Effect of Fluticasone Furoate on Interleukin 6 Secretion From Adenoid Tissues in Children With Obstructive Sleep Apnea

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Objective: To determine the effect of intranasal corticosteroid therapy on T-regulatory cells and other inflammatory cytokines in adenoid tissues in children with obstructive sleep apnea syndrome.

Design: Randomized, prospective, exploratory study.

Setting: Academic pediatric otolaryngology practice in a tertiary care children’s hospital.

Patients: Participants included 24 children between the ages of 2 and 12 years who were undergoing adenotonsillectomy for polysomnogram-documented obstructive sleep apnea syndrome.

Intervention: Children were randomized to either no treatment (n = 13) or treatment with fluticasone furoate nasal spray, 55 µg/nostril daily (n = 11), for 2 weeks before adenotonsillectomy. Adenoid tissue was obtained at the time of the procedure.

Main Outcome Measures: The number of tissue T-regulatory cells, as determined by staining with FOXP3, CD4, and CD25, was the primary outcome measure. Staining for interleukin (IL)–10 and transforming growth factor β protein by immunohistochemistry, and adenoid mononuclear cell spontaneous and induced release of cytokines (IL-10, IL-6, IL-12, IL-13, tumor necrosis factor, and transforming growth factor β) were secondary outcomes.

Results: Cells isolated from fluticasone furoate nasal spray–treated adenoid tissue released significantly less IL-6 spontaneously as well as upon stimulation with anti-CD3 monoclonal antibody (P = .05) compared with non-treated adenoid tissue. There were no significant differences in the number of CD4/FOXP3–, CD25/FOXP3–, or transforming growth factor β–positive cells. Intensity of staining for IL-10 was also comparable between the groups.

Conclusions: In this study, we show reduction of IL-6, a proinflammatory cytokine, in adenoid tissue obtained from children with obstructive sleep apnea syndrome treated with fluticasone furoate nasal spray. This reduction could contribute to the clinical efficacy of this class of medications in the treatment of childhood obstructive sleep apnea syndrome.


Obstructive sleep apnea syndrome (OSAS) occurs in 2% to 3% of children, has a significant detrimental effect on quality of life, and can lead to undesirable sequelae, such as cardiovascular complications, failure to thrive, excessive daytime sleepiness, behavioral disturbances, hyperactivity, attention problems, and poor school performance.

Obstructive sleep apnea syndrome is characterized by prolonged partial upper airway obstruction and/or intermittent complete obstruction with disruption of normal ventilation during sleep and normal sleep patterns. Multiple factors contribute to the pathophysiologic characteristics of this disease, such as adenotonsillar hypertrophy, obesity, neuromuscular disorders, craniofacial abnormalities, and abnormal dilator tone of the upper airway muscles. Recent evidence has suggested a role for inflammation in OSAS based on several interesting observations. These include histopathologic evidence of inflammation, detection of proinflammatory cytokines and markers in the serum of patients with OSAS, and demonstration of increased release of certain cytokines as well as higher proliferation of tonsillar cells in patients with OSAS compared with recurrent infection. Furthermore, intranasal corticosteroid therapy has been shown to improve mild OSAS in children.

Intranasal corticosteroids are potent anti-inflammatory agents that are useful in the treatment of upper airway diseases, including allergic rhinitis and nasal polyposis. Recent studies have suggested that the inhibitory actions of glucocorticoids in inflammatory diseases are

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partially mediated by upregulation of T-regulatory (T-reg) cells and the secretion of interleukin (IL–10) by these cells. T-regulatory cells are a subset of helper T cells and constitute 5% to 10% of the CD4+ T-cell population; they are known to have an anti-inflammatory, immunoregulatory role. They are CD4+CD25+ cells that usually secrete IL-10 and transforming growth factor β (TGF-β) and express a transcription factor known as forkhead box P3 (FOXP3).22

We undertook this exploratory investigation to study the mechanism of action of intranasal corticosteroids in the treatment of OSAS in children. We hypothesized that treatment with an intranasal corticosteroid, fluticasone furoate nasal spray (FFNS), would result in an increased number of T-reg cells in the adenoid tissue of children with OSAS as well as increased secretion of the T-reg–associated cytokines IL–10 and TGF–β. Furthermore, we hypothesized that treatment with FFNS would downregulate the secretion of proinflammatory cytokines important in OSAS.

METHODS

STUDY DESIGN

We performed a randomized, prospective, open-label, parallel-group study in an academic pediatric otolaryngology practice in a tertiary care children's hospital. Children with OSAS diagnosed by polysomnogram who were candidates for adenotonsillectomy were recruited. The parents were approached in the clinic at the time that adenotonsillectomy was recommended. The study protocol was explained, and parental consent for their child to participate was obtained. The patients were then randomized to 2 groups: 1 received treatment with FFNS, 50 μg/nasal, once daily for 2 weeks, and the other group received no treatment. All children underwent adenotonsillectomy 2 weeks after initiation of therapy. The adenoids were removed with an adenoid curette and weighed before and after therapy. They were then taken directly to the laboratory on ice and divided into 2 portions, 1 for immunohistochemistry testing and 1 for cellular processing, as detailed in the “Immunohistochemistry” subsection. Bottles containing FFNS were weighed before and after use to monitor adherence. Parents received a telephone call 2 to 3 days before the scheduled procedure to discuss their child's symptoms of OSAS.

In preparation for double staining, the slides were treated with ET buffer in the microwave for 10 minutes at maximal power. The primary mouse antibody FOXP3 (catalog No. ab20034; Abcam, Cambridge, Massachusetts) was applied at a 1:100 dilution in phosphate-buffered saline for 1 hour at room temperature. Antibody binding was visualized with anti-mouse polymer–labeled HRP-bound secondary reagent (EnVision+; Dako, Carpinteria, California). For CD4/FOXP3 double staining, the secondary mouse antibody used was CD4 (catalog No. VP-C319; Vector Laboratories, Burlingame, California) applied at a 1:50 dilution and visualized (catalog No. sk-5300, Vector Blue Alkaline Phosphatase Substrate Kit III; Vector Laboratories). For CD25/FOXP3 double staining, the secondary mouse antibody, CD25 (catalog No. NCL-CD25-305; Novocastra Lab, Newcastle upon Tyne, England), was applied at a dilution of 1:25. Antibody binding was visualized with anti-mouse polymer–labeled HRP-bound secondary reagent (EnVision+). Corresponding negative control experiments were performed by omitting the incubation step with the primary antibody for TGF–β, CD4, FOXP3, and CD25.

For IL–10 staining, tissue slides were treated in EDTA buffer in the microwave for 10 minutes at maximal power. Goat anti–IL–10 antibody at a 1:20 dilution (catalog No. AF-217-NA; R&D Systems) was applied on the tissue sections for 1 hour at room temperature. Antibody binding was visualized with anti-goat IgG–HRP (1:100 dilution, catalog No. HAF017; R&D Systems) and DAB detection system (catalog No. K3466; Dako). For negative controls, goat IgG was used (catalog No. I-5000; Vector Laboratories) at the same concentration as the primary antibody.

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All immunohistochemistry, cytokine stimulation, and cell counting were performed with investigators masked as to whether the participants had received FFNS. One portion of the adenoid specimen was paraffin–embedded, sectioned, mounted on slides, and stored until all specimens were collected. We then double-stained all specimens with CD4/FOXP3 and CD25/FOXP3 and to evaluate TGF–β and IL–10. Antibigen was retrieved on deparaffinized sections, and endogenous peroxidase activity was quenched in hydrogen peroxide, 3%, and blocked in milk peroxidase. For TGF–β-3 detection, slides were treated in ET buffer (EDTA, pH 9.0; and TRIS, pH 7.0; mixed with water) in a microwave at maximal power for 10 minutes to improve antigen retrieval. Mouse primary antibody (catalog No. MAB1835; R&D Systems, Minneapolis, Minnesota) was applied at a dilution of 1:20 in phosphate-buffered saline for 1 hour at room temperature. Antibody binding was visualized with the anti-mouse/rabbit immunodetector horseradish peroxidase (HRP)/3',3' diaminobenzidine detection system (Bio SB, Inc, Santa Barbara, California).

PATIENTS

Children were included if they were between the ages of 2 and 12 years and had OSAS documented by polysomnography showing an apnea–hypopnea index of 5/h or more, regardless of minimal oxygen saturation. Children were excluded if they (1) had significant medical problems such as uncontrolled asthma, (2) had received long-term medication therapy other than bronchodilators and leukotriene receptor antagonists, (3) had received systemic corticosteroids in the past month or intranasal corticosteroids within 2 weeks, (4) had neurologic and craniofacial abnormalities, (5) had a body mass index greater than the 95th percentile for age, and (6) were girls who already had their first menstrual cycle.
The cellular comparisons were made using a nonpaired t test and are reported as mean (SEM). The number of CD4+/FOXP3-, CD25/FOXP3-, and TGF-β-stained cells was compared between patients receiving treatment and those not receiving treatment. Similarly, the intensity of IL-10 staining between the groups was compared in the germinal centers, in the interfollicular areas, and in the combined areas. The cytokine data were not normally distributed and were analyzed using nonparametric statistics. The spontaneous release of cytokines by the cultured cells was compared between the groups. To evaluate stimulated release, we subtracted spontaneous release from stimulated release and compared the groups. The comparisons were performed using a Mann-Whitney test. The data are reported as median (range) and depicted as individual data. All analyses were performed using statistical software (Systat 12 for Windows 2007; Systat Software, Inc, Chicago, Illinois).

**RESULTS**

Twenty-four children were randomized to receive either FFNS (n=11) or no treatment (n=13). Their demographic characteristics and sleep study results are reported in **Table 1**. Not all analyses were performed on all specimens because of technical reasons; thus, the number of observations for the different sets of data reported here vary. The number of samples for the different experiments was as follows: cytokine release (no treatment, 12; FFNS, 11); IL-10 staining (no treatment, 10; FFNS, 9); TGF-β staining (no treatment, 9; FFNS, 11); and double staining (no treatment, 10; FFNS, 12). The children in both groups had clinically significant OSAS, and there were no statistically significant differences between the groups in any of the recorded variables, except that the group receiving no treatment had a significantly higher rapid eye movement apnea-hypopnea index (P=.05). To adjust for different weights of the children, the volume of the adenoids, estimated by water displacement in the operating room, was divided by the respective weights of the patients and multiplied by 100. There was no significant difference in the adjusted adenoid weight between the no-treatment group (16.8 [3.8] mL/kg × 100; n=9) and the FFNS group (13.0 [2.7] mL/kg × 100; n=9) (P=.43). Patient adherence was confirmed by a statistically significant decrease in the mean weight of FFNS bottles from 54.6 (0.45) g before treatment to 52.8 (0.5) g after treatment (P=.02). The mean reduction of the medication bottle weights for the group was 1.77 (0.11) g. As a positive check, we weighed a bottle of medication before and after 28 sprays (the number of doses needed for a 2-week course); the difference was 1.56 g, which suggests that our study group was adherent to medication use.

**CYTOKINE RELEASE STUDIES**

Spontaneous release of cytokines from adenoid mononuclear cells was highest for IL-6, IL-10, TNF, and TGF-β and showed very low levels for IL-12 and IL-13 (**Table 2**). Lower levels were measured in adenoid mononuclear cells...
from the FFNS-treated patients for IL-6, IL-13, IL-10, and TNF, but the reduction was significant only for IL-6 (Table 2, Figure 1).

Stimulated cytokine release was calculated by subtracting spontaneous release levels from levels obtained after stimulation with either PHA (Table 3) or OKT3 (Table 4). Again, there was very little release noted with either of the stimulants for IL-12 (Tables 3 and 4). There were higher levels of IL-6, IL-10, IL-13, and TNF after stimulation with OKT3 than with PHA. Higher levels of TGF-β were measured after stimulation with PHA. Pretreatment with FFNS resulted in lower OKT3-induced IL-6 release compared with no treatment (Table 4, Figure 1) and tended to approach statistical significance for PHA-induced IL-6 release (Table 3). Pretreatment with FFNS had no significant effect on stimulated release of any of the other cytokines (Tables 3 and 4).

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Most of the double-stained cells clustered in the interfollicular area, with few cells in the germinal centers. There was no significant difference in the number of CD4/FOXP3-positive cells or CD25/FOXP3-positive cells between the 2 groups (Figure 2 and Figure 3). There were few TGF-β-positive cells, and they were present mostly in the interfollicular areas. Twenty HPFs were evaluated for these cells. The mean number of TGF-β-positive cells was 1.7 (0.3) cells/HPF in the no-treatment group (n=9) and 2.3 (0.3) cells/HPF for patients in the FFNS group (n=11), with no significant difference between the groups (P = .22). Because of the diffuse character of the staining with IL-10, we analyzed brown staining intensity using the automated cellular imaging system and measured the integrated optical density (IOD) per 10 µm². A control slide stained for every patient. There were clear and significant differences in the number of TGF-β-positive cells and they were present mostly in the interfollicular areas. Most of the double-stained cells clustered in the interfollicular area, with few cells in the germinal centers. There was no significant difference in the number of CD4/FOXP3-positive cells or CD25/FOXP3-positive cells between the 2 groups (Figure 2 and Figure 3). There were few TGF-β-positive cells, and they were present mostly in the interfollicular areas. Twenty HPFs were evaluated for these
tables.
The FFNS did not control for in our parallel study design. However, we did attempt to adjust for one of these variables—the weight of the subjects—by considering it when calculating the estimate of adenoid size. Another reason for the discrepancy is that the measurements in other studies were performed on the adenoids in situ, using radiographs or nasal endoscopy; we measured the weight after adenoidectomy. Although adenoidectomy was performed with the standard curette technique, there is always some degree of residual adenoids that are removed using suction electrocautery. This amount could vary from patient to patient, thus increasing the level of variability in our measurement. The increased variability between adenoid measurements, the parallel study design, and the small sample size all probably made it more difficult to detect a significant effect of treatment on adenoid size in our study. Adherence did not seem to be an issue, because there was a significant reduction in the weight of the bottles from before to after treatment.

Our results show that pretreatment with FFNS inhibited spontaneous and OKT3-induced IL-6 release from adenoid mononuclear cells. The same therapy had no effect on the other cytokines measured. The cytokine IL-6 is secreted by T cells and mononuclear phagocytes and induces T- and B-cell growth and differentiation as well as the production of acute-phase reactants, such as C-reactive protein, by the liver. Both C-reactive protein and IL-6 are important predictors of cardiovascular risk and morbidity. Levels of both proteins are elevated in the serum of children with sleep-disordered breathing, higher with increasing severity of disease, and reduced after adenotonsillectomy. Therefore, evidence supports the role of IL-6 in the pathophysiologic basis of OSAS, and the reduction observed after FFNS in our study might partially explain the mechanism for the beneficial effect of intranasal corticosteroid therapy. It is certainly plausible to speculate that the mechanism of action of intranasal corticosteroids is mediated via a reduction in the production of IL-6 by adenoid lymphocytes that, in turn, leads to decreased growth and differentiation of these adenoid cells and a decrease in adenoid size. It is also plausible to speculate that a reduction of IL-6 in adenoid tissues could lead to a parallel decrease in serum levels and a reduction of the pro-inflammatory effects of the cytokine observed in children with OSAS. Unfortunately, serum levels were not measured in our study.

The FFNS did not result in significant inhibition of the release of any other cytokines compared with the no-treatment group. Levels of IL-12 were generally low in our study; among the other cytokines, IL-13 and TNF were reduced in the group using FFNS compared with the no-treatment group, but the difference was not statistically significant. Previous studies in adults with OSAS have shown serum levels of TNF and IL-6 to be elevated in patients with OSAS compared with controls; however, in those studies, the effect of corticosteroid therapy was not investigated. One in vitro study in children examined the effect of corticosteroid therapy on cytokine release from tonsillar cells. The results showed an inhibitory effect of active therapy with corticosteroids on levels of TNF, IL-8, and IL-6, agreeing somewhat with...
our study (relating to IL-6 levels), albeit in a different experimental system.

Our main study hypothesis was that administration of intranasal corticosteroids would result in an increase in T-reg cells in the adenoids of the treated children. This was based on observations\(^{10,21}\) of the effects of corticosteroid treatment in the context of other inflammatory diseases, such as asthma and nasal polypsis. One such study\(^{21}\) showed an increase in FOXP3 messenger RNA and tissue IL-10 levels in patients with chronic rhinosinusitis after administration of intranasal corticosteroids. We chose to test this hypothesis by measuring se-cretion of the cytokines IL-10 and TGF-\(\beta\) from adenoid mononuclear cells and quantification of cells stained with both these cytokines as well as by CD4 or CD25 and the T-reg cell marker FOXP3 in adenoid sections. There were no significant differences in either of these measures be-tween the treatment group and the no-treatment group.

In other reports on OSAS, there is some evidence point-ing to lower activity of the cytokine IL-10. This is sup-portted by reports of lower levels of this cytokine in the serum of patients with OSAS compared with controls\(^6\) and by a lower level of this cytokine in the supernatants of control and stimulated tonsillar cells obtained from children with OSAS compared with those obtained from children with recurrent infections.\(^{12}\) Our results were internally consistent, as there was no evidence of an increase in T-reg cells between the groups using 2 differ-ent approaches, namely, cell staining and functional cytokine secretion of cultured cells.

The reason underlying the lack of statistically signifi-cant differences in T-reg cell markers between the groups in our experiments is not clear. This could be related to the small sample size or to technical problems. It would probably have been more reliable, and more indicative of T-reg cell numbers, if we had triple-stained the adenoid cells with CD4, CD25, and FOXP3 simultane-ously, using cell sorting. Another way to evaluate T-reg cells and their cytokines that might have been more re-liable would have been to examine levels of mRNA for FOXP3, TGF-\(\beta\), and IL-10. Finally, the beneficial role of intranasal corticosteroid therapy in improving pediatric OSAS might not be mediated by T-reg cells.

Our study had several limitations that might have af-fected the results. The sample size was small and the study was open-label, although randomized. However, the find-ings were objective, and the absence of a placebo arm is not expected to affect such results to a significant ex-tent. The duration of treatment was only 2 weeks—a longer treatment period might have enhanced our results in line with our hypothesis. Because the wait time for opera-tions in patients with OSAS is approximately 2 to 3 weeks at our institution, we did not believe that it would be ethically proper to withhold surgery for much longer than that for an unproved therapy for children with mod-erate disease. Use of a higher dosage of corticosteroids might have provided greater effect, as seen in nasal polypsis studies.\(^{17}\) Finally, although the bottle weights were reduced after treatment, this does not ensure complete adherence.

In summary, in this exploratory study of children with OSAS, pretreatment with FFNS compared with no treat-ment resulted in inhibition of IL-6 secretion by adenoid cells in the corticosteroid-treated group, without affect-ing other cytokine levels significantly. This might par-tially explain the mechanism by which intranasal corticosteroid therapy provides relief for children with OSAS. Our results failed to demonstrate an upregulation of T-reg cells or their cytokines after treatment with FFNS for 2 weeks.

Submitted for Publication: September 21, 2010; final re-vision received December 10, 2010; accepted March 6, 2011.

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Author Contributions: Drs Esteitie and Emani contrib-uted equally to this work. Drs Esteitie, Suskind, and Baroody had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Baroody. Acquisition of data: Emani, Sharma, Suskind, Baroody. Analysis and interpretation of data: Esteitie, Baroody. Drafting of the manuscript: Esteitie, Emani, Sharma, Baroody. Critical revision of the manuscript for important intellectual content: Suskind, Baroody. Statistical analysis: Baroody. Obtained funding: Baroody. Ad-ministrative, technical, and material support: Emani, Suskind, Baroody. Study supervision: Emani, Baroody.

Financial Disclosure: None reported.

Funding/Support: This study was funded by an investi-gator-initiated grant from GlaxoSmithKline.

Previous Presentation: This study was presented at the Annual Meeting of the American Society of Pediatric Oto-laryngology; May 2, 2010; Las Vegas, Nevada.

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