Impact of Gastro-esophageal Reflux on Mucin mRNA Expression in the Esophageal Mucosa

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Abstract

Introduction Changes in the expression of mucin genes in the esophageal mucosa associated with uncomplicated gastro-esophageal reflux disease have not been evaluated even though such changes could be associated with reflux-induced mucosal damage. We therefore sought to identify reflux-induced changes in mucin gene expression using a cell line and biopsies from the esophageal mucosa in patients with and without reflux.

Methods MUC-1, MUC-3, MUC-4, and MUC-5AC gene expressions were investigated in the HET-1A cell line following exposure to acid (pH 4) and/or bile (120 µM of a bile salt milieu), and in esophageal mucosal biopsies from controls, subjects with non-erosive gastro-esophageal reflux, and subjects with reflux associated with ulcerative esophagitis (erosive). The mucosal biopsies were also evaluated for IL-6 mRNA expression (inflammatory marker) and CK-14 mRNA expression (mucosal basal cell layer marker). Gene expression was determined using real-time reverse transcriptase-polymerase chain reaction analysis.

Results In the cell line studies, there were differences in mRNA levels for all of the evaluated mucins following treatment with either acid or the acid and bile combination. In the studies which evaluated tissue specimens, IL-6 and CK-14 mRNA levels increased according to degree of reflux pathology. The expression of MUC-1 and MUC-4 in mucosa from patients with erosive reflux was lower than in subjects without reflux and in patients with non-erosive reflux, whereas the expression of MUC-3 and MUC-5AC was increased (although these differences did not reach significance at $p<0.05$). When mRNA expression data for tissue samples from all groups were combined, significant correlations were identified between IL-6 vs. CK-14 and IL-6 vs. MUC-3, MUC-3 vs. CK-14 and MUC-3 vs. MUC-5AC, and for MUC-1 vs. MUC-5AC. The correlation between IL-6 and CK-14 was also significant within the control and non-erosive reflux groups. The correlation between IL-6 and MUC-3 was significant within the control and erosive reflux groups, and the correlation between MUC-1 and MUC-5AC was significant within the erosive reflux group.

Conclusions The results of this study suggest that the profile of mucin expression in the esophageal mucosa is influenced by the pH and composition of the gastro-esophageal reflux. Further work should explore the response of these genes to acid and bile reflux, and their role in the etiology of mucosal damage in gastro-esophageal reflux.

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Introduction

Gastro-esophageal reflux disease is a common problem that affects up to 50% of Western populations. It occurs when excessive quantities of gastric contents, with or without duodenal contents, reflux into the esophageal lumen. This is characterized by an inflammatory response in the esophageal mucosa, and in some patients who experience prolonged pathological reflux, other pathology such as columnar metaplasia (Barrett's esophagus) or adenocarcinoma can arise. Some patients with gastro-esophageal reflux have a microscopically visible esophageal mucosal injury, but no endoscopically visible mucosal ulceration, whereas other patients with reflux have endoscopic evidence of ulcerative esophagitis. Notably, duodeno-gastro-esophageal reflux is more common in patients who have ulcerative esophagitis, i.e., erosive reflux disease, compared to patients who have non-erosive reflux disease.[1] It is believed that the combination of acid and bile in the refluxate could be responsible for a more severe mucosal injury, and this probably contributes to the development of mucosal ulceration.[2] Some patients with reflux will progress to Barrett's esophagus, which is the only known precursor lesion to esophageal adenocarcinoma.[3]

The normal esophagus is not exposed to excessive amounts of gastric acid or bile, and it lacks a viscous adherent mucus gel barrier.[4] The esophageal submucosal glands do, however, secrete soluble mucus to aid in lubrication,[5] and in response to excessive exposure to acid, the esophageal mucosa secretes increased quantities of viscous mucus. This response is impaired in patients with ulcerative esophagitis.[6,7] The main components of the mucus layer are large glycoproteins: the so-called mucins (MUC),[8] which are produced by specialized epithelial cells. Mucin genes are expressed throughout the human gastrointestinal tract (including the esophageal epithelium) in a site-specific manner.[9] Advances in the detection of the expression of mucin genes and the proteins they produce have provided new insights into the role of the mucus layer and its potential relevance to gastrointestinal disease.[8] For instance, MUC-3 mRNA expression is much stronger in biopsies collected from Barrett's esophagus mucosa, yet virtually absent in normal mucosal biopsies.[10] It is certainly possible that changes in the expression of mucin genes could be associated with the development of the mucosal damage caused by gastro-esophageal reflux, and even its progression to Barrett's esophagus.

The expression of mucin genes has not been evaluated in the context of gastro-esophageal reflux disease in patients who do not have Barrett's esophagus, even though this uncomplicated reflux almost certainly precedes the development of metaplasia in the reflux–Barrett's esophagus–adenocarcinoma sequence. Furthermore, changes in mucin

mRNA expression, if present, might provide clues to biological processes associated with reflux-induced esophageal mucosal damage, and it is also conceivable that alterations in mRNA expression in the esophageal squamous epithelium might precede the development of intestinal metaplasia. Furthermore, alterations in mucin mRNA expression might also provide specific biomarkers for testing the efficacy of treatments for gastro-esophageal reflux.

For these reasons, we sought to identify changes in mucin gene expression which are associated with gastro-esophageal reflux, using an esophageal mucosal cell line and esophageal mucosal biopsies from patients with and without reflux.

Methods

Mucin gene expression was initially investigated in an esophageal cell line which was exposed to acid and bile, and then in a panel of biopsies taken from control subjects, subjects with non-erosive gastro-esophageal reflux disease, and subjects with gastro-esophageal reflux disease associated with ulcerative esophagitis. The specific genes of interest were MUC-1, MUC-3, MUC-4, and MUC-5AC. When examining the mucosal biopsy specimens, we also evaluated IL-6 mRNA expression, as this has previously been shown to correlate with reflux-induced mucosal inflammation,[11–13] and we evaluated CK-14 mRNA expression, an increase of which has been shown to correlate with hyperplasia of the basal cell layer of the esophageal mucosa, i.e., one of the earliest reflux-induced histopathological changes.[14–16]

Cell Culture and Acid/Bile Treatment

The HET-1A cell line was used to model the response of normal human esophageal epithelium to acid and/or bile exposure. This is a keratinocyte cell line that was derived from the esophagus of a human male and then immortalized with SV-40 large T antigen.[17] HET-1A cells were seeded at a density of 10^5 cells/well (in 2 mL LHC-9 medium) in six-well plates. The cells were cultured for 2 days until they reached approximately 40–50% confluence. At this stage, the cells were subjected to 3 days of pulsatile acid and/or bile treatment, with three 5-min periods of exposure per day, each pulse separated by approximately 4 h. The acid pulse medium consisted of LHC-9 medium acidified to pH 4 using hydrochloric acid (HCl). The bile pulse medium consisted of LHC-9 medium adjusted to pH 7 using HCl and containing 120 μM of a bile salt milieu. The bile salt milieu contained glycocholate, taurocholate, glycocheno-deoxycholate, taurochenodeoxycholate, glycodeoxycholate,

and taurodeoxycholate (Sigma-Aldrich®, USA) in a molar ratio of 20:3:15:3:6:1. This concentration and molar ratio mimics the median seen in reflux esophagitis.[18] The acidified bile medium consisted of LHC-9 medium adjusted to pH 4 containing the previously mentioned molar concentrations of bile milieu. After each 5-min period of exposure, the cells were rinsed with untreated LHC-9 medium, this was discarded, and then 2 mL of untreated LHC-9 medium was added to the cells. After the last 5-min period of exposure on day 3, the cells were left for 3 h to recover and then lysed in 1 mL of TRIzol® (Invitrogen, USA) for RNA extraction. The cells were approximately 90% confluent at this time. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was performed for all mucin genes (as described below). Experiments were repeated a minimum of three times.

Subjects and Tissue Collection

Since 2004, as part of an ongoing tissue collection protocol, patients who are undergoing upper gastrointestinal endoscopy for a range of clinical conditions have been invited to provide additional endoscopic biopsy samples for laboratory research. For the current study, we selected individuals from whom we had collected fresh tissue samples who also met the following criteria:

1. Normal subjects ($n=12$)—Individuals who at endoscopy had a visibly normal esophageal mucosa, no other endoscopic indicators of gastro-esophageal reflux disease, and no symptoms (or previous history) of gastro-esophageal reflux disease.
2. Non-erosive reflux disease ($n=15$)—Individuals who at endoscopy had no evidence of ulcerative esophagitis, but had typical symptoms of gastro-esophageal reflux disease (heartburn and regurgitation), and at endoscopy they had evidence of mechanical incompetence of the gastro-esophageal junction—Hill grade III or IV.[19]
3. Erosive reflux disease ($n=12$)—Individuals who had typical symptoms of gastro-esophageal reflux disease (heartburn and regurgitation), and mucosal ulceration (ulcerative esophagitis—Savary Miller grade I to IV) was visible at endoscopy.

The biopsies used for this study were taken from the distal esophagus, 5 cm proximal to the squamo-columnar junction. Biopsies were placed immediately into RNA-later® (Ambion, USA) for storage using the manufacturer's protocol. The biopsies were stored at −20°C until required for this study. Biopsies from metaplastic columnar mucosa from five additional patients with non-dysplastic Barrett's esophagus were also analyzed for comparative purposes. In samples from these patients, Barrett's esophagus with

intestinal metaplasia was verified by ABPAS/D histochemical staining and histopathological evaluation. Biopsies from the duodenal mucosa and proximal gastric mucosa were obtained from three further subjects, and these samples served as positive control tissues for the expression of intestinal and gastric mucins, respectively.

All biopsies were removed from storage and thawed. A small piece (20% to 30%) of the biopsy tissue was removed from each biopsy sample, placed in formalin, and then assessed using routine histochemical and histopathological methods to ensure that the tissue of origin was correctly identified and that it consisted only of esophageal squamous epithelium. The remainder of the biopsy was used for gene expression analysis. Tissue collection was approved by the Flinders Clinical Research Ethics Committee and the Repatriation General Hospital Clinical Research Ethics Committee.

RNA Extraction and Reverse Transcription

Following removal of 20% to 30% of each tissue biopsy for histopathology, the remainder of the biopsy was transferred to a 1.5-mL snap-top tube containing 500 μL of TRIzol® (Invitrogen Life Technologies, NY, USA). Tissue was homogenized using a plastic pestle attached to a Dremmel® MultiPro™ drill, and total RNA was extracted according to the manufacturer's protocol. The concentration of RNA was determined using a Biophotometer (Eppendorf®, North America Inc, Westbury, USA). RNA quality was determined by electrophoresis through a 1% agarose gel. All RNA samples were confirmed to be undegraded by visualization of distinct 28S and 18S rRNA species. The final RNA solution was stored at −80°C until required for cDNA synthesis.

DNAse treatment of total RNA was performed prior to reverse transcription in order to minimize PCR signal arising from carry over genomic DNA. The Ambion DNAfree™ kit was used. To 1 μg of each RNA sample (i.e., 5 μL of 200 ng/μL RNA), 2 μL of sterile water, 1 μL 10× DNase I buffer, 1 μL tRNA (2.5 μg/μL), and 1 μL r-DNAse I were added. After a quick spin, the samples were incubated for 30 min at 37°C in an Eppendorf® Mastercycler. Two microliters of DNAse inactivation reagent was added to a total volume of 12 μL in each tube, and the samples were centrifuged at 10,000 rpm for 5 min.

A new set of tubes was prepared, which contained 1 μL dNTPs (10 mM each in stock), 1 μL pd(N)6 (250 ng/μL stock), and 10 μL of the corresponding centrifuged RNA (equal to 1 μg) per sample. These samples were incubated at 65°C for 5 min then incubated on ice for at least 1 min. A second mastermix was added, which contained 4 μL 5× first strand buffer, 1 μL 0.1 M DTT, 2 μL sterile water, and 1 μL Superscript III™ RT (200 U/μL; Invitrogen™, USA)

per sample. Reactions included a reverse transcriptase negative control sample, i.e., containing 1 μL sterile water instead of 1 μL Superscript III RT enzyme. Reverse transcription was performed in an Eppendorf® Master-cycler, with an initial incubation at 25°C for 5 min, followed by incubation at 50°C for 60 min. The reaction was terminated by heating at 70°C for 15 min.

Real-time Polymerase Chain Reaction

Real-time PCR was performed using 1/130th of the cDNA reaction (all amplicons except MUC3 and MUC5AC), or 1/13th of the cDNA reaction (MUC3 and MUC5AC). PCR amplification was performed in 20-μL final volumes containing 3 μL of cDNA template, 2 μL of each forward and reverse primer, 3 μL of sterile water, and 10 μL of 2× Quantitect SYBRGreen Master Mix (Qiagen, Germany). Thermocycling utilized a Rotorgene 6000 cycler (Corbett, Australia) with an initial denaturation at 95°C for 15 min, followed by 45 cycles with different annealing temperatures for every amplicon (Table 1), and with a final extension at 72°C for 4 min. Triplicate reactions were performed on all samples. PCR products were verified by nucleotide sequencing.

Cycle thresholds were determined using the relative quantitation analysis module in the Rotorgene 6000 Series software (Corbett Research, Australia). The amplification efficiency of each primer pair was estimated from a real-time PCR dilution curve generated using serial twofold dilutions of genomic DNA, and 95% confidence intervals for the amplification efficiencies were estimated from the mean least squares slope of the standard curves and their

associated standard errors. Quantitative real-time RT-PCR analysis was then performed using Q-Gene software,[20,21] with the amplification efficiency confidence intervals applied to the relative concentration analyses of both the genes of interest and the housekeeping gene. Gene of interest expression data was normalized by dividing by the corresponding levels of beta-actin for each sample. Expression values for the tissue samples were set relative to expression in the HT-29 colon adenocarcinoma cell line, and the HET-1A cell line samples were set relative to gDNA. In cases where more than one biopsy per patient was analyzed, the results were averaged.

Statistical Analysis

Dot plots of gene expression from the normal, non-erosive reflux disease, and erosive reflux disease groups were generated, and statistical analyses were performed using KaleidaGraph® (Synergy Software). Apparent reflux-related changes in gene expression between these three groups were assessed for statistical significance using the Kruskal–Wallis rank sum test with a post hoc Bonferroni adjustment. Spearman rank sum correlations between the expression levels of two markers were calculated using the online program Spearman Rank Sum Correlation Tester (http://www.wessa.net/rankcorr.wasp).

Results

Mucin Expression in Cell Lines: Acid and/or Bile Treatments

Figure 1 shows the results of a typical HET-1A acid and bile treatment experiment performed in triplicate. Compared with the pH 7 control, mean MUC-1 and MUC-4 mRNA levels were raised (but this response was variable across triplicates for MUC-1) following treatment with either bile alone or acid (pH 4) alone (Fig. 1a and c). However, the combined treatment with both acid and bile was followed by a decrease in MUC-1 and MUC-4 mRNA. MUC-3 expression was markedly higher in cells treated with either acid or the acid and bile combination (Fig. 1b). Responses varied within triplicates for the pH 4 treatment group of MUC-3. MUC-5AC mRNA levels were also increased following treatment with either acid or the acid and bile combination (Fig. 1d), although MUC-5AC mRNA levels were lower following treatment with the acid and bile combination compared to treatment with the acid only. Overall, there were differences in mRNA levels for all of the evaluated mucins following treatment of the cells with either acid or the acid and bile combination.

Table 1 PCR Conditions and Primer Sequences

Amplicon	Primer sequence	Annealing temperature (°C)
MUC-1	F GGTGGCAGCAGCCTCTCTTA	55
	R CCCTGAAGAACCTGAGTGGA	
MUC-4	F GGCCAGGTTCTCCTATTTCC	54
	R TCCAGTCTCCCAAAAGCAAT	
MUC-3	F CGCTCCCACGGGCTATGAAG	60
	R AGAACGCACTGGCCCTGGTGA	
MUC-5AC	F CTGAGGGTCTCAGGAATGACGC	60
	R TGTACTGAGGAGGGGAGCGC	
IL-6	F GCAATAACCACCCCTGACC	55
	R TAAAGCTGCGCAGAATGAGA	
CK-14	F ACGATGGCAAGGTGGTGT	54
	R GGGATCTTCCAGTGGGATCT	
β-actin	F TTGCCGACAGGATGCAGAAG	59
	R GCCGATCCACACGGAGTACT	

Figure 1 MUC-1 (**a**), MUC-3 (**b**), MUC-4 (**c**), and MUC-5AC (**d**) expression in HET-1A cells following exposure to acid and bile. Data is expressed as mean ± SEM.
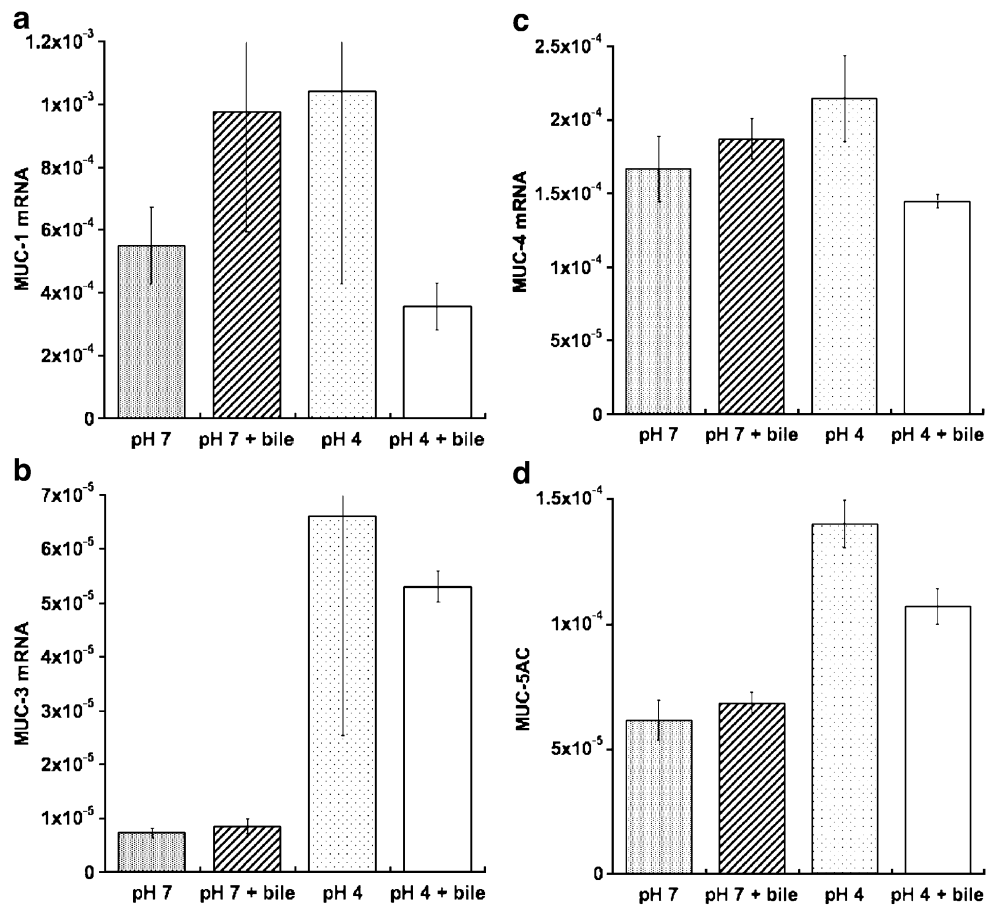
IL-6 Expression in Esophageal Mucosal Biopsies

IL-6 mRNA levels in biopsies from the esophageal mucosa in patients without reflux, patients with non-erosive reflux disease, and patients with erosive reflux disease were measured, and the results are shown in Fig. 2a. Generally, levels increased according to the degree of reflux pathology, i.e., consistent with increasing inflammation. The median values were 1, 3, and 7 for the normal, non-erosive reflux, and erosive reflux groups, respectively. Comparison of the three groups showed significant differences in IL-6 mRNA levels ($p=0.010$, Kruskal–Wallis test). Post hoc testing demonstrated significant differences between the normal and non-erosive reflux groups ($p=0.006$) and the normal and erosive reflux groups ($p=0.013$). There was no significant difference between the non-erosive and erosive reflux groups ($p=0.526$).

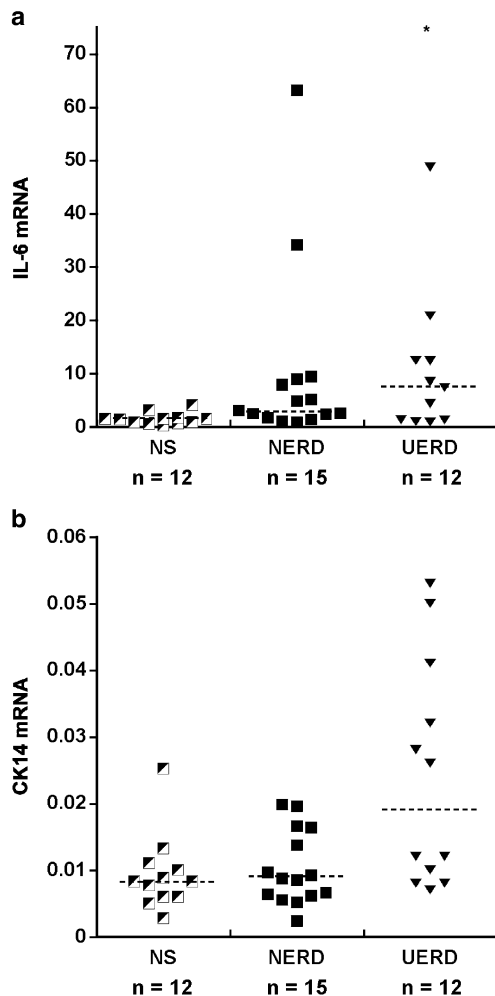## CK-14 Expression in Esophageal Mucosal Biopsies

Figure 2b depicts the sample values and medians for CK-14 mRNA levels in the three groups. The levels of CK-14 were similar in the samples from patients without reflux vs. patients with non-erosive reflux (median=0.008 and 0.009, respectively). The levels in the samples from

patients with erosive reflux were increased threefold (median=0.024, $p=0.02$ Kruskal–Wallis test). Post-testing showed no significant difference between the normal and non-erosive reflux groups ($p=0.591$), but significant differences between the normal and erosive reflux groups ($p=0.014$) and between the non-erosive and the erosive reflux groups ($p=0.019$).

Mucin Expression in Squamous Epithelium from Control and Reflux Subjects

The expressions of MUC-1, MUC-3, MUC-4, and MUC-5AC measured in the mucosal biopsy specimens from patients without reflux, patients with non-erosive reflux disease, and patients with erosive reflux disease groups are summarized in Fig. 3. While the mean and median values for MUC-1 (Fig. 3a) and MUC-4 (Fig. 3c) expression in mucosa from patients with erosive reflux were lower than in patients without reflux and in patients with non-erosive reflux, the differences in MUC-1 and MUC-4 expression for the three groups were not statistically significant ($p=0.055$ and $p=0.133$, respectively, Kruskal–Wallis test). Similarly, the increases in mean MUC-3 (Fig. 3b) and MUC-5AC (Fig. 3d) expression for the non-erosive reflux vs. no reflux groups and for the erosive reflux vs. no reflux

Figure 2 IL-6 (**a**) and CK-14 (**b**) mRNA mRNA levels in esophageal mucosal biopsies from controls (*NS*), patients with non-erosive reflux (*NERD*), and erosive reflux (*UERD*). All data points shown and median for each group (*asterisk*, outlying data point).

groups were also not statistically significant ($p=0.077$ and $p=0.390$, respectively, Kruskal–Wallis test).

Correlations Between Gene Expression Levels

Correlation between the expression levels for different genes was tested for all possible combinations using the Spearman rank order correlation test. When mRNA expression data for all tissue samples from all three groups were combined, significant correlations were identified between IL-6 vs. CK-14 and IL-6 vs. MUC-3, MUC-3 vs. CK-14 and MUC-3 vs. MUC-5AC, and for MUC-1 vs. MUC-5AC. The Spearman rho values for these comparisons are shown in Table 2. Correlations which did not reach statistical significance for any combination are not shown. Correlations for all three tissue groups combined have 37 degrees of freedom. The significant correlations from the larger combined group of samples were further investigated to determine if the correlation was confined to

one or more tissue subgroups (also shown in Table 2). Once separated into the separate groups, there were 13 degrees of freedom for results from the non-erosive reflux group and 10 degrees of freedom for results from each of the non-reflux and erosive reflux groups.

The IL-6 vs. CK-14 correlation was only significant for the non-reflux and the non-erosive reflux groups. A significant correlation between IL-6 and MUC-3 was present for the non-reflux and the erosive reflux groups. For MUC-1 vs. MUC-5AC, the correlation was only significant for the erosive reflux group. The MUC-3 vs. CK-14 and the MUC-3 vs. MUC-5AC correlations were not significant when analyzed for specific groups.

Mucin Expression in Gastric, Duodenal, and Barrett's Esophagus Epithelium

Esophageal biopsies from patients without reflux, as well as esophageal biopsies from patients with Barrett's esophagus, mucosal biopsies from the proximal stomach, and duodenal mucosal biopsies were tested for mRNA levels of MUC-1, MUC-3, MUC-4, and MUC-5AC to validate the assays for the expression of these genes. Mean mucin mRNA levels, according to tissue type, are depicted in Fig. 4. MUC-1 expression was increased threefold in biopsies from Barrett's esophagus mucosa and approximately 15-fold in proximal gastric mucosa, compared with esophageal biopsies from patients without reflux (Fig. 4a). MUC-4 mRNA levels are shown in Fig. 4c. All tissue types demonstrated a decrease in expression compared to esophageal biopsies from patients without reflux. Figure 4b shows that MUC-3 expression levels are higher in proximal gastric mucosa, much higher in Barrett's esophagus mucosa and greatest in duodenal mucosa, compared with esophageal biopsies from patients without reflux. Figure 4d indicates that MUC-5AC expression was increased in Barrett's esophagus mucosa and even more so in proximal gastric mucosa and that MUC-5AC was not expressed in duodenal epithelium.

Discussion

Mucin gene function is important for the integrity of mucosal cell function. These genes can be classified into two main families: (1) mucins which are secreted and may participate in mucus gel formation (including MUC-2, MUC-5AC, MUC-5B, and MUC-6), and (2) membrane-bound or signaling mucins (including MUC-1, MUC-3, and MUC-4). Membrane-bound mucins are involved in initiating or modulating intracellular signals and are important for cell function.[22,23] Mucin genes MUC-1 to MUC-6 have all been shown to be expressed in some or all of the following tissues: normal esophageal mucosa, metaplastic columnar

Figure 3 MUC-1 (**a**), MUC-3 (**b**), MUC-4 (**c**), and MUC-5AC (**d**) mRNA levels in esophageal mucosal biopsies from controls (*NS*), patients with non-erosive reflux (*NERD*), and erosive reflux (*UERD*). All data points shown and median for each group (*asterisk*, outlying data point).
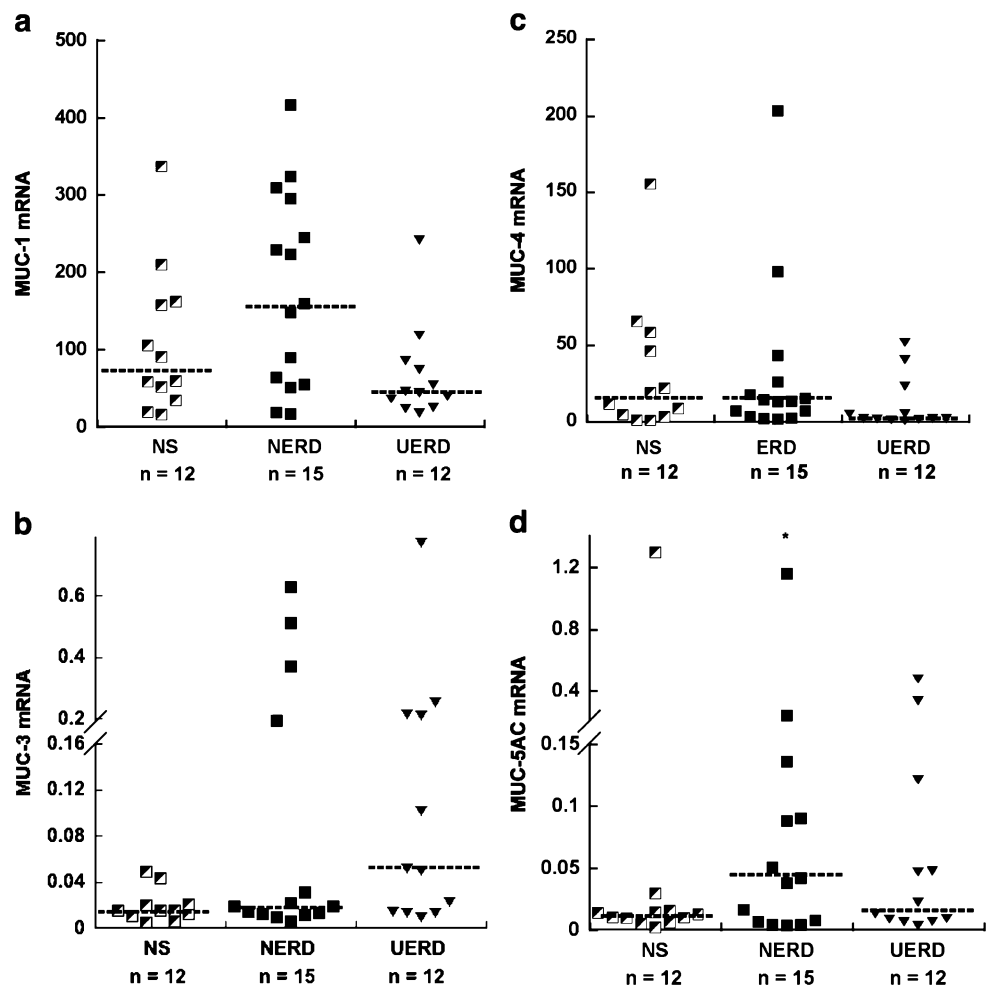
esophageal mucosa (with or without intestinal metaplasia), and in esophageal adenocarcinoma.[10,24–26] However, to date, the function of these genes in the context of uncomplicated gastro-esophageal reflux disease, either erosive or non-erosive, has not been investigated. This is perhaps surprising because gastro-esophageal reflux is the precursor of intestinal metaplasia (Barrett's esophagus), and this is the major risk factor for the development of esophageal adenocarcinoma. Hence, early changes in the way in which genes function in the esophageal mucosa in the presence of pathological gastro-esophageal reflux might help us to understand how metaplasia occurs and perhaps allow us to eventually identify individuals who are at risk of progression to Barrett's esophagus and eventually cancer.

For these reasons, we studied mucin gene expression in the esophageal mucosa in control subjects without gastro-esophageal reflux and compared this to expression in the mucosa of patients with non-erosive and erosive gastro-esophageal reflux disease. We specifically chose to investigate the expression of four mucin genes. The secreted mucin gene MUC-5AC was selected because MUC-5AC mRNA expression has not been previously detected in the superficial epithelium or deep glands of the normal

esophageal mucosa, whereas it is strongly expressed in the superficial layers of metaplastic columnar epithelium and to a lesser extent in the deep glands in metaplastic columnar epithelium with or without specialized intestinal metaplasia.[24] Furthermore, MUC-5AC expression is markedly higher in mucosa of the proximal stomach compared to Barrett's esophagus with intestinal metaplasia.[25,26]

We also selected three signaling mucins: MUC-1, MUC-3, and MUC-4 for investigation. MUC-1 mRNA is strongly expressed in the superficial epithelium and the deep glands of the gastric cardia,[26] and it is also expressed in the mid layer of the stratified squamous epithelium of the normal human esophagus, but its expression is absent in the deep submucosal glands of the esophagus.[24] Furthermore, MUC-1 mRNA levels are elevated in the superficial epithelium and deep glands of metaplastic columnar epithelium in patients with Barrett's esophagus.[24] MUC-3 is not expressed in the normal esophagus. It appears to only be expressed in the superficial columnar epithelium in Barrett's esophagus in the presence of specialized intestinal metaplasia.[10,24,25] MUC-4 mRNA is expressed in the mid layer of the stratified squamous epithelium of the human esophagus, but is absent from the deep submucosal

Table 2 Rho and *p* Values for Expression Correlations for All Genes Across All Tissue Groups and Separate Tissue Groups (*$p<0.05$)

| mRNA	All tissue groups combined	No reflux	Non-erosive reflux	Erosive reflux
IL-6 vs. CK-14	0.531 ($p=0.001$)*	0.712 ($p=0.018$)*	0.604 ($p=0.024$)*	0.340 ($p=0.254$)
IL-6 vs. MUC-3	0.654 ($p<1.000\times10^{-6}$)*	0.630 ($p=0.037$)*	0.493 ($p=0.064$)	0.734 ($p=0.015$)*
MUC-3 vs. CK-14	0.336 ($p=0.038$)*	0.337 ($p=0.258$)	−0.014 ($p=0.952$)	0.554 ($p=0.064$)
MUC-3 vs. MUC-5AC	0.412 ($p=0.011$)*	0.154 ($p=0.603$)	0.457 ($p=0.085$)	0.336 ($p=0.263$)
MUC-1 vs. MUC-5AC	0.356 ($p=0.028$)*	0.056 ($p=0.849$)	0.407 ($p=0.126$)	0.678 ($p=0.024$)*

glands.[24] MUC-4 expression is lower in the superficial epithelium and deep glands of metaplastic columnar epithelium.[10]

Other groups have studied the relative pattern of mucin mRNA expression in the normal esophagus and stomach, as well as in Barrett's esophagus.[10,24–26] Buisine et al.[27] showed that MUC-3 is highly expressed in the adult duodenum, but by comparison MUC-1, MUC-4, and MUC-5AC are virtually absent. These studies used in situ hybridization, Northern analysis, and endpoint RT-PCR. These techniques are semi-quantitative and are unable to detect subtle changes in mRNA levels. Our study used the more sensitive and quantitative real-time RT-PCR technique, designed specifically to detect small changes in mucin expression that may occur in reflux subjects.

Because quantitative RT-PCR has not been used in this setting elsewhere, we validated the methodology used in our current study by testing mucin mRNA expression in tissue samples from stomach, duodenum, and Barrett's esophagus, and the patterns of expression were found to be comparable to the outcomes of other studies.

Overall, our results support the hypothesis that the expression of mucin genes in the esophageal mucosa is influenced by the composition (pH and bile) of the refluxate in gastro-esophageal reflux. In the cell culture study, we observed increases in the expression of all tested mucin genes in cells exposed to pH 4. These differences were similar to the trends observed in the studies undertaken using biopsy samples, i.e., the median MUC-1, MUC-3, and MUC-5AC mRNA levels were higher in the espoha-

Figure 4 Mean MUC-1 (**a**), MUC-3 (**b**), MUC-4 (**c**), and MUC-5AC (**d**) mRNA levels in normal esophageal mucosal biopsies (*NS*), Barrett's esophagus, proximal stomach, and duodenum.
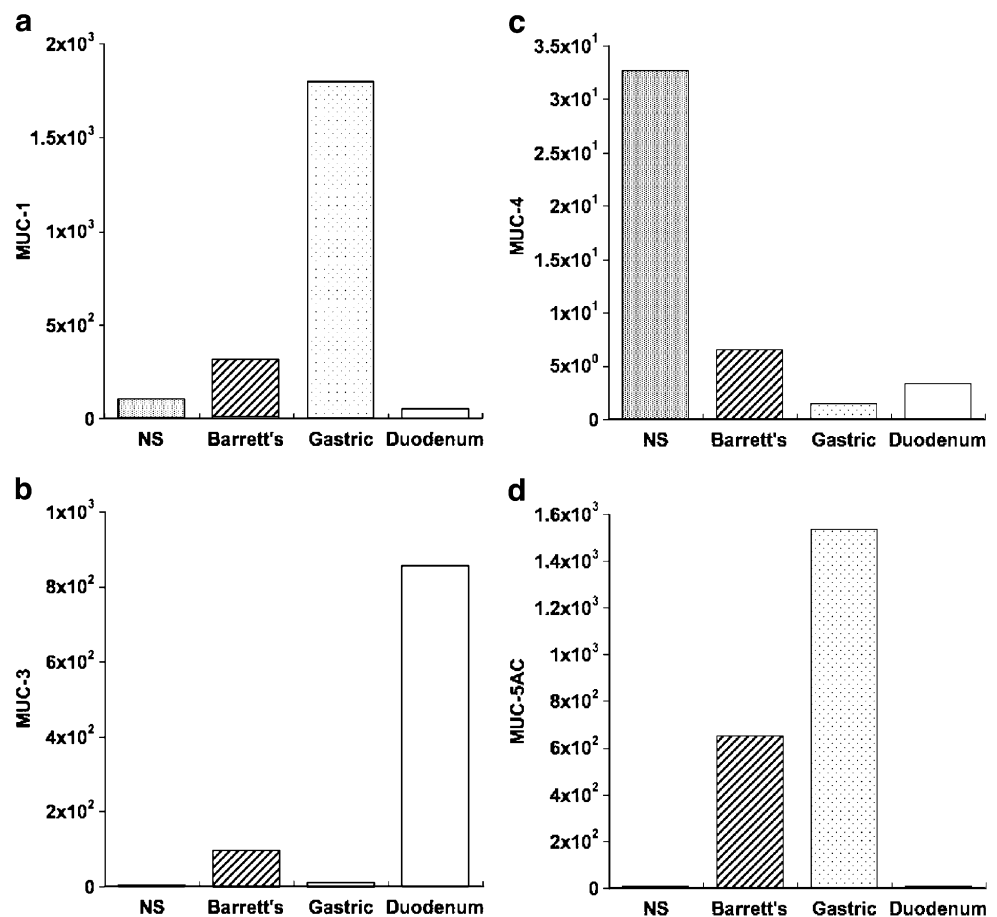
geal mucosa from patients with non-erosive reflux although the difference between this group and the control group did not reach statistical significance. However, it is possible that increases in the expression of these mucin genes are occurring in the esophageal epithelium due to exposure to intra-luminal acid. With the exception of MUC-3, we also observed decreases in mucin gene expression in the Het-1A cells which were treated with a combination of acid and bile. Similar differences were also seen in the esophageal mucosa from patients with erosive reflux, with the expression levels for MUC-1, MUC-4, and MUC-5AC less than those seen in samples from control subjects and patients with non-erosive reflux. While again the differences between groups did not reach statistical significance, it is conceivable that protective roles of MUC-1, MUC-4, and MUC-5AC in the esophageal epithelium are abolished or reduced when gastro-esophageal reflux is associated with ulcerative esophagitis, and this could be associated with the combination of acid and bile in the refluxate.

The significant correlations between MUC-3 expression and IL-6 (a marker of inflammation) and MUC-3 expression and CK-14 (a basal epithelial layer marker) suggest that MUC-3 could have a role in esophageal mucosal protection during inflammation, and this may therefore have implications for our understanding of the repair of reflux-induced mucosal damage. Furthermore, co-expression of MUC-1 and MUC5-AC in mucosa from patients with erosive reflux disease may indicate the metaplastic potential of esophageal epithelium in this state. This is because MUC-1 and MUC-5AC are known to be co-expressed in proximal gastric mucosa and Barrett's esophagus, but not in normal esophageal mucosa. Our results, which show for the first time that these two markers are co-expressed in esophageal epithelium from patients with erosive reflux (but not controls or patients who did not have ulcerative esophagitis), indicate possible progression or an early step at the molecular level towards columnar metaplasia.

Unlike previous reports, our study has investigated the effect of gastro-esophageal reflux on the expression of mucin genes. This is probably the earliest step in the progression to esophageal adenocarcinoma via the Barrett's esophagus to cancer sequence. We took active steps to ensure that the tissues used in this study were correctly classified, and the potential for biopsy sampling error has been largely eliminated by obtaining histopathology on part of each biopsy sample. However, we did not use 24 h ambulatory pH monitoring to categorize patients, but rather relied on endoscopic criteria and clinical symptoms. While it has been argued that pH monitoring is the "gold standard" for the identification of patients with gastro-esophageal reflux, it should be recognized that this test can be associated with false positive and false negative outcomes. Some studies have shown that up to 50% of patients with reflux can have a normal 24-h pH test.[28] Furthermore, we have collected tissue samples from many patients undergoing endoscopy in our institution, and for the current study we were conservative in our classification of patients, and when there was doubt about the clinical diagnosis we excluded the specimens from the individuals concerned. In addition, the endoscopic criteria for identifying ulcerative esophagitis are well accepted in clinical practice, and there is little doubt that this category of patients had gastro-esophageal reflux disease. An additional potential confounder is the possible use of proton pump inhibitor medication. However, as most of the patients in the two reflux categories were taking this medication at the time of endoscopy, the gradation of severity was probably not influenced by medication use.

Previous studies using animal models or clinical tissue samples have shown that IL-6 levels are elevated in the esophageal mucosa in the presence of reflux esophagitis.[11–13] For this reason, we evaluated IL-6 as a possible surrogate tissue marker of reflux. Our results demonstrated an increase in IL-6 levels across the three study groups, in a manner which was consistent with increasing severity of reflux. Hyperplasia of the basal cell of the esophageal mucosa is also evident in patients with gastro-esophageal reflux, and it is probably caused by epithelial repair mechanisms in response to reflux-induced damage.[14] The presence of CK-14 has been shown to be restricted to the basal cell layer within the esophageal mucosa, and hyperplasia of this layer is associated with increased expression.[15,16] Hence, we evaluated CK-14 as another potential surrogate tissue marker of reflux. Its expression also correlated with increasing severity of reflux. These results provide additional support to the hierarchy of our study groups—controls vs. non-erosive vs. erosive reflux. Also supporting this is the significant correlation between IL-6 and CK-14 mRNA levels.

The differences between gene expression levels for the patient groups failed to reach statistical significance at the level of $p<0.05$. However, the data trends suggest that if the sample size was larger, many of these trends might have reached statistical significance. When we commenced this study, we were not sure what magnitude of difference (if any) we were likely to find, and for this reason a sample size calculation was not feasible. Hence, a larger sample size may yield significant differences, and further work is warranted to explore this possibility.

Overall, our results suggest that the profile of mucin expression in the esophageal mucosa is influenced by the pH and composition of gastro-esophageal reflux. Further work should explore the response of these genes to acid and bile reflux and explore their role in the etiology of mucosal damage in gastro-esophageal reflux, as well as its progression to Barrett's esophagus and beyond.

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