binding or uptake even after contact lens challenge with multiple invasive strains. By contrast, normal surface corneal epithelial cells express rafts and PA internalization only after contact lens wear.

A possible explanation for the observed difference between conjunctival and corneal epithelial cells to bind and internalize PA through transplasma membrane transport involving lipid rafts are potential differences in the molecular structure of rafts for each cell type. The presence of GM1-BCT staining demonstrates the presence of individual membrane regions rich in ceramide-sphingolipids, but does not establish equivalent functional raft substructure for different cells. Liquid and ordered (raft) domains have different thicknesses and, to prevent exposure of the underlying hydrophobic core of the plasma membrane bilayer to water, lipid molecules at boundary domains must dynamically stretch and/or compress to maintain membrane integrity. This process requires energy that can be reduced or minimized by reducing the area of the interface between liquid and ordered (raft) domains by forming an inwardly directed bud or a separate vesicle. The cost of forming a vesicle is less than the cost of maintaining a boundary between liquid and ordered domains, thus vesicle formation and intracellular transport can be triggered by anything that favors the formation of curved membrane structures, such as the COP I coat in Golgi membranes or potentially by binding of PA to GM1 or other raft-associated sites in surface corneal epithelial cells. This type of mechanism could bypass the need for a unique protein trigger, but requires initial PA binding to some site(s) on the cell membrane.

The presence of serum under in vitro cell culture conditions may alter normal in vivo raft structure and functions in mediating PA binding or uptake. Clearly, future studies are needed to establish why constitutively expressed rafts in rabbit conjunctival epithelial cells in vivo do not bind or take up PA, whereas either Chang conjunctival cells expressing rafts in serum-containing cultures exhibit avid PA binding and uptake. Such studies may also shed considerable light on the nature and molecular basis of raft-mediated intracellular transport processes as well.

In contrast, the concentration dependent inhibition of PA uptake by whole LPS in two test strains is not explained by cytotoxic effects or by the presence or absence of serum in cell culture assays over the 0- to 100-µg/mL concentration range studied. However, LPS is known to have complex proinflammatory and broad immunoregulatory effects in many cell types which may be influenced by serum-containing culture conditions. Tang et al. have recently shown that LPS-mediated, caspase-independent apoptosis in cultures of human lung epithelial cells culture requires 48 hours and the presence of serum.

In view of the failure of CFTR/LPS homologous peptides to block PA uptake, LPS may act to prevent binding of PA through other structural sequences or by broad downstream effects mediated through more complex mechanisms. An attractive candidate for LPS-targeted suppression of PA uptake is the regulation of the important protective agent surfactant protein D that is present in normal human tears. Recently, Evans et al. have shown that exposure to PA upregulates surfactant protein D production by human corneal epithelial cells, and LPS is essential in this process.

A final remaining question that must be resolved by further experimentation is to determine what percentage of the corneal epithelial cell surface is or can be occupied by lipid rafts. Realizing that rafts are dynamic structures that form, aggregate, and transfer internally, the diameter of sphingolipid/cholesterol rafts on the outer surface of plasma membrane has been estimated by several investigators to be very small (tens of...
hundreds of nanometers) compared with that of cells (tens of micrometers) and to occupy some 10% of the cell surface.23,54,55 By contrast, sphingolipids comprise 20% to 50% of the total polar lipids of the plasma membrane, are concentrated in the outer bilayer leaflet, and thus cover the apical surface of most epithelial cells. Additional complexity is introduced by the finding that small unique ganglioside-rich microdomains can exist within larger ordered domains. Flotillin-positive caveoleae are examples of such super rafts and remain one of the least-understood membrane domain structures. Recently, Anderson et al.56 have proposed that the molecular address for proteins targeted to lipid domains is a unique lipid at this time.

esis and maintenance of these proposed shells remain unclear in diameter. However, the mechanisms underlying the biogenesis and maintenance of these proposed shells remain unclear at this time.

In summary, our results demonstrate distinct differences in the immunolocalization of membrane lipid rafts and CFTR in ocular surface cells in vivo. In the rabbit, surface conjunctival epithelial cells express rafts and CFTR but do not bind or internalize PA even when challenged by hypoxic contact lens wear. By contrast, rabbit corneal surface epithelial cells express CFTR after PA exposure and/or contact lens wear, and internalize PA. Topical blockade of PA uptake by the anti-rat-inhibitor filipin prevents PA uptake by corneal wholemounts after in vivo contact lens wear, confirming and extending previous in vitro findings.25

By contrast, CFTR localization in vivo and in vitro does not directly correlate with PA binding; and, CFTR peptides previously reported to block PA uptake in primary human and rabbit corneal epithelial cultures containing serum failed to do so under serum-free conditions in hTCEpi cells. Taken together, these results suggest that CFTR is not the predominant receptor that mediates PA uptake in vivo in corneal epithelial cells; and, immunologic detection of membrane lipid rafts and CFTR alone is not sufficient to predict PA uptake in vivo by normal ocular surface cells. The results further suggest that future experiments are needed to assess the effect(s) of serum in studies of PA uptake in in vitro assays of all cell lines studied.

References