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Esophageal Pepsin and Proton Pump Synthesis in Barrett’s Esophagus and Esophageal Adenocarcinoma

Tina L. Samuels, MS ©; Kenneth W. Altman, MD, PhD ©; Jon C. Gould, MD; Tammy Kindel, MD, PhD; Matthew Bosler, BA; Alexander MacKinnon, MD; Catherine E. Hagen, MD; Nikki Johnston, PhD

**Objectives/Hypothesis:** Gastroesophageal reflux disease and associated metaplasia of the esophagus (Barrett’s esophagus [BE]) are primary risk factors for esophageal adenocarcinoma (EAC). Widespread use of acid suppression medications has failed to stem the rise of EAC, suggesting that nonacid reflux may underlie its pathophysiology. Pepsin is a tumor promoter in the larynx and has been implicated in esophageal carcinogenesis. Herein, specimens from the esophageal cancer spectrum were tested for pepsin presence. Pepsin-induced carcinogenic changes were assayed in an esophageal cell culture model.

**Study Design:** Laboratory analysis.

**Methods:** Pepsin was assayed in reflux and cancer-free esophagi, BE, EAC, and esophageal cancer lacking association with reflux (squamous cell carcinoma [SCC]). Revisited or locally synthesized pepsin was assayed by Western blot. Local synthesis of pepsin and proton pumps was assayed via reverse transcription–polymerase chain reaction. The effect of pepsin on BE and EAC markers was investigated via enzyme-linked immunosorbent assay and quantitative polymerase chain reaction in human esophageal epithelial cells treated with pepsin or control diluent.

**Results:** Pepsinogen and proton pump mRNA were observed in BE (3/5) and EAC (4/4) samples, but not in normal adjacent specimens, SCC (0/2), or reflux and cancer-free esophagi. Chronic pepsin treatment (0.1–1 mg/mL, 4 weeks) of human esophageal cells in vitro induced BE and EAC markers interleukin 8 and KRT8 and depleted normal esophageal marker KRT10 (P < .05) expression.

**Conclusions:** Local synthesis of pepsin and proton pumps in BE and EAC is not uncommon. Absence of these molecules in normal (noncancer) esophagi, SCC, and in vitro data support a role for pepsin in reflux-attributed carcinogenic changes in the esophagus.

**Key Words:** Pepsin, gastric proton pump, interleukin 8, cytokeratin profile, Barrett’s esophagus, esophageal adenocarcinoma.

**Level of Evidence:** NA

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**INTRODUCTION**

Esophageal adenocarcinoma (EAC) incidence has risen faster than any other cancer in the United States since the 1970s. With a 5-year survival of <20%, it boasts the highest mortality rate among US cancers and represents one of few contributing to increasing death rates in white males, the highest risk demographic. No medical or surgical intervention has proven substantially preventive or therapeutic to date.

Primary risk factors for EAC include gastroesophageal reflux disease (GERD) (relative risk: 43.5, 95% confidence interval [CI]: 18.3–103.5), Barrett’s esophagus (BE) reflux-attributed intestinal metaplasia (relative risk: 11.3, 95% CI: 8.8–14.4), and to a lesser extent obesity, smoking, and low fruit and vegetable intake. The current screening program for EAC involves endoscopic surveillance of BE patients with symptoms reminiscent to proton pump inhibitors (PPIs). Yet, BE is commonly an undiagnosed benign condition, and fewer than 0.5% of BE patients develop EAC. The screening program fails to identify >90% of EAC cases, contributing to late-stage detection and poor prognosis. Improved screening indicators are clearly needed to identify GERD and BE patients at highest risk of neoplastic progression.

Surprisingly little is known of the molecular events and progenitor cells involved in progression of EAC. Acid has historically been considered the most damaging element in gastric reflux, and acid-suppressing PPIs are the most common medical therapy for GERD. Given that ~25% of the US population suffers from GERD, PPIs have represented the third highest-selling drug category for >20 years. Despite decades of data on such widespread use, evidence for their cancer preventive benefit is inconclusive. Large population studies and meta-analyses

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N.J. devised the project and main conceptual ideas and directed the project. A.M. contributed to the concept. J.C.G. and T.K. contributed to concept and obtained patient specimens. M.B. aided in obtaining patient specimens and clinical research coordination. T.L.S. designed and performed experiments and drafted the manuscript. A.M. and C.E.H. performed histopathological analyses. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

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have demonstrated a direct association between PPI use and EAC risk, and evidence supports a range of possible mechanisms by which PPIs may contribute to carcinogenesis. This and experimental data suggest that acid is not the primary agent responsible for the association of GERD with EAC, implicating nonacid reflux in carcinogenesis instead.

The gastric enzyme pepsin has previously been shown to promote chronic inflammation and carcinogenesis in the upper airways during extraesophageal reflux, which is typically weak or nonacidic and similar to that of patients taking PPIs. Furthermore, parietal and chief cells observed in BE, in the background of EAC and in heterotopic gastric mucosa patches in the esophagus, represent a potential source of locally synthesized pepsin and acid that may contribute to disease progression. We recently reported pepsinogen synthesis in BE mucosa in a small cohort of BE patients, and demonstrated pepsin-induced inflammatory changes in esophageal cells in vitro. The purpose of this study was to continue investigation of the contribution of pepsin to molecular and morphological changes associated with BE and EAC in vitro, and to survey esophageal pepsinogen and gastric proton pump synthesis in clinical specimens from the normal-metaplasia-adenocarcinoma spectrum.

MATERIALS AND METHODS

Human Biopsy Specimens

Two to three adjacent intraoperative pinch biopsies (1–2 mm³) were obtained through upper endoscopy during elective general surgery (gastric bypass, sleeve gastrectomy, incisional hernia, Nissen fundoplication) from patients with diagnosis of BE from June 2015 to June 2016 (Medical College of Wisconsin [MCW] Institutional Review Board [IRB] PRO00006838). Patients who underwent ablation and subsequently tested BE-negative were excluded. Patients with short segments (<1 cm) of BE were excluded to reduce the likelihood of non-BE or a gastric contaminant. Biopsies were obtained from BE and neighboring normal esophageal tissue visually identified by the surgeon in accordance with American College of Gastroenterology guidelines for BE (i.e., “the anatomic region where the distal extent of the tubular esophagus is in contact with the proximal extent of the gastric folds”). Adjacent normal (non-BE) esophageal biopsies were obtained at minimum 5 cm proximal to the proximal extent of BE. Esophageal pinch biopsies were obtained from control subjects undergoing similar procedures and having no evidence of gastric reflux indicated by past medical history or negative esophagogastroduodenoscopy, and no indication of esophageal malignancy under the same IRB protocol from November 2015 to June 2016. From each BE or normal region of each patient, one biopsy was immediately placed in 10% formalin for transport and stored at −80 °C. Tissue from five EAC cases with paired adjacent normal specimens, two squamous cell carcinoma (SCC) cases with paired adjacent normal specimens, normal (noncancer) esophageal tissue from two patients, and normal gastric tissue from two patients were obtained through the MCW Tissue Bank (IRB PRO00017015).

Cell Culture

As described, primary esophageal epithelial (EE) cell culture was established from a pinch biopsy from the esophagus of a patient undergoing elective general surgery procedures (MCW IRB PRO00004777) with no history or preoperative symptoms of reflux and no indication of esophageal malignancy. Immortalized esophageal cells (Het-1A cells; American Type Culture Collection, Manassas, VA) cultured per supplier instructions were utilized for experiments requiring extended time in culture (i.e., chronic pepsin treatment). Cells were cultured in normal growth media (pH 7.4) or media containing porcine pepsin (0.1 or 1 mg/mL; Sigma-Aldrich, St. Louis, MO) in triplicate. Media was changed every 2 to 3 days, with freshly diluted pepsin added each change and cells passaged per supplier instructions at 1:4 dilution upon reaching 75% culture confluence. Culture supernatants and cells were collected at 75% culture confluence following 2 and 4 weeks treatment.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

To examine the effect of pepsin on mitochondrial function as an indicator of cell damage and potential oxidative stress, EE cells were seeded at 7.5 × 10⁴ cells per well in a 96-well plate and cultured to 40% confluence. Cells were incubated in normal growth media (pH 7.4) containing 0.1 mg/mL porcine pepsin (Sigma-Aldrich) for 20, 40, or 60 minutes or incubated in normal growth media alone (control) in 12 replicate wells per condition. Treatment media was aspirated, wells rinsed with phosphate-buffered saline, and normal growth media replaced. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reagent was added immediately, and the assay was performed per kit instructions including media-alone and background absorbance correction (Sigma-Aldrich).

Interleukin 8 Enzyme-Linked Immunosorbent Assay of Pepsin-Treated Het-1A

To examine whether pepsin exposure could induce expression of interleukin 8 (IL-8), a cytokine mediator of inflammation and carcinogenesis elevated in GERD, BE, and EAC, IL-8 was assayed in pepsin or diluent-treated Het-1A cell culture supernatants described above in duplicate per manufacturer instructions (Human IL-8 Enzyme-Linked Immunosorbent Assay [ELISA]; MyBioSource, San Diego, CA). Blank-subtracted mean absorbance of standards was plotted against a concentration of IL-8 to generate a standard curve from which the IL-8 concentration of specimens was interpolated.

Cytokeratin Real-Time Quantitative Polymerase Chain Reaction of Pepsin-Treated Het-1A

Quantitative polymerase chain reaction (qPCR) was performed to determine whether pepsin exposure could induce a change in cytokeratin expression similar to that observed during progression to BE (i.e., from normal [noncancer] esophageal cytokeratin KRT10 to Barrett’s esophageal cytokeratin KRT8). One hundred fifty nanograms of RNA from pepsin or diluent-treated Het-1A described above was extracted (TRIZOL; ThermoFisher Scientific, Waltham, MA), DNased (RNaseasy Mini Kit with DNase, Qiagen, Germantown, MD), and reverse transcribed using oligo d(T) primers (Superscript III Reverse Transcription kit, ThermoFisher Scientific). qPCR was performed in triplicate using Taqman gene expression assays in a Viia7 instrument (ThermoFisher Scientific) per manufacturer’s instructions. Threshold cycle (Ct) values >35 were used for analysis, and cytokeratin expression in each specimen was normalized to the housekeeping gene (HPRT1).

Histopathological Analysis of BE Specimens

A single formalin-fixed esophageal biopsy from each subject was paraffin embedded, sectioned, hematoxylin and eosin stained, and assessed by board-certified pathologists (A.M., C.E.H.).
Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis/Western Blot

Biopsies were homogenized, 30-μg total protein separated via 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis alongside human pepsin 3b and human pepsinogen I, and probed with mouse anti-human pepsin monoclonal antibody and mouse anti-actin antibody (loading control; EMD Chemicals, Gibbstown, NJ) as described. Anti-pepsin antibody designed for specificity to mature pepsin demonstrates approximately 100-fold greater sensitivity for pepsin relative to pepsinogen.

Pepsinogen and Proton Pump Reverse Transcription-Polymerase Chain Reaction in Clinical Specimens

RNA was extracted and reverse transcribed as above. Gastric RNA (Agilent Technologies, Santa Clara, CA) was reverse transcribed as a positive control. Primers and conditions are shown in Table I. All primers spanned >100 bp introns. Amplicon was separated on 2% agarose alongside 50-1,000 bp DNA Marker (Cambrex, East Rutherford, NJ).

Statistical Methods

MTT assay was evaluated using a linear trend test via linear regression. For ELISA and qPCR, data from each gene/protein of interest were analyzed using a repeated measures analysis of variance for paired samples followed by Student’s t test. Analyses were performed using SAS 9.3 (SAS Institute, Cary, NC).

RESULTS

MTT Assay of Primary Esophageal Cell Culture Following Acute Pepsin Treatment

Time-dependent reduction in mitochondrial dehydrogenase activity was observed, suggesting that pepsin elicits mitochondrial dysfunction (Fig. 1). Mean normalized absorbance (standard error) decreased from 0.168 (0.009) in untreated control cells, to 0.146 (0.015), 0.123 (0.005), and 0.066 (0.006), at 20, 40, and 60 minutes treatment, respectively. Regression coefficient equaled −0.00163 (P < .0001) (i.e., mean absorbance decreased 0.00163 units per minute of pepsin treatment).

Expression of BE Markers in Het1-A Cells Following Chronic Pepsin Treatment

HPRT1 is a housekeeping gene, constitutively expressed and required for the maintenance of a basic cellular function, specifically generation of purine nucleotides. The median Ct of HPRT1 in 1 μL cDNA from the 150 ng RNA per group was not significantly changed in pepsin treatment groups relative to control/untreated cells at the maximum treatment duration of 4 weeks, supporting the stability of basic cellular functions throughout the maximum duration of pepsin treatment (median Ct ± standard deviation [SD], P relative to control: 0.1 mg/mL pepsin group = 27.51 ± 0.146, P = .728; 1 mg/mL pepsin group = 26.86 ± 0.349, P = .085; control/untreated = 27.46 ± 0.136). Total RNA yield was similar in pepsin-treated and control groups, supporting similar culture densities at time of harvest (median RNA yield ± SD, P relative to control: 0.1 mg/mL pepsin group = 703.7 ± 66.8 ng, P = .611; 1 mg/mL pepsin group = 798.7 ± 54.4 ng, P = .078; control/untreated cells = 670.1 ± 54.7 ng).

The effects of 0.1 mg/mL pepsin treatment for 2 and 4 weeks on IL-8 secretion relative to control cells cultured without pepsin were P = .0430 and .0682 at 2 and 4 weeks, respectively, with significance defined as P = .05 (Table II).

### Table I. Reverse Transcription-Polymerase Chain Reaction Primers, Reaction Conditions and Predicted Amplicon Size.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Primer Sequence</th>
<th>Reaction Conditions (Cycles, Annealing Temperature)*</th>
<th>Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsinogen A</td>
<td>PGA5</td>
<td>F: ACCG TGGAC AGCAT CAACCA TG R: TCTCT CGTGG AGGTG CICTG</td>
<td>30–35 cycles, 62°C</td>
<td>437</td>
</tr>
<tr>
<td>Hypoxanthine-guanine</td>
<td>HPRT1</td>
<td>F: TGCTC GAGAT GTGAT GAAGG R: CCGA CCAG GAAAG CAGAG</td>
<td>35 cycles, 55°C</td>
<td>307</td>
</tr>
<tr>
<td>phosphoribosyltransferase 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATPase H+/K+ transporting alpha subunit</td>
<td>ATP4A</td>
<td>F: CCTGCT GTGCC TACTC CTACT R: GATGCG AGACCC TGGAA GACGA</td>
<td>30–35 cycles, 55°C</td>
<td>352</td>
</tr>
<tr>
<td>ATPase H+/K+ transporting beta subunit</td>
<td>ATP4B</td>
<td>F: GCAG AGGC CCGT GACAC R: CGAC ATATC TGCCG TGAC</td>
<td>30–35 cycles, 55°C</td>
<td>287</td>
</tr>
</tbody>
</table>

*Reaction conditions = 94°C, 5 minutes; listed number of 30-second cycles of 94°C, annealing temperature and 72°C; and 72°C, 5 minutes.

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A 1 mg/mL pepsin treatment for 2 or 4 weeks increased IL-8 secretion relative to control cells (2-week \( P < 0.001 \), 4-week \( P = 0.0028 \)).

BE-type cytokeratin, KRT8, was elevated following a 4-week 1 mg/mL pepsin treatment of Het-1A (\( P = 0.0123 \)) (Table III). Noncancer esophagus cytokeratin, KRT10, was depleted by a 4-week treatment with 0.1 mg/mL or 1 mg/mL pepsin (\( P = 0.0005 \) and \( 0.0094 \), respectively). A higher-dose pepsin treatment resulted in greater effect on both keratins.

### Pepsinogen/Pepsin and Gastric Proton Pump Expression in Pathologist Examined Clinical Specimens

Between June 2015 and June 2016, six consecutive BE patients consented for study; collection error led to the exclusion of one. Normal adjacent specimens were obtained from two of the five BE patients included for study. Three consecutive control subjects were consented for study between November 2015 and June 2016; all were included for study. Of specimens obtained from the MCW Tissue Bank, one EAC case was excluded from the study due to poor quality indicated by low RNA yield and inability to detect the housekeeping gene (HPRT1). The patients or cases included for the study were therefore five BE patients (two with paired adjacent normal tissue); three control patients (referred to as GERD and esophageal cancer free [G/C-free]); four EAC cases, each with paired adjacent normal; two SCC cases, both with paired adjacent normal; normal esophagus from two patients, and normal gastric tissue from two patients.

The results are summarized in Table IV. Pepsin protein in biopsies lacking pepsin precursor (pepsinogen) mRNA could be presumed to be of gastric origin, deposited during a reflux event. Pepsin in biopsies that contained pepsinogen mRNA could be of local and/or gastric origin.

Western blot revealed pepsin protein in 5/5 BE, 4/4 EAC, and 1/2 SCC (Fig. 2). Pepsin was observed in adjacent normal tissue from 1/2 BE and 0/4 EAC, and was absent in 3/3 G/C-free. Pepsin protein was observed in 2/2 normal gastric specimens (positive control) and absent from 2/2 normal esophageal specimens.

RT-PCR was used to detect mRNA corresponding to pepsinogen (PGA5) and the \( \alpha \) (ATP4A) and \( \beta \) (ATP4B) sub-units of the gastric H+/K+-ATPase (proton pump) in esophageal specimens. Presence of mRNAs supports potential de novo synthesis. RT-PCR demonstrated coexpression of pepsinogen and proton pump mRNAs in 3/5 BE, 4/4 EAC, and 2/2 normal gastric (positive control) specimens (Fig. 3). Pepsinogen and proton pump mRNAs were absent from paired

### Table II.

Chronic Pepsin Treatment-Induced Barrett’s Esophagus Prognostic Biomarker IL-8 Protein Expression in Het-1A Esophageal Cells.

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>0.1 mg/mL pepsin</th>
<th>1 mg/mL pepsin</th>
<th>Adjusted ( P ), Relative to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks</td>
<td>IL-8 pg/mL (SE)</td>
<td>41.8 (2.2)</td>
<td>67.0 (8.5)</td>
<td>0.043</td>
</tr>
<tr>
<td></td>
<td></td>
<td>174.4 (6.3)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>4 weeks</td>
<td>Control</td>
<td>42.3 (2.0)</td>
<td>93.7 (23.9)</td>
<td>0.0662</td>
</tr>
<tr>
<td></td>
<td>1 mg/mL pepsin</td>
<td>167.7 (11.3)</td>
<td>0.0028</td>
<td></td>
</tr>
</tbody>
</table>

IL-8 = interleukin 8; SE = standard error.

### Table III.

Chronic Pepsin Treatment Induces Barrett’s Esophagus–Like Cytokeratin Phenotype in Het-1A Cells.

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>0.1 mg/mL pepsin</th>
<th>1 mg/mL pepsin</th>
<th>Adjusted ( P ), Relative to Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks</td>
<td>KRT8 Ct</td>
<td>-1.463 (0.211)</td>
<td>-1.890 (0.069)</td>
<td>1.34 ( .1985 )</td>
</tr>
<tr>
<td></td>
<td>HPR7 (SE)</td>
<td>-1.681 (0.044)</td>
<td>1.16 ( .4692 )</td>
<td>.4807</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-3.355 (0.097)</td>
<td>1.39 ( .1201 )</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 mg/mL pepsin</td>
<td>-3.813 (0.126)</td>
<td>1.79 ( .0123 )</td>
<td>.1708</td>
</tr>
<tr>
<td></td>
<td>-4.193 (0.070)</td>
<td>1.79 ( .0123 )</td>
<td>.1708</td>
<td></td>
</tr>
<tr>
<td>4 weeks</td>
<td>KRT70</td>
<td>-1.471 (0.116)</td>
<td>-1.365 (0.102)</td>
<td>-1.08 ( .6517 )</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-1.191 (0.045)</td>
<td>-1.21 ( .2158 )</td>
<td>.4664</td>
</tr>
<tr>
<td></td>
<td>1 mg/mL pepsin</td>
<td>-2.139 (0.061)</td>
<td>-1.33 ( .0094 )</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-1.727 (0.025)</td>
<td>-1.33 ( .0094 )</td>
<td>.0094</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-1.325 (0.035)</td>
<td>-1.76 ( .0005 )</td>
<td>.0094</td>
<td></td>
</tr>
</tbody>
</table>

SE = standard error.
adjacent normal esophagus from BE and EAC patients, G/C-free control patients, and normal esophageal specimens. Pepsinogen and proton pump mRNAs were also absent from SCC, which is not associated with GERD. De novo pepsin and proton pump synthesis in the esophagus was therefore observed only in metaplasia and dysplasia attributed to GERD.

Independent histopathological examinations of a single biopsy from each patient undergoing resection for BE by two certified pathologists (A.M., C.E.H.) confirmed presence of columnar-lined epithelium of the esophagus (CLE) in all BE specimens and specialized intestinal metaplasia (SIM) (defined by presence of goblet cells) in 3/5 BE cases. Absence of SIM in the remaining two cases was inconclusive given inadequate sampling density (see Discussion). Three BE cases demonstrated PGA5, ATP4A, and ATP4B co-expression: one was CLE with SIM and two were CLE without identifiable SIM (Table IV). All adjacent normal esophagus specimens from BE cases were confirmed normal squamous epithelium on histopathological exam and did not express pepsinogen or proton pump mRNA. PGA5, ATP4A, and ATP4B expression was observed in all EAC cases regardless of differentiation status.

**DISCUSSION**

Although the requirement of pepsin to incur reflux-associated damage was proven in animal models in the 1960s and 1980s, successful treatment of peptic ulcer by acid reducing H2 receptor antagonists during the same period led to the current clinical focus on acid and application of acid-suppressing drugs to other gastroenterological illnesses. Now, more than 113 million PPI prescriptions are written in the United States annually, 70% of which represent inappropriate or overuse, with significant implications for patient health given associated risks.

Although PPIs reduce gastric acid, they do not reduce the number or frequency of reflux events. Breakthrough symptoms and esophageal damage continue to occur in 40% of GERD patients taking PPIs. Mature pepsin is produced within the stomach of patients taking PPIs, and similar concentrations of pepsin have been observed in saliva or laryngeal aspirates of GERD and laryngopharyngeal reflux patients regardless of PPI use.

In the context of acid reflux, pepsin incurs immediate erosive damage to esophageal tissue. However, during weak and nonacid reflux commonly experienced by patients taking PPIs, the enzymatic activity of pepsin is transiently inhibited, allowing interaction with an unidentified cell surface receptor, endocytosis, and retention in acidic intracellular vesicles where its enzymatic activity would be restored. Pepsin has been observed in a variety of airway and esophageal epithelial cells where it initiates a cascade of inflammation and cancer-associated changes. We previously demonstrated that <24-hour pepsin exposure induces a cancer-promoting gene expression profile, anchorage-independent growth, and cell migration in hypopharyngeal and laryngeal cells in vitro, and that pepsin promoted oral tumor growth in vivo. More recently, we described the effect of acute nonacid pepsin exposure on proinflammatory signaling cascades and cell migration in esophageal cells in vitro, and demonstrated that BE commonly expresses pepsinogen mRNA.

Our previous study demonstrated that pepsin increased esophageal cell migration, a multistep process that plays an important role in both protective responses such as wound healing and the progression of cancer. Herein, the same treatment yielded mitochondrial dysfunction, indicative of a detrimental role for pepsin in the esophagus. Degradation of

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Pathology Status</th>
<th>Western Blot</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>BE1</td>
<td>BE</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>BE1-N</td>
<td>Normal squamous</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>BE2</td>
<td>CLE, SIM not observed</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>BE2-N</td>
<td>Normal squamous</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>BE3</td>
<td>BE, no dysplasia</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>BE4</td>
<td>BE, no dysplasia</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>BE5</td>
<td>CLE, SIM not observed</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>EAC1</td>
<td>EAC malignant</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EAC1-N</td>
<td>Normal squamous</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EAC2</td>
<td>EAC malignant, moderately differentiated</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>EAC2-N</td>
<td>Normal squamous</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EAC3</td>
<td>EAC malignant, poorly differentiated</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>EAC3-N</td>
<td>Normal squamous</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EAC4</td>
<td>EAC malignant, moderately differentiated</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>EAC4-N</td>
<td>Normal squamous</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>TABLE IV.</strong> Pepsin and Proton Pump mRNA Expression in Graded Metaplastic and Dysplastic Clinical Specimens.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 Sampling density too low to conclude absence of SIM.

ATP4A = ATPase H+/K+ transporting α subunit; ATP4B = ATPase H+/K+ transporting β subunit; BE = Barrett’s esophagus; CLE = columnar-lined esophagus; EAC = esophageal adenocarcinoma; PGA5 = pepsinogen A; RT-PCR = reverse transcription-polymerase chain reaction; SIM = specialized intestinal metaplasia.
mitochondrial cristae and mitochondrial dysfunction appear to be hallmarks of nonacid pepsin-mediated injury\textsuperscript{18,19,28} that are potentially mediated through pepsin-suppression of Bcl-2 family proteins involved in maintaining cristae structure and mitochondrial homeostasis.\textsuperscript{20} Mitochondrial dysfunction is an indicator of mitochondrial DNA damage by reactive oxygen species, which is a common occurrence during chronic inflammation and is implicated in the etiology of metaplastic and neoplastic progression including BE.\textsuperscript{44,45}

GERD is a chronic condition often persisting for years, during which the esophagus is bathed in up to 3 mg/mL pepsin >73 times per day. Exposure of the esophageal mucosa is not limited to the duration of the reflux event. Bile and acid persist in the esophagus several minutes following a reflux event, and refluxed pepsin can be retained by esophageal cells for up to 12 hours if endocytosed during a reflux event.\textsuperscript{19} Carcinogenesis, the transformation of normal cells to cancer cells, is characterized by distinct changes at cellular, genetic, and epigenetic levels in defined phases of initiation, promotion, and progression. Depending on the dose and frequency of exposure and carcinogenicity of the compound, this process typically requires months to years. Reflux-associated cancer typically occurs in patients following years of medically treated reflux, the treatment being PPIs, despite successful symptom resolution.\textsuperscript{3,9} Although early changes in cancer initiation may be observed following a brief exposure to a carcinogen as we reported following acute pepsin treatment of esophageal cells,\textsuperscript{25} other changes require repeated or prolonged exposure. Two- and 4-week exposure to 0.1 to 1 mg/mL pepsin were selected as chronic pepsin treatment conditions for experiments herein based on similar in vitro studies of IL-8 and KRT\textsuperscript{46,47} response to components of reflux.\textsuperscript{46,47} By dose and duration, these conditions are considerably milder than physiological pepsin exposure of the typical GERD patient who develops cancer; however, the continuous exposure conditions of our in vitro model differ from the transient, repeated exposures experienced by the typical chronic GERD patient. This limitation should be considered when interpreting the in vitro data herein.

As demonstrated herein, chronic (2 to 4 weeks) pepsin exposure elicited secretion of IL-8. IL-8 is a neutrophil chemoattractant and stimulant of cellular proliferation and angiogenesis, and therefore uniquely poised to mediate the transition from inflammation to carcinogenesis. IL-8 is elevated in GERD patients, highest in patients with BE and EAC, and reduced in BE patients following antireflux surgery.\textsuperscript{48}

Furthermore, chronic pepsin treatment induced transition from a noncancer esophageal cytokeratin profile (KRT\textsuperscript{10} high, KRT\textsuperscript{8} low) to a BE-associated profile (KRT\textsuperscript{10} low, KRT\textsuperscript{8} high).
Interestingly, a similar cytokeratin profile shift occurs following 3-week treatment with all-trans retinoic acid, a key player in columnar differentiation during BE development, but not given pulses of acid (pH 3.5 for 1 hour, 3 days per week), supporting the hypothesis that nonacid rather than acid reflux constituents underlie the association of GERD with metaplastic or neoplastic changes.

Herein, pathologists confirmed columnar-lined epithelium (CLE) in all suspected BE cases. SIM was observed in three of five cases. Goblet cells are currently used to define SIM and BE per the American College of Gastroenterology (ACG), yet goblet cell findings are prone to false negative detection. Eight biopsies are required to sufficiently reduce risk of false negative detection as reflected by ACG guidelines; however, fewer than four are evaluated in routine practice.

Given that similar immunohistochemical markers, DNA content abnormalities, and rates of progression to dysplasia and malignancy have been observed in CLE, irrespective of goblet cell presence, considerable controversy exists regarding the use of goblet cells as diagnostic criteria for BE or inclusion criteria for EAC screening. Several CLE-positive cases in this study expressed pepsinogen and gastric H+/K+-ATPase proton pump mRNA, consistent with previous reports of pepsin and acid production in BE. Both heterotopic gastric mucosa in the esophagus and BE contain diverse cell types, including oxyntocardiac mucosa characterized by parietal (acid secreting) and chief (pepsin secreting) cells. Evidence suggests that oxyntocardiac mucosa is a precursor of intestinal metaplasia and EAC, and it is commonly found in the background of EAC as observed herein. Accordingly, pepsin and proton pump mRNA was expressed exclusively in GERD-associated metaplasia and dysplasia in this study, and was absent from noncancer esophagus and squamous cell carcinomas, which bear no association with GERD. These findings support our hypothesis that transition of esophageal mucosa to a gastric-like phenotype characterized by pepsin and proton pump expression may represent a step in the development and progression of BE and EAC.

One may speculate that gastric proton pump expression in the esophagus could be advantageous or deleterious, aiding maintenance of intracellular pH during excessive acid exposure similar to pH-inducible esophageal Na+/H+ exchange pumps, or contributing to hypersecretion and edema in response to inflammatory signaling events akin to inflammation-induced ion transport of respiratory tract mucosa. Cleavage of pepsinogen to mature pepsin requires an acidic environment; pepsin is enzymatically active from pH 2 to 6.5 and reversibly inactivated at the pH of esophageal mucosa.

**Fig. 3.** Reverse transcription–polymerase chain reaction demonstrated pepsinogen and proton pump mRNA in esophageal biopsies from patients with Barrett’s esophagus (BE) and esophageal adenocarcinoma (EAC), but none in esophageal cancer not associated with reflux (squamous cell carcinoma [SCC]). Esophageal specimens from gastroesophageal reflux disease and esophageal cancer-free control patients (G/C-free Esoph) were used as a negative control for BE; cancer-free gastric (Norm Gastric) and esophageal (Norm Esoph) specimens were used as positive and negative controls, respectively, for EAC. Hypoxanthine-guanine phosphoribosyltransferase 1 (HPRT) was used as a positive control. BE-N and EAC-N were adjacent normal. Pepsinogen and proton pump mRNA were coexpressed in 3/5 BE samples, 4/4 EAC samples, but not in the SCC or normal esophageal biopsy controls. Pepsinogen or proton pump mRNA presence in esophageal biopsies suggests local synthesis.
Refluxed pepsin can be retained in esophageal cells for up to 12 hours in acidic intracellular vesicles where its activity would be restored. Esophageal acid production may therefore allow conversion of locally synthesized pepsinogen to active pepsin and provide another means of reactivation of intracellular stores of refluxed pepsin. Contrary to intermittent reflux, pH monitoring of patients with heterotopic gastric mucosa confirms that esophageal proton pumps can produce a constant sequence of acid, which may in turn elicit pepsinogen conversion to pepsin and increase pH-dependent pepsin activity, leading to tissue disruption and cell death, thereby perpetuating local acidity and upregulation of proton pumps to maintain intracellular pH. Intriguingly, esophageal proton pumps could be a site of pharmacological action of PPIs.

Continued study is warranted to investigate whether a phenotypic change characterized by pepsinogen and proton pump expression is a conserved response of aerodigestive tract mucosa to chronic reflux and the implications of local synthesis on inflammation and carcinogenesis. To this end, we recently produced esophageal cell lines designed to ectopically express pepsinogen and gastric proton pump subunits. We plan to examine their capacity to produce functional proton pumps and pepsin and the impact of expression on mitochondrial function, proliferation, and inflammation and cancer-associated signaling pathways.

Limitations should be considered when interpreting the findings herein. First, patients in the GC-free control population were undergoing obesity-related surgeries. Obesity is a risk factor for GERD and esophageal cancer. However, criteria such as indication of esophageal malignancy and evidence of gastric reflux indicated by past medical history or esophageagastroduodenoscopy were used to exclude potential control subjects presently or historically affected by GERD, Barrett’s esophagus, or esophageal cancer. MI-pH testing, which detects acid and nonacid reflux, would be useful to demonstrate absence of reflux, yet this test is expensive, invasive, and was not considered standard of care for these patients. However, mature pepsin protein, which is present in all reflux regardless of pH, was absent from the GC-free control esophagi and normal esophageal specimens obtained from our institutional tissue bank yet present in patients with Barrett’s esophagus and esophageal adenocarcinoma, supporting the absence of reflux in the GC-free control population.

Although IL-8 was measured in chronically pepsin-treated and control-culture supernatants harvested at a visualized concentration of acid, which may in turn elicit pepsinogen conversion to pepsin and increase pH-dependent pepsin activity, leading to tissue disruption and cell death, thereby perpetuating local acidity and upregulation of proton pumps to maintain intracellular pH. Intriguingly, esophageal proton pumps could be a site of pharmacological action of PPIs.

CONCLUSION

These data demonstrate that BE and EAC frequently synthesize pepsinogen and proton pump mRNAs, and that chronic nonacid pepsin exposure in vitro induces cellular changes associated with BE and EAC. These data support a causal role for pepsin in progression of EAC and warrant validation in a greater number of clinical specimens, more in-depth investigation of the effect of pepsinogen and proton pump expression on the molecular biology of the esophagus, and examination of causation in in vivo models of disease.

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