

Pepsin and Carbonic Anhydrase Isoenzyme III as Diagnostic Markers for Laryngopharyngeal Reflux Disease

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Objectives/Hypothesis: The objective was to investigate the potential use of pepsin and carbonic anhydrase isoenzyme III (CA-III) as diagnostic markers for laryngopharyngeal reflux disease. **Study Design:** Prospective cell biological investigation was conducted of laryngeal biopsy specimens taken from 9 patients with laryngopharyngeal reflux disease and 12 normal control subjects using antibodies specific for human pepsin (produced in the authors' laboratory within the Department of Otolaryngology at Wake Forest University Health Sciences, Winston-Salem, NC) and CA-III. **Methods:** Laryngeal biopsy specimens were frozen in liquid nitrogen for Western blot analysis and fixed in formalin for pepsin immunohistochemical study. Specimens between two groups (patients with laryngopharyngeal reflux disease and control subjects) were compared for the presence of pepsin. Further analyses investigated the correlation between pepsin, CA-III depletion, and pH testing data. **Results:** Analysis revealed that the level of pepsin was significantly different between the two groups ($P < .001$). Secondary analyses demonstrated that presence of pepsin correlated with CA-III depletion in the laryngeal vocal fold and ventricle ($P < .001$) and with pH testing data in individuals with laryngopharyngeal reflux disease. **Conclusion:** Pepsin was detected in 8 of 9 patients with laryngopharyngeal reflux disease, but not in normal control subjects (0 of 12). The presence of pepsin was associated with CA-III depletion in the laryngeal vocal fold and ventricle. Given the correlation between laryngopharyngeal reflux disease and CA-

III depletion, it is highly plausible that CA-III depletion, as a result of pepsin exposure during laryngopharyngeal reflux, predisposes laryngeal mucosa to reflux-related inflammatory damage. **Key Words:** Laryngopharyngeal reflux, pepsin, carbonic anhydrase.

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INTRODUCTION

An increasing weight of clinical evidence suggests that reflux of gastric contents into the laryngopharynx (laryngopharyngeal reflux [LPR]) contributes to the pathophysiological characteristics of a number of nonspecific otolaryngological inflammatory and neoplastic disorders.^{1–7} It has been demonstrated that the laryngeal epithelium is more sensitive than the esophagus to injury by gastric reflux. Based on pH monitoring studies, up to 50 reflux episodes per day into the esophagus (below pH 4.0) are considered normal. In contrast, it has been shown experimentally that as few as three reflux episodes per week can produce severe laryngeal damage.⁴ This increased sensitivity to damage may be an underlying cause of many diseases of the airway; however, the biological basis for this sensitivity to damage is not well understood. We propose that reflux-related laryngeal disease results when there is a breakdown in the defense mechanisms that normally protect against damage by corrosive refluxate.

One such defense mechanism that would protect the laryngeal epithelium against damage by LPR is carbonic anhydrase. Carbonic anhydrase (CA) catalyzes the reversible hydration of carbon dioxide, producing bicarbonate ions that are actively pumped into the extracellular space where they can neutralize refluxed gastric acid. By increasing pH, CA would also play an indirect role in reducing peptic activity. Eleven catalytically active isoenzymes have been isolated to date, each showing differences in activity, susceptibility to inhibitors, and tissue-specific distribution.⁸ The esophagus has been shown to express CA-I to CA-IV in the epithelium. The presence of CA in the esophagus is physiologically important because endogenous bicarbonate secretion is capable of increasing the pH of gastroesophageal reflux-derived residual acid from 2.5 to almost neutrality.^{9,10}

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We and other authors have reported an increase in the expression of CA-III protein in esophageal epithelial specimens taken from patients with gastroesophageal reflux disease (GERD) compared with normal control subjects. This is thought to be a compensatory attempt to counteract damage by reflux.^{8,11} In addition, immunofluorescence studies have revealed a re-localization of CA-III from the basal and lower prickle cell layers in normal esophageal tissue to the suprabasal compartment in inflamed esophageal tissue.^{8,11} Together, these findings suggest that the expression of CA-III is modified in patients with GERD and may act as an important protective mechanism by increasing the buffering capacity of inflamed tissue.

In contrast, the localization of CA-III in laryngeal epithelia is unaffected by LPR.⁸ In fact, CA-III, which is present in normal laryngeal epithelia, was found to be absent or expressed at low levels in 64% (47 of 73) of samples taken from patients with LPR. Further analyses of CA levels in different laryngeal sites (vocal fold, ventricle, and posterior commissure) revealed that CA-III protein was absent in 63% (15 of 24) of the vocal fold samples, with a further 21% (5 of 24) of the samples exhibiting low levels. The mean CA-III protein level in the vocal fold epithelia was found to be significantly ($P < .001$) less than that in the posterior commissure epithelium. Furthermore, a correlation between patient severity and posterior commissure CA-III levels was noted ($P < .05$); higher symptom scores were associated with higher CA-III levels.¹² These results suggest that as LPR severity increases, vocal fold CA-III may be depleted, whereas CA-III in the posterior commissure may increase. Finding increased levels of CA-III in the posterior commissure epithelium suggests that this epithelium has increased protection against damage by refluxed acid, which may be one reason why neoplasia rarely develops in the posterior commissure.

The studies just described have all examined expression levels and localization of CA-III in human mucosal biopsy specimens taken from patients with reflux disease. In support of those findings, it has been demonstrated, using a porcine in vitro organ culture model, that exposure of the laryngeal, but not esophageal, epithelium to pepsin (pepsin A, 0.1%) results in irreversible depletion of CA-III.¹² The objective of the present study is to link these observations. Our aims were, first, to demonstrate the presence of pepsin in laryngeal mucosal specimens taken from patients with LPR and compare levels with those found in a control population with no evidence of GERD or LPR, and second, to show a correlation between presence of pepsin and absence or depletion of CA-III protein. Establishment of a direct link between pepsin and inflammatory disease of the airway could lead to new therapeutic approaches.

MATERIALS AND METHODS

Tissue Samples

Laryngeal epithelial biopsy specimens were taken from patients with documented LPR ($n = 9$), as determined by ambulatory 24-hour double-probe pH monitoring and from control sub-

jects ($n = 12$) with no evidence of LPR or GERD. The control population had to demonstrate clinically normal ranges on transnasal fiberoptic laryngoscopy, esophageal and upper esophageal sphincter/pharyngeal manometry, and ambulatory 24-hour double-probe pH monitoring. Biopsy specimens were taken from the posterior larynx in normal control subjects, and from three different laryngeal sites (the vocal fold, posterior commissure, and ventricle regions) in patients undergoing suspension microlaryngoscopy for known laryngeal lesions at Wake Forest University Health Sciences (Winston-Salem, NC). The study was approved by the Institutional Review Board, and all subjects gave written informed consent. Following excision, laryngeal biopsy specimens were temporarily placed in phosphate-buffered saline (PBS) on ice, before being snap-frozen in liquid nitrogen and stored at -80°C for Western blot analysis. For immunohistochemical studies, specimens were fixed in 10% neutral-buffered

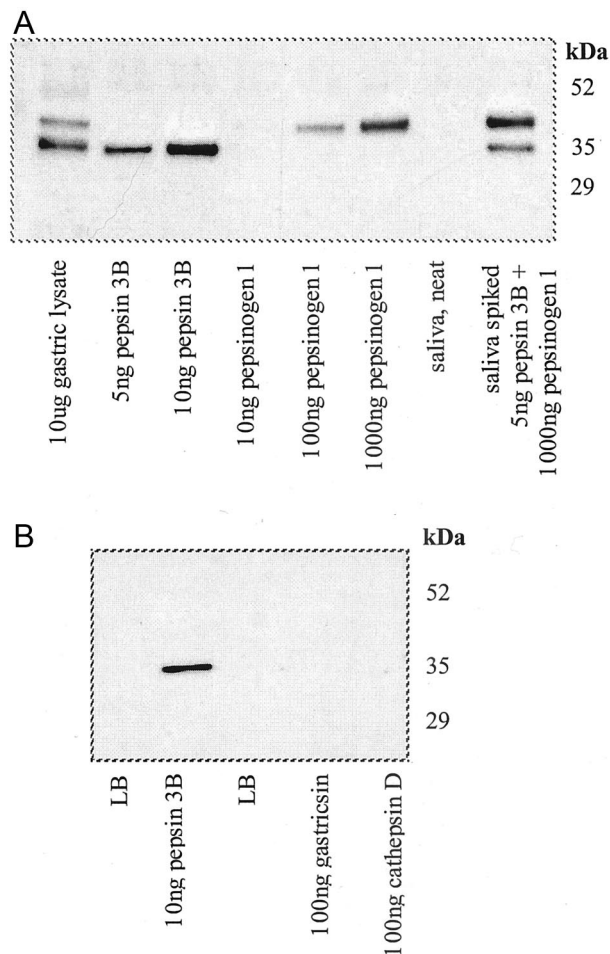


Fig. 1. Specificity of polyclonal anti-human pepsin antibody by Western blot analysis. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel and immunoblotted with an anti-pepsin peptide antibody. (A) Peptide antibody detects both pepsin (lower band, 35 kDa) and pepsinogen (higher band, 43 kDa) in a gastric lysate. This antibody is 100 times more sensitive for pepsin than for pepsinogen. Complete resolution occurs when a highly viscous saliva sample is spiked with both pepsin 3B and pepsinogen 1. (B) Peptide antibody shows specificity for pepsin, showing no cross-reactivity with either gastricsin or cathepsin D (both of which share sequence identity with pepsin). Pepsin 3B = purified human pepsin 3B; pepsinogen 1 = purified human pepsinogen 1; LB = loading buffer.

formalin (Sigma Chemical Co., St. Louis, MO) and dehydrated in various grades of alcohol before being embedded in paraffin wax. Five-micrometer hematoxylin and eosin-stained, paraffin-embedded sections were examined by a histopathologist (J. A. Garvin, MD, Wake Forest University Health Sciences) using light microscopy to confirm tissue type of lining epithelium (respiratory or stratified squamous) and pathological features.

Rabbit Anti-Pepsin Immunoglobulin G

Chemically synthesized peptides were derived from the sequence of human prepepsinogen (SwissProt accession No. P00790) corresponding to amino acid residues 296 to 311. Peptides were conjugated to Keyhole limpet hemocyanin, and rabbits were immunized with the conjugates. Ammonium sulfate precipitated serum was affinity purified using a column with immobilized pepsin (Pierce, Rockford, IL).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Tissue specimens were homogenized and lysed as previously described.⁸ Ten micrograms of total protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel, transferred to a nitrocellulose membrane (Amersham International, Little Chalfont, UK), and probed with a murine monoclonal anti-human CA-III diluted 1:1500 (Spectral Diagnostics, Toronto, Ontario, Canada), a murine monoclonal anti-human actin diluted 1:10,000 (Oncogene Research Products, San Diego, CA), or a rabbit polyclonal anti-human pepsin diluted 1:1500 (developed in our laboratory). All antibodies were diluted in PBS containing 1% (vol/vol) Tween-20. Detection was by enhanced chemiluminescence. The specificity of the anti-human CA-III antibody has been described previously.⁸ The specificity of

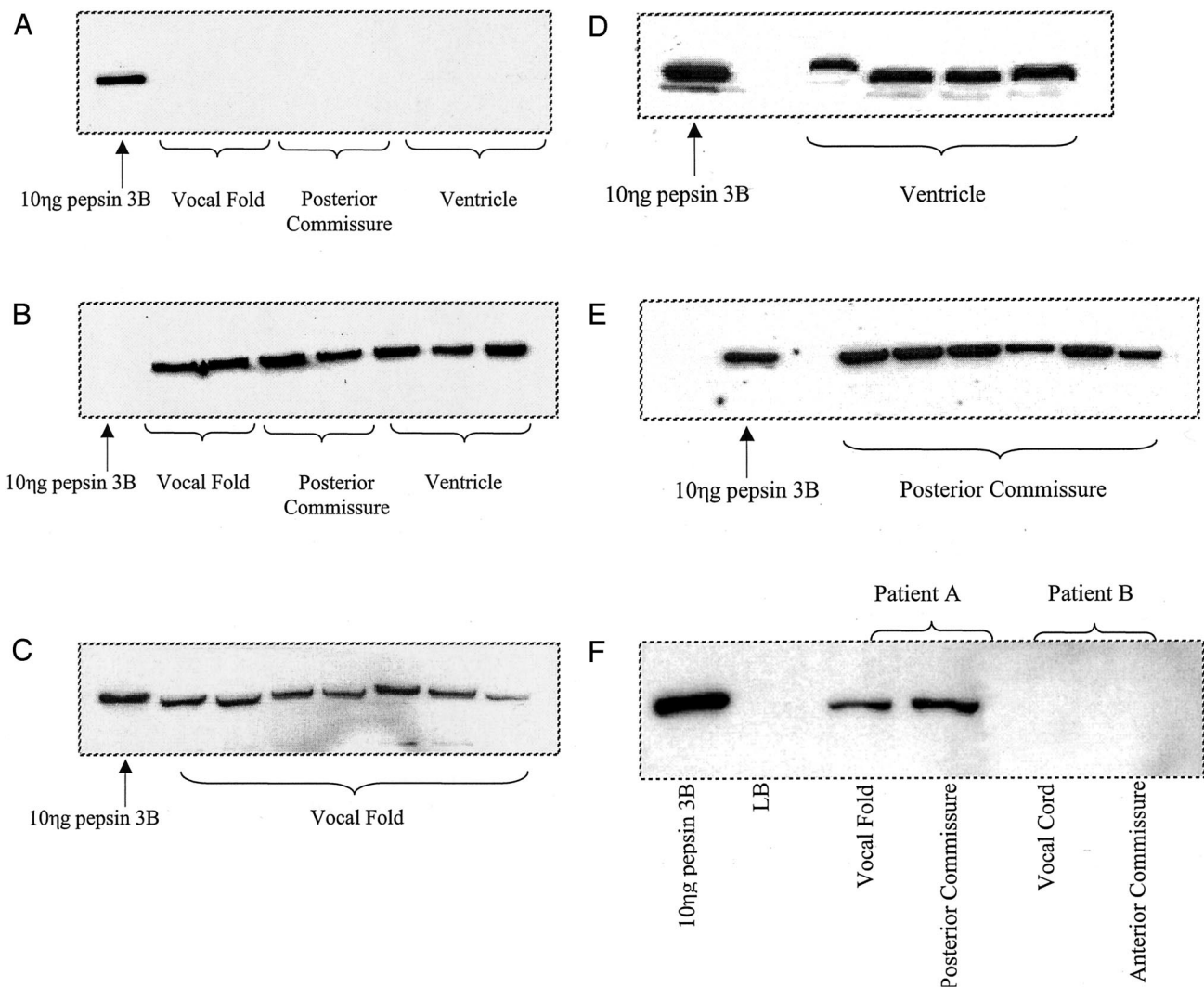


Fig. 2. Western blot analysis for pepsin in laryngeal epithelial specimens taken from normal control subjects and patients with laryngopharyngeal reflux (LPR). Proteins (10 μ g) were separated on a 12% gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with either an anti-pepsin or an anti-actin antibody (A). Pepsin was not detected in any of the samples taken from normal control subjects (n = 12). (B) Repeat probes of blots were performed for actin to show equal loading of total protein. (C, D, E, and F) Pepsin was detected in all of the (C) vocal fold samples (7 of 7), (D) ventricle samples (4 of 4), and (E) posterior commissure (n = 4) samples taken from patients with LPR. (F) Pepsin was detected in samples (2 of 2) taken from patient A, but not in those (0 of 2) from patient B. Patient A had a greater number of pharyngeal reflux episodes (13 episodes at pH < 4 and 194 episodes at pH < 5) detected by double-probe 24-hour pH monitoring than patient B (4 episodes at pH < 4 and 8 episodes at pH < 5). Pepsin 3B = purified human pepsin 3B (positive control); LB = loading buffer.

the polyclonal pepsin antibody was determined by Western blot analysis (Fig. 1).

Immunohistochemical Detection of Pepsin

Slides with 5- μ m formalin-fixed, paraffin-embedded tissue sections were placed in an incubator at 60°C for 30 minutes to secure the tissue sections to the slide. Tissue sections were deparaffinized in xylenes and dehydrated in various grades of alcohol. Endogenous peroxidase activity was blocked by incubating the sections in 3% (vol/vol) hydrogen peroxide for 5 minutes at room temperature. Antigen retrieval was enhanced by placing the sections in a heat-induced epitope retrieval solution (Reveal, Biocare Medical, Walnut Creek, CA) in a digital pressure cooker (Biocare Medical) for approximately 35 minutes. Slides were allowed to cool in Reveal before further treatment. Nonspecific sites were blocked by incubating the sections in 1% bovine serum albumin for 1 hour at room temperature. Primary antibody was then applied to each section for 1 hour at room temperature. A goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Jackson Immuno Research Laboratories Inc., West Grove, PA) was then applied to the sections for 30 minutes at room temperature. Subsequent development of a colored reaction product was achieved by treating the slides with 3, 3'-diaminobenzidine for 1 minute. Sections were counterstained with hematoxylin before mounting for microscopic examination. Normal gastric tissue sections were used for positive control samples. Sections of lymphatic tissue were used for negative control samples. Negative control sections for laryngeal tissue had the primary antibody omitted.

Statistical Analyses

All data were recorded into SPSS 11.0.2 for the Macintosh (Chicago, IL). The association between the levels of pepsin and CAIII in normal control subjects and patients with LPR was evaluated using the Pearson Chi-square. Statistical significance was considered for p-values greater than 0.05.

RESULTS

Figure 1 demonstrates the specificity of the polyclonal anti-human pepsin antibody. The polyclonal antiserum recognized two immunoreactive bands in a gastric lysate. Polypeptide bands were detected at 35 kD, corresponding to the molecular weight of pepsin, and at 43 kD, corresponding to the molecular weight of pepsinogen. The

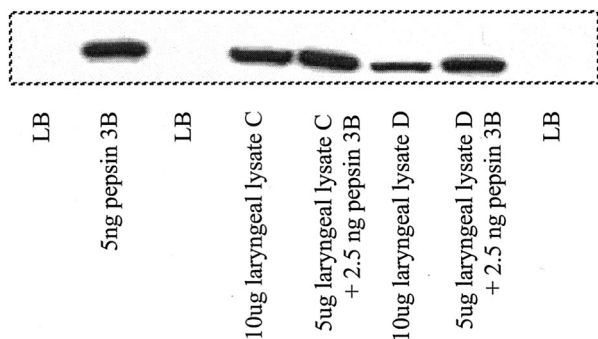


Fig. 3. Western blot analysis of pepsin in laryngeal epithelial lysates. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel and immunoblotted with an anti-pepsin peptide antibody. A single polypeptide band was detected when laryngeal lysates were mixed with purified pepsin 3B. Pepsin 3B = purified human pepsin 3B; LB = loading buffer.

antibody was 100 times more sensitive for pepsin than for pepsinogen. Furthermore, complete resolution occurred when a highly viscous saliva sample was spiked with both purified human pepsin 3B and pepsinogen 1. The anti-human pepsin antibody showed no cross-reactivity with either gastricsin or cathepsin D, both of which are homologous aspartic proteases.

An investigation was performed to examine the presence of pepsin in laryngeal epithelial tissue specimens taken from patients with documented LPR and to compare pepsin levels found with that in specimens taken from normal control subjects (Fig. 2A). The polyclonal antiserum recognized a single immunoreactive band at 35 kD, corresponding to the correct molecular weight of pepsin. Pepsin was not detected in any of the specimens taken from control subjects (n = 12) (Fig. 2A and B). However, pepsin was detected in 8 of 9 patients with LPR ($P < .001$) (Fig. 2C, D, E, and F).

The present study revealed a possible correlation between pepsin levels and pH testing data (Fig. 2F).

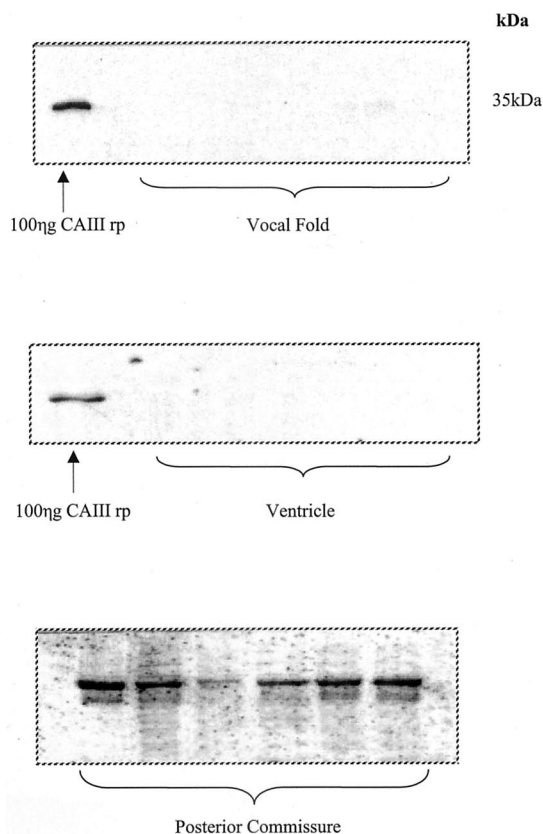


Fig. 4. Expression of carbonic anhydrase isoenzyme III (CA-III) in laryngeal epithelial specimens taken from patients with laryngopharyngeal reflux (LPR). Proteins (10 μ g) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel and immunoblotted with an anti-CA-III antibody. A single polypeptide band was detected at 35 kDa, corresponding to the correct molecular weight of the antigen. Carbonic anhydrase isoenzyme III was not detected in any of the vocal fold (0 of 7) or ventricle samples (0 of 4) in which pepsin was detected (Fig. 2). CA-III protein was detected in samples taken from the laryngeal posterior commissure (6 of 6). CAIII rp = CA-III recombinant protein (positive control).

Pepsin levels were compared between samples taken from two patients. Pepsin was detected in samples taken from patient A but not in those from patient B. Patient A had a significantly greater number of reflux episodes detected in the pharynx by double-probe 24-hour pH monitoring (13 episodes at pH<4 [11 with patient in upright and 2 with patient in supine position] and 194 episodes at pH<5 [113 in upright and 81 in supine position]) than patient B, who had 4 episodes at pH<4 (4 in upright and 0 in supine position) and 8 episodes at pH<5 (8 in upright and 0 in supine position).

To ensure that the protein detected by ECL was, indeed, pepsin, two of the laryngeal lysates were mixed

with purified pepsin 3B (Fig. 3). Consistent with our hypothesis, a single band was detected at 35 kD.

Western blot analysis confirmed a correlation between the presence of pepsin and absence of CA-III protein expression in the laryngeal vocal fold and ventricle of patients with LPR (Fig. 4). CA-III protein, which was expressed to high levels in normal control subjects, was not detected in any of the vocal fold or laryngeal ventricle samples taken from patients with LPR ($P < .001$). No significant difference was found in expression levels of CA-III in the posterior commissure between normal subjects and patients with LPR ($P > .05$).

The affinity purified peptide antibody against human pepsin has also been successfully used to identify pepsin

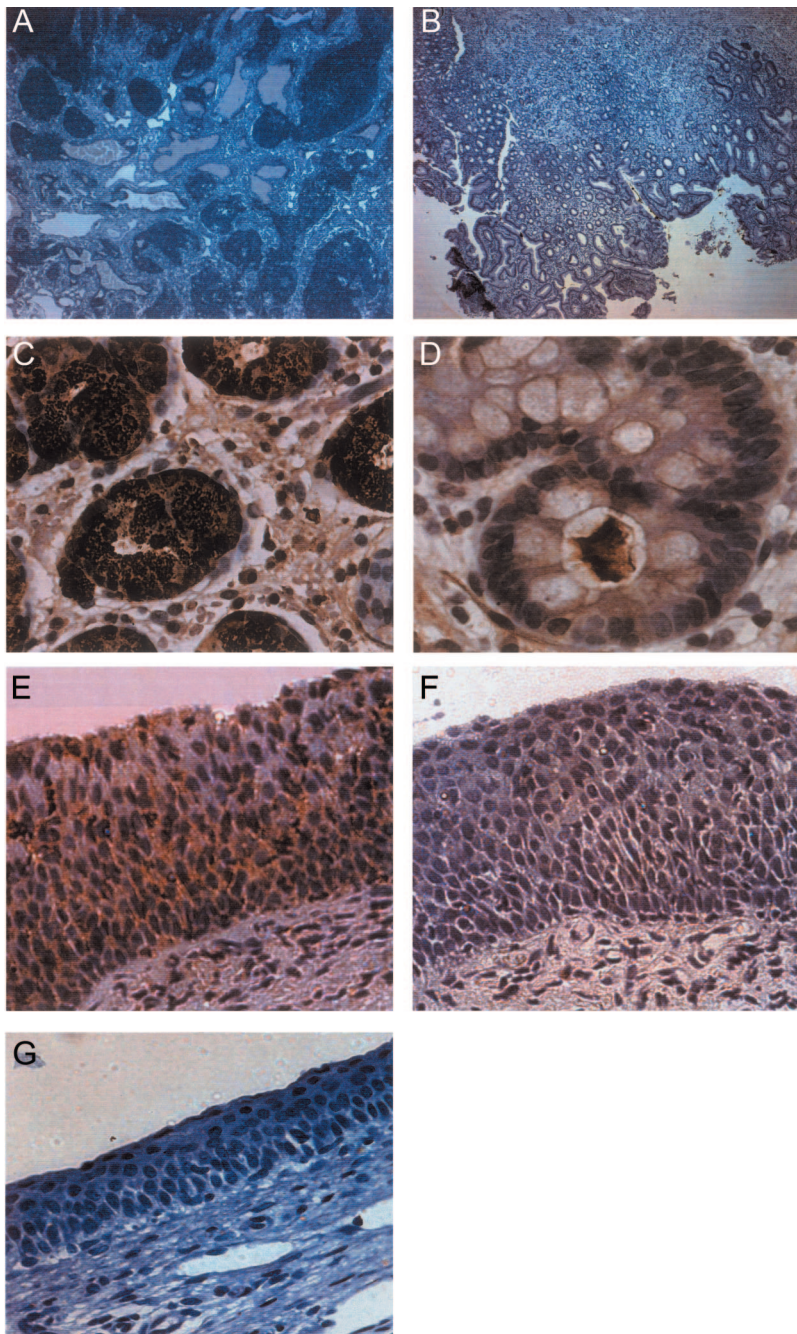


Fig. 5. Immunohistochemical detection of pepsin. The affinity purified anti-pepsin antibody was used in conjunction with a diaminobenzidine staining kit. Five-micrometer tissue sections were counterstained with H&E. (A) Pepsin is not present in lymphatic tissue (negative control) (original magnification $\times 29$). Gastric tissue. (B) There is no staining when the primary antibody is omitted (negative control) (original magnification $\times 29$). (C) The peptide antibody detects pepsinogen in the cytoplasm of chief cells at the base of the gastric glands (original magnification $\times 450$). (D) Pepsin in the gastric pits before being secreted into the lumen of the stomach (original magnification $\times 450$). Tissue specimen taken from a patient with laryngopharyngeal reflux. (E) The peptide antibody detects pepsin both in the interstitium and in the cytoplasm of laryngeal epithelial cells (original magnification $\times 179$). (F) There is no staining when the primary antibody is omitted (negative control) (original magnification $\times 179$). (G) Tissue specimen taken from a normal control subject. There is no staining when the anti-pepsin antibody is applied (original magnification $\times 179$).

in formalin-fixed, paraffin-embedded tissue sections by immunohistochemical analysis (Fig. 5). Pepsin was not detected in lymphatic tissue (Fig. 5A) or in gastric tissue when the primary antibody was omitted (Fig. 5B) (negative control sample). Incubation of gastric tissue sections with the pepsin antibody (positive control sample) revealed staining at the base of the gastric glands where pepsin-secreting cells, called chief cells, are located. Both the cytoplasm of chief cells, where pepsinogen is located (Fig. 5C), and the gastric pits, where pepsin is secreted (Fig. 5D), were positively stained. Figure 5E demonstrates the presence of pepsin both in the interstitium and in the cytoplasm of laryngeal epithelial cells from a patient with reflux-related laryngeal disease. No staining was observed when the primary antibody was omitted (Fig. 5F). Pepsin was not detected in laryngeal sections taken from normal control subjects (Fig. 5G).

DISCUSSION

Although the present study is small, detection of pepsin in the laryngeal epithelium of 8 of 9 patients with pH-documented LPR and 0 of 12 normal control subjects is highly significant ($P < .001$). The fact that CA-III, which has been shown to be present in normal laryngeal epithelia,^{8,12} is depleted in the laryngeal vocal fold and ventricle of these patients, together with the previously observed depletion of laryngeal CA-III protein following exposure to pepsin in our *in vitro* model,¹² suggests that the pepsin present in the laryngeal epithelium of these patients is likely to be the causal factor for CA-III depletion. As a result, the laryngeal epithelium may not have been able to produce sufficient bicarbonate to neutralize gastric acid during reflux events causing a decrease in extracellular pH. Therefore, pepsin would have remained active and, because it is known to cause cellular damage, is likely to have been a factor contributing to reflux-associated laryngeal injury and disease. Several investigators have demonstrated peptic activity at pH levels as high as 6.5.^{13,14} Furthermore, we have previously shown by electron microscopy that exposure of the laryngeal epithelium to pepsin A (0.1%) breaches cell membrane integrity and increases intracellular space.⁸ These results demonstrate the ability of pepsin to penetrate and thus injure the laryngeal epithelium.

In the present study, CA-III was detected in the laryngeal posterior commissure, despite the presence of pepsin. This finding supports the previously reported increased buffering capacity of the laryngeal posterior commissure thought to be provided by CA-III. These observations highlight the differences between these tissues and the possible protective role of CA-III in the posterior commissure against reflux-related damage and development of disease, which is rarely reported in this region.¹² We hypothesize that exposure of laryngeal epithelia to pepsin during LPR results in injury by causing a breakdown in epithelial defense (specifically, CA-III) and suggest that the combination of presence of pepsin and absence of CA-III protein in the laryngeal vocal fold and ventricle is diagnostic of LPR.

Reflux has been reported to be associated with, to complicate, and possibly even to cause not only laryngopharyngeal but also respiratory tract diseases.⁴ The results from the present study support the hypothesis that pepsin is refluxed into the upper aerodigestive tract, where it is likely to cause inflammatory disease. Thus, a large-scale study is currently being performed to assess pepsin levels in airway secretions taken from patients with reflux-related disease and to correlate the presence of pepsin and the depletion of CA-III with the inflammatory process and markers for neoplasia. We have optimized an enzyme-linked immunosorbent assay for the detection of pepsin (U.S. Patent No. 5,879,897).¹⁵ The limit of detection using trimethylsilyl bromide (TMB) as a substrate for horseradish peroxidase is 10 pg. At present, the most sensitive diagnostic test for LPR is 24-hour ambulatory double-probe pH monitoring. This test is invasive and often poorly tolerated with a sensitivity of only 75%. If a correlation is found between the presence of pepsin and reflux-related inflammatory disease, this newly developed, noninvasive biochemical test may provide a method for assessment of reflux in infants, children, and adults with laryngopharyngeal and respiratory tract diseases.

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