Actin cytoskeleton disruption by ExoY and its effects on *Pseudomonas aeruginosa* invasion

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Abstract

Three of the Type III-secreted effectors of *Pseudomonas aeruginosa* (ExoS, ExoT, and ExoY) each alter mammalian cell morphology in culture without causing a loss of cell viability. For ExoS and ExoT this property involves RhoGAP activity, and leads to actin cytoskeleton disruption and a reduced capacity for internalizing bacteria. ExoY does not possess RhoGAP activity. Instead, cell rounding depends upon its adenylate cyclase catalytic region. Since anti-phagocytic activities of ExoS and ExoT are associated with cell rounding and cytoskeleton disruption, we hypothesized that ExoY would also inhibit *P. aeruginosa* invasion of epithelial cells coinciding with adenylate cyclase-mediated cytoskeleton disruption. The results showed actin disruption of epithelial cells at 2 h post-infection associated with both adenylate cyclase-active ExoY and its catalytic mutant form ExoYK81M, and which coincided with inhibition of bacterial invasion (76% inhibition by ExoY, and 37% by ExoYK81M). Surprisingly, at 4 h post-infection, neither form of ExoY inhibited invasion despite extensive actin disruption. These data suggest that ExoY, like ExoS and ExoT, contains more than one active domain affecting mammalian cell function. The data also suggest that cytoskeleton disruption does not necessarily predict invasion inhibitory activity, supporting the recently proposed model that *P. aeruginosa* internalization can proceed through more than one pathway.

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1. Introduction

The opportunistic bacterial pathogen, *Pseudomonas aeruginosa*, is a leading cause of morbidity and mortality in humans from nosocomial and community-acquired pneumonia’s, burn-wound infections, corneal infections, and sepsis [1–4]. Effector proteins secreted by a Type III Secretion System (TTSS), and controlled by the transcriptional activator ExsA, have been shown to contribute significantly to the virulence of this bacterium in multiple in vivo animal models [5–11]. Furthermore, analysis of *P. aeruginosa* clinical isolates has also shown a clear correlation between expression of TTSS effectors and increased disease severity and patient mortality [12].

ExoY is one of four TTSS effectors identified to date; the others being ExoU, ExoS and ExoT [13–15]. Host
cell contact or low environmental calcium (Ca\(^{2+}\)) causes *P. aeruginosa* to secrete TTSS effectors; either direct injection into the host cell cytoplasm or secretion to the external environment, respectively. ExoU is a phospholipase that is acutely cytotoxic to mammalian cells within 3–4 h of host cell contact [16,17]. ExoS and ExoT each expresses N-terminal RhoGAP activity associated with mammalian cell rounding, cytoskeleton disruption, and reduced capacity for internalizing bacteria (i.e., they exert an anti-phagocytic effect), but without causing a loss of cell viability over 3–4 h of bacterial–host cell contact [7,18–20]. ExoS and ExoT also express a C-terminal ADP-ribosyltransferase activity that also affects mammalian cell morphology, the actin cytoskeleton, and epithelial wound-healing, but without anti-internalization effects [21–24]. ExoY is an adenylate cyclase [25] activated by an unknown host cell factor, which also mediates rounding of cultured mammalian cells without causing a loss of viability [20,26]. A recent study showed that ExoY promoted cyclic AMP accumulation in vascular endothelial cells, and increased vascular permeability [27]. Although the majority of *P. aeruginosa* isolates contain the *exoY* ability [27], ExoY is a functional TTSS effector with adenylate cyclase function. This was tested using the double mutant PA103*ΔexoUΔexoT::Tc* (which expresses a functional TTSS, but no known effectors) as a vehicle to deliver ExoY, or its catalytically-inactive adenylate cyclase form ExoYK81M, into epithelial cells in vitro.

2. Materials and methods

2.1. Bacterial strains and inocula

Experiments were performed using a double mutant of *P. aeruginosa* strain PA103 (*PA103ΔexoUΔexoT::Tc*) (serogroup O11) which does not express any known TTSS effector proteins [20]. This mutant was complemented with the plasmid pUCPexoY allowing it to express ExoY with adenylate cyclase activity, or the plasmid pUCPexoYK81M allowing it to express the catalytically-inactive form of ExoY without adenylate cyclase activity, or with pUCP18 as a plasmid control [20].

Bacteria were grown overnight at 37 °C on trypticase soy agar (TSA) plates. These were supplemented with carbenicillin (300 \(\mu\)g/mL) for selection of the complemented plasmids. For use in experiments, bacteria were resuspended in modified Eagle’s medium (MEM) with Earle’s salts and l-glutamine, buffered with 1 M HEPES–NaOH, pH 7.6, 0.35 g/L NaHCO\(_3\), and 6 g/L bovine serum albumin (BSA). Bacterial concentrations were determined by spectrophotometry (OD\(_{650}\) of 0.1 was equivalent to \(~1\times10^8\) cfu/mL) and confirmed by viable counting.

2.2. Cell culture

Immortalized rabbit corneal epithelial cells (RCE) were grown on small glass coverslips or 24-well tissue culture plates (Corning, New York, NY, USA), and fed on alternate days with modified supplemented hormonal epithelial medium (SHEM) containing bovine pituitary extract (5 \(\mu\)g/mL) instead of cholera toxin [29]. Experiments used cells grown for 3–6 days after passage. Results presented were obtained from cells grown between passages 6 and 20.

2.3. Reagents

Rhodamine–phalloidin and SlowFade® Light Antifade were obtained from Molecular Probes (Eugene, OR, USA). DPX mountant was obtained from Fluka Chemicals (St. Louis, MO, USA). All other reagents were obtained from Sigma (St. Louis, MO, USA) unless otherwise stated.

2.4. Invasion assays

Gentamicin survival assays were used to quantify *P. aeruginosa* invasion of epithelial cells grown on 24-well tissue culture plates as previously described [30]. Before addition of bacteria, cells were washed once with MEM (500 \(\mu\)L). A bacterial inoculum of \(~2\times10^6\) cfu in 200 \(\mu\)L of MEM was added per well of cells. Since each well was calculated to contain \(~10^6\) cells, the multiplicity of infection (MOI) was \(~2\). Following 2, 3, 4, and 5 h of incubation at 37 °C (5% CO\(_2\)), loosely adherent bacteria were rinsed off by washing with 500 \(\mu\)L of PBS (138 mM NaCl, 2.7 mM KCl, 8.1 mM Na\(_2\)HPO\(_4\), 1.47 mM KH\(_2\)PO\(_4\), pH 7.4) prior to killing remaining adherent extracellular bacteria with 1 mL of gentamicin solution (200 \(\mu\)g/mL in MEM) for 1 h. Survivors of gentamicin treatment (intracellular bacteria) were enumerated by viable counts. Prior to viable counting, cells were washed once with PBS (1 mL) to remove residual antibiotic, and lysed by treatment with 200 \(\mu\)L of PBS containing Triton X-100 (0.25% vol/vol). Three to four wells were used for each sample, and all experiments were repeated at least once.

2.5. Microscopy

Epithelial cells cultured on glass coverslips were washed with PBS then inoculated with 0.5 mL of bacterial suspension (MOI = 10). Infected cells were incubated for 2 or 4 h at 37 °C in 5% CO\(_2\). The bacterial
suspension was then aspirated, and wells washed with two sequential aliquots of PBS (2 mL) to remove non-associated bacteria. Cells were fixed with formaldehyde solution (4% wt/vol) for 10 min at room temperature (RT), washed twice with PBS, then permeabilized with Triton X-100 (0.1% vol/vol in PBS) for 5 min (RT). Coverslips were again washed twice with PBS, before blocking with BSA (1% wt/vol in PBS) for 30 min. Actin microfilaments were labeled with rhodamine–phalloidin (5 U/mL in PBS with 1% wt/vol BSA for 20 min RT) in the dark. After two washes with PBS, coverslips were mounted on glass slides using 1 drop of SlowFade® Light Antifade reagent and sealed with DPX mountant before assessment by fluorescence microscopy and photography of representative samples.

2.6. Statistical analysis

ANOVA and Fisher’s PLSD were used to compare groups of data. P values <0.05 were considered significant.

3. Results

3.1. ExoY and ExoYK81M each disrupt the actin cytoskeleton of epithelial cells

Fluorescence microscopy was used to compare the actin cytoskeleton of epithelial cells at 2 h (Fig. 1, upper panels) and 4 h (Fig. 1, lower panels) post-infection by PA103ΔexoU/exoT::Tc (ΔU/T) complemented with pUCP18 (control) or its ExoY- and ExoYK81M-complemented forms. As expected, the double mutant, which does not secrete any known TTSS effectors, had no visible effect on cell morphology or the actin cytoskeleton (Fig. 1A and D). At 2 h post-infection, however, both ExoY and ExoYK81M-complemented P. aeruginosa each caused visible actin redistribution to the cell margins that was accompanied by some cell rounding (Fig. 1B and C, respectively). After 4 h infection, ExoY-complemented bacteria caused significant rounding of the epithelial cells accompanied by actin cytoskeleton disruption (Fig. 1E). At the same time point, ExoYK81M-complemented bacteria continued to show altered actin distribution, but with less visible cell rounding (Fig. 1F).

3.2. ExoY and ExoYK81M inhibit early (2 h) P. aeruginosa invasion

Invasion of epithelial cells by PA103ΔexoU/exoT::Tc (U/T) complemented with pUCP18 (control) was compared to that of ExoY- and ExoYK81M-complemented bacteria at 2, 3, 4, and 5 h post-inoculation (Fig. 2). At the earliest (2 h) time-point, ExoY reduced epithelial cell invasion by P. aeruginosa (76% inhibition of invasion compared to the ΔU/T mutant, P = 0.0008, Fisher’s PLSD). The catalytically-inactive ExoYK81M also reduced P. aeruginosa invasion at 2 h (37% inhibition of invasion compared to the ΔU/T mutant, P = 0.025, Fisher’s PLSD). Inhibition of invasion caused by complete ExoY was significantly greater than that caused by the catalytic mutant ExoYK81M (p = 0.019, Fisher’s PLSD). Interestingly, only bacteria expressing the active adenylate cyclase form of ExoY showed reduced

Fig. 1. Fluorescence microscopy (rhodamine–phalloidin labeling) showing the actin cytoskeleton of epithelial cells after 2 h (upper panels A–C) or 4 h (lower panels D–F) infection with PA103ΔexoU/exoT::Tc complemented with pUCP18 (A, D) or the same mutant complemented with pUCPexoY (B, E) or pUCPexoYK81M (C, F). Magnification ~1200×.
invasion compared to the ΔU/T mutant by 3 h post-infection \((p = 0.017, \text{ Fisher’s PLSD})\), and neither form of ExoY significantly affected \(P.\ aeruginosa\) invasion at 4 h post-infection or later \((p > 0.05, \text{ ANOVA})\). Control experiments confirmed that bacterial growth rates were similar over the experimental period. For example, from a starting inoculum of \(\sim 2.76 \times 10^7\) cfu/ml, each of the complemented ΔU/T mutant bacteria grew to a concentration of \(\sim 1.63 \times 10^8\) cfu/ml over a 4 h period.

4. Discussion

This study showed that ExoY-induced epithelial cell rounding was associated with actin cytoskeleton disruption that was preceded by actin redistribution to the cell margins. The data also showed that an adenylate cyclase catalytic mutant of ExoY disrupted the actin cytoskeleton. Both forms of ExoY inhibited early (2 h) \(P.\ aeruginosa\) invasion of epithelial cells, but at later times (4 h), \(P.\ aeruginosa\) effectively invaded epithelial cells despite continued cytoskeleton disruption and cell rounding.

ExoY-mediated cell rounding associated with adenylate cyclase activity has been shown previously [20]. The present study shows that, as might be expected, this involves significant changes in actin distribution and cytoskeleton integrity. It was notable, however, that the catalytic mutant of ExoY also caused some disruption to the cytoskeleton that was more evident at 2 h than at 4 h post-infection. It is possible that this latter effect involves low-level residual adenylate cyclase activity within the K81M catalytic mutant of ExoY. However, there may be other functional domains of ExoY that can also interfere with the actin cytoskeleton, as has been shown for other Type-III secreted effectors of \(P.\ aeruginosa\). For example, ExoS and ExoT each have dual functional domains (N-terminal RhoGAP activity; C-terminal ADP-ribosyltransferase activity) each of which is capable of interfering with mammalian cell signaling affecting the function and integrity of the cytoskeleton [7,18,21–24,31].

Adenylate cyclase activity of ExoY causes elevated intracellular cyclicAMP in mammalian cells [25]. Complete ExoY was more effective at inhibiting bacterial invasion of epithelial cells at 2 h post-infection than the adenylate cyclase catalytic mutant. This invasion inhibitory activity is consistent with the actions of other bacterial toxins, e.g., \(Bordetella\ pertussis\) that directly or indirectly elevate intracellular cyclicAMP, and exert antiphagocytic effects towards human neutrophils and monocytes [32,33]. Other studies have also shown correlations between elevated intracellular cyclicAMP and reduced phagocytic ability of human neutrophils [34,35]. Since cyclicAMP is a versatile intracellular second messenger that can modulate multiple signal transduction pathways and cellular activities, further studies are needed to determine the mechanism(s) by which the adenylate cyclase activity of ExoY inhibits \(P.\ aeruginosa\) invasion.

Previous studies have shown that actin cytoskeleton disruption mediated via toxins such as cytochalasin D or latrunculin A or other TTSS effectors, e.g., ExoT and ExoS has significant invasion-inhibitory activity towards \(P.\ aeruginosa\) [7,30,36–38]. Consistent with those earlier studies, ExoY-mediated actin disruption at 2 h coincided with inhibition of bacterial invasion for both adenylate cyclase active and inactive forms of the molecule. Surprisingly, by 4 h, actin cytoskeleton disruption no longer corresponded with reduced bacterial invasion. Other studies have shown circumstances (highly polarized epithelial cells) under which \(P.\ aeruginosa\) can be internalized by a pathway(s) insensitive to actin-cytoskeleton disruption [39]. The epithelia used in the present study were not highly polarized, and thus it is likely that inhibition of invasion by ExoY or ExoYK81M is associated with the observed cytoskeleton disruption. It is possible, however, that at later time points (4 h), \(P.\ aeruginosa\) internalization “switch”s to an alternate pathway(s) independent of actin cytoskeleton function thereby overriding the ExoY-mediated effects on invasion observed at earlier times. Alternatively,
another domain of ExoY might itself promote invasion to effectively override invasion-inhibition caused by cytoskeleton disruption.

In conclusion, this study demonstrates that ExoY can temporarily inhibit the internalization of P. aeruginosa by epithelial cells when expressed in the background of a P. aeruginosa mutant lacking other known Type-III secreted effector proteins. This early invasion inhibitory action corresponds in time with cytoskeleton disruption and is associated with adenylate cyclase-active and catalytic mutant forms of this effector. Loss of invasion inhibition by ExoY at later time points supports results of earlier studies showing circumstances exist in which P. aeruginosa is capable of invading epithelial cells after cytoskeleton disruption. Further studies are needed to elucidate the mechanisms(s) by which catalytic and “non-catalytic” actions of ExoY disrupt the mammalian cytoskeleton and modulate invasion, and how those activities contribute to the pathogenesis of P. aeruginosa infection.

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