Increased Upper Airway Cytokines and Oxidative Stress in Severe Obstructive Sleep Apnea

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Abstract:

Inflammation may contribute to upper airway pathophysiology in obstructive sleep apnea (OSA). Our objective was to compare upper airway pro-inflammatory cytokine expression, oxidative stress and connective tissue deposition in severe (n=25) versus mild (n=17) OSA patients.

Upper airway surgical specimens were separated by predominance of either mucosal or muscle tissue. Expression levels for Interleukin-1α, Interleukin-6, Interferon-γ, RANTES, Transforming Growth Factor-β, and L-Selectin were measured by Ribonuclease Protection Assay. Oxidative stress was assessed via protein carbonyl group detection by immunoblotting. Histochemistry was employed for immunolocalization of selected cytokines, and connective tissue morphometry.

In the severe OSA group, expression of Interleukin-1α, Interleukin-6 and Transforming Growth Factor-β was significantly higher in mucosa-predominant tissues, whereas in muscle-predominant specimens, RANTES expression was greater in severe OSA. Increased protein carbonylation was observed in severe OSA within both mucosal and muscle compartments. Immunohistochemistry localized Transforming Growth Factor-β to submucosal and perimuscular inflammatory cells, while Interleukin-6 was primarily localized to myocytes. Consistent with the pro-fibrotic cytokine profile observed in mucosa-predominant tissue, morphometric analysis revealed greater submucosal and perimuscular connective tissue in severe OSA subjects.

There is increased pro-inflammatory and pro-fibrotic cytokine expression, oxidative stress, and connective tissue deposition in upper airway tissues from severe versus mild OSA patients.

Keywords: Cytokine; Fibrosis; Inflammation; Oxidative Stress; Sleep Apnea, Obstructive
**Introduction:**

There is growing evidence that systemic (1;2) and upper airway (1;3-5) inflammation are increased in obstructive sleep apnea (OSA). Upper airway inflammation in OSA may be related to mechanical trauma and/or increased tissue oxidative stress (1)(4)(6). Inflammatory markers are increased in induced sputum, exhaled breath condensate and nasal lavage specimens in OSA (1)(3)(7)(8)(9)(10). There is also increased inflammatory cell infiltration within upper airway tissue (4)(11)(12). We reported differences in inflammatory cell subsets within the mucosal versus muscular compartments of palatal tissue in OSA (4), suggesting that the inflammatory process may differ between upper airway tissue compartments.

Altered tissue mechanics and neuromuscular function are clearly important factors in the pathophysiology of OSA (13). Exposure of upper airway tissues to pro-inflammatory mediators, such as cytokines and reactive oxygen species, could alter tissue structure and function and impair skeletal muscle contractility (14)(15)(16). Moreover, sustained exposure to certain cytokines can lead to fibrosis, which could adversely affect upper airway collapsibility through changes in tissue compliance and/or mechanical coupling of dilator muscles (12)(17)(18)(19)(20)(21). Therefore, evaluation of pro-inflammatory mediators within upper airway tissues of OSA patients may provide new insights into the mechanisms underlying inflammation as well as the potential sequelae for upper airway tissue structure and function.

The primary objective of this study was to compare levels of cytokine/chemokine expression and oxidative stress present within upper airway tissues obtained from severe versus mild OSA patients. We hypothesized that severe OSA would be associated with: 1) higher levels of pro-inflammatory and/or pro-fibrotic cytokine expression; 2) increased oxidative stress; and 3) greater connective tissue deposition, within upper airway structures. We further hypothesized that the pro-inflammatory mediator profile would differ between the mucosal versus muscular compartments of the upper airway.
Methods:

Subjects: Adults with untreated OSA scheduled to undergo upper airway surgery for OSA were recruited from the McGill University system. Subjects had either an apnea-hypopnea index of 10-25 events per h of sleep (Mild group) or > 40 events/h (Severe group) on an overnight polysomnogram performed within 8 weeks of surgery. Exclusion criteria included previous upper airway surgery (excluding remote tonsillectomy), active smoking, oral or inhaled anti-inflammatory medication, upper respiratory tract infection within 8 weeks, diabetes, any known neuromuscular disease or inflammatory condition. This study was approved by our Research Ethics Board, and informed consent was obtained from all subjects.

Polysomnography: Diagnostic polysomnography was performed as previously described either in the Sleep Laboratory (Sandman, Tyco Inc., Ottawa) (4), or at home, using the Suzanne portable complete polysomnography device (Tyco, Inc., Ottawa, Canada) (22). The same signals were recorded during both types of studies, and analysis was performed on the same computerized system (Sandman version 6.2) using identical scoring criteria. Polysomnographic recordings had to conform to “very good” or “excellent” recording quality criteria as previously described (22) and show total sleep time >4 h. Sleep-wake state and arousals were scored using conventional criteria (23)(24) and respiratory events were scored according to Chicago criteria (25). Standard measures of sleep structure, respiratory disturbance and oxygenation were derived as previously described (4)(22) for correlation with tissue findings.

Surgical Tissue Procurement: Patients underwent conventional uvulopalatopharyngoplasty (UPPP) and/or tonsillectomy. The time of surgery was dictated by the clinical operating room schedule with procurement typically occurring between 10 a.m. and 2 p.m. There was no systematic difference in a.m. vs p.m. harvesting between the mild vs. severe group. Tissues were excised via scalpel prior to use of electrocautery to avoid burn-related tissue alterations. UPPP specimens consisted of the uvula and contiguous resected palatal tissue. For tonsillectomy specimens, the resection margin included a segment of the anterior tonsillar pillar, which was dissected off the tonsil itself and harvested for analysis. The tonsillar tissue per se was not analyzed. A study technologist procured tissues in the operating theatre immediately upon
resection. Specimens were oriented in OCT embedding medium, snap-frozen in isopentane and stored at – 80°C.

*Tissue Evaluation:* Six micron frozen sections were stained with hematoxylin and eosin to determine tissue content prior to further processing. Sections with intact squamous epithelium and underlying submucosal connective tissue and mucus glands were identified as “mucosa-predominant”, while specimens with >50% muscle content were identified as “muscle predominant”, as previously described (4). A majority of mucosa-predominant samples were uvulopalatal specimens, while a majority of muscle-predominant samples were tonsillar pillars; these proportions were not significantly different for mild vs. severe groups. In addition, although it was not possible to perform all of the gene expression and biochemical analyses in every patient due to limited amounts of tissue, the clinical characteristics and tissue proportions for each subgroup analysis did not differ significantly from those observed in the mild vs. severe groups as a whole.

*Ribonuclease Protection Assay:* For RNA extraction, serial frozen sections were processed as previously described (26). Total RNA was extracted using Trizol Reagent (Invitrogen). ³²P-labeled riboprobes were synthesized using a commercial human multiprobe kit (BD Bioscience, Pharmingen, San Diego, CA) containing templates against gene transcripts for a selected series of cytokines/chemokines known to be involved in leukocyte trafficking, inflammatory responses and tissue repair and fibrosis: RANTES (CCL5), L-Selectin, Interleukin-1α (IL-1α), Interleukin-6 (IL-6), Transforming Growth Factor-β (TGF-β) and Interferon-γ (IFN-γ). The riboprobes were hybridized with each RNA sample overnight at 56°C, using 20 μg of RNA per sample. Protected RNA fragments were separated using 5% polyacrylamide gel and detected using autoradiography. Bands were then quantified using an image analysis system (FluorChem 8000; Alpha Innotech Corp, San Leandro, CA) and the signals normalized to an L32 housekeeping gene.

*Immunocytochemistry:* In order to localize the sites of cytokine expression, immunocytochemistry was performed as previously described (27) using the alkaline phosphatase technique and visualization with Fast Red (Dako, Inc). Primary antibodies (mouse
anti-human) for IL-6 (Serotec) and TGF-β (R & D Systems) were used at dilutions of 1:50 and 1:10, respectively. Two sets of control slides were processed in parallel: slides incubated with no primary antibody, and slides incubated with a primary mouse anti-human antibody to an irrelevant antigen (“isotype” control).

**Detection of Protein Carbonylation:** Formation of protein carbonyls was assessed by immunoblotting as a marker of oxidative stress as previously described (28). Serial sections of mucosa- and muscle-predominant tissues were homogenized in 6 volumes (wt/vol) of buffer (pH 7.4, 10 mM HEPES buffer, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mg/ml of phenylmethylsulfonyl fluoride, 0.32 mM sucrose, and 10 µg/ml each of leupeptin, aprotinin, and pepstatin A). Crude homogenates were centrifuged at 4°C for 15 min at 5,000 rpm. Supernatants were collected for immunoblotting. Protein carbonyls were detected using a commercial kit (Oxyblot Protein Oxidation Detection, Intergen Inc, Purchase NJ). Aliquots (15 µg) of protein were denatured with 12% SDS; protein side chains were derivatized to 2,4-dinitrophenylhydrazone by reaction with 10 µl of 1X 2,4-dinitrophe nylhydrazine (DNPH), followed by addition of 7.5 µl neutralizing solution and 2-mercaptoethanol. Derivatized proteins were separated on 12% SDS-polyacrylamide gel. Proteins were then transferred to polyvinylidene difluoride membranes, which were blocked and incubated with anti-DNP moiety antibody overnight. Following application of horse radish peroxidase-conjugated secondary antibody, densities of protein bands were quantified with an optical densitometer and the optical densities of protein bands were quantified. Loading of equal amounts of proteins was confirmed by stripping the membranes and re-probing with anti-α-tubulin antibody (Sigma-Aldrich Co.).

**Morphometric Analysis of Connective Tissue Content:** Frozen sections were stained with van Gieson’s stain for connective tissue (27). Representative fields (≥ 5 per slide) of van Gieson-stained sections were captured using the 10X objective on the microscope using a computerized image system (ImagePro Plus, Media Cybernetics, Bethesda MD). A standardized grid was superimposed on the image and point-counting was used to identify tissue content. Connective tissue content was expressed as percentage of total counts per grid, and values are reported as the mean for all grids from a given tissue specimen. Tissue elements included in counts were muscle, vessels, nerve, mucous glands, connective tissue and fat. (As fat per se was not preserved/stained
in the van Gieson sections, fat was identified in these sections as typical thin-rimmed empty vacuoles within tissue.) For muscle-predominant specimens, the connective tissue content within muscle fascicles was also analyzed separately from connective tissue in regions surrounding muscle tissue.

Statistical Analysis: Between-group comparisons were made using t-tests. Correlation analysis was performed using Pearson product correlation. A p value of < 0.05 was used for statistical significance, while a Bonferroni-type correction was applied for multiple comparisons.
**Results:**

*Subject Characteristics:* As shown in Table 1, subject characteristics were similar in the two groups, while apnea severity was significantly greater in the severe OSA group. Neither group had significant medical co-morbidities, and medications were similar in the two groups.

*Cytokine mRNA Expression:* Figure 1A shows that while detectable levels of mRNA expression were present for all cytokines studied in the mucosa-predominant compartment for both mild and severe OSA groups, the group mean values for IL-1α, IL-6, and TGF-β were significantly higher in the severe vs. mild OSA subjects. A different pattern was observed in the muscle-predominant compartment, where RANTES expression was significantly higher for severe OSA patients, while IL-1α, IL-6, and TGF-β were not significantly different between the 2 groups (Fig. 1B).

Correlation analyses were performed between polysomnographic measures of respiratory event frequency, duration and O₂ desaturation, and cytokine expression in both mucosa- and muscle-predominant tissue. A positive correlation was identified between TGF-β mRNA levels in mucosa-predominant tissue and AHI (r = 0.54, p = 0.01). No other significant correlations were identified.

*Immunohistochemistry:* Increased IL-6 expression has been shown to occur within skeletal muscle in association with increased muscle usage (29), while TGF-β upregulation is closely linked with tissue fibrosis in several diseases (30;31). Therefore, immunocytochemistry was performed for these two prototypical cytokines, as shown in Figs. 2 and 3. IL-6 staining was primarily localized to muscle fibers in either a peripheral sub-sarcolemmal or diffuse pattern (Fig. 2). In addition, some areas of sub-epithelial connective tissue also appeared to demonstrate positive immunoreactivity for IL-6, although this was not a consistent finding. As shown in Fig. 3, positive staining for TGF-β was localized to the cytoplasm of mononuclear inflammatory cells. In mucosa-predominant sections, these TGF-β positive cells were observed in the sub-epithelial connective tissue as well as in deeper connective tissue layers. Within the muscle-predominant specimens, TGF-β positive mononuclear cells were seen in the connective tissue both around and within muscle fascicles.
**Connective Tissue:** To determine the amount of connective tissue present within upper airway tissue specimens, van Gieson staining was performed. Representative van Gieson-stained sections are shown in Fig. 4, while group mean data are shown in Fig. 5. In both mucosa- and muscle-predominant specimens, there was significantly more connective tissue in severe OSA subjects. For muscle-predominant specimens, within-fascicle connective tissue content was not significantly different between the two OSA groups, while connective tissue outside of muscle fascicles (analogous to mucosa-predominant connective tissue) was significantly increased in severe OSA. Counts for other tissue elements including mucous glands, blood vessels and muscle content were not significantly different between the 2 groups. For the two groups combined, a positive correlation was identified between connective tissue content in mucosa-predominant tissue and AHI (r = 0.45, p = 0.04). No other significant correlations were identified.

**Detection of protein carbonylation:** For both mucosa- and muscle-predominant specimens, there was evidence for increased oxidative modification (carbonylation) of upper airway tissue proteins in the severe OSA group. This was revealed in the form of several prominent bands, with molecular weights of approximately 26 kD, 39 kD and 68 kD (Fig. 6). As illustrated in Fig. 7, the changes were significantly different between mild and severe OSA subjects for the 26 and 68 kDa protein bands in mucosa-predominant specimens, and for all three molecular weight species in muscle-predominant specimens.
Discussion:

Several studies have demonstrated increased inflammation in the upper airway in OSA. For instance, Series et al (12) reported increased CD4+ and CD8+ lymphocytes and macrophages, in uvular tissue from morbidly obese, but not non-obese OSA patients compared with snoring controls. We recently compared inflammatory cells within mucosal and muscular compartments of upper airway specimens from OSA patients and non-apneic controls of similar BMI (4). OSA patients had significantly more activated (CD25+) T lymphocytes within both compartments, but with differing T cell populations in the muscular (CD4+) versus mucosal (CD8+ as well as CD4+) compartments. These findings suggest that inflammation within these two compartments may be regulated by different pro-inflammatory mediators in OSA.

There is currently little published data on upper airway tissue cytokine expression in adult OSA. Loubaki et al (32) reported that TNF-α levels are increased in uvular muscle tissue from obese OSA patients compared to both non-obese OSA patients and controls. Unfortunately, that study had no obese, non-OSA controls to clarify the role of obesity. In our study, the mild and severe OSA groups were well-matched for BMI (with mean BMI closest to the non-obese OSA of Loubaki (32)), yet we found significant differences in cytokine levels between mild and severe groups.

The cytokines we selected in this study modulate leukocyte trafficking, tissue repair and fibrosis. RANTES is a chemoattractant for inflammatory cells including CD4+ lymphocytes (33), and is upregulated in skeletal muscle cells by pro-inflammatory cytokines including TNF-α, IL-1α, and IFN-γ (33). L-Selectin is an adhesion molecule involved in leukocyte migration into injured tissues (34). In vivo, expression of RANTES and other CC chemokines increases greatly after skeletal muscle injury (33)(35). In addition, cellular receptors for RANTES are found on both leukocytes and myoblasts (35), suggesting that RANTES may influence muscle regeneration (35). Thus our finding of increased RANTES expression in severe vs mild OSA could be consistent with increased upper airway muscle loading, injury and repair in severe OSA.

To our knowledge, expression of IL-6 has not previously been demonstrated in upper airway muscles of OSA patients. IL-6 production by skeletal muscle is a well-recognized normal response to loading and exercise (29). Thus our finding of IL-6 expression within muscle fibers of both groups is again consistent with loading of upper airway muscles, although we did not find significant differences between mild and severe OSA.
TGF-β has anti-inflammatory properties and is a prototypical pro-fibrotic cytokine which plays a key role in post-inflammatory fibrosis (31)(30). In our study, TGF-β expression was upregulated in severe vs. mild OSA, with immunohistochemical findings suggesting that inflammatory cells were the primary source. IL-1α and IL-6 can also promote fibrosis, whereas IFN-γ is generally considered to have anti-fibrotic effects (31)(30). Therefore, our demonstration of a “pro-fibrotic” cytokine profile (i.e., significantly increased IL-1α, IL-6 and TGF-β, but no increase in IFN-γ) in the mucosa in severe OSA is consistent with the greater upper airway connective tissue content observed in this group.

These findings suggest that ongoing inflammation and repair may lead to a form of upper airway “remodeling” with increased tissue connective tissue deposition in severe OSA. Previous studies have reported increases (17)(18) or unchanged (11) upper airway mucosal connective tissue in OSA. Disorganization of the elastic fibrillar network in uvular mucosa in severe OSA has also been described (12). In upper airway muscles, OSA has been associated with increased connective tissue in the English bulldog (36), and in human palatal (19) and pharyngeal constrictor (20), but not in uvular (37) or genioglossus muscles (37)(38). The differences in findings among studies are likely due to variations in OSA severity, tissue sampling, and the techniques applied to assess connective tissue characteristics.

Increased fibrosis within upper airway tissue could affect airway patency in several ways. Connective tissue deposition could contribute to increased wall thickness and reduced intraluminal cross-sectional area. Fibrosis could also impede mechanical coupling between upper airway contractile and non-contractile elements. Indeed, Series et al (21) showed that equivalent intensities of stimulated muscle contraction produced less uvular tissue displacement in OSA than normal (21). Furthermore, upper airway collapsibility could be adversely affected, in that analysis of tissue changes following oropharyngeal laser procedures suggests that excessive connective tissue responses are associated with OSA worsening (39).

Another potential source of upper airway dysfunction in OSA is increased oxidative stress. Increased inflammation and recurrent hypoxia-reoxygenation are both potential sources of increased oxidative stress in OSA (6)(40). NF-kappaB, which is a key transcription factor upregulated in intermittent hypoxia (6)(40) has potent pro-inflammatory effects, upregulating cytokines such as IL-1α, IL-6, and RANTES (41). Thus, the pro-inflammatory cytokine profile observed in our tissues, while not specific, is consistent with a response to increased oxidative stress.
stress. Moreover, our demonstration of increased carbonyl modification of proteins within the upper airway tissues is consistent with this scenario (28). Reactive oxygen species have a well-established ability to impair skeletal muscle force-generating capacity (15;16), and could also adversely affect upper airway function by contributing to previously described upper airway neuropathy (5).

Several limitations of our study should be acknowledged. In particular, use of clinical specimens precluded our studying 3 potentially important groups: non-obese, non-apneic normals, and morbidly obese subjects (both non-apneic and apneic). We were unable to identify non-apneic individuals among patients undergoing upper airway surgical intervention, and could not ethically biopsy tissue of this magnitude from normals. Morbidly obese patients rarely undergo upper airway surgery at our institution. Thus, while clear differences emerged between mild and severe OSA patients in our study, it may be that cytokine expression in mild OSA subjects would also differ from normals, and/or that morbidly obese subjects would show even greater inflammation. Other tissue sampling techniques and/or validation of non-invasive measures will be required to address these possibilities. Other limitations of our study are that immunostaining provides localization data but is difficult to quantify (42;43), and that only a small minority of our subjects were female, so that potential sex differences could not be addressed.

In summary, we demonstrate that severe OSA is associated with increased expression of several pro-inflammatory and/or pro-fibrotic cytokines within the mucosal and muscular compartments of upper airway tissue, with a concomitant increase in connective tissue deposition. To our knowledge, we also show the first evidence for increased oxidative stress within the upper airway muscles in severe OSA. The extent to which these abnormalities contribute to upper airway dysfunction in OSA, and their relationship to systemic and other tissue inflammatory changes in this condition, remains to be determined. However, it seems likely that these alterations would affect upper airway mechanics and function.
Acknowledgements:

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Reference List:


Table 1
Subject Characteristics and Sleep Data

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Values are Mean ± SE
Figure Legends:

Figure 1. Group mean values (± SE) for cytokine mRNA levels, normalized to housekeeping gene expression, for the severe (n = 17) versus mild (n = 10) OSA groups. Figure 1A. Mucosa-predominant tissue; Figure 1B: Muscle-predominant tissue. *p < 0.05
Figure 2: Immunohistochemical staining for IL-6. Unequivocal positive staining was localized to myocytes, while some areas of sub-epithelial connective tissue also appeared positive. The immunostaining pattern of myocytes was either peripheral (panel A) or diffuse (panels B, C). Both patterns could be observed within the same section. Panel D demonstrates the absence of myocyte staining when using an irrelevant isotype negative control antibody.
Figure 3: Immunohistochemical staining for TGF-β. Positive staining was localized to the cytoplasm of inflammatory cells. Panel A illustrates staining of cells in the sub-epithelial connective tissue layer in a mucosa-predominant specimen. Panel B shows absence of staining in the same patient with isotype negative control antibody. In muscle-predominant sections, positively staining inflammatory cells were also seen in connective tissue surrounding (panel C) or within (panel D) muscle fascicles.

Figure 4: Photomicrographs of representative van Gieson-stained sections. Panel A: Mucosa-predominant tissue specimen with counting grid over sub-epithelial area. In this particular image, 85% of counts fell on stained connective tissue. Panel B: Muscle-predominant specimen illustrating connective tissue staining within muscle fascicles and in the surrounding tissue.

Figure 5: Group mean values (± SE) for connective tissue counts in van Gieson-stained mucosa- and muscle-predominant sections. Mucosal specimens: mild (n = 10); severe (n = 17); Muscle specimens: mild (n = 8); severe (n = 10).*p <0.01
Figure 6. Representative immunoblot (OxyBlot) for carbonylated proteins from muscle-predominant samples, indicating the presence of carbonylated protein bands which are increased in the severe OSA group. Equal protein loading between lanes was verified using α-tubulin as a reference protein standard.
Figure 7. Group mean optical density values (± SE) for carbonylated protein bands in severe versus mild OSA subjects (n = 5 per group) for mucosa- and muscle-predominant tissues specimens. *p < 0.05