SA1

Alterations in airway epithelial cell function by Pseudomonas secretory products

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Pseudomonas aeruginosa causes severe acute nosocomial pneumonias and chronic lung infections in patients with cystic fibrosis (CF). Chronic lung injury is the primary cause of death in CF and is linked to co-existent P. aeruginosa infection. The mechanisms involved in P. aeruginosa-mediated lung damage remain uncertain.

Pyocyanin (1-hydroxy-5-methylphenazine), one of several small phenazine compounds synthesized and secreted by most P. aeruginosa strains, has been linked to organism virulence. Pyocyanin undergoes eukaryotic cell-mediated aerobic redox cycling, resulting in formation of reactive oxygen species (ROS) such as superoxide (O2.-) and hydrogen peroxide (H2O2). How pyocyanin enters eukaryotic cells, the site(s), mechanism(s), and nature of the oxidants produced during cellular exposure to pyocyanin, the cellular targets modified and their functional consequences are each poorly understood. There is even less known about other related phenazines produced by P. aeruginosa, such as 1-hydroxyphenazine (1-HP) and phenazine-1-carboxylic acid (PCA).

We are testing the hypothesis that during P. aeruginosa lung infection pyocyanin release leads to site-specific oxidant-mediated effects on airway epithelial cells that disrupt cellular energy generation, activate oxidant-sensitive signaling pathways, and promote inflammation in the lung. We have shown that mitochondria are a key site of pyocyanin-mediated ROS production and a target of cytotoxicity. We have also found that pyocyanin negatively affects two critical antioxidant defense mechanisms in epithelial cells, catalase and glutathione (GSH). It decreases steady state catalase MRNA, and protein and also directly inhibits the activity of the enzyme. It decreases cellular GSH levels in part by leading to GSH oxidation and export from the cell. Furthermore, paradoxically, GSH appears to be capable of accelerating the formation of ROS by pyocyanin by directly reducing pyocyanin. Furthermore, pyocyanin exposure alters a number of oxidant-sensitive transcription factors (e.g. NF-κB), as well as gene products influenced by them.

We have also employed [14C]pyocyanin to assess the ability of pyocyanin to cross intact airway epithelial cell monolayers; Calu3 and primary human epithelial cells cultured on millicell filters. When [14C]pyocyanin and [3H]mannitol were placed in the upper chamber (apical side), steady movement of [14C]pyocyanin from the apical to basolateral fluid occurred over time. In contrast, <1% of the membrane impermeable [3H]mannitol was detected on the basolateral side of the monolayer, indicating that movement of pyocyanin did not occur via paracellular leak. When human fibroblasts were placed below the epithelial cell monolayer, pyocyanin that had traversed the monolayer was able to induce ROS production in the fibroblasts as assessed by oxidation of intracellular DCF. This suggests that luminal pyocyanin may be able to influence the function of cell types located beneath the epithelium.

We also find that PCA has significant biologic effects on airway epithelial cells, the type, magnitude, or mechanism of which in some cases vary from those of pyocyanin. PCA increased epithelial cell IL-8 and ICAM-1 expression (4-5- and 3-fold, respectively) and decreased TNF-dependent RANTES and MCP-1 release 50-90% in a ROS-dependent process. In contrast to results with pyocyanin, PCA did not appreciably oxidize NADH or NADPH at pH 7 and oxidation was considerably slower than that caused by pyocyanin at pH 5. However, the addition of PCA (10-100 μM), like pyocyanin, increased ROS formation in epithelial cells. Interestingly, despite ROS production by both pyocyanin and PCA, we have observed significant differences in the ability of some antioxidants to inhibit their pro-inflammatory effects.

We are seeking to develop systems to confirm the in vivo relevance of many of our in vitro observations. We find that when 50μl of HBSS, 50μM pyocyanin, or 50 μM PCA was injected intratracheally in mice, both pyocyanin and PCA markedly increased the number of PMNs in BAL over the ensuing 24h. Interestingly, the time course for PMN influx was different for pyocyanin (peak = 48 h) and for PCA (peak = 24 h). Moreover, increased influx of PMNs was accompanied by increased levels of PMNchemokines and of intercellular adhesion molecule-1 (ICAM-1).

These findings support the hypothesis that pyocyanin and other P. aeruginosa-derived phenazines can participate in biologically important redox reactions that may significantly alter the function of airway epithelial cells during acute and chronic P. aeruginosa lung infections.

SA3

Secretory IgA: Preventing (and Promoting) Pathogen-Epithelial Interactions

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Secretory IgA antibodies are heavily glycosylated, protease resistant, polymeric immunoglobulins. As the predominant antibody class in respiratory and gastrointestinal sections, sIgA represents a first line of defense against mucosal pathogens. Here we demonstrate that sIgA can function in both innate and acquired immunity to the shiga-like toxin ricin. Ricin, a member of the A-B family of toxins, is comprised of an enzymatic A subunit and a galatose-specific lectin B subunit. In solid phase binding assays, ricin bound to N- and O-linked oligosaccharide side chains on secretory component and the heavy chains of human IgA1 and IgA2, independent of the antibody variable domains. Ricin had no detectable affinity for human IgG. sIgA (but not IgG) reduced ricin attachment to the apical surfaces of polarized intestinal epithelial cells grown in culture and to the lumenal surfaces of human duodenum in tissue section overlay assays. These data indicate that oligosaccharide side chains on sIgA may serve as 'decoy' receptors for ricin, thereby reducing (but not completely eliminating) the effective dose of toxin that gains access to the intestinal epithelium. Furthermore, these results suggest that

acquired immunity may be necessary to completely safeguard against toxin exposure in vivo. To test this we produced a panel of monoclonal IgA antibodies against the ricin A and B subunits. Although neutralizing antibodies against both toxin subunits were identified, only those directed against the B subunit completely prevented ricin attachment to sections of human duodenum. We are currently testing whether these antibodies are protective in an animal model of gastrointestinal ricin poisoning. While we assume that the formation of SIgA-ricin complexes in vivo will prevent the absorption of ricin by the intestinal tract, we have recently shown that Peyer's patch M cells can mediate the uptake and transepithelial transport of sIgA (and possibly sIgA-antigen complexes). This raises the possibility that sIgA antibodies could actually promote the transport of a small amount of toxin from the intestinal lumen to underlying gut-associated leukocytes.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

SA5

Enteropathogenic E.coli (EPEC) interference of epithelial cell function

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The human intestinal pathogen, enteropathogenic Escherichia coli (EPEC), causes a diarrhoeal disease in infants through a mechanism that is dependent on the injection of effector proteins into epithelial cells of the small intestine. Studies with the HeLa (adenocarcinoma human cervix derived epithelial) cell line have allowed the identification of several EPEC encoded bacterial proteins (Tir, Map, EspF-H) that are delivered into host cells (1). Further work with such cells has revealed the multifunctional nature of both Tir (Translocated intimin receptor) and Map (Mitochondria-associated protein) proteins as well as the ability of these effector molecules to induce signalling events within host cells by independent, synergistic and/or antagonistic mechanisms (2,3). The genes encoding the delivery system and the listed effector molecules are encoded on a chromosomally located 'pathogenicity island' called LEE (Locus of Enterocyte Effacement; 1). An outer membrane protein, Intimin, is also encoded on LEE and serves as a receptor for Tir following Tir's delivery and insertion into the host plasma membrane (1). However, the use of such cell lines, although useful, is somewhat limited since they do not possess many of the characteristics of gut epithelial cells, such as a polarised nature and the presence of absorptive microvilli. This deficiency is illustrated by the finding that the EspF effector molecule is required for EPEC-mediated disruption of tight junction integrity of polarised cells (4) – a process likely to contribute to diarrhoea. Recent studies in our laboratory, using polarised Caco-2 cells (colorectal adenocarcinoma) has revealed both the cryptic nature of EPEC effector molecules and the complex nature of EPEC signalling mediated into host cells, illustrating the need to carry out studies using the most appropriate cells. These conclusions arose from our finding that EspF is not the only EPEC-encoded factor required to disrupt intestinal barrier function, but that two additional LEE-encoded factors are also involved. Thus, we found that the Map effector molecule i) is as essential as EspF for disrupting intestinal barrier function ii) can function independently of EspF, iii) functions to alter tight junction structure and iv) mediates these effects in the absence of its mitochondrial targeting sequence (5). Additionally, the outer membrane protein intimin is shown to be crucial for both EspF and Map to exhibit their intestinal barrier disrupting activities, with this Intimin-mediated activity shown to be independent of interaction with its known receptor, Tir. Indeed, Map retains its ability to induce Cdc42-dependent filopodia formation following delivered into host cells by the intimin mutant but can not exhibit its tight junction disrupting activity (5). Thus, this work not only reveals the cryptic nature of EPEC effector molecules and the complex involvement of multiple molecules in disrupting barrier function, but also shows that EPEC can control effector activity within host cells from an extracellular location. Additionally, this study indicates that Intimin interacts with non-Tir receptor(s) to mediate its controlling influence on Map and EspF's tight junction disrupting activities.

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SA6

Dysfunctional signalling from epithelial to mesenchymal cells in Helicobacter pylori infection

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Epithelial organisation depends on interactions between epithelial and sub-epithelial cells including myofibroblasts, and control of these interactions is likely to be important in responses to injury, infection, inflammation, and the progression to cancer. In the gastric epithelium exposure to Helicobacter pylori is associated in some patients with increased plasma gastrin, increased acid secretion and a progression to peptic ulcer. In other subjects, infection of the gastric corpus leads to a progression to gastric cancer via the premalignant condition of chronic atrophic gastritis. The latter is characterized by loss of gastric glands and acid-secreting parietal cells, hyperproliferation, inflammation and fibrosis consistent with disruption of epithelial — mesenchymal signaling.

The mechanisms by which H.pylori disrupts tissue organization are not well understood. Recent work suggests epithelial responses to H.pylori include increased expression of genes associated with control of extracellular matrix (Varro et al., 2004). For example, we and others have shown that MMP-7 is increased in gastric epithelial cells in response to H.pylori infection. Using an MMP-7-promoter/luciferase reporter construct in gastric epithelial AGS cells we showed that H.pylori strains with the cag pathogenicity island stimulated expression by a mechanism involving NF?B and the Rho family small GTPase RhoA (Wroblewski et al, 2003).

We have now examined the hypothesis that MMP-7 plays a role in epithelial mesenchymal signaling. Using human primary gastric glands and gastric myofibroblasts we found that epithelial cells released MMP-7 which stimulated myofibroblast migration. Recombinant MMP-7 stimulated by both [3H] thymidine incorporation into myofibroblasts and increased migration in Boyden chamber assays. These responses were significantly reduced by specific inhibitors of MMP-3 and -8 but not MMP-2/9 suggesting selective activation of protease cascades by MMP-7. The capacity of MMP-7 to increase secretion of MMP-3 and -8 was confirmed using Western blot and fluorogenic substrate analysis. In addition, MMP-7 increased phosphorylation of p42/44 (max at 10min) and Akt (Ser473, max at 2h) in gastric myofibroblasts and inhibitors of MEK (UO126), PI3K (LY294002) and Akt significantly reduced myofibroblast migration and proliferation. The data suggest that H.pylori increases epithelial MMP-7 which in turn acts as a signalling molecule to increase myofibroblast migration and proliferation. We suggest this pathway plays a role in tissue responses to injury and infection. It may also contribute to stromal formation in epithelial tumours.

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SA8

Enteropathogenic E. coli and the host cell - Molecular Intimacy

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Many pathogens infect the host via the intestine. We use a combination of whole animal imaging approaches together with defined tissue-based assays to investigate how virulence factors integrate during colonisation and infection.

While remaining extracellular the human pathogens enteropathogenic and enterohaemorrhagic Escherichia coli (EPEC and EHEC respectively) and the mouse pathogen Citrobacter rodentium establish direct links with the cytoskeleton of the target epithelial cell leading to formation of actin- and cytokeratin-rich pedestals underneath attached bacteria. In these microorganisms effector proteins are translocated to the cytosol of the host cell through a type III secretion system (TTSS). TTSS is common among human, animal and plant pathogens and consists of a multi protein assembly, referred to as the needle complex, which spans the bacterial cell wall. The TTSS of EPEC, EHEC and C. rodentium is unique as one of the translocator proteins, EspA, form a filamentous extension to the needle complex. By expressing EspAEHEC in EPEC we have now demonstrated the mechanism by which monomeric EspA subunits are polymerised to form a hollow filament. Recently, a number of new type III effector proteins were identified in EHEC, EPEC and C. rodentium; their contribution to infection and colonisation will be discussed

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SA9

Regulation of transport, apoptosis and fibrosis during lung infection

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The course of infectious disease critically depends on host cell mechanisms including transport. Recent evidence highlights the significance of CFTR as a receptor for Pseudomonas aeruginosa. CFTR seems to be involved in the internalization of P. aeruginosa and the induction of apoptosis of the host cell during the course of infection (1-3). Internalization requires lipid rafts and the transformation of those rafts into large ceramide-enriched membrane platforms (1, 2) that initiate intracellular signalling including the activation of src like kinases (3). Apoptosis of infected cells is mediated by a stimulation of the CD95/CD95 ligand system (4), which has been shown to result in a src like kinase-dependent activation of outwardly rectifying Cl- channels in lymphocytes (5). Exposure of Chang cells to P. aeruginosa indeed leads to activation of outwardly rectifying Cl- channels, an effect, however, not required for induction of apoptosis (6). Lack of CD95 or of its ligand abrogates host cell apoptosis in vitro and leads to lethal lung infection in vivo (2, 4, 7). Thus, pathogen induced host cell apoptosis is an important defence mechanism against infection with P. aeruginosa. Further evidence points to a role of the serum and glucocorticoid inducible kinase SGK1 in cystic fibrosis and lung infection. SGK1 is known to upregulate the epithelial Na+ channel ENaC (8-10) and the Cl- channel CFTR (11). The expression of SGK1 is heavily upregulated in fibrosing tissue such as diabetic nephropathy (9), liver scirrhosis (12), Crohn's disease (13), and lung fibrosis (14). Most recent observations indicate that the kinase may indeed be required for the induction of fibrosis. In conclusion, host cells play a decisive role in the course of lung infection. The mechanisms triggered in host cells include activation of kinases and subsequent stimulation of ion channels. Those mechanisms may participate in the triggering of pathogen internalization, host cell apoptosis and/or enhanced matrix protein deposition.

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