Differential Expression of Cysteinyl Leukotriene Receptors 1 and 2 in Tonsils of Children With Obstructive Sleep Apnea Syndrome or Recurrent Infection*

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Background: Recurrent tonsillitis and sleep apnea are the major indications for tonsillectomy in children. We hypothesized that the recurrent vibration in the upper airway of snoring children would promote inflammatory changes in the tonsillar tissue and would lead to the up-regulation of cysteinyl leukotriene (LT) receptors (Rs).

Objective: To assess the expression patterns of the human LT-Rs in children undergoing tonsillectomy, and compare those patterns in children having recurrent throat infections (RIs) and children with obstructive sleep apnea syndrome (SA).

Methods: Tonsillar tissue from 17 children with SA and 13 with RIs was subjected to quantitative polymerase chain reaction using specific primers for LT1-R and LT2-R, and to immunohistochemistry and Western blotting for protein expression of LT1-R and LT2-R.

Results: Messenger RNA encoding for the expression of LT1-R and LT2-R was detected in the tonsils of all children. Immunoblots revealed significantly higher expressions of LT1-R and LT2-R in the tonsils of children with SA. The topographic pattern of both receptors differed among the tonsils of children with SA and RI.

Conclusion: LT1-R and LT2-R are expressed in pediatric tonsillar tissue, are more abundant in SA patients, and demonstrate a specific topographic pattern of expression. These findings suggest that an inflammatory process involving LT expression and regulation occurs in children with SA.

Key words: leukotriene receptor; obstructive sleep apnea syndrome; recurrent tonsillitis

Obstructive sleep apnea syndrome (SA) is a highly prevalent health problem, occurring in at least 2 to 3% of all children.1 It has become apparent that this frequent condition carries substantial cardiovascular, metabolic, and neurocognitive morbidities.2-5 In children, SA is almost universally associated with hypertrophy of the tonsils and adenoids, such that removal of such tissues is usually the first line of therapy.6,7 In addition, tonsillectomy is also frequently performed in...
children who sustain recurrent throat infections (RIs) requiring multiple courses of antibiotics.8

In adults with SA, evidence9–11 suggesting that oropharyngeal mucosal inflammation is present in patients and contributes to the pathophysiologic mechanisms mediating the intermittent obstruction of the upper airway during sleep has emerged over the last few years. It has been further postulated12 that the increased inflammatory markers found in the upper airway of adult patients with SA were due to the inflammatory responses elicited by the presence of recurrent vibratory damage occasioned by snoring. However, the expression of leukotrienes in the upper airways of SA patients was not examined. Considering that LTs play major roles as inflammatory mediators in the human upper airway,13 we hypothesized that the inflammatory processes triggered by snoring may involve the cysteinyl LT receptors (Rs) LT1-R and LT2-R. Thus, LT1-R and LT2-R would be expressed in human tonsillar tissue, and their expression would be greater in the tonsils of SA pediatric patients than in those of patients with recurrent tonsillitis.

**Materials and Methods**

The study was approved by the University of Louisville Human Research Committee, and informed consent was obtained from the legal caretaker of each participant. Consecutive children undergoing tonsillectomy for either SA or RI were identified prior to surgery and were recruited into the study. The diagnosis of SA was established by overnight polysomnography in the sleep laboratory and required the presence of an apnea-hypopnea index more than five events per hour of sleep.14 Patients referred for RI were selected based on a history of at least five tonsillar infections in <6 months and the absence of any symptoms suggestive of SA using a previously validated questionnaire.15 However, they were not evaluated by an overnight polysomnogram since our questionnaire-based evaluation is highly sensitive and specific in ruling out sleep-disordered breathing in children.15 RI children received their last dose of antibiotic therapy 6 weeks from the day of surgery. Children with known asthma, allergic rhinitis, a history of allergies, and/or having received corticosteroid or LT-modifier therapy within 1 year from surgery were excluded.

**Tissue Collection and Processing**

After both palatine tonsils were removed by a pediatric ear, nose, and throat specialist (JLG), a portion of each tonsil was snap-frozen in liquid nitrogen and stored at −80°C. Another portion of the tonsils was fixed in 4% formalin, cryoprotected with 30% sucrose, and kept at 4°C.

**Quantitative (Real-Time) Polymerase Chain Reaction**

Total RNA was prepared from tonsillar tissue using a reagent (TRIzol; Invitrogen; Carlsbad, CA) following the manufacturer’s instructions. Isolated total RNA was quantified using a spectrophotometer (model DU-530; Beckman; Fullerton, CA). Aliquots of total RNA (1 μg) were reverse-transcribed using random primers and a reverse transcriptase (Superscript II-Reverse Transcriptase; Invitrogen) according to the manufacturer’s protocol. Complementary DNA equivalent to 20 ng total RNA were subjected to real-time polymerase chain reaction (PCR) analysis (MX4000; Stratagene; La Jolla, CA) following the manufacturer’s protocol. PCR primers (Invitrogen) and Taqman probes (Biosearch Technologies; Novato CA) for LT1-R, LT2-R, and β-actin were designed using appropriate software (Beacon Designer, version 2.0; Premier Biosoft International; Palo Alto, CA). The primer and probe for LT1-R were as follows: forward primer, 5'-TTATGTTCCG-AAAAGCATTTG-3'; reverse primer, 5'-GACTAGCCTGGTGATCTAAAAG-3'; and Taqman probe, 5'-FAM-TGAGGAAACCCGGCCCTC-BHQ-1-3'. The primer and probe for LT2-R were as follows: forward primer, 5'-GACTATATGCTTGGTGGTTG-3'; reverse primer, 5'-ATGATGGTTGTTGACTGCTTC-3', and Taqman probe, 5'-FAM-TGAGGAAACCCGGCCCTC-BHQ-1-3'. Each reaction (25 μL) contained 2.5 μL reaction buffer (10×), 6 mmol/L MgCl₂, 0.2 μmol/L deoxynucleoside triphosphate, 0.6 μmol/L each primer, 0.25 μL Taq DNA polymerase (SureStart; Stratagene), and 2 μL complementary DNA dilutions. The cycling condition consisted of 1 cycle at 95°C for 10 min and 40 two-segment cycles (ie, 95°C for 30 s and 55°C for 60 s). Standard curves for the target gene (ie, LT1-R or LT2-R) and the housekeeping gene (ie, β-actin) were performed for each assay. Briefly, 10-fold serial dilutions of control complementary DNA were amplified (MX-4000 PCR machine; Stratagene). The cycle time (ie, the initial amplification cycle) of each standard dilution was plotted against standard complementary DNA copy numbers. Based on the standard curves for each gene, the sample complementary DNA copy number was calculated according to the sample cycle time value. Finally, each of the calculated copy numbers for either LT1-R or LT2-R was normalized against the corresponding β-actin copy numbers. Standard curves and PCR results were analyzed using the software of the PCR machine (MX4000; Stratagene).

**Immunohistochemistry**

Coronal sections (30 μm) were initially incubated in 0.3% H₂O₂ for 30 min, washed several times in phosphate-buffered saline solution (PBS), and blocked with PBS/0.4% wetting agent (Triton X-100; PerkinElmer Life and Analytical Sciences; Boston, MA)/0.5% tyramide signal amplification (TSA) (Perkin Elmer Life and Analytical Sciences) blocking reagent/10% normal goat serum (Vector Laboratories; Burlingame CA) for 1 h. Sections were then serially incubated with LT1-R antibody (1:1500) [Cayman; Ann Arbor MI] or LT2-R antibody (1:4000) [Cayman] at 4°C for 24 h, and then were washed in PBS six times for 5 min each time. Sections then were incubated at room temperature for 1 h in biotinylated antirabbit antibody (1:600) [Vectastain Elite ABC kit; Vector Laboratories] in a PBS/0.5% TSA blocking reagent/10% goat serum solution. After three 5-min washes, sections were incubated at room temperature with streptavidin-horseradish peroxidase diluted 1:100 in PBS/0.5% TSA blocking reagent. Subsequently, the sections were incubated with tetramethyl rhodamine tyramide diluted 1:50 in amplification diluent (PerkinElmer Life and Analytical Sciences) for 2 min. Sections then were washed in PBS and mounted onto glass slides. Negative controls were prepared by omitting either the primary
or the secondary antibodies for both receptors. Sections were prepared from five sets of tonsils from either the SA or RI groups and were visualized using a fluorescent microscope by an investigator who was blinded to the sample source.

Western Blotting

Tonsils were homogenized in a lysis buffer (50 mmol/L Tris [pH 7.5], 0.4% NP-40, 10% glycerol, 150 mmol/L NaCl, 10 mg/mL aprotinin, 20 mg/mL leupeptin, 10 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L sodium orthovanadate, and 100 mmol/L sodium fluoride), and the protein concentration was determined using the Bradford method (Bio-Rad; Hercules, CA). Samples (40 µg protein) were resolved on 12% sodium dodecyl sulfate-polyacrylamide gels using electrophoresis (Invitrogen) for 90 min and electroblotted onto 0.2-µm nitrocellulose membranes for 2 h at 120 V. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline solution and 0.05% Tween 20, and then were incubated overnight at 4°C with commercially available primary antibodies recognizing the LT1-R (1:500) [Cayman] or the LT2-R (1:500) [Cayman], and later with anti-β-actin (1:20,000) [Sigma; St. Louis, MO], both diluted in 5% milk. Lanes also were incubated with a mixture of the primary antibody and the receptor-blocking peptide (1:2 ratio) in order to ascertain the specificity of the antibody using this competition assay. Membranes then were washed with Tris-buffered saline solution and 0.05% Tween 20, and were incubated with either horse radish peroxidase-linked antirabbit or antimouse antibodies (for LT-Rs and β-actin, respectively). Proteins were visualized by enhanced chemiluminescence (Amersham; Piscataway, NJ). The intensities of the bands corresponding to the protein of interest were quantified using scanning densitometry and were compared using t tests or analysis of variance, as appropriate.

Statistical Analysis

All data were subjected to statistical analysis using either t tests or analysis of variance followed by post hoc tests, as appropriate. A p value of < 0.05 was considered to be statistically significant. Results are presented as the mean ± SD.

Results

Of the 30 children recruited for the study, 17 were SA patients and 13 were RI patients. The mean age was 5.7 ± 3.2 years (range, 2 to 12 years; 14 male children), 80% were white, and the remaining 20% were African-American (Table 1). LT1-R and LT2-R messenger RNA were present in all 16 tonsils studied, but no differences in gene expression emerged between the SA and the RI groups (Fig 1). In the palatine tonsils excised from SA patients, both LT1-R and LT2-R were primarily and abundantly expressed in the tonsillar epithelial layer and in the extrafollicular area within the tonsillar parenchyma. Clusters of LT1-R and LT2-R positively labeled cells were also present within blood vessels. No staining was detected in the tonsillar germinal centers (Fig 2). In contrast, in tonsils obtained from RI patients, only reduced numbers of cells expressed the LT1-R and LT2-R, and these cells were primarily restricted to the epithelial layer, with no expression detected in the germinal centers (Fig 2).

Immunoblots of tonsillar lysates for LT1-R detected a protein with a molecular weight of about 38 kD (Fig 1), which was confirmed by the competition assay with the immunogenic peptide. LT1-R expression was significantly greater in the SA group than in the RI group (eight patients; p < 0.05) [Fig 1]. LT2-R appeared as a single immunoreactive band with a molecular weight of about 59 kD (Fig 1), which was further confirmed by competition with the specific blocking peptide. The expression of the LT2-R was also significantly greater in the SA group than in the RI group (eight patients; p < 0.05) [Fig 1].

Discussion

Some studies16,17 have characterized the expression of LT1-R and LT2-R in several human tissues at both the gene and protein levels. Although LT1-R expression was identified in human nasal mucosa,18 and in lymphoid organs such as the spleen,19 the expression of LT-Rs in human tonsils, which is a key lymphoid organ, has not been heretofore assessed. The present study conclusively demonstrates that human tonsils express both LT1-R and LT2-R, and that their expression is regulated by two distinctly different disease processes, namely, sleep apnea and recurrent tonsillitis.

LT1-R antagonists such as montelukast are widely used in the treatment of asthma and allergic rhinitis in children, while no antagonist for the LT2-R has been developed thus far. While the implications of these antagonists for the clinical management of children with sleep apnea that is primarily attributable to enlarged adenotonsillar tissue remains unknown and clearly merits further investigation, the cloning of the human genes for LT1-R19 and LT2-R20 enabled us to explore the levels of gene expres-

| Table 1—Demographic Characteristics in 30 Pediatric Patients Undergoing Tonsillectomy for Either SA or RIs |
|------------------|------------------|
| Characteristics  | Values           |
| SA, No.          | 17               |
| RI, No.          | 13               |
| Age, *yr         | 5.7 ± 3.2        |
| Sex, No.         |                  |
| Female           | 16               |
| Male             | 14               |
| Race, %          |                  |
| White            | 80               |
| African-American | 20               |

*Values given as mean ± SD.
sion in the tonsillar tissue of children. We could not obtain tonsillar tissue from healthy children for obvious ethical reasons. Nevertheless, current findings indicate that both LT1-R and LT2-R are expressed in human tonsils, and that different disease states alter the patterns of expression, suggesting that LT1-R and LT2-R may underlie components of the pathophysiologic mechanisms linking the enlargement of the tonsillar tissue to the emergence of sleep apnea in snoring children, possibly through increased upper airway inflammation linked to mechanical irritation of the upper airway mucosa due to snoring, up-regulation of LT-R expression, and ultimately to accelerated growth of the tonsillar tissue leading to upper airway obstruction during sleep. Interestingly, the immunostaining of tonsillar sections identified unique disease-dependent distribution patterns of the receptors with high abundance in the epithelial layer and in the parenchyma, including blood vessels, and almost no expression in cells located in the germinal centers. These findings would suggest that the cells that positively label for LT-Rs either have acquired them in the late stages of maturation or, as proposed by Ebenfelt and Ivars-son, may have migrated from the vasculature to occupy their sites within the tonsils.

The rapidly accumulating body of evidence in adult SA patients lends credible support to the theory that the recurrent vibration of the air column in the upper airway due to snoring will induce mechanical trauma. This recurrent vibratory trauma will in turn promote the development of an inflammatory response leading to mucosal swelling and subsequently to upper airway obstruction. Although the evidence supporting such a mechanical damage-inflammatory pathway is currently unavailable in children with SA, current findings would support the contention that the up-regulation of LT1-R and LT2-R, which are deeply involved in inflammatory and allergic responses, is somehow linked to enhanced local upper airway inflammation in pediatric cases of SA. The specific mediators leading to the increased expression of LT-Rs await further investigation. Surprisingly, lower expression levels were present in those children with recurrently infected tonsils, and this was somewhat unexpected, by virtue of the episodic infectious processes afflicting these children’s
tonsils. However, it should be stressed that surgery was performed only in children with RIs during periods of quiescence in which no evidence was present for ongoing inflammatory processes.

In summary, we have delineated the expression and tissue distribution of LT1-R and LT2-R in human tonsils, and have shown that they are regulated differentially in two frequent disease states, namely, RIs and SA, leading to the need for their surgical removal. We postulate that, based on such intriguing, albeit preliminary, observations, the use of LT-R antagonists may be a potential future therapeutic consideration in treating children with SA.

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