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A Mixed Cell Culture Model for Assessment of Proliferation in Tonsillar Tissues from Children with Obstructive Sleep Apnea or Recurrent Tonsillitis

Laura D. Serpero, PhD, Leila Kheirandish-Gozal, MD, Ehab Dayyat, MD, Julie L. Goldman, MD, Jinkwan Kim, PhD, and David Gozal, MD

Division of Sleep Medicine and Kosair Children's Hospital Research Institute, Departments of Pediatrics and Surgery, University of Louisville, Louisville, KY

Abstract

Background—Recurrent infective tonsillitis (RI) and obstructive sleep apnea (OSA) are the major indications for adenotonsillectomy (T&A) in children. However, little is known on the determinants of lymphadenoid tissue proliferation in the pediatric upper airway.

Aim—To develop an in vitro culture system allowing for assessment of tonsillar or adenoidal proliferation under basal or stimulated conditions.

Methods—Tonsils surgically removed from pediatric patients with obstructive sleep apnea and recurrent tonsillitis during T&A, were dissociated using standard methods. Whole cell tonsillar cultures were either maintained in normal medium or stimulated with LPS (25 µg/ml) and concanavalin A (10 µg/ml) for 24 hours (STIM). Cellular proliferation was evaluated by [³H] thymidine incorporation. In parallel, supernatants were collected after 48 hours, and concentration of cytokines was measured using standard ELISA procedures.

Results—Basal proliferative rates were increased in the OSA group (305.2 ± 40.6 cpm; n=31) compared to RI group (232.8 ± 31.9 cpm; n=26; p < 0.001). No significant differences in proliferative rates emerged after STIM between OSA and RI. Furthermore, basal TNF-α, IL-6, and IL-8 concentrations in the supernatant were increased in OSA-derived cultures compared to RI, but IL-8 was higher after STIM in RI, while IL-6 remained increased in OSA.

Conclusions—The proliferative rates and concentrations of inflammatory mediators in tonsillar cell cultures from children with OSA and RI suggest that lymphadenoid tissue proliferation in these 2 conditions may be regulated by different mechanisms. This novel method may allow for future development of specific therapeutic interventions aiming to curtail and reverse tonsillar and adenoidal hypertrophy in children in a disease-specific manner.

Keywords

obstructive sleep apnea; recurrent tonsillitis; tonsillar hypertrophy; adenotonsillectomy; cytokines

INTRODUCTION

Obstructive sleep apnea (OSA) in children is a significant and frequent health problem, with a prevalence currently estimated at 1-3% of all children in the general population (1,2). This clinical syndrome is characterized by repeated episodes of partial or complete upper airway

obstruction during sleep resulting in disruption of normal ventilation, hypoxemia, hypercapnia and sleep fragmentation (3). As a consequence of sleep-disordered breathing, OSA in children is associated with important cardiovascular and neurobehavioral morbidities (4-7). Adenotonsillectomy (T&A) is the treatment of choice since the most common cause of OSAS in children is adenotonsillar hypertrophy. However, persistence of abnormal polysomnographic findings after surgery is reported in approximately 20% to 40% of cases, particularly when obesity is present (8-13). Another frequent condition in children for which T&A remain the standard therapeutic approach is recurrent tonsillitis (RI), currently defined as “3 or more infections of tonsils and/or adenoids per year despite adequate medical therapy” (14). Indeed, tonsillectomy remains the most common surgical operation among United States children, with an estimated 290,000 children under 15 years of age undergoing tonsillectomy with or without adenoidectomy (15).

Thus, for both childhood OSA and RI, adenotonsillar hypertrophy is present, and constitutes the primary basis for the decision to conduct T&A (16-19). However, the pathophysiological mechanisms underlying upper airway lymphadenoid tissue hypertrophy remain unknown. Therefore, to elucidate the etiology of tonsillar hypertrophy, and to add new insights into the role of inflammatory processes, we developed a dissociated tissue cell culture system of tonsils and adenoids, which closely mimics the complexity of in vivo conditions.

METHODS

The study was approved by the University of Louisville Human Research Committee, and informed consent was obtained from the legal caregiver of each participant. Consecutive children who underwent tonsillectomy for either OSA or RI were identified before surgery and recruited into the study. The diagnosis of OSA was established by overnight polysomnography in the sleep laboratory and required the presence of an apnea-hypopnea index ≥ 5 events/hour of total sleep time (20). Children with recurrent tonsillitis (RI) were selected based on a history of at least 5 tonsillar infections requiring administration of antibiotic course over a period of less than 6 months, as well as the absence of any symptoms suggestive of OSA using a previously validated questionnaire highly sensitive and specific in ruling out sleep disordered breathing in children, and whose polysomnographic findings showed an apnea-hypopnea index < 1 event/hour of total sleep time (1,20).

Children with known chronic conditions such as asthma, allergic rhinitis, or history of allergies were excluded from the study. None of the children displayed symptoms of acute infection at the time of the surgery, and none of them had received any antibiotic treatment for at least 6 weeks before the day of the surgery. Apart from the tonsillar hypertrophy-related symptoms, all patients were otherwise healthy and did not receive any medication.

Tissue collection and processing

Both palatine tonsils and adenoids were removed by a pediatric ENT surgeon using a uniformly and standardized approach using the harmonic scalpel for subcapsular dissection and removal of tonsils for all subjects. Tissues that were not required for diagnostic purposes were immediately collected by one of the investigators, and delivered in icy phosphate-buffered saline (PBS) plus antibiotics to our laboratory within 10 minutes after excision for immediate processing under aseptic conditions. The palatine tonsils were washed thoroughly with PBS containing antibiotics, and single-cell suspensions were prepared from tissue blocks by mechanical dissociation. Briefly, the outer layers of mucosal tissue were removed with a scalpel and the central parts of the specimens were dissected in 2-3 pieces before the aseptic mechanical dissociation of the tissue. The dissected tissue pieces were then placed into a Petri dish on ice with sterile icy PBS plus antibiotics, and then dissociated by grinding

the tissue through the sieve with a syringe plunge and transferring the contents into a cell strainer (Cell strainer: 70 μ m; BD Biosciences, San Jose, CA). The cells were released into phosphate buffer saline (PBS) supplemented with penicillin (100 IU/ml), streptomycin (100 μ g/ml) and gentamicin (50 μ g/ml). To avoid loss of cells during standard purification procedures (which could change the ratio of lymphocytes subsets) we used for analysis the whole cell suspension obtained from the tissue pieces. Dendritic cells, macrophages, endothelial cells and lymphocytes were identified by morphology and were present in the newly established primary cell cultures. Erythrocytes were lysed with ACK cell lysing buffer (Cambrex, Walkersville, MD). The resulting cell suspension was washed twice with PBS plus antibiotics. Then, cells were pelleted (2500 rpm for 5 minutes), and counted in a Neubauer chamber. As evaluated by the trypan blue exclusion test, viability of cells in specimens used in the experiments was approximately 70-80%.

Cells were transferred into a 96-round bottom well plates at the concentration of 2×10^5 viable cells/well and at the final volume of 200 μ l and cultured in complete RPMI 1640 medium supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; HyClone, Utah), 100 U/mol penicillin, 100 μ g/ml streptomycin, 2.5 μ g/ml fungizone, 2mM L-glutamine, 1mM sodium pyruvate and 1% nonessential aminoacids (all from Gibco, Carlsbad, CA), and placed in a 5% CO₂ incubator at 37° C. Whole tonsil-derived cells were incubated either alone (CO) or stimulated with 25 μ g/ml LPS plus 10 μ g/ml concanavalin A to test proliferation under stimulated conditions (STIM). LPS from *Escherichia coli* 055:B5 and Concanavalin A from *Canavalia ensiformis* were purchased from Sigma Chemical Co. (St. Louis, MO).

Proliferation Assay

Cells were incubated for the final 18 to 20 hours with 0.0185 MBq (0.5 μ Ci) ³H-thymidine in complete medium (Amersham Biosciences, UK). Cells were then harvested onto glass-fiber filters with a cell harvester, and radioactivity was measured in a liquid scintillation counter. All experimental conditions were always performed in triplicate, and ³H-thymidine uptake results were expressed as the average of the 3 wells in counts per minute (cpm).

Cytokine Assays

Concentrations of TNF- α , IL-8 and IL-6 were measured using commercially available ELISA kits from the supernatants of either CO or STIM conditions. To determine cytokine production, cells were incubated in 24-well flat-bottom plates in complete RPMI medium supplemented with 10% FBS with or without addition of 25 μ g/ml LPS plus 10 μ g/ml concanavalin A. Supernatants were collected after 48 hour, and stored at -80°C until assay. TNF- α levels were measured according to manufacturer's instructions using a high-sensitivity ELISA assay able to detect concentrations as low as 0.09 pg/ml (BioSource Europe S.A., Belgium). IL-8 was evaluated using a commercial ELISA kit (R&D Systems; Minneapolis, MN.) with a detection range between 0 and 2000 pg/ml. To determine IL-6 concentrations was used the IL-6 EASIA assay (BioSource Europe S.A., Belgium). The concentrations of cytokines in the supernatants were normalized to the number of cells plated, and expressed as pg/10⁶ cells. For all assays, calibration curves were performed in duplicate for each experiment.

Data Analysis

All data are expressed as mean $\pm$ SD. Data from OSA and RI derived tonsils were compared using independent t-tests or analysis of variance procedures followed by post-hoc tests as appropriate. A p value of less than 0.05 was considered as achieving statistical significance.

RESULTS

Study population

A total of 112 children with either habitual snoring and a putative clinical diagnosis of OSA (n=72) or with RI (n=40) were prospectively consented and underwent an overnight sleep study. Of these, 46 children with habitual snoring did not fulfill OSA criteria and 9 children with RI who also had polysomnographic evidence of OSA were excluded, such that 57 children undergoing T&A ultimately completed the study. Of these, 31 children were diagnosed with OSA and 26 fulfilled all of the criteria for RI. The demographic characteristics and overnight polysomnographic findings for these 2 groups are shown in Table 1.

Cell Proliferation Assay

Basal proliferative rates were significantly higher in children with OSA (305.2 ± 40.6 cpm) compared to those found in the RI group (232.8 ± 31.9 cpm; $p < 0.001$). However, after stimulation with LPS and concanavalin A, no significant differences emerged between the 2 groups (1307 ± 95.5 cpm in OSA vs 1234 ± 112.9 cpm in RI; $p > 0.05$). In general for both OSA and RI groups, the proliferative responses to the mitogen LPS alone (from 287.8 ± 38.7 cpm in basal conditions to 411.1 ± 42.9 cpm; $p < 0.01$; $n = 12$) were markedly reduced compared to those following addition of concanavalin A (conA) alone (from 267.1 ± 24.7 cpm to 825.4 ± 62.1 cpm; $p < 0.0001$; LPS vs ConA – $p < 0.001$).

Cytokine Assays

Basal release of TNF- α , IL-8, and IL-6 to the supernatants was increased in tonsillar cultures from children with OSA compared to the children with RI (Figure 1). Following stimulation with LPS and ConA (STIM), the concentrations of the 3 cytokines increased in both groups (Figure 1). However IL-6 concentrations after STIM were higher in OSA-derived samples, TNF- α were similar, and IL-8 concentrations were higher in RI (Figure 1).

DISCUSSION

In the present study, we show a novel approach for assessment of the tissue properties of tonsils and adenoids derived from children with either OSA or RI. This *in vitro* model permits reproducible evaluation of differential proliferative responses of upper airway lymphadenoid tissues originating from different disease conditions, and also allows for dynamic assessments of cytokine production. Current findings further reinforce the concept that proliferative mechanisms appear to be differentially regulated in pediatric OSA and RI, thereby suggesting the presence of distinct pathogenetic processes.

Before we discuss the potential implications of our study, several technical issues deserve comment. First, we can not ascertain that the processing of the tonsillar tissues harvested during surgery did not alter the intrinsic properties of the constitutive cell populations of these tissues. However, the standardized procedures used herein should affect the tissues harvested from children with OSA and those from children with RI in a similar and equivalent fashion, such that we believe that the differences in proliferation and cytokine release do indeed reflect divergent properties related to the 2 conditions. Nevertheless, differences in lymphocyte properties have been reported in ex-vivo cultures of tonsils either in block preparations or as dissociated lymphocytes (21). Secondly, we used only palatine tonsils in this study. However, we should point out that differences in cytokine networks have been postulated between nasopharyngeal lymphadenoid tissues and palatine tonsils (22). In our hands, we found the same differences in proliferative rates and cytokine release

between OSA and RI in adenoid tissue cultures (data not shown), suggesting that our approach is applicable to both tissues when comparing the 2 clinical diagnostic entities of OSA and RI. Our patient cohorts were matched for age, gender, and ethnicity, as well as for body mass index, and overnight sleep studies were conducted in all subjects to rule out potentially overlapping conditions, as well as to confirm the diagnostic category assignment. Thus, the clinical phenotype characteristics of the 2 groups were distinct, and permitted improved exploration of differences between the 2 major diagnoses leading to T&A in children. The selection of LPS and ConA as the stimulus for proliferation assays was based on the previously reported lymphocyte populations that are present in tonsillar tissues (23-26). Indeed, these 2 substances have marked effects on most of the immune cell types represented in tonsillar tissues.

In previous work from our laboratory, we identified several fundamental differences between OSA and RI in the expression of inflammatory mediators such as leukotrienes. Indeed, both upper airway condensates and tonsillar tissues showed higher concentrations of leukotrienes and their receptors in children with OSA when compared to children with RI, and treatment with the leukotriene receptor antagonist, montelukast, was associated with improvements in the severity of sleep disordered breathing (27-29). Similarly, the expression of glucocorticoid receptors in tonsils differs between OSA and RI (30), such that treatment of mild OSA with intranasal corticosteroids resulted in significant improvements in the severity of the respiratory disturbance during sleep (31). Furthermore, it is possible that respiratory viruses during early post-natal life may modify the neuroimmunomodulatory networks within the tonsillar tissues, and promote different patterns of proliferation in response to various exogenous stimuli (32). The increased inflammation in clinically diagnosed OSA has been noted before, with histological specimens revealing enlargement of follicles compared with chronic tonsillitis, thereby suggesting a hyperplastic condition of lymphoid cells in the germinal centers (33). Of note, the underlying muscles however do not appear to be affected (34). Locally enhanced inflammatory events in tonsils of children with OSA could also be triggered and maintained by snoring and associated vibration frequencies along with recurring upper airway collapse promoting soft-tissue damage (35). Notwithstanding aforementioned considerations, it can be stated with confidence that whereas chronic inflammation of palatine tonsils in children usually leads to tissue hyperplasia and hypertrophy, the mechanisms underlying the regulation of benign follicular lymphoid proliferation and hyperplasia are still poorly understood in pediatric OSA and RI. Several epidemiological studies have further revealed that factors such as environmental smoking, allergies, and intercurrent respiratory infections are all associated with either transient or persistent hypertrophy of lymphadenoid tissue in the upper airways of snoring children. (36-40). Therefore, development of *in vitro* models allowing for determination of the potential effects of particular stimuli and of newly developed pharmacological agents on proliferative patterns and inflammatory pathway responses in tonsils of children with either OSA or RI was clearly needed and is now available, as shown in the present study.

Immunohistochemical examination of tonsils and adenoids of children with putative RI (overnight sleep studies were not performed in this study), revealed a persistent hyperplasia of B lymphocytes in the germinal centers, and decreased T-cell compartment (41). However, in the presence of concanavalin A, a mitogen that mimics the stimulation of lymphocytes by antigens (42), the proliferative responses were higher than with LPS stimulation for both OSA and RI groups. Although LPS characteristically acts as a mitogen for B lymphocytes and conA operates as a mitogen for T lymphocytes, it is possible that LPS stimulation will increase production of cytokines, which in turn may lead to T cell proliferation. Evidence for a preferential T cell response is not unexpected, particularly in the OSA-derived tonsillar cultures, considering the recently described enhanced expression of these lymphocytes in tonsils of children with OSA (43).

Both the local and systemic inflammatory processes elicited by the presence of OSA are associated with enhanced release of potent pro-inflammatory mediators, such as TNF-alpha, IL-6, IL-8 and other cytokines (44-49), and these cytokines will lead to recruitment of lymphocytes and macrophages that play an essential role in the host immune response to inflammatory and infective processes. Our current findings are in agreement, and further add to the conceptual framework that local inflammation is enhanced in OSA, even when compared to RI. However, in a previous study in which tonsillar mononuclear cell cultures were analyzed for cytokine production in children with recurrent tonsillitis and in children with tonsillar hypertrophy associated with symptoms of obstructive sleep apnea, higher spontaneous production of TNF-alpha, IL-1 alpha and beta, and IL-8 were measured in the patients with RI (50). These *a priori* contradictory findings could possibly be due to the different approaches in cell culture and measurement techniques. Indeed, our technique included the unaltered and integral cell population of the tonsils thereby allowing for cell-cell interactions, and permitting the promotion of T-cell B-cell activation and differentiation, all of which closely mimic the *in vivo* situation, and ultimately reveal different global immunological regulatory pathways in OSA and RI. In this context, it can be speculated that the elevated concentrations of cytokines in the supernatants from OSA patients as compared to RI may reciprocally reflect the more pronounced cell proliferative processes in OSA. In contrast, the selective lymphocyte cytokine measurements, as performed by Agren and colleagues (50), may have skewed the global pattern of inflammation in favor of more localized assessments of lymphocyte-lymphocyte interactions, particularly when considering the topographic distribution of cytokine-producing cells in the tonsils (51). As such, the elevated IL-8 concentrations found in RI in the present study after stimulation, may indeed underlie the differences in immune pathway recruitments that occur in OSA and RI. We should also comment that the high concentrations of tumor necrosis-alpha and IL-6 in OSA-derived tonsillar mixed cell cultures may also reflect enhanced monocyte-macrophage activation, most likely elicited by persistent inflammatory processes, which could have a long-term damaging effect, since they induce the activation and the proliferation of endothelial cells and fibroblasts, leading to progressive replacement of immunological active tissue with fibrotic tissue.

Conclusions

The different proliferative rates and production of inflammatory mediators in tonsillar cell cultures from children with OSA and RI suggest that lymphadenoid tissue hypertrophy in these 2 conditions may be regulated by divergent mechanisms. The growing understanding in recent years of the immunologic functions of both tonsils and adenoids has led to substantial scientific debate in relation to the indications for T&A (52). The novel *in vitro* system presented herein may allow for future testing of the efficacy of selectively targeted compounds against the proliferation of the specific cellular elements in tonsils that should enable marked reductions in the cytokine networks activated by OSA or RI, and ultimately lead to the selective involution of these tissues in a disease specific manner.

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Figure 1.

Concentrations of TNF- α (panel A), IL-6 (panel B), and IL-8 (panel C) in the supernatants of tonsillar cell cultures in basal conditions (CO) or after stimulation with LPS and conA (STIM) in children with OSA (filled columns) and RI (hatched columns). The y axis in all graphs is displayed in a logarithmic scale.

Table 1

Demographic and polysomnographic characteristics in 31 children with obstructive sleep apnea and 26 children with recurrent tonsillitis.

	OSA (n=31)	RI (n=26)	P value
Age (years)	5.9±0.5	5.7±0.6	NS
Gender (% female)	45	50	NS
African American (%)	23	23	NS
BMI z score	1.17±0.33	1.01±0.24	NS
AHI (/hTST)	10.3±2.3	0.2±0.1	<0.0001
Nadir SaO2 (%)	82.6±3.3	94.9±0.3	<0.0001
Arousal Index (/hTST)	18.4±4.3	7.8±0.3	<0.0001

NS- not significant; BMI - body mass index; AHI – obstructive apnea hypopnea index; TST – total sleep time;