Leukotriene Pathways and *In Vitro* Adenotonsillar Cell Proliferation in Children With Obstructive Sleep Apnea*

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Introduction: **The abundant expression of leukotrienes (LTs) and their receptors in adenotonsillar tissues of children with obstructive sleep apnea (OSA) suggest that LT antagonists could be useful in treating OSA.**

Methods: **The effects of LTD4 and of LT receptor antagonists zileuton, montelukast, and BAY u9773 were examined on mixed cell cultures prepared from dissociated tonsils or adenoids harvested intraoperatively from children with polysomnographically diagnosed OSA. Proliferation was assessed by ^{3}[H]-thymidine incorporation, and inflammatory cytokine production (tumor necrosis factor [TNF]-α, interleukin [IL]-6, IL-8, IL-10, and IL-12) was assessed in supernatants using enzyme-linked immunosorbent assay.**

Results: **LTD4 elicited dose-dependent increases in adenotonsillar cell proliferation ($p < 0.001$; $n = 12$). All LT antagonists exhibited dose-dependent reductions in adenotonsillar cellular proliferation rates, with montelukast more than BAY u9773 more than zileuton ($n = 14$/group; $p < 0.001$). However, BAY u9773 showed partial agonist effects and increased cellular proliferation at higher concentrations (10^{-4} mmol/L; $p < 0.01$; $n = 12$). LTD4 effects were partially blocked by montelukast and BAY u9773 but not by zileuton. All three antagonists reduced TNF-α, IL-6, and IL-12 concentrations, with selective changes in IL-8 and no effects on IL-10 levels.**

Conclusions: **LT pathways mediate intrinsic proliferative and inflammatory signaling pathways in adenotonsillar tissues from children with OSA, and targeted pharmacologic disruption of these pathways may provide nonsurgical alternatives for prevention and treatment of this disease.** ***(CHEST 2009; 135:1142–1149)***

Abbreviations: cysLT = cysteinyl leukotriene; ELISA = enzyme-linked immunosorbent assay; LT = leukotriene; OSA = obstructive sleep apnea; PBS = phosphate-buffered saline; T&A = tonsillectomy and adenoidectomy; TNF = tumor necrosis factor

Obstructive sleep apnea (OSA) is a frequent condition in children characterized by habitual snoring and increased upper airway resistance during sleep, leading to partial or complete intermittent obstructive events of the upper airway, hypoxemia and hypercapnia, and recurrent arousals.[1] It has now become clear that although craniofacial, structural, and neuromuscular factors also play a role, hypertrophy of adenotonsillar tissues is by far the predominant etiologic factor involved in pediatric OSA, even if obesity has emerged as another major contributor to pediatric OSA.[2,3] As such, the severity of OSA correlates with adenoid and tonsillar size, and surgical excision of these tissues are consequently accompanied by significant clinical improvements.[4–7]

In the past few years, we and others have shown[8,9] evidence of inflammation in both nasal and oropharyngeal mucosa in children with OSA, and we surmised that inflammatory processes may underlie increased adenotonsillar proliferation. Indeed, intranasal corticosteroids have shown favorable outcomes in children with OSA, and their use for periods of 4 to 6 weeks has been associated with improvements in the respiratory disturbance during sleep and partial involution of adenoidal hypertrophy.[10–14] Furthermore, increased concentrations of leukotrienes (LTs) in

tonsils and upper airway condensate in children with OSA along with a relatively high abundance of LT receptors in these tissues suggested that LT pathways may contribute to the proliferative status of adenotonsillar tissues,[15,16] and in fact, improvements in sleep disturbances occurred after treatment in an open-label trial of children with mild OSA.[17]

We recently developed[18] a novel method allowing for *in vitro* cell culture of tonsils and adenoids derived from children undergoing tonsillectomy and adenoidectomy (T&A). We hypothesized that LT antagonists would lead to dose-dependent reductions in cellular proliferation and increased apoptosis in whole tonsillar and adenoid cell cultures obtained from children with OSA, and that these effects would be associated with a decreased production of proinflammatory cytokines.

Materials and Methods

Subjects

The study was approved by the University of Louisville Human Research Committee, and informed consent was also obtained from the legal caregiver of each participant. Assent was also obtained from children > 7 years of age. Consecutive children who underwent tonsillectomy for OSA were identified before surgery and recruited into the study. Overnight polysomnography was performed using standard methods that have been published in detail elsewhere.[19] OSA was considered to be present when the obstructive Apnea-Hypopnea Index was ≥ 5 h of total sleep time in the context of habitual snoring in otherwise healthy children without any chronic disorders requiring treatment with medications (including topical or systemic antiinflammatory or antihistaminic medications), or without any known genetic or craniofacial syndromes.

Cell Culture

Surgically removed tonsils and adenoids from children with OSA were immediately placed in ice cold phosphate-buffered saline (PBS) solution plus antibiotics, Sample processing was initiated within 30 min under aseptic conditions. Briefly, tonsils or adenoids were washed thoroughly with PBS solution, manually dissected into Petri dishes, and gently grounded with a syringe plunger through a 70-μ mesh screen to obtain a mixed cell suspension through mechanical dissociation. RBCs were removed by lysis buffer. Cell viability of all specimens was determined by trypan blue exclusion. Specimens with a viability of < 75% were discarded. Cell cultures were established in standard medium RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum plus antibiotics, which included streptomycin, Fungizone, gentamicin, and penicillin to prevent bacterial and fungal contamination. Mixed cell suspensions were transferred onto 96-round bottom-well plates at a concentration of 1×10^6 cells/well. Cells were cultured in a 5% CO_2 incubator at 37°C for 72 h. Cells were also cultured using 24-well plates to determine proinflammatory cytokine levels. Cultures were also exposed to LTD4 or to LT antagonists, with control conditions corresponding to the addition of the diluent alone. LTD4 was purchased from Cayman Pharma (catalog No. 20310; Neratovice, Czech Republic). A lipoxygenase inhibitor (zileuton; Sigma-Aldrich; St. Louis, MO), a cysteinyl LT (cysLT) receptor 1 antagonist (montelukast; Sigma-Aldrich), and a dual cysLT receptor 1 and 2 antagonist (BAY-u9773; Biomol Research Laboratories; Plymouth Meeting, PA),[20] were added to the medium 24 h after plating to achieve final concentrations ranging from 10^{-3} to10^{-8} mmol/L.

Proliferation Assay

Cells were incubated for the final 18 to 20 h of the 72-h culture with 0.0185 MBq (0.5 μCi) ^{3}[H]-thymidine in complete medium (Amersham Biosciences; Little Chalfont, 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 ^{3}[H]-thymidine uptake results were expressed as the average of the three wells in counts per minute.

Immunohistochemistry

Coronal sections (40 μm) of tonsils were initially incubated in 1xcitrate buffer (Lab Vision Corporation; Fremont, CA) at 95°C for 45 min, washed several times in PBS solution, and blocked with a PBS/0.4% Triton X-100/0.5% signal amplifier solution (TSA; Perkin Elmer Life Sciences; Boston, MA) blocking reagent/10% normal horse serum for 1 h. Sections were then serially incubated with anti-CD4 antibody (1:300; Santa Cruz Biotechnology; Santa Cruz, CA) or anti-cysLT1 receptor antibody (1:1000; BD Pharmagen; San Jose, CA) at 4°C for 24 h, and then washed in PBS solution six times for 5 min each wash. Sections were incubated at room temperature for 1 h in horse antimouse biotinylated antibody (1:400; Vector Laboratories; Burlingame, CA) in a PBS/0.4% TSA blocking reagent/10% horse serum solution, and then with streptavidin-horseradish peroxidase diluted 1:100 in PBS/0.5% TSA blocking reagent solution. Subsequently, the sections were incubated with TSA fluorescein reagents diluted 1:50 in amplification diluent (Perkin Elmer Life Sciences) for 2 min. Sections were then washed and mounted onto glass slides. Negative controls were prepared by either omitting the primary or the secondary antibody. Sections were prepared from five sets of tonsils from OSA subjects, and they were visualized using a fluorescent microscope by an investigator who was blinded to the sample source.

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