Understanding the Role of Biofilms in Chronic Tonsillitis and Adenoidal-tonsillar Hyperplasia

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Introduction

There has been a growing recognition that biofilms are implicated in recurrent otolaryngologic diseases of children. Biofilms have been described in chronic middle-ear disease [1-3], chronic rhinosinusitis, device infections, cholesteatoma, adenoiditis and chronic tonsillitis.[4-5.] The biofilm phenotype enhances resistance to antimicrobials as the metabolic heterogeneity and polymicrobial infections may act in a synergistic manner. The biofilm phenotype facilitates genetic material transfer and signaling from one bacterium to another, while facilitating bacterial evasion of the host immune system. Finally, the biofilm can act as a reservoir of chronic disease, with recurring, acute exacerbations. Given that these characteristics are found in chronic disease states of the tonsils, it is not unreasonable to hypothesize that bacterial biofilms play a role. This paper presents the current thinking regarding the role of bacterial biofilms in chronic adenotonsillitis and adenoidal-tonsillar hyperplasia, and concludes with suggestions for future research efforts.

Anatomy and physiology of the tonsils

The palatine tonsils are located on either side of the pharynx and are part of Waldeyer's ring, which consists of the nasopharyngeal tonsil (adenoid), the lingual tonsil on the dorsal surface of the tongue base, and the tubal tonsils, near the torus tubarius. These tissues are components of the mucosa-associated lymphoid tissues (MALT), and play a role in mucosal immunity, thus protecting the body from microorganisms entering through the upper respiratory tract. The tonsils contain germinal centers, which are a component of the B cell humoral immune response.

The Clinical Problem

More than 530,000 tonsillectomies and adenoidectomies (T&A) are performed annually in the United States, making it one of the most common surgical procedures in children [6]. Historically, most T&As were performed for chronic tonsillitis, but the most common indication today is obstructive sleep apnea (OSA). OSA is the obstruction of the upper airway by large tonsils and adenoids. While many different medical and oral appliances, including positive airway pressure therapy, have been advocated, T&A generally results in relieving the obstruction and resolving the OSA, however, long-term results show that some children can regress. A recent Cochran review demonstrated that T&A not only eliminated sore throat from tonsillitis, but the procedure reduces the number of episodes of sore

throat from pharyngitis in children in the first year of surgery, compared to non-surgical treatment [6-7]. In a child with Obstructive Sleep apnea with Hypoponea Syndrome (OSAHS), surgical alleviation can have a significant positive impact on self-control, attention, and hyperactivity [8]. Both obese and non-obese children with OSA have improvements on apnea-hypopnea index, quality of life measurements, and behavior. [9]. Balanced against these benefits is the small but real mortality of the procedure, as well as the postoperative morbidity [7]. Variations exist among surgeons in terms of the optimum techniques for tonsillectomy. In terms of cost, significant variation exists among different surgeons and hospitals, even within a multihospital network [10]. Given these issues, it is important to better understand the underlying pathophysiology of chronic tonsil disease.

Sleep Disordered Breathing

It is important to note that sleep disordered breathing (SDB) is a spectrum of disorders affecting both children and adults, ranging from snoring, upper airway resistance syndrome, obstructive hypoventilation, through OSA and OSAHS. SDB can be caused by both peripheral and central disorders. This review will focus on pediatric SDB caused by enlarged tonsils and adenoids.

There are several components to the diagnosis of OSA, including history, physical examination, questionnaires such as the Pediatric Sleep Questionnaire, and sleep studies. Children with OSA can have irritability and behavioral problems, daytime sleepiness, trouble concentrating in school, hyperactivity, growth retardation, delayed development , hypertension, sleep walking and enuresis (intermittent urinary incontinence during sleep in a child at least five years of age) [11]. The child may exhibit long pauses in breathing, much tossing and turning in the bed, chronic mouth breathing during sleep, and night sweats (most likely secondary to an increased effort to breathe).There is a strong association between OSA and obesity [12], and sleep disorders can play a role in the development of insulin resistance, and Type 2 diabetes [13].

One useful questionnaire is the OSA-18, a validated, disease-specific quality of life (QOL) survey consisting of 18 questions regarding sleep disturbance, physical symptoms, emotional symptoms, daytime function, and caregiver concerns. The gold standard for diagnosis is a sleep study, or polysomnography (PSG), however they are infrequently used in clinical practice because of cost, a relatively low number of pediatric sleep centers, and lack of normative data. PSGs are generally used in children with severe OSA, or comorbidities such as craniofacial disorders, bleeding dyscrasias, or cardiopulmonary compromise. During a PSG, a number of electrophysiologic signals are recorded, and can include an electroencephalogram, airflow measurements, expired carbon dioxide, oxygenation levels, ventilation efforts and limb movements. Video recording is generally employed. The severity of sleep apnea can be quantified by the apnea-hypopnea index (AHI), which is the number of complete cessations in airflow (apnea) or partial obstructions in airflow (hypopnea) events per hour of sleep. In the pediatric population, an AHI greater than 1.5 is abnormal, and a child with an AHI greater than 5 is generally treated.

Physiological Effects of OSA

There are increased levels of oxidative stress markers in children with obstructive adenotonsillar hypertrophy [14]. A prospective, controlled study in 30 children with obstructive adenotonsillar hypertrophy and 25 controls demonstrated elevated 8-hydroxy 2-deoxyguanosine (8-OhdG) and malondialdehyde (MDA) concentrations in urine and blood in the affected children.

In another study, insulin-like growth factor-1 (IGF-1) and insulin-like growth factor-binding protein-3 (IGFBP-3) concentrations (both mediators of growth and development) were assessed in 51 children with OSAHS. Levels of both increased after T&A, but to a greater degree in younger children. The degree of increase was not associated with severity of obstruction. The authors suggest that the levels of IGF-1 and IGFBP-3 were affected more by duration than severity of OSAHS [15].

The role of T&A in reducing asthma symptomatology was examined in a very large US study. Using data from the 2003-2010 MarketScan database, 13,506 children with asthma underwent T&A, while 27,012 asthmatic children, matched for age, sex and geography, without T&A acted as controls. The children who underwent T&A had significant improvements in several asthma outcomes, including acute asthma exacerbation, acute status asthmaticus, asthma-related emergency room visits, and asthma -related hospitalizations. In addition, reduction in prescription refills were noted for bronchodilators, inhaled corticosteroids, leukotriene receptor antagonists, and systemic corticosteroids. The asthmatic children who did not undergo surgery did not enjoy any significant reductions in these outcome measures.[16]

Myofunctional Therapy

Myofunctional therapy is designed to correct the function of the tongue and facial muscles by employing various repatterning techniques and behavior modification, with the goal of improving tongue position, chewing, breathing and swallowing. A systematic review of the literature demonstrated that myofunctional therapy reduced apnea-hypopnea indices (AHI) in children. Additionally, pediatric patients with OSA who were treated with T&A and palatal expansion and initially cured were divided into 2 groups. 11 children who continued myofunctional therapy remained cured of their OSA, whereas 13 controls had recurrence of their OSA. The authors suggest that myofunctional therapy could be a useful adjunct to other OSA treatments. [17].

Given the magnitude of the problem, the adverse consequences, and a paucity of effective medical management options, a better understanding of the underlying pathophysiology of tonsillar infection and enlargement is needed. Understanding the microbiology and pathophysiology of such diseases represents an important step in the management of biofilm-related infections.

WHAT ARE BIOFILMS?

Biofilms are complex, highly organized communities of bacteria, which can form on both biotic and abiotic surfaces. Frequently polymicrobial in nature, biofilms are encased in a self-produced polymeric extracellular matrix, which is self-secreted and includes proteins, polysaccharides, extracellular DNA, peptidoglycans, lipids and phospholipids. The matrix is negatively charged and allows

the bacteria to resist host immune strategies, while promoting bacterial interaction with synergistic capabilities that enable them to adapt to local environmental conditions. The biofilm has a distinctive mushroom-like form with more elaborate structures on the inside, including channels for nutrients and waste to travel through the biofilm. Their internal architecture can also be re-arranged to control delivery of nutrients to the biofilm's core. There are many sub-environments within the larger biofilm, which vary according to a variety of factors including nutrient availability, pH, and oxygen levels. The bacteria in a biofilm live more as a society than as individual microorganisms. (**Figures 1A** and **B**).

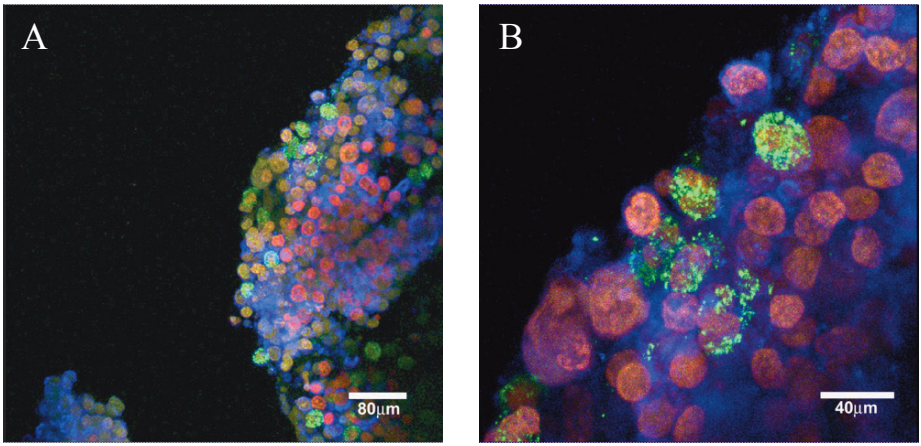

Figure 1A. Human tonsillar tissue with biofilms. BLUE: Phalloidin for filamentous actin in the cytoskeleton of the human cells. RED: Propidium iodide that stains the nuclei. GREEN: Eubacterial FISH probe. **1B**. Zoom photograph of A.

The phenotype of biofilms dramatically amplifies their antimicrobial resistance, enabling bacteria to survive in many environments, even hazardous ones. Biofilms are protected from radiation, extreme weather conditions, and a person's immune system. The matrix surrounding a biofilm acts as a barrier, preventing white blood cells from infiltrating the biofilm. As the mechanisms of resistance have become better understood, a search has begun for methods to eradicate infections in hospitals, medical devices, and within the body itself. "Nowadays it's known that some anaerobic bacteria are also able to grow as biofilm even if this feature and its role in the healthcare-associated infections (HAIs) are still poorly characterized. As consequence, the involvement of biofilm-forming anaerobic bacteria in infections related to healthcare procedures, including surgery and medical devices implantation, is underestimated." [18]

Although traditional microbiological techniques of culture and staining have been successful in identifying bacteria in the planktonic state, bacteria encased in biofilms cannot be identified in this manner, since the reduced metabolic rate of the core bacteria makes them more difficult to culture. This marked underestimation of the bacterial population led to the concept of "culture negative infections"

associated with chronic human diseases. Today, however, with modern day nucleic acid amplification techniques in combination with advanced imaging techniques that can more fully capture the architecture of biofilms, we now have the ability to detect and identify biofilm bacteria, enabling us to better understand their role in the pathogenesis of many chronic diseases.

Much of the early work on biofilm examination involved use of the scanning electron microscope (SEM). SEM technology uses accelerated electrons as a source of illumination to provide high resolution spatial images of biofilms. A limitation of this approach was the morphologically disruptive nature of the dehydration required for sample preparation. The confocal laser scanning microscope (CLSM) is a non-destructive technique that utilizes a laser source in combination with a light microscope that can image biofilm in the x, y, and z axis to a resolution of one micron. These high-resolution 3-D images can be obtained without having to physically slice the specimen, enabling more accurate identification of bacterial species. Combined with fluorescent in situ hybridization (FISH), such imaging permits not only more accurate species identification but a more detailed understanding of the spatial arrangement of the biofilm. The development of the CLSM in the 1980s provided researchers with the ability to examine biofilms *in situ* without the limitations encountered by the SEM, albeit at lower magnifications. The trade-off in resolution was more than offset by the ability to examine the biofilm matrix unaltered and intact. [19].

What is the evidence that biofilms are found in tonsils?

The first study to evaluate tonsils for the presence of biofilms used light microscopy and SEM to examine tonsils removed from 19 patients, 15 with chronic tonsillitis and 4 with OSA. Biofilms were found in 11 of the 15 chronic tonsillitis specimens, and 3 of the 4 tonsils removed for OSA. The authors concluded that chronic tonsil infections may not respond to treatment because the bacteria form biofilms that protect them from antibiotics and the immune system. [20].

In a study from Leiden University Medical Center, 24 tonsils were obtained from children who underwent tonsillectomy for chronic or recurrent tonsillitis. Tonsils were examined by SEM, and CLSM. For the CLSM analysis, a double fluorescent staining technique was employed. Propidium iodide was used to detect bacteria, and fluorescein isothiocyanate concanavalin A staining was employed to detect the glycocalyx matrix. SEM results were consistent with biofilms, by showing bacteria aggregated in microcolonies, while the CLSM double-staining technique allowed visualization of bacteria and the glycocalyx matrix. Using these techniques, the investigators demonstrated the presence of biofilms on 17 of the 24 specimens (70.8%). [21]

A study from the Catholic University of Córdoba, Córdoba, Argentina, evaluated the tonsils from 36 children (age 1-6) undergoing surgery for obstruction as opposed to chronic, overt infection. The tonsils were evaluated with hematoxylin-eosin and Gram staining, as well as fluorescent microscopy, and CLSM. 77.28% of the tonsils had biofilms, and symptoms such as raucous breathing, tonsillar and adenoid hypertrophy, apnea and cervical adenopathy were related to the presence of biofilms in the tonsils. The authors propose that biofilms are involved in the

pathogenesis of tonsil and adenoid hypertrophy, and recommended that strategies focusing on preventing bacterial attachment to the mucosa be developed. [22].

Researchers at the Gil Hospital, Graduate School of Medicine, Gachon University, Incheon, Korea, conducted a study in an effort to determine if there is an association between tonsillar biofilms and recurrent tonsillitis. A group of 40 patients was analyzed: Group 1 consisted of 20 patients with recurrent tonsillitis with at least 5 episodes of disease a year over the previous 2 years; and Group 2 (controls matched for age and gender) consisted of 20 patients undergoing laryngeal microsurgery who had no episodes of tonsillitis in the previous 2 years. SEM was used to examine the tonsillar tissue for biofilms, which were classified into 5 categories. The findings demonstrated that biofilms were significantly more prevalent, and of a higher grade, in the recurrent tonsillitis group than in the control group [23].

A study from the Universidade Federal do Triângulo Mineiro examined the tonsils removed from 46 children for histopathological changes that would differentiate tonsils removed for hypertrophy versus recurrent tonsillitis. Group I consisted of 22 children with hypertrophy and obstruction as the major indication for tonsillectomy, while Group II consisted of 24 children with recurrent tonsillitis. Histopathological features evaluated included lymph follicles, germinal centers, fibrosis, necrosis, reticulation, infiltration by plasma cells and neutrophils. No mention was made regarding a bacterial evaluation. The results indicated that Group I (obstruction) had a higher number of germinal centers, and the authors concluded that the number of germinal centers is the only histopathological criterion that can be used to differentiate between the two groups [24].

Specimens obtained during tonsillectomy performed on 22 children in Milan, Italy, with recurrent exacerbations of chronic hyperplastic tonsillitis were analyzed by spectrophotometry. Biofilm-producing bacteria were found in 50% of the 44 tonsillar specimens, and there was a significant relationship between the grade of tonsillar hyperplasia and the presence of biofilms. *Staphylococcus aureus* was the most frequent pathogen, found in 81.8 percent of the specimens. [25].

In a study from the Institute of Otorhinolaringology, Catholic University of the Sacred Heart, Rome, Italy, 16 surgical samples of tonsils and adenoids and 24 samples of ethmoid mucosa from CRS patients were cultured and examined with a SEM to detect evidence of biofilm formation. 57.5% of chronically infected mucosa had biofilms and marked destruction of ciliated epithelium was noted in 41.7% of ethmoid mucosa in the CRS patients. The authors suggest that biofilm formation is responsible for the resistance of these infections to antibiotic therapy, as well as the persistence of a chronic inflammatory reaction. [26].

In a study from the University of Malaya Medical Centre, 70 patients with recurrent tonsillitis, chronic tonsillitis, or obstructive sleep apnea underwent tonsillectomy, thus a total of 140 palatine tonsils were available for evaluation. The tonsils were evaluated by SEM and CLSM, and bacterial biofilms were present in 60% of tonsils: 30 out of 49 patients with recurrent tonsillitis; 5 out of 9 patients with chronic tonsillitis; and 7 out of 12 patients with obstructive sleep apnea. *S. aureus* (39.65%) was the most frequently isolated bacteria, followed by *Haemophilus influenzae* (18.53%), and the majority of the visualized bacteria were cocci shaped

with some bacilli indicating a polymicrobial biofilm community. The authors also noted an association between the presence of biofilms with clinical symptoms such as snoring, apnea, and nasal obstruction, and tonsillar hypertrophy. They concluded that the presence of biofilms explains the recalcitrant nature of tonsillar diseases. [27]

Researchers from Denmark utilized an in-depth 16SrRNA gene-based pyrosequencing approach to compare the microbiota of the tonsillar crypts in children and adults with recurrent tonsillitis or tonsillar hypertrophy. Molecular mapping to the species level revealed a complex microbiota composed of between 42 and 110 taxa., with a core microbiome of 12 genera. Unifrac analysis (a distance measure between organismal communities using phylogenetic information) revealed that recurrent tonsillitis is a polymicrobial infection associated with a shift in the microbiota, and that interactions within consortia of taxa and between the host and bacteria play a role in the disease [28].

Adenoids, Biofilms and Ear Infections

It has long been recognized that adenoidectomy is beneficial in reducing the incidence of chronic otitis media with effusion in children [29], although it is unclear the mechanism of action: specifically whether adenoidectomy improves Eustachian tube function versus removing a bacterial reservoir. A recent study provides strong evidence that adenoids act as a chronic reservoir for pathogenic biofilm bacteria [30]. Interestingly, biopsies obtained from the adenoid surface at the nasopharyngeal dome (ND) and near the ostium of the Eustachian tube (ET) in 45 children with chronic ear infections demonstrated that bacteria-producing biofilms were detected significantly ($p = 0.04$) more frequently in the ET samples than in the ND samples. The most common organism isolated was *S. aureus*. The authors suggested that these results indicate that the adenoids are a reservoir for bacteria, and that hypertrophic adenoids play a role in recurrent AOM and/or OME. [31]. A Cochrane review confirms the significant benefit of adenoidectomy in the resolution of pediatric otitis media with effusion, but does not support the role of adenoidectomy in the treatment of acute otitis media. [32]. These results suggest, but do not prove, that biofilms may also be implicated in tonsillitis and tonsillar hypertrophy.

Conclusion and future directions

Studies to date have resulted in several important findings. It is clear that biofilms are associated with tonsillar hyperplasia, as well as recurrent tonsillitis. There appears to be an association between the presence of biofilms with clinical symptoms of obstruction. Using sophisticated bacterial identification techniques, recurrent tonsillitis has been shown to be a polymicrobial infection. The palatine tonsils are continuously engaged in local immune responses to microorganisms.

The presence of bacterial biofilms help explain the observation that diseases of the tonsils can be difficult to treat medically. Bacteria in biofilms are known to be resistant to antibiotics, and are protected from the host immune system. In addition, bacterial biofilms can contribute to a chronic inflammatory state. The difficulty comes in that the studies to date cannot determine causality, i.e. whether bacterial biofilms are a causative factor, or just a consequence of recurrent exacerbations of chronic hyperplastic tonsillitis. For example, tonsils of asymptomatic individuals can harbor biofilms of bacteria associated with tonsillitis patients.

Using the well-developed chinchilla model of otitis media, it has been shown that the interactions between bacteria species influences host -to-host variations [33]. It is critical to understand the intricate interactions between bacteria and the host during biofilm formation, maturation and dispersal. Dysbiosis (defined as a microbial imbalance) could well play a role in chronic tonsillitis. A state of chronic inflammation could provide an opportunity for some species (or strains) of bacteria to flourish.

To achieve this understanding, studies that simply visualize biofilms on tonsils *ex vivo* will be of less value. There is a need for a technology to rapidly detect biofilms *in vivo*, which would enable researchers to develop a set of control patients. Serial examinations of affected and control would be of exceptional value. Researchers must delineate the tonsillar bacteria more thoroughly, down to the level of strains, serotypes, whole genome sequences, and how they influence biofilm formation. Molecular-based strategies will give further insights into the consequences of host-microbial interactions. A deeper understanding of the balance between pro-inflammatory and anti-inflammatory cytokines, in healthy and chronically-infected tonsils, will provide insights into the underlying mechanisms of these disorders. It will be important to delineate the interactions between immunocompetent cells and the various strains of bacteria in the tonsillar biofilm. An appreciation of the complex structure of bacterial biofilms and the ultrastructural and biochemical mechanisms responsible for its evasion of the immune system and resistance to treatments is currently lacking.

There is a need to develop prevention strategies, as well as better therapeutic management of biofilm-related infections. It may be possible to hinder the adherence of bacterial cells to mucosal tissues, or to develop polymeric nanocomposites with antimicrobial properties. It is conceivable that probiotics may play a role in managing tonsillar disease. A more nuanced understanding of the role of biofilms in chronic tonsillitis and tonsillar hypertrophy has the potential to reduce the need for surgical intervention, and enable us to better serve our patients.

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