Role of the Corneal Epithelial Basement Membrane in Ocular Defense against *Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa* can invade corneal epithelial cells and translocates multilayered corneal epithelia in vitro, but it does not penetrate the intact corneal epithelium in vivo. In healthy corneas, the epithelium is separated from the underlying stroma by a basement membrane containing extracellular matrix proteins and pores smaller than bacteria. Here we used in vivo and in vitro models to investigate the potential of the basement membrane to defend against *P. aeruginosa*. Transmission electron microscopy of infected mouse corneas in vivo showed penetration of the stroma by *P. aeruginosa* only where the basement membrane was visibly disrupted by scratch injury, suggesting that the intact basement membrane prevented penetration. This hypothesis was explored using an in vitro Matrigel Transwell model to mimic the corneal basement membrane. *P. aeruginosa* translocation of multilayered corneal epithelia grown on Matrigel was ~100-fold lower than that of cells grown without Matrigel (P < 0.005, t test). Matrigel did not increase transepithelial resistance. Matrigel-grown cells blocked translocation by a *P. aeruginosa* protease mutant. Without cells, Matrigel also reduced traversal of *P. aeruginosa* and the protease mutant. Fluorescence microscopy revealed a relative accumulation of bacteria at the superficial epithelium of cells grown on Matrigel at 3 h compared to cells grown on uncoated filters. By 5 h, bacteria accumulated beneath the cells, suggesting direct trapping by the Matrigel. These findings suggest that the basement membrane helps defend the cornea against infection via physical barrier effects and influences on the epithelium and that these roles could be compromised by *P. aeruginosa* proteases.

*Pseudomonas aeruginosa* is an important opportunistic pathogen, commonly affecting burn victims, individuals with cystic fibrosis, patients in hospital intensive care units, and contact lens wearers (9–11). In the absence of contact lens wear, the cornea is remarkably resistant to infection, with *P. aeruginosa* effectively colonizing this tissue only if it is injured or otherwise compromised (41). To initiate clinically significant corneal pathology, *P. aeruginosa* (and almost all other microbes) must first access the corneal stroma, which is normally protected by a multilayered epithelium and associated basement membrane (1).

The corneal epithelial basement membrane is secreted by the overlying epithelia and is comprised of sheets of extracellular matrix constituents, including type IV collagen, heparan sulfate proteoglycan, and various glycoproteins (laminin, entactin, nidogen, and fibronectin) and growth factors that mediate cellular function (1). Quantitative imaging of the corneal epithelial basal membrane in the rhesus macaque has shown a complex cross-linking of fibers and proteins intermingled with pores ranging from 30 to 400 nm in size (2). As shown for other basement membranes in the body (17, 19, 33), the corneal basement membrane anchors epithelial cells and provides positional information for healing, tissue regeneration, and repair (44). In vitro, Matrigel forms an artificial basement membrane with pores ranging from 26 to 359 nm in size, is composed of laminin, collagen IV, heparan sulfate proteoglycans, entactin, nidogen, and naturally occurring growth factors, and closely resembles the natural corneal basement membrane (2, 26).

It has been shown that *P. aeruginosa* isolates can translocate MDCK cell monolayers (6, 22) and that translocation and virulence were reduced by mutation of genes encoding multidrug resistance efflux systems (23). Purified elastase and exotoxin A from *P. aeruginosa* have each been shown to increase the permeability of MDCK cell monolayers (4), and purified elastase increases alveolar permeability in vivo (5). However, the role of the basement membrane in *P. aeruginosa* translocation has not been studied.

We have previously shown that *P. aeruginosa* can translocate multilayered corneal epithelia in vitro and that human tear fluid reduced both translocation in vitro and virulence in vivo in murine models of corneal infection (28). While this and other studies have focused on the role of the tear film and corneal epithelium in defense against *P. aeruginosa* keratitis (14), little attention has been given to the basement membrane. Previous studies have reported that the epithelial basement membranes of other tissues can form a physical barrier to potential pathogens, including human papillomavirus, herpes simplex virus, and Rift Valley fever virus (25, 42, 43, 47). In this study, it was hypothesized that the corneal basement membrane forms a physical barrier to defend against the penetration of *P. aeruginosa*, since its pores are smaller than the size of bacteria, and that *P. aeruginosa* proteases can functionally overcome that defense. This hypothesis was examined correl-
ately using transmission electron microscopy of in vivo-infected mouse corneas and tested directly using a quantitative in vitro Matrigel-based model system to mimic the corneal epithelium and its associated basement membrane.

MATERIALS AND METHODS

Bacterial strains and mutants. The invasive P. aeruginosa strains 6294, PA01, and an isogenic triple protease mutant of PA01 (lack lasBA pre) (which lacks LasA, LasB, and alkaline protease) (12) were used. Strains 6294 and PA01 behave similarly with respect to invasion and translocation of corneal epithelial cells in vitro and are equally virulent in murine models of corneal infection (28, 30). Bacteria were cultured on Trypticase soy agar plates at 37°C and then resuspended in SHEME (supplemented hormon epithelial media) without antibiotics to a concentration of 10^6 CFU/ml (optical density at 650 nm of 0.1) for use during in vivo experiments. For in vitro assays, the bacterial suspension was subsequently diluted to a final concentration of 10^5 CFU/ml. Bacteria were enumerated by viable counts after serial dilution to confirm bacterial concentration.

Murine in vivo infection model and transmission electron microscopy. Female C57BL/6 mice (5 to 7 weeks old) were infected using the scarification model of corneal infection as described previously (40). Briefly, three full-thickness epithelial abrasions were produced on the left corneas of six mice with a 26-gauge needle after anesthesia. Eyes were immediately inoculated with 5 μl of SHEME inoculum containing strain 6294. After 24 h, mice were euthanized and the left eye enucleated. Eyes were then prepared for transmission electron microscopy. Briefly, whole eyes were fixed in 3% gluteraldehyde in 0.1 M phosphate buffer (pH 7.2) at room temperature for 3 h and then postfixed in 1% osmium tetroxide in phosphate buffer at room temperature for 2 h. Samples were dehydrated in a graded series of ethanol and embedded in Spurr’s resin. Thin sections of the cornea were examined with a Zeiss EM 100A electron microscope set at 60 kV.

Cell culture. Immortalized rabbit corneal epithelial cells (36) were grown to confluence on 8-μm-pore-size filters that were either uncoated or coated with Matrigel (BD Biosciences, Bedford, MA) and then air-lifted as previously described (27). Briefly, the cells were cultured on the filters for 3 days with both sides of the filter submerged in SHEM and then air-lifted by removing media from the top chamber from day 4 to day 7. Cells were used for experiments on day 7 after passage.

In vitro assay of bacterial traversal. Corneal epithelial cells were grown on Transwell filters with or without Matrigel-coating (BD Biosciences, San Jose, CA). Bacterial inocula were added only to the apical chamber, such that bacteria could reach the basal chamber only by translocating the cells and the matrix on which they were grown. The use of 8-μm-pore-size filter supports ensured that bacteria could reach the lower chamber if they were able to penetrate the cells and/or Matrigel coating. Matrigel-coated and uncoated filters used without cells were also included as controls. At hourly time points after inoculation, viable bacteria in the upper and lower chambers were enumerated by viable counts. Transepithelial resistance (TER) was also recorded using an Evom meter (World Precision Instruments Inc., Sarasota, FL). Additional samples were included to enumerate bacteria retained by the cells and/or Matrigel at various time points postinfection. For this purpose, Transwell filters were removed from their plastic inserts after one wash with phosphate-buffered saline (PBS) and then treated with 0.25% (vol/vol) Triton X-100 in PBS for 15 min to lyse cells before enumerating them by viable counts.

Fluorescence microscopy. PA01 and its protease mutant (laxA lasBA preA) were transformed with plasmid pSMC2, constitutively expressing green fluorescent protein (GFP). At 3 h and 5 h postinfection, filters were rinsed once with PBS and fixed for 5 min with 4% (wt/vol) paraformaldehyde in PBS. The filters were then washed three times (for 5 min each) with PBS, excised, and flat-mounted for microscopy. Z-stack analysis using the Velocity software (Improvision Inc., Lexington, MA) and an IX-70 microscope (Olympus, Melville, NY) were used to visualize bacteria expressing GFP relative to the apical cell surface, the underlying filter, and the Matrigel coating between them if present.

Statistical analysis. Data were expressed as means ± standard deviations. Statistical significance of differences between groups was determined using Student’s t test. P values of <0.05 were considered significant. Experiments were repeated at least three times with at least three samples used in each group.

RESULTS

In vivo, P. aeruginosa bacteria were detected in the underlying stroma only where the basement membrane was discontinuous. Transmission electron microscopy images of infected corneas showed extensive epithelial and basement membrane disruption where the scratches of the central cornea had been made to enable the initiation of disease (Fig. 1). Areas of basement membrane disruption were characterized by massive stromal swelling, epithelial detachment, and polymorphonuclear (PMN) leukocyte infiltration (Fig. 1A and B). In areas where the basement membrane was discontinuous, bacteria were seen both in the subbasal epithelia and within the stroma (Fig. 1A and B). Regions distant from the scratched area showed intact basement membrane with or without epithelial disruption (Fig. 1C, D, and E). While bacteria were found both inside and between intact epithelial cells (Fig. 1C), most were found adjacent to the upper (epithelial) side of the intact basement membrane (Fig. 1B and E). In these areas where the basement membrane was intact, bacteria were not found in the underlying stroma (Fig. 1C, D, and E).

Matrigel reduced P. aeruginosa traversal of corneal epithelial cells in vitro. Qualitative evidence from in vivo experiments suggested that the corneal basement membrane could restrict bacterial penetration into the corneal stroma. To test this directly, we developed an in vitro model system of bacterial traversal that enabled quantification of data (see Methods). Artificial basement membrane (Matrigel) was used since it has been shown to resemble corneal basement membrane in vivo both physically and biochemically. Bacterial traversal rates through corneal epithelium grown on Transwell filters were compared for cells grown with or without a Matrigel coating on the filter. The number of bacteria that reached the basal chamber when cells were grown on Matrigel was reduced ~100-fold at each time point up to 4 h postinoculation and by ~10-fold at the 5-h time point compared to the number for cells grown without Matrigel (Fig. 2A). Matrigel alone (without cells) also significantly reduced bacterial traversal through the filters (~175-fold at 1 h and ~5-fold at each of the remaining time points) (Fig. 2B). Growth of cells on Matrigel did not significantly affect TER at most time points (Table 1).

Matrigel promotes retention of P. aeruginosa within the Transwell filter system with and without epithelial cells. Reduced penetration of P. aeruginosa through Matrigel-coated filters (irrespective of whether cells were present) suggested that Matrigel could physically retain the bacteria. To test that directly, the numbers of bacteria retained by filter-grown corneal epithelial cells (or filters alone) were compared with and without Matrigel (Fig. 3). Retention of viable bacteria was observed at 3 h postinfection for cells grown on Matrigel compared to results for cells grown without Matrigel, which showed no retentive effect when normalized to retention by filters alone (Fig. 3A). By 5 h, no significant difference in retention of viable bacteria was found, suggesting that bacteria could overcome this retentive effect with time. Matrigel-coated filters (without cells) also showed greater accumulation of bacteria than uncoated filters at 3 h (Fig. 3B). However, in this instance, the retentive effect of Matrigel was also observed at 5 h postinfection.

Fluorescence microscopy of Matrigel-grown cells (Fig. 4)
showed two distinct zones of bacterial retention, the first being at the apical cell surface (Fig. 4A) and the other at the filter surface (Fig. 4B). Without Matrigel, apical cell surface retention of bacteria was also observed (Fig. 4C) but filter surface retention was absent (Fig. 4D).

*P. aeruginosa* protease activity modulates traversal through epithelial cells and Matrigel. Since basement membrane proteins and epithelial cells could be susceptible to *P. aeruginosa* protease activity, we compared traversal of corneal epithelial cells grown with and without Matrigel by wild-type *P. aeruginosa* and a protease mutant (PA01 lasA lasBA prA). Wild-type bacteria showed increased traversal of corneal epithelial cells compared to results for the protease mutant with and without Matrigel (Fig. 5A). It was also observed that the presence of Matrigel significantly reduced the traversal of both the wild-type and mutant bacteria. Indeed, in the presence of Matrigel, traversal of the protease mutant was almost completely blocked (Fig. 5A). Wild-type and protease mutants were also

FIG. 1. Transmission electron micrograph of scratch-injured and infected mouse cornea after 24 h of incubation with *P. aeruginosa* invasive strain 6294. (A) Scratched area where the basement membrane had been breached (1, 2) and the stroma infiltrated by PMNs. (B) Section showing discontinuous and intact basement membrane, the former correlating with bacterial and PMN infiltration of the stroma. (C to E) Different sections peripheral to the scratched area showing intact basement membrane and the absence of bacterial infiltration of the stroma.
compared for their ability to traverse Matrigel without cells (Fig. 5B). Matrigel was equally effective at blocking traversal of both wild-type and protease mutant bacteria for the first 3 h. Thereafter, wild-type bacteria had a significant advantage in their ability to penetrate the Matrigel (Fig. 5B).

Matrigel promotes retention of the protease mutant within the Transwell filter system with and without epithelial cells. Matrigel was associated with increased retention of the protease mutant on the filter system with cells (Fig. 6A) and without cells (Fig. 6B). Growth of cells on Matrigel was associated with 1.54-fold and 1.87-fold increases in bacterial retention at 3 and 5 h, respectively, compared to results for cells grown without Matrigel (Fig. 6A). In the presence of corneal epithelial cells, retention of the protease mutant also significantly increased between 3 and 5 h with and without Matrigel (Fig. 7).

**DISCUSSION**

Microscopy of in vivo-infected eyes suggested that bacteria penetrated into the corneal stroma from the overlying corneal epithelium only in regions where the basement membrane had become discontinuous. That data supported the hypothesis that the basement membrane acts as a barrier against bacterial traversal in vivo. Quantitative in vitro experiments confirmed that the basement membrane could reduce bacterial penetration, that the mechanism involved a physical barrier effect, and that *P. aeruginosa* proteases have the potential to reduce this protective activity.

Our results are consistent with those of previous studies, which have reported that epithelial basement membranes of other tissues, such as columnar genital epithelium and epidermal layers of the skin and lining of the gut, can act as a barrier to herpes simplex virus and Rift Valley fever virus to limit initial disease onset and dissemination in mouse and insect models of flank scarification and Rift Valley fever infection.

**TABLE 1.** TER across multilayers of rabbit corneal epithelial cells grown with or without Matrigel and previously inoculated with *P. aeruginosa* strain PA01

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean TER reading (Ω·cm²) from cells infected with PA01 at indicated time point postinoculation</th>
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<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>With Matrigel</td>
<td>141±14</td>
</tr>
<tr>
<td>Without Matrigel</td>
<td>161±11</td>
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*TER of Matrigel-grown cells was significantly reduced from that of cells grown without Matrigel only at 2 and 3 h (*P = 0.004 and 0.017 respectively, t test). Baseline readings for Matrigel only or filters alone were significantly lower than TER values for cells grown with and without Matrigel (data not shown).
respectively (25, 43, 47). Our results also add to our knowledge of *P. aeruginosa* translocation gained from previous studies (4, 6, 22, 23) by showing that the basement membrane contributes to reducing *P. aeruginosa* translocation of multilayered corneal epithelial cells and directly showing the involvement of *P. aeruginosa* proteases in the bacterial translocation process.

Our data comparing wild-type PA01 and the triple protease mutant in their ability to penetrate the basement membrane in vitro directly show that *P. aeruginosa* protease expression can enhance bacterial penetration through the basement membrane alone and in combination with cells (Fig. 5). Protease expression by *P. aeruginosa* is an important virulence mechanism (8, 13, 24, 45). Previous studies have examined the roles of elastase (LasB) and alkaline protease on corneal pathology using purified proteases and/or bacterial mutants deficient in one or multiple proteases to establish those contributing to *P. aeruginosa*’s virulence in vivo (32, 35, 46). While the mechanism for involvement of *P. aeruginosa* proteases in vivo has not been established, basement membrane components are known to be targeted (7, 20, 21, 34). In addition, purified *P. aeruginosa* elastase increases airway epithelial permeability in vivo (5) and in vitro (6), the latter through an effect on tight junctions. Thus, it has been assumed that proteases allow the pathogen to gain access to tissues.

Bacterial traversal across Transwell filters was shown to be most effectively reduced when Matrigel and cells were both present (Fig. 2A). Yet bacterial retention by cells grown on filters was increased by Matrigel for only the first 3 h; by 5 h, similar numbers of bacteria were retained with or without Matrigel (Fig. 3A). Other data collected in this study could explain this apparent paradox. As discussed above, protease activity enhanced bacterial penetration through both cells and Matrigel. Indeed, for Matrigel used alone, traversal was effectively prevented only for the first 3 h (Fig. 5B). After that, wild-type bacteria showed a significant increase in penetration compared to protease mutants (Fig. 5A and Fig. 2A), which corresponded to a reduced retentive capacity of Matrigel for wild-type bacteria at 5 h compared to that in the initial 3-h period (Fig. 3A) and to an increased retentive capacity of Matrigel for the protease mutant at the same time points (Fig. 6A). Taken together, those data suggested that after the first 3 h, proteases had compromised the protective activity of Matrigel against bacterial penetration in these assays. Since protease production by *P. aeruginosa* is regulated by quorum sens-

![Fluorescence microscopy with Z-stack analysis of corneal epithelial cells grown with and without Matrigel at 5 h postinfection with *P. aeruginosa* strain PA01 expressing GFP. Cells grown on Matrigel showed bacterial retention in two distinct zones: at the apical cell surface (A) and the filter surface (B). Cells grown without Matrigel showed apical surface retention of bacteria (C) but no filter surface retention (D). Arrows indicate filter pores.](http://iai.asm.org/)

FIG. 4. Fluorescence microscopy with Z-stack analysis of corneal epithelial cells grown with and without Matrigel at 5 h postinfection with *P. aeruginosa* strain PA01 expressing GFP. Cells grown on Matrigel showed bacterial retention in two distinct zones: at the apical cell surface (A) and the filter surface (B). Cells grown without Matrigel showed apical surface retention of bacteria (C) but no filter surface retention (D). Arrows indicate filter pores.
ing and other regulatory systems (16, 37, 38), the capacity to penetrate might depend upon the bacterial concentration and the surrounding environmental conditions. Indeed, all of the experiments performed with wild-type bacteria, with or without epithelial cells, showed that traversal was delayed rather than prevented; once traversal began, additional traversal at each hourly time point appeared quantitatively similar. Thus, by 5 h, bacterial traffic in and out of the cell layers in these in vitro assays might have reached a steady state.

It is also possible that Matrigel contributed to protecting cells against traversal by mechanisms independent of direct physical trapping, for example, by modulating antimicrobial activity of epithelial cells. Epithelial cells can make antimicrobial peptides in response to bacteria (31). Basement membrane proteins are known to play roles in epithelial growth, differentiation, and polarization that could influence antimicrobial activity, or other epithelial changes. These data suggest that a deep penetrating injury to the anterior stroma is required to make otherwise healthy corneas susceptible to infection by a wide range of microbes, including bacteria, viruses, amoebas, and fungi (18, 29, 39, 48). The data are

than-expected bacterial retention by cells/Matrigel several hours postinfection could be that protease production influences other cellular responses to bacteria which are in turn impacted by the Matrigel. For example, if bacterial proteases enhance the innate immune response(s) of the epithelial cells to enable them to more easily resist bacteria (3), then protease mutants would be expected to persist longer via their reduced ability to trigger a robust cellular response.

In conclusion, the data collected in this study suggest that the corneal epithelial basement membrane may function as an important defense against infection. In vivo observations strongly suggest that the intact basement membrane prevents bacteria from entering the corneal stroma. Quantitative data collected in vitro showed that an artificial basement membrane (Matrigel) that is physically and biochemically similar to the corneal basement membrane can provide a barrier to bacterial traversal when used alone or in combination with corneal epithelial cells. While the mechanism for protection was found to involve direct physical trapping within the basement membrane, which is likely due to a filtering effect of its small pore size, there may also be effects of epithelial cell permeability, antimicrobial activity, or other epithelial changes. These data are consistent with animal models of ocular infection, which have shown that a deep penetrating injury to the anterior stroma is required to make otherwise healthy corneas susceptible to infection by a wide range of microbes, including bacteria, viruses, amoebas, and fungi (18, 29, 39, 48). The data are
also consistent with clinical observations of patients with corneal disease which support the probability of a broad-spectrum defense against infection independent of epithelial cell barrier function, since not all types of epithelial injury/disease predispose to corneal infection (15). However, our results also show that basement membrane defenses against infection can be compromised, by P. aeruginosa proteases in this instance. Further in vivo studies would help elucidate the role of the basement membrane and multilayered epithelia in defending the cornea and other tissues against infection. However, given the difficulty of in vivo basement membrane studies, the in vitro Matrigel system used in the present study provides a useful model for investigating the cellular and molecular basis of epithelial barrier function. Research aimed at exploring the role of barriers that prevent bacterial penetration could lead to a better understanding of how tissues become susceptible to infection and may ultimately lead toward a means for restoring resistance.

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