Chronic Mucosal Disease and the Role of Intracellular Infection and Biofilm

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Introduction

Chronic otitis media with effusion (OME) and recurrent acute otitis media (rAOM) are the most common reasons children will undergo surgery and respond poorly to antibiotic treatment. More than 33% of children will experience three or more episodes of AOM by three years of age [1]. Increases in the numbers of children suffering from rAOM have been observed [2]. Chronic OME is often unresponsive to treatment and rAOM often relapses within a month of completing antibiotics [3, 4]. While the important role of bacteria in OM development is acknowledged, these are often unable to be cultured from middle ear effusions and the mechanisms of recurrence and persistence are not well understood. Indirect evidence suggests bacterial biofilm and intracellular infection are involved.

Biofilm

Biofilm formation is a universal mechanism used by bacteria for survival in the environment [5]. The bacteria within a biofilm have an altered phenotype with respect to growth rate and gene transcription that their planktonic or free floating counterparts [6-9]. Biofilm bacteria are difficult to culture, resistant to antibiotics and are the preferred bacterial phenotype adopted for chronic persistence [10]. *In vivo* and *in vitro* data suggest that otopathogens are capable of forming biofilms and also of invading and surviving within cells. Bacteria can use these mechanisms to evade immune responses, both cellular and humoral, and to subsequently survive and persist within the host. Bacteria within biofilms are resistant to antimicrobial treatment due to their nutrient limited physiology and their adoption of the biofilm phenotype. Furthermore, bacteria residing within cells are protected from antibiotics incapable of penetrating the cell membranes (including those in the β-lactam group).

Intracellular infection

Intracellular persistence represents an additional mechanism by which bacteria, viruses and parasites are able to persist in the body and can act as an infection reservoir in persistent conditions. When bacteria become intracellular they are able to evade host immune responses and many antimicrobial therapies which do not penetrate the cell membrane well. There are a range of pathogens that which exploit intracellular environments including *Mycobacterium tuberculosis* in lung infections, *Streptococcus pyogenes* in tonsillitis, *Escherichia coli* in recurrent

urinary tract infections, *Helicobacter pylori* in chronic gastric infection and *Salmonella* in gastrointestinal infections [11-16].

Intracellular infection in otitis media

Using transmission electron microscopy, our group was the first to demonstrate intracellular bacterial persistence in the middle ear mucosa of children with chronic OME [17]. Based on PCR results positive for the presence of pneumolysin (a pneumococcal endotoxin) and morphological criteria these were identified as *S. pneumoniae*. However, due to limitations of electron microscopy methodology, biofilm was not demonstrated and the bacterial species present within the epithelial cells were unable to be definitively identified.

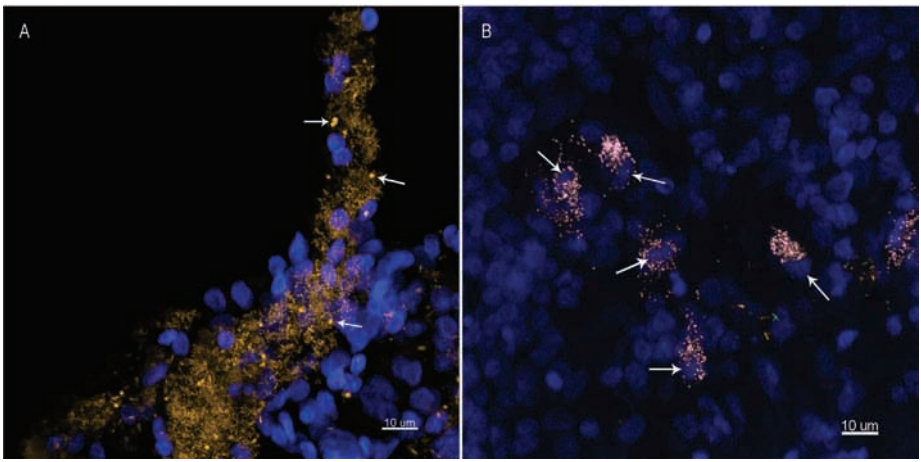
Biofilm in otitis media

Most direct evidence of biofilms in OM has been from animal models [10, 18]; however, recent studies have demonstrated bacterial biofilm in the middle ears of children with chronic suppurative OM (CSOM), rAOM or OME [19-21]. With the exception of the study by Hall-Stoodley et al who identified bacterial species in a subset of children [19], biofilms were demonstrated using mainly non-specific imaging techniques including scanning electron microscopy and LIVE/DEAD viability staining. This lack of pathogen identification limits the ability to determine the role that biofilm plays as a mechanism of persistence in chronic OME and rAOM and it was important to demonstrate otopathogens in these structures.

Current work into biofilm and intracellular infection in otitis media

Our group in Perth, Western Australia, has established fluorescent *in situ* hybridisation (FISH) techniques to identify otopathogens in biofilm and intracellularly in the middle ear mucosa of children suffering from rAOM and chronic OME. FISH is a technique which can be used to identify bacterial species using fluorescently labelled oligonucleotide probes targeting the 16S ribosomal RNA. A universal eubacterial probe and species specific probes for *S. pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* have been established in our laboratory to assess middle ear mucosal biopsy samples using FISH and confocal laser scanning microscopy (CLSM). Using these techniques we have successfully identified both biofilm and intracellular infection containing known otopathogens (**Figure 1**). *M. catarrhalis* and *S. pneumoniae* were seen in both biofilm formations and intracellularly, however, *H. influenzae* appeared to be present more often intracellularly. Biofilm and intracellular bacteria were often present concurrently in the middle ear biopsies. Other bacteria for which species were not identified were also often present, this is indicative of the polymicrobial nature of this persistence mechanism.

Figure 1. Representative images taken from a mucosal biopsy of a child suffering from rAOM; FISH: Universal probe, *M. catarrhalis, S. pneumoniae* and Hoechst 33342 (nuclei stain). A) Extensive bacterial biofilm covering the middle ear mucosa. B) intracellular bacteria in middle ear mucosal cells, arrows point to the host cell nuclei surrounded by bacterial cells. Many of the bacteria evident were unidentified. However, *S. pneumoniae* were observed both intracellularly and in biofilm throughout the sample and *M. catarrhalis* were observed throughout the biofilms. Bars = 10μm.



Treatment implications

These findings indicate that intracellular infection and biofilm containing known otopathogens may contribute to bacterial persistence, leading to the failure of conventional therapies and to the recalcitrance of infection. These biofilms are often polymicrobial in nature and are likely to contain species other than those commonly identified as otopathogens. This has implications for designing future treatment strategies as bacteria within biofilms are largely resistant to antimicrobials and many antibiotic treatments fail to target intracellular bacteria. It is apparent that both of these persistence mechanisms need to be targeted if treatments are to be effective. Methods to disrupt biofilms and to target intracellular bacteria need to be considered and combination treatments are likely to be necessary.

Future Work

Similarly to what we have observed in middle ear infections, intracellular infection may be present in the tissues of the upper and lower respiratory tracts providing an additional mechanism by which bacteria can persist and reinfect the host. This persistence mechanism may also be important in other chronic conditions including chronic rhinosinusitis, obstructive sleep apnoea and cystic fibrosis. While most studies have focussed on the presence of biofilm in these conditions little work has been conducted considering the role that intracellular infection may play. The concurrent presence of biofilm and intracellular infection with known pathogens needs to be explored as it may have major implications in tailoring new treatments to eradicate underlying infection reservoirs.

References

1. Giebink GS. Vaccination against middle-ear bacterial and viral pathogens. Ann N Y Acad Sci. 1997; 830:330-52.
2. Joki-Erkkila VP, Laippala P, Pukander J. Increase in paediatric acute otitis media diagnosed by primary care in two Finnish municipalities -1994-5 versus 1978-9. Epidemiol Infect. 1998; 121(3):529-34.
3. Pichichero ME. Recurrent and persistent otitis media. Pediatr Infect Dis J. 2000; 19(9):911-6.
4. Vlastarakos PV, Nikolopoulos TP, Maragoudakis P, et al. Biofilms in ear, nose, and throat infections: how important are they? Laryngoscope. 2007; 117(4):668-73.
5. Post JC, Stoodley P, Hall-Stoodley L, et al. The role of biofilms in otolaryngologic infections. Curr Opin Otolaryngol Head Neck Surg. 2004; 12(3):185-90.
6. Costerton JW, Lewandowski Z, Caldwell DE, et al. Microbial biofilms. Annu Rev Microbiol. 1995; 49:711-45.
7. Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev. 2002; 15(2):167-93.
8. Sauer K, Camper AK, Ehrlich GD, et al. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. J Bacteriol. 2002; 184(4):1140-54.
9. Stoodley P, Sauer K, Davies DG, et al. Biofilms as complex differentiated communities. Annu Rev Microbiol. 2002; 56:187-209.
10. Ehrlich GD, Veeh R, Wang X, et al. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. Jama. 2002; 287(13):1710-5.
11. Amano A, Nakagawa I, Yoshimori T. Autophagy in innate immunity against intracellular bacteria. J Biochem (Tokyo). 2006; 140(2):161-6.
12. Cheminay C, Mohlenbrink A, Hensel M. Intracellular Salmonella inhibit antigen presentation by dendritic cells. J Immunol. 2005; 174(5):2892-9.
13. Marouni MJ, Barzilai A, Keller N, et al. Intracellular survival of persistent group A streptococci in cultured epithelial cells. Int J Med Microbiol. 2004; 294(1):27-33.
14. Osterlund A, Popa R, Nikkila T, et al. Intracellular reservoir of *Streptococcus pyogenes* in vivo: a possible explanation for recurrent pharyngotonsillitis. Laryngoscope. 1997; 107(5):640-7.
15. Schmid D, Dengjel J, Schoor O, et al. Autophagy in innate and adaptive immunity against intracellular pathogens. J Mol Med. 2006; 84(3):194-202.
16. Shin JS, Abraham SN. Cell biology. Caveolae--not just craters in the cellular landscape. Science. 2001; 293(5534):1447-8.
17. Coates H, Thornton R, Langlands J, et al. The role of chronic infection in children with otitis media with effusion: evidence for intracellular persistence of bacteria. Otolaryngol Head Neck Surg. 2008; 138(6):778-81.

18. Reid SD, Hong W, Dew KE, et al. *Streptococcus pneumoniae* Forms Surface-Attached Communities in the Middle Ear of Experimentally Infected Chinchillas. J Infect Dis. 2009.
19. Hall-Stoodley L, Hu FZ, Gieseke A, et al. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. Jama. 2006; 296(2):202-11.
20. Homoe P, Bjarnsholt T, Wessman M, et al. Morphological evidence of biofilm formation in Greenlanders with chronic suppurative otitis media. Eur Arch Otorhinolaryngol. 2009.
21. Lee MR, Pawlowski KS, Luong A, et al. Biofilm presence in humans with chronic suppurative otitis media. Otolaryngol Head Neck Surg. 2009; 141(5):567-71.