Pepsin as a Causal Agent of Inflammation during Nonacidic Reflux

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ABSTRACT

Objective
To investigate the contribution of pepsin to inflammation attributed to nonacidic gastric reflux via analysis of inflammatory cytokine and cytokine receptor gene expression in pepsin-treated human hypopharyngeal epithelial cells *in vitro*.

Methods
Human hypopharyngeal epithelial cells were incubated with or without pepsin (0.1mg/ml) at pH7.4, 37ºC, overnight. Expression of 84 inflammatory cytokines and cytokine receptors was analyzed via RT2 qPCR array.

Results
Expression of a number of inflammatory cytokines and receptors was altered in human hypopharyngeal epithelial cells following overnight treatment with pepsin at neutral pH. >1.5-fold change in gene expression was detected for CCL20, CCL26, IL8, IL1F10, IL1A, IL5, BCL6, CCR6 and CXCL14 (p<0.05).

Conclusion
Exposure of hypopharyngeal cells to pepsin in a nonacidic environment induces the expression of several pro-inflammatory cytokines and receptors, including those known to be involved in inflammation of esophageal epithelium in response to reflux and which contribute to the pathophysiology of reflux esophagitis. These data indicate that refluxed pepsin may contribute to laryngeal inflammation associated with nonacidic gastric reflux including that experienced by patients undergoing maximal acid suppression therapy.

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INTRODUCTION

●Common clinical manifestations and endoscopic findings of laryngopharyngeal reflux (LPR) are primarily attributed to mucosal inflammation[1].

●Inflammation and other immune responses initiated within airway mucosa are a fundamental determinant of tissue damage during reflux and are predicted to give rise to the diverse phenotypes characteristic of reflux-attributed disease[2].

● Frequency, duration, and volume of refluxate vary widely relative to the severity of reflux disease[3]. The expression profile of proinflammatory cytokines within esophageal mucosa, however, is highly correlative with severity of gastroesophageal reflux disease (GERD)[4,5].

● Investigation of the molecular events triggering inflammation during LPR has thus far focused on a limited number of proinflammatory molecules or attempted to measure the response of gene expression to acid suppression medication, which has been shown to have limited efficacy in the treatment of LPR[6].

● Weakly and nonacidic gastric reflux is associated with persistent symptoms observed in as many as 20% of acid-suppressed patients[7]. Nonacid components of gastric refluxate, such as pepsin and bile, are predicted to underlie these symptoms.

● Our previous work indicates that in a nonacidic environment pepsin is endocytosed leading to altered gene expression and cell damage[8]. Therefore, we predict that pepsin may alter inflammatory gene expression and contribute to laryngoscopic findings and symptoms associated with weakly and nonacidic LPR.

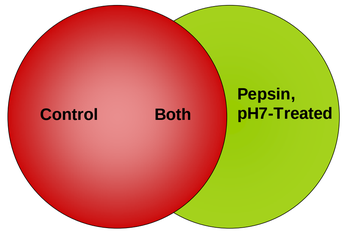
● The aim of this study was to investigate the potential of pepsin to contribute to mucosal inflammation, and thereby mucosal damage, via change in cytokine and receptor gene expression during nonacidic extra-esophageal reflux.

● Expression of 84 inflammatory cytokine and receptor genes was compared in human hypopharyngeal epithelial cells treated with and without pepsin at neutral pH *in vitro* via real-time PCR array technology.

MATERIALS & METHODS

Cell Culture
FaDu cells, a human epithelial cell line derived from hypopharyngeal squamous cell carcinoma (ATCC, Manassas, VA), were grown in Eagle's Minimum Essential Medium (ATCC, Manassas, VA) containing 10% Fetal Bovine Serum, (ATCC, Manassas, VA) to a density of 70% confluency. Cells were incubated at 37°C overnight with normal growth media or media containing 0.1mg/ml porcine pepsin (Sigma, St. Louis, MO). Cells were washed in PBS, harvested, snap frozen, and stored at -80ºC until use.

Table 1. Cytokine and Receptor Expression of Control and Pepsin-treated Human Hypopharyngeal Cells



Expressed in Both Control and Pepsin-Treated Cells
ABCF1,BCL6,C3,C4A,C5,CCL20,CCL24,CCL26,CCL5,CCR6 ,CCR7,CEBPB,CXCL1,CXCL11,CXCL14,CXCL2,CXCL3, IL10RB,IL13RA1,IL1A,IL1B,IL1F10,IL1F5,IL1F7,IL1F9,IL1R1, IL1RN,IL5,IL8,IL8RB,LTA,LTB4R,MIF,SCYE1,TOLLIP
Expressed Only in Control Cells
ICEBERG, IL17C
Expressed Only in Pepsin-Treated Cells
CCR9, CXCL10, LTB, TNF
Not Detected in Control or Treated Cells
CCL1,CCL11,CCL13,CCL15,CCL16,CCL17,CCL18,CCL19, CCL2,CCL21,CCL23,CCL25,CCL3,CCL4,CCL7,CCL8,CCR1, CCR2,CCR3,CCR4,CCR5,CCR8,CRP,CX3CR1,CXCL10, CXCL12,CXCL13,CXCL5,CXCL6,CXCL9,IFNA2,IL10,IL13, IL1F6,IL1F8,IL22,IL5RA,IL8RA,IL9,IL9R,SPP1,CD40LG, XCR1

RNA Isolation and Reverse Transcription
RNA was isolated from treated FaDu cells as directed using the SuperArray RT2 qPCR-Grade RNA Isolation Kit (SuperArray, Frederick, MD). RNA concentration and quality were assessed by UV spectroscopy and agarose gel electrophoresis. RNA was stored at -80ºC until use. RNA was reverse transcribed using the SuperArray RT2 First Strand Kit (SuperArray, Frederick, MD) and stored at -80ºC up to one day until use.

RT2 qPCR Array
FaDu cell cDNA was diluted in RT2 SYBR Green/Fluorescein qPCR Master Mix (SuperArray, Frederick, MD) as directed and aliquoted into a 96-well RT2 Profiler PCR Array (Human Inflammatory Cytokines & Receptors RT² Profiler™ PCR Array, SuperArray, Frederick, MD). Real-time PCR was performed in an iCycleriQ Multicolor Real Time PCR Detection System (Bio-Rad Life Science, Hercules, CA). Real-time PCR conditions were 95°C for 10m and 40 cycles of 95°C for 15s and 60°C for 1m, immediately followed by a melt curve of 95°C for 1m, 65°C for 2m, and sixty 10s-cycles of 0.5°C increase. Three biological replicates were performed for each condition.

Data Analysis
Samples exhibiting more than one peak within the melt curve or Ct > 35 were excluded from analysis. The means of the housekeeping genes for each replicate was subtracted from each gene value to normalize the data. Differential expressionwas evaluated by fitting a mixed model to the entire data set with fixed gene and gene-treatment interaction effects, and random intercept and treatment effect for each biological replicate. The p-values for the gene-specific treatment effects were adjusted using the Benjamini-Hochberg procedure using a 5% cutoff.

Table 2. Change in Gene Expression of Human Hypopharyngeal Cells Exposed to Pepsin at Neutral pH

Unigene ID	Symbol	Description	Fold Change	p-value
Positively regulated by pepsin				
Hs.75498	CCL20	Chemokine (C-C motif) ligand 20	6.12	<.0001
Hs.131342	CCL26	Chemokine (C-C motif) ligand 26	3.68	0.0001
Hs.624	IL8	Interleukin 8	3.35	0.0004
Hs.306974	IL1F10	Interleukin 1 family, member 10 (theta)	3.05	0.0192
Hs.1722	IL1A	Interleukin 1, alpha	2.65	0.0039
Hs.2247	IL5	Interleukin 5 (colony-stimulating factor, eosinophil)	2.31	0.0128
Hs.478588	BCL6	B-cell CLL/lymphoma 6 (zinc finger protein 51)	2.02	0.0364
Negatively regulated by pepsin				
Hs.46468	CCR6	Chemokine (C-C motif) receptor 6	0.39	0.0057
Hs.81134	IL1RN	Interleukin 1 receptor antagonist	0.53	0.0576
Hs.483444	CXCL14	Chemokine (C-X-C motif) ligand 14	0.47	0.0253

RESULTS

● Of 84 cytokine and receptor genes analyzed, 49 were excluded from analysis due to C_t>35. 37 genes were detectable (C_t $^{>}/_{=}$ 35 in 2 of 3 replicates) in untreated cells, 39 were detectable in treated cells, 35 were detectable in both, and 43 were undetectable (C_t $^{<}/_{=}$ 35 in 2 of 3 replicates) in either (see Table1).

● Expression of CCR9, CXCL10, LTB, and TNF was detectable in pepsin-treated but not in untreated cells (see Table1).

● IL17C and ICEBERG expression was detected in untreated but not in pepsin-treated cells (see Table1).

● Expression of 7 genes was increased by >1.5-fold (p<0.05) relative to control cells incubated overnight without pepsin: CCL20, CCL26, IL8, IL1F10, IL1A, IL5, BCL6 (see Table2).

● Expression of two genes was reduced >1.5-fold (p<0.05) relative to control: CCR6 and CXCL14 (see Table 2).

DISCUSSION

● Pepsin induced hypopharyngeal cell expression of members of the prototypical proinflammatory IL1 and TNF cytokine gene families while reducing expression of a competitive antagonist of IL1 signaling, IL1 receptor antagonist (IL1RN), trending towards significance (p=0.058).

●Similarly, IL1 family member IL1β is elevated in esophageal biopsies of patients with reflux esophagitis (RE)[3,9] and TNF and IL1 are elevated in the esophageal mucosa of patients with non-erosive esophageal reflux disease (NERD)[10].

●In contrast, progression of GERD to Barrett's esophagus, the intestinal dysplasia of esophageal epithelia characterized by plasma and mast cell infiltration rather than inflammation, is associated with expression of anti-inflammatory IL10 and the humoral response (Th2) cytokine IL4[3].

● The two most highly elevated mRNAs in hypopharyngeal cells exposed to pepsin, CCL20 and CCL25, are Th2 cytokines and potent chemoattractants of lymphocytes. Th2 cytokines CCL20 and BCL6 are also increased in the esophageal mucosa of a rat model of RE as well as in NERD patients[11,10].

● Interestingly, CD8+ T lymphocytes, a subset of which express the CCL20 receptor, CCR6, are significantly increased in the luminal layer of the laryngeal epithelium of LPR patients relative to controls[12].CCR6, the sole receptor for CCL20, was significantly reduced in hypopharyngeal cells exposed to pepsin, which may serve to enhance the efficiency of recruitment of CCR6-expressing lymphocytes to mucosa exposed to refluxate *in vivo*.

● Pepsin also induced hypopharyngeal cell expression of the strong neutrophil chemoattractant IL8. IL8 expression is closely correlated with the severity and progressio n of GERD:

-expression is significantly higher in esophageal biopsies of NERD relative to control and in RE relative to NERD patients[5],
-decreases in patients with Barrett's esophagus relative to those with RE[3],
-parallels grading of disease severity (Los Angeles classification) of RE and NERD[5,13],
-and is reduced upon esophageal healing and symptom resolution following treatment with the proton pump-inhibitor lansoprazole[14].

IL8 expression may also be an indicator of disease relapse, as RE recurrence is more frequently observed in patients with higher levels of esophageal IL8 protein[6].

CONCLUSIONS

● Increased expression of IL8 and other proinflammatory cytokines by hypopharygeal cells exposed to pepsin in a nonacidic environment adds to the existing body of literature which suggests that pepsin, and endocytosis thereof, plays a key role in mucosal injury attributed to LPR, including weakly and nonacidic LPR.

● The observations presented here, in conjunction with our previous findings of pepsin in middle ear biopsies of otitis media patients[14] and subglottic biopsies from patients with subglottic stenosis (unpublished observations) suggests that adherence and endocytosis of refluxed pepsin by various regions of the respiratory mucosa contributes to numerous inflammatory diseases of the upper airway.

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