CELL BIOLOGY OF LARYNGEAL EPITHELIAL DEFENSES IN HEALTH AND DISEASE: FURTHER STUDIES

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This is the second annual report of an international collaborative research group that is examining the cellular impact of laryngopharyngeal reflux (LPR) on laryngeal epithelium. The results of clinical and experimental studies are presented. Carbonic anhydrase (CA), E-cadherin, and MUC gene expression were analyzed in patients with LPR, in controls, and in an in vitro model. In patients with LPR, we found decreased levels of CAIII in vocal fold epithelium and increased levels in posterior commissure epithelium. The experimental studies confirm that laryngeal CAIII is depleted in response to reflux. Also, cell damage does occur well above pH 4.0. In addition, E-cadherin (transmembrane cell surface molecules, which have a key function in epithelial cell adhesion) was not present in 37% of the LPR laryngeal specimens. In conclusion, the laryngeal epithelium lacks defenses comparable to those in esophageal epithelium, and these differences may contribute to the increased susceptibility of laryngeal epithelium to reflux-related injury.

KEY WORDS — carbonic anhydrase, cell biology, cellular defenses, E-cadherin, extraesophageal reflux, gastroesophageal reflux, laryngopharyngeal reflux, mucin, mucus, reflux, vocal fold.

INTRODUCTION

An estimated 15% to 44% of American adults experience the gastroesophageal reflux disease (GERD) symptom, heartburn, on a monthly basis.[1] Patients with long-term GERD may develop esophagitis, peptic stricture, and/or Barrett's esophagus. The last is a premalignant condition with an associated increased risk of esophageal adenocarcinoma.[2,3]

Over the past 2 decades there has been increasing clinical evidence to suggest that retrograde reflux of gastric contents into the laryngopharynx, or laryngopharyngeal reflux (LPR), may play an important role in the development of laryngeal disease. This is supported by observations of pH-documented LPR in 50% of patients with laryngeal and voice disorders.[4,5]

Laryngopharyngeal reflux contributes to the patho-physiology of a number of nonspecific otolaryngological inflammatory and neoplastic disorders.[4-11] The most common symptoms appear to be hoarseness, globus pharyngeus, dysphagia, chronic cough, and throat clearing.[4,10] Additionally, LPR has been reported to be associated with paroxysmal laryngospasm.[12-14] These findings, along with others, raise the question of an association of reflux-related laryngospasm with sudden infant death syndrome, with LPR playing a significant role.[15-18]

Reflux has also been reported to be associated with, to complicate, and possibly even to cause asthma.[19,20] The prevalence of LPR among asthma patients has been estimated to lie between 60% and 80%.[20,21] Furthermore, aggressive antireflux therapy has been shown to improve asthma symptoms in approximately 70% of asthma patients.[22] Finally, LPR has also been implicated in the development of la-

ryngeal carcinoma.[23,24]

The manifestations of LPR differ significantly from those of GERD. Patients with LPR typically present with hoarseness, chronic cough, and throat clearing, but uncommonly with heartburn.[4,5,10,11,25] Patients with GERD, on the other hand, have esophagitis and heartburn, but rarely have throat symptoms.[4,11,25] This discrepancy exists because the patterns and mechanisms of LPR and GERD are different. Patients with LPR typically have upright (daytime) reflux with good esophageal motor function and no esophagitis, whereas GERD patients have supine (nocturnal) reflux and esophageal dysmotility.[4,11,25]

Not only are the mechanisms and manifestations of LPR and GERD different, but it has been shown that the laryngeal epithelium is more sensitive to reflux-related injury than is esophageal epithelium.[4] It appears that the two epithelial tissues are protected differently against gastric reflux. One such mucosal defense mechanism that may be used to counteract damaging factors is provided by a family of metalloenzymes called carbonic anhydrases (CAs). Carbonic anhydrase (EC 4.2.1.1) is ubiquitously expressed in mammalian tissues and catalyzes the reversible hydration of carbon dioxide[26-28] as follows:

$$\text{CA}$$
$$CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$$

The bicarbonate ions thereby produced are actively pumped out of the cell, via anion exchange, into the extracellular space, in which they can then neutralize hydrogen ions and lead to an increase in extracellular pH. Carbonic anhydrase may also play an important role in controlling internal pH by neutralizing intracellular protons. Eleven catalytically active isoforms have been isolated and characterized, as well as several other CA-related proteins that share sequence homology but do not posses catalytic activity. All isoforms catalyze the same reaction, but with differing activities, and all demonstrate expression in specific tissue and subcellular localizations.[29] The role of CA in the larynx is undefined, but it may act as an intrinsic defense mechanism against LPR by generating a bicarbonate barrier. This role has been suggested for the esophagus.[30]

We have previously shown that esophageal samples taken from patients with GERD demonstrate an increased expression of CAIII in the inflamed squamous epithelium.[29] In addition, we demonstrated that the enzyme was redistributed to the suprabasal layers of the epithelium. These perturbations are presumably directly induced by the refluxate and may represent compensatory attempts to counteract damage.

Preliminary studies showed that CA isoenzymes in the squamous epithelium of the larynx do not have the same pattern of response. As a result, laryngeal epithelium may be less resistant to damage by LPR. Furthermore, absence of CAIII in the vocal fold epithelium may result in a reduced ability to protect against LPR.[29]

To substantiate our preliminary data, we examined the laryngeal CA isoform status of 26 patients with pH-documented LPR. In addition, an in vitro organ culture system was used to determine whether the absence of CAIII observed in patients with LPR is a direct result of the refluxate.

Two other sets of experiments were performed. First, an in vitro model was developed using the porcine larynx to assess the damage potential of the refluxate. Second, we investigated the expression of genes encoding mucin, the major glycoprotein in mucus,[31] the E-cadherin cell-cell adhesion molecule, and cytokeratins in order to evaluate their roles in laryngeal epithelial protection.

Mucus is a complex mixture of biological materials secreted by the cells of the mucosa that acts to protect the delicate mucosal surfaces of the body. Mucus is composed of water (approximately 95%), proteins and glycoproteins (3%), lipids (1%), and electrolytes (1%)[32]; however, the exact composition of mucus alters from site to site throughout the body. Mucin is coded for by a selection of more than 10 genes encoding a product that may be either gel-forming (eg, MUC2, 5AC, 5B, 6) or membrane-bound (eg, MUC1, 3, 4).[33] Each mucosal tissue exhibits its own specific expression of mucin genes, from the gel-forming mucins found in the stomach[34] to the cell surface–associated mucins found coating the esophagus.[35]

At present, the expression of MUC genes in the larynx is unknown. Therefore, depending on the epithelial type, ie, squamous or respiratory, it is proposed that the products of each mucin gene perform a specific physiological function, one of which may be to provide luminal protection against gastric reflux during LPR. In this study, the expression and subcellular localization of each individual mucin molecule was investigated in the lining epithelium of laryngeal biopsy specimens and in the underlying fibrous connective tissue.

The architecture of a tissue is defined by the nature and the integrity of its cellular and extracellular compartments, and is based on proper adhesive cell-cell and cell–extracellular matrix interactions. Because cell adhesion complexes are linked to the cytoskele-

ton, they represent checkpoints for regulation of cell shape and gene expression and thus are instructive for cell behavior and function. This organization allows a reciprocal flow of mechanical and biochemical information between the cell and its microenvironment, and necessitates that cells actively maintain a state of homeostasis within a given tissue context. The loss of the ability of epithelial cells to establish correct adhesive interactions with their microenvironment results in disruption of tissue architecture with often fatal consequences for the host organism.

Loss of E-cadherin–mediated adhesion has emerged as a key element of the neoplastic process[36] leading to increased invasiveness and decreased differentiation.[37-39] Mutations in E-cadherin and mutations in α, β, and γ catenins and the activation of several receptor tyrosine kinases (epidermal growth factor receptor and receptor for hepatocyte growth factor, resulting in catenin phosphorylation) have all been shown to result in the perturbation of cellular adhesion.[38,40,41] Expression of α catenin has been shown to be reduced or absent in a number of primary tumors of the esophagus, stomach, and colon, with E-cadherin–mediated adhesion being abrogated by the down-regulation of α catenin.[42-44]

Cytokeratins are intermediate filaments that form the basis of the cytoskeletal structure in epithelial cells. Twenty subsets have been characterized to date; subsets 7 and 20 have the most restricted expression. The patterns of cytokeratin expression can be regarded as specific markers of epithelial differentiation status. The structure of cytokeratin is based on rodlike subparticles.[45] Each single polypeptide chain has amino and carboxy-terminal domains of characteristic size, composition, and sequence that are separated by an α-helix–rich domain with a heptad structure. Two polypeptide chains spontaneously form coiled-coil dimers in solution, by interfacing their respective apolar areas of the α-helix.[45-47] In this study we have also conducted preliminary investigations of the expression of E-cadherin and pan-cytokeratin in human biopsy specimens from normal laryngeal mucosa and from patients with LPR to determine whether gastric reflux has any effect on the cellular expression of these proteins.

MATERIALS AND METHODS

Human Tissue Samples. Laryngeal epithelial biopsy specimens (n = 78) were obtained from patients (n = 26) undergoing suspension microlaryngoscopy at Wake Forest University School of Medicine, Winston-Salem, North Carolina. The study was approved by the Institutional Review Board, and all patients gave written informed consent. After excision, the laryngeal biopsy specimens were placed in phosphate-buffered saline solution (PBS) on ice, snap frozen in liquid nitrogen, and stored at −80°C for Western blot analysis. For immunohistochemical and in situ hybridization studies, specimens were fixed in 10% neutral-buffered formalin (Sigma, St Louis, Missouri) and dehydrated in various grades of alcohol before being embedded in paraffin wax. Hematoxylin and eosin staining of paraffin-embedded sections (5 µm) was used to classify the type of lining epithelium (respiratory or stratified squamous) by light microscopy. Formalin-fixed, paraffin-embedded sections (5 µm) of normal human laryngeal tissue were obtained from Peterborough Hospital Human Research Tissue Bank, Peterborough, England.

SDS-PAGE and Western Blot Analysis. Tissue samples were homogenized and lysed as previously described.[29] Ten micrograms of total protein was separated on a 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred to a nitrocellulose membrane (Amersham International, Little Chalfont, England), and probed with antibodies against human CA isoenzymes (sheep anti-human CAI immunoglobulin G [IgG] diluted 1:2,000, The Binding Site, Birmingham, England; rabbit anti-human CAII IgG peptide antibody diluted 1:1,000; or murine monoclonal anti-human CAIII diluted 1:1,500, Spectral Diagnostics, Toronto, Canada). All antibodies were diluted in PBS containing 1% (vol/vol) Tween-20. Detection was by enhanced chemiluminescence (ECL). The specificity of polyclonal anti-human CAI and monoclonal CAIII antibodies has previously been described.[29] The specificity of the polyclonal CAII antibody was determined by Western blot analysis. Fifty nanograms of CAI and CAII purified proteins and CAIII recombinant protein were separated by SDS-PAGE. After transfer of proteins onto nitrocellulose membrane via Western blotting, the membrane was probed with anti-CAII IgG.

Rabbit Anti-CAII IgG. Phosphopeptides from residues 127 through 139 of human CAII were synthesized by Dr Graham Bloomberg (University of Bristol, Bristol, England). Polyclonal antibodies against CAII were developed by coupling peptides to carrier proteins with glutaraldehyde.[48] Crude IgG was affinity-purified against CAII purified protein (Sigma, Dorset, England) with Reacti-Gel supports (Pierce, Rockford, Illinois).

Organ Culture of Porcine Tissue Samples. The esophagus and larynx from pigs were obtained from a local abattoir within minutes of slaughter and transported in oxygenated PBS on ice to the laboratory. Within 1 hour of death, both the esophageal mucosa and the posterior commissure laryngeal mucosa were

dissected and cut into 2-mm^2 sections that were then placed luminal surface–down on a stainless steel wire grid. The wire grid was positioned over the central well of an organ culture dish (Fred Baker Scientific, Runcorn, England), and Ham's F-10 nutrient medium (pH 7.4; Gibco BRL, Paisley, Scotland), supplemented with penicillin (200 IU/mL) and streptomycin (0.2 mg/mL) and prewarmed to 37°C, was added to the well up to the level of the luminal surface of the epithelium to generate a fluid-gas interface.[49] This tissue was left for 15 minutes to allow equilibration. The nutrient medium was then replaced by acidified Ham's F-10 medium adjusted to pH 7, pH 5, pH 4, or pH 2 with 0.1 mol/L hydrochloric acid, with or without 0.1% (wt/vol) porcine pepsin A (Sigma). The tissue was incubated for 20 minutes at 37°C in a humidified incubator with 95% oxygen and 5% carbon dioxide. For recovery studies, the treatment medium was removed and the tissue was gently washed before incubation in fresh Ham's F-10 medium (pH 7.4) for a further 4 hours at 37°C. After treatment, the tissue was either snap frozen in liquid nitrogen for Western blot analysis or fixed for analysis by electron microscopy.

Damage Model of Porcine Larynx. Krebs-Ringer solution (pH 2.0 to pH 8.0) alone or containing 0.1 to 1.0 mg/mL porcine pepsin A was used as a test solution to mimic gastric reflux contents. A control solution of 1 mg/mL denatured porcine pepsin A was also used at pH 2.0. Porcine larynges were trimmed of extraneous material and sealed tracheally by means of a bijou tube with cyanoacrylate adhesive. The model was then immersed in Krebs-Ringer solution, and a rinse of Krebs-Ringer solution was instilled for 15 minutes. The rinse solution was removed and replaced with the test solution, which was incubated in the model for 1 hour. During the experiment, the model was maintained at 37°C and aerated luminally and serosally with 95% oxygen–5% carbon dioxide. The instillates were characterized for damage quantitatively via a fluorimetric DNA assay.[50]

Electron Microscopy. Porcine esophageal and laryngeal mucosal sections were fixed in 3% (vol/vol) glutaraldehyde and embedded in epoxy resin. Ultrathin resin-embedded sections (50 to 100 nm) were cut on a Reichert OMU3 microtome with glass knives and then transferred onto 3-mm copper grids. After staining with uranyl acetate–lead citrate, the sections were visualized with a Philips EM-2085 transmission electron microscope at the appropriate magnification.

In Situ Hybridization. In situ hybridization was carried out on formalin-fixed 5-μm serial sections of human laryngeal tissue samples with 48 bp 3'5' digoxigenin-labeled antisense oligonucleotide probes to the tandem repeat regions of each of the mucin genes (1 through 8) in a method modified from Aust et al[51] as described by Severn et al.[52]

Immunohistochemistry. The avidin-biotin indirect immunoperoxidase method was used. To enhance E-cadherin antigen retrieval in formalin-fixed, paraffin-embedded tissues, we used a microwave antigen retrieval technique. Sections were pretreated with 0.01 mol/L citrate buffer at pH 6.0 and microwave-treated for five 2-minute cycles at 750 W, with pauses to ensure that there was no fluid loss due to evaporation. The slides were then allowed to cool in buffer before further treatment. Blocking of endogenous peroxidase was achieved by incubating the sections in a 0.3% (vol/vol) solution of hydrogen peroxide for 30 minutes. Nonspecific sites were blocked for 15 minutes with 1 mL of normal rabbit serum. Primary antibody (100 μL) was then added to each section and left to incubate overnight at 4°C in a humidified chamber. A streptavidin-biotin immunoperoxidase method was used to amplify epitope recognition. Sections were incubated with 100 μL biotinylated rabbit anti-mouse IgG (Dako Ltd, High Wycombe, England) for 1 hour at room temperature and then incubated with 100 μL streptavidin peroxidase conjugate (streptavidin-biotin complex/horseradish peroxidase, Dako Ltd) for a further hour at room temperature. Subsequent development of a colored reaction product was achieved by treatment of the slides with a solution of 1 mL of 3,3'-diaminobenzidine (DAB; concentration 25 mg/mL in 100 mL of PBS and 100 mL of hydrogen peroxide) for as long as 5 minutes. Sections were counterstained with hematoxylin before mounting for microscopic examination. Normal colonic tissues of homogeneous immunophenotype for the studied antigens were included as positive control sections. Negative control sections had the primary antibody omitted.

Monoclonal Antibodies. Monoclonal antibody for E-cadherin was purchased from Transduction Laboratories (distributed by Affinity, Exeter, England). Anti-cytokeratin antibody was obtained from Novocastra Laboratories (Newcastle Upon Tyne, England). The E-cadherin antibody was used at a dilution of 1:1,000, and the pan-cytokeratin antibody at 1:100.

Evaluation of Immunohistochemistry. Each tissue specimen was examined for E-cadherin and pan-cytokeratin by 2 independent observers who were blinded to the clinical and pathological diagnosis. Expression and subcellular localization (eg, membranous, cytoplasmic, nuclear) were evaluated in stratified squamous epithelium and respiratory-type epithelium.

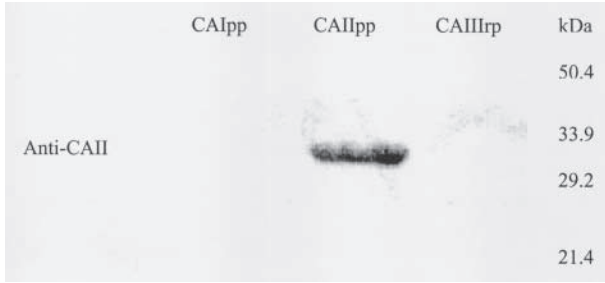*Statistical Methods*. Statistical analysis was per-

Fig 1. Specificity of polyclonal anti-human carbonic anhydrase (CA) II antibody by Western blotting. Fifty nanograms of CAI and CAII purified protein (CAIpp and CAIIpp, respectively) and CAIII recombinant protein (CAIIIrp) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with 12% gel. After electrophoresis, proteins were visualized by immunoblotting with anti-CAII antibody. Polypeptide band was detected at 30 kd. Results show that this antibody is specific for its isoenzyme, showing no cross-reactivity with either CAI or CAIII isoforms.

formed to compare CA protein expression in different laryngeal epithelial tissues and to examine whether CAIII expression levels in posterior commissure epithelium correlated with clinical symptoms. The data were recorded and coded into SPSS 6.1.1 for Macintosh (Chicago, Illinois). The χ^2 test was used to evaluate the statistical associations among abnormal symptom indices, reflux finding scores, and the presence of CA. The matched-pairs *t*-test was used to evaluate differences between levels of CA in the vocal fold and posterior commissure. A p value of <.05 was regarded as significant for all statistical parameters tested.

RESULTS

Figure 1 demonstrates the specificity of the polyclonal anti-human CAII antibody. The polyclonal an-
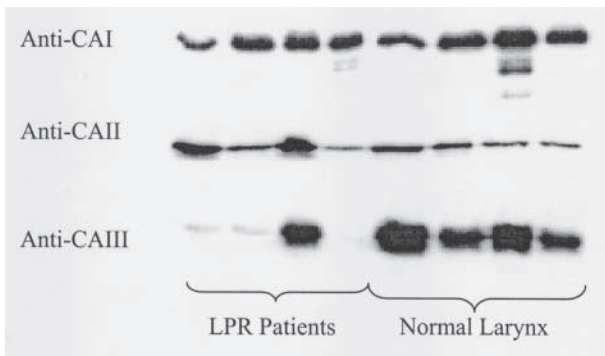

Fig 2. Expression of CAI, CAII, and CAIII in samples of normal laryngeal epithelium and in patients with laryngopharyngeal reflux (LPR). Proteins (10 μg total protein) were visualized by SDS-PAGE on 12% polyacrylamide gel and immunoblotted with anti-CAI, -CAII, or -CAIII. Both CAI and CAII protein levels were similar in LPR patients and normal laryngeal epithelium. CAIII was expressed to high levels in normal laryngeal samples, but only in small percentage of LPR patients. Remaining LPR patients exhibited very little CAIII protein.
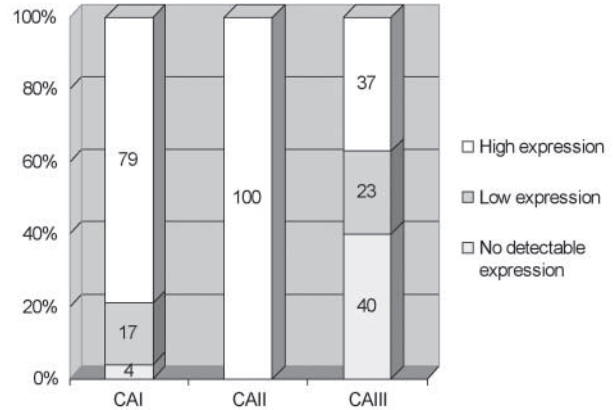

Fig 3. Expression of CAI, CAII, and CAIII in laryngeal epithelium of patients with documented LPR. Results from 3 different sites from which biopsy specimens were taken have been combined for each isoenzyme. Twenty-one percent (16/75) of samples had very little or no CAI. All samples analyzed (18/18) expressed CAII to high levels. Sixty-four percent (47/73) of samples had very little or no detectable CAIII protein.

tiserum recognizes a single immunoreactive band at 30 kd and is highly specific for its isoenzyme, showing no cross-reactivity with the CAI or CAIII isoforms.

Preliminary investigations were carried out to determine the expression levels of CAI, CAII, and CAIII in normal human laryngeal tissue as compared with tissue from LPR patients (Fig 2). Normal samples and those from LPR patients expressed both CAI and CAII to similar levels. All normal samples possessed CAIII, whereas 3 of 4 LPR patients showed very little or no CAIII expression.

The CAI and CAIII protein levels were analyzed in 26 patients with documented LPR by Western blot analysis, and the results were confirmed by immunofluorescence by the method described by Axford et al.[29] Each patient had a biopsy specimen taken from 3 different laryngeal sites: the vocal fold, the posterior commissure (both of which are covered by squamous epithelia), and the ventricle (lined by a ciliated columnar respiratory-type epithelium). The expression levels of both CAI and CAIII were investigated in a total of 75 and 73 laryngeal samples, respectively. The results are summarized in Fig 3. Twenty-one percent (16/75) of samples showed very little or no CAI expression, and 64% (47/73) of samples demonstrated very little or no detectable expression of CAIII protein. The CAII expression was determined in 18 laryngeal samples: 1 vocal fold, 11 posterior commissure, and 6 ventricle. All samples were positive for CAII protein.

Table 1 shows all of the CA data of the LPR patients. Sixty-three percent (15/24) of the vocal fold epithelial samples showed no CAIII to be present,

TABLE 1. EXPRESSION OF CAI AND CAIII IN LARYNGEAL MUCOSA OF PATIENTS WITH LPR

Patient No.	CAI			CAIII		
	Vocal Fold	*Posterior Commissure*	*Ventricle*	*Vocal Fold*	*Posterior Commissure*	*Ventricle*
1	+	+ +	+	−	+	−
2	+ +	+ +	+ +	−	−	−
3	+ +	+ +	+	−	+ + +	+
4	+ + +	+ +	+ +	+ +	+ +	+ + +
5	+ + +	+ +	+ +	−	+ +	+ +
6	+ +	+ +	+ +	+	−	−
7	+ +	+ +	+ +	+	+ + +	+
8	+ +	−	+	−	+	−
9	−	+ +	+	−	+ +	−
10	+ + +	+ +	+ +	−	+ +	+ +
11	+ + +	+ + +	+ +	+ +	+ +	−
12	+ +	+ +	+ +	−	−	−
13	−	+ +	+ +	−	+ +	−
14	+ +	+ +	+ +	+ + +	+ +	+
15	+ + +	+ + +	+ + +	+	−	+ +
16	+	+ +	+ +	−	+ +	−
17	ND	+ +	ND	ND	+	ND
18	+ + +	+ +	+ + +	−	+ +	+
19	+	+ +	+ + +	ND	+ +	+
20	+ +	+ +	+ + +	+	+	+ +
21	+ +	+ +	+ +	−	−	−
22	+ + +	+ +	+ +	+ +	+ +	+
23	+	+	ND	−	−	ND
24	+ +	+ +	+ +	+	+ +	+ + +
25	+	+	+	−	ND	−
26	+ +	+ +	+	−	+ +	+ +

CA — carbonic anhydrase; LPR — laryngopharyngeal reflux; ND — not determined; − — no detectable expression; + — low level of expression; ++ and +++ — high levels of expression.

and another 21% (5/24) had severely decreased levels of CAIII. With the data in numerical form (− = 0; + = 1; ++ = 2; +++ = 3), the mean (±SD) CAIII level for vocal fold epithelium was 0.6 ± 0.8, and it was 1.6 ± 0.9 for posterior commissure epithelium. This difference was significant at $p < .001$. In addition, there was a correlation between the patients' symptom severity and the posterior commissure CAIII levels ($p < .05$); ie, higher symptom scores were associated with higher CAIII levels.

The CAI and CAIII expression levels were investigated in porcine esophageal and laryngeal mucosae after acid or acid and pepsin stress with an in vitro organ culture system (Figs 4 and 5). After 20 minutes of acid stress at pH 4 and pH 2, a decreased CAIII expression was observed in the porcine laryngeal mucosa (Fig 4A), but not in the esophageal mucosa (Fig 4B). Both laryngeal (Fig 4A) and esophageal (Fig 5A) CAI protein levels were unaffected. When the laryngeal tissue was incubated for a further 4 hours in fresh medium, corresponding to the recovery phase, the CAIII protein returned to basal levels (Fig 4C).

An increase in CAI was observed at all pH levels in both the esophagus and the larynx after the addition of pepsin (Fig 5A). In contrast to treatment with acid alone (Fig 4A,B), a decrease in esophageal CAIII was observed at pH 4 and pH 2 with the addition of pepsin. Moreover, expression levels returned to basal levels after a recovery phase (Fig 5B). This return to basal levels does not occur in the larynx when pepsin is present (Fig 5C). Electron microscopy revealed morphological changes commonly associated with esophagitis after 20 minutes of acid stress at pH 4 in the larynx. Similar changes were observed in esophageal tissue, but only after treatment at pH 2 (Fig 6).

Figure 7[50] shows the results from the porcine laryngeal damage model and demonstrates that the effect of pepsin in releasing cellular DNA is significantly greater than that of the control Krebs-Ringer solution alone from pH 2 to pH 6. At pH 2, 1 mg/mL pepsin produces 2.35 times more mucosal damage, measured as DNA release, than does a pH 2 solution alone, and pepsin at pH 6 produces 1.67 times more damage than does a pH 6 solution alone. Over the pH range, pepsin treatment has a more damaging effect on the laryngeal mucosa than does acid treatment
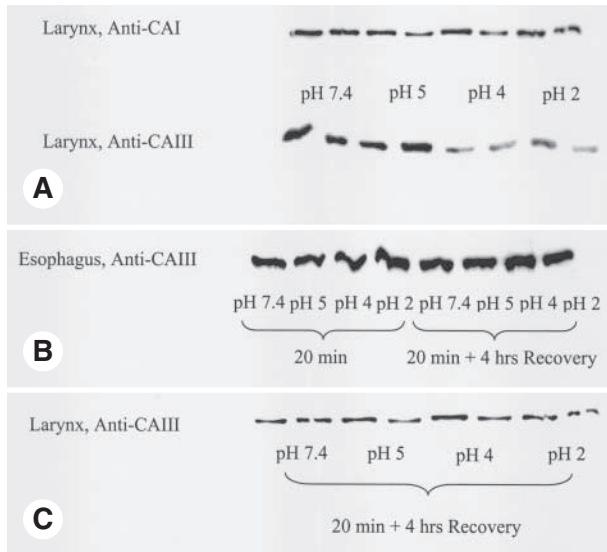
Fig 4. Expression of CAI and CAIII in porcine laryngeal and esophageal mucosae after 20 minutes of acid stress and after recovery phase. Proteins (10 µg) were separated by SDS-PAGE on 12% gel and immunoblotted with anti-CAI and -CAIII antibodies. Tissues were incubated for 20 minutes with Ham's F-10 medium adjusted to pH indicated, and then either harvested for lysis (denoted by 20 minutes) or removed into fresh pH 7.4 medium for 4-hour recovery phase before harvesting. **A)** Laryngeal CAI protein levels were unaffected by changes in pH. CAIII protein levels decreased after 20 minutes at pH 4 and pH 2. **B)** Esophageal CAIII protein levels were unaffected by changes in pH. CAI protein levels were also unaffected by changes in pH in esophagus (Fig 5A). **C)** Laryngeal tissue was exposed to acidified medium for 20 minutes and then incubated in fresh pH 7.4 medium for 4 hours (recovery phase). CAIII protein levels returned to their basal levels.

Fig 5. Expression of CA isoforms after acid and pepsin stress. Proteins (10 µg) were separated on 12% SDS-PAGE gel and immunoblotted with anti-CAI and -CAIII antibodies. Tissues were incubated for 20 minutes in Ham's F-10 medium at indicated pH, with or without 0.1% porcine pepsin A, then harvested for lysis or removed into fresh pH 7.4 medium for 4-hour recovery phase before harvesting. **A)** Increase in CAI was observed after 20 minutes of stress with 0.1% pepsin at all pH levels in both esophagus and larynx. **B)** Decrease in CAIII expression was seen in esophagus at pH 4 and pH 2 in presence of pepsin. Recovery to basal levels was observed after incubation for 4 hours in fresh medium. **C)** Decrease in CAIII protein levels was observed at pH 4 and pH 2 in larynx, with no recovery taking place at pH 4 and pH 2 after pepsin treatment.

alone. The ability of pepsin to damage the mucosa at pH up to 6 would indicate that the refluxate even at pH above 4 could still result in damage to the larynx. Figure 8[50] demonstrates that damage to the porcine larynx was also pepsin concentration–dependent. Damage only occurred in the time course of the incubation (1 hour) with pepsin levels of 0.75 mg/mL and above. These levels are in the range reported for human gastric juice.[53]

In situ hybridization studies have shown that both MUC5AC and MUC4 are expressed in normal human laryngeal mucosa. However, the biopsy specimens from LPR patients (n = 10) demonstrated expression of only the cell surface–associated mucin MUC4, and not the secreted mucin MUC5AC (Table 2).

Preliminary investigations were carried out to determine the expression and subcellular localization of E-cadherin in normal human laryngeal tissues as compared with those found in LPR patients. Pan-cytokeratin immunoreactivity was also used to character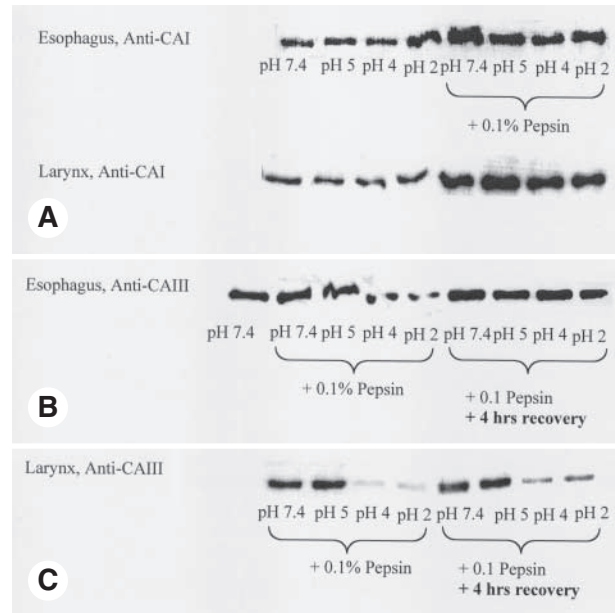ize the differentiation and phenotype of the la-ryngeal epithelium. The expression of E-cadherin was strongly seen at the cell-cell junctions in normal stratified squamous and respiratory-type laryngeal epithelia. All normal tissues were positive with a pan-cyto-keratin antibody. E-cadherin expression and cellular localization was evaluated in laryngeal tissues from the vocal fold (n = 14), posterior commissure (n = 18), and ventricle (n = 19) in patients with documented LPR. Twenty (5 from the vocal fold, 6 from the posterior commissure, and 9 from the ventricle) of 51 laryngeal samples (37%) showed partial or complete loss of E-cadherin membranous expression at the cell-cell junctions. There was no difference in the pan-cytokeratin immunoreactivity in the biopsy specimens from LPR patients as compared to normal laryngeal mucosa.

DISCUSSION

The damaging factors and protective mechanisms relating to GERD have been studied previously in the esophagus,[54-56] but until now, the larynx has remained largely uninvestigated. With the increasing interest in LPR as a possible etiologic factor in the
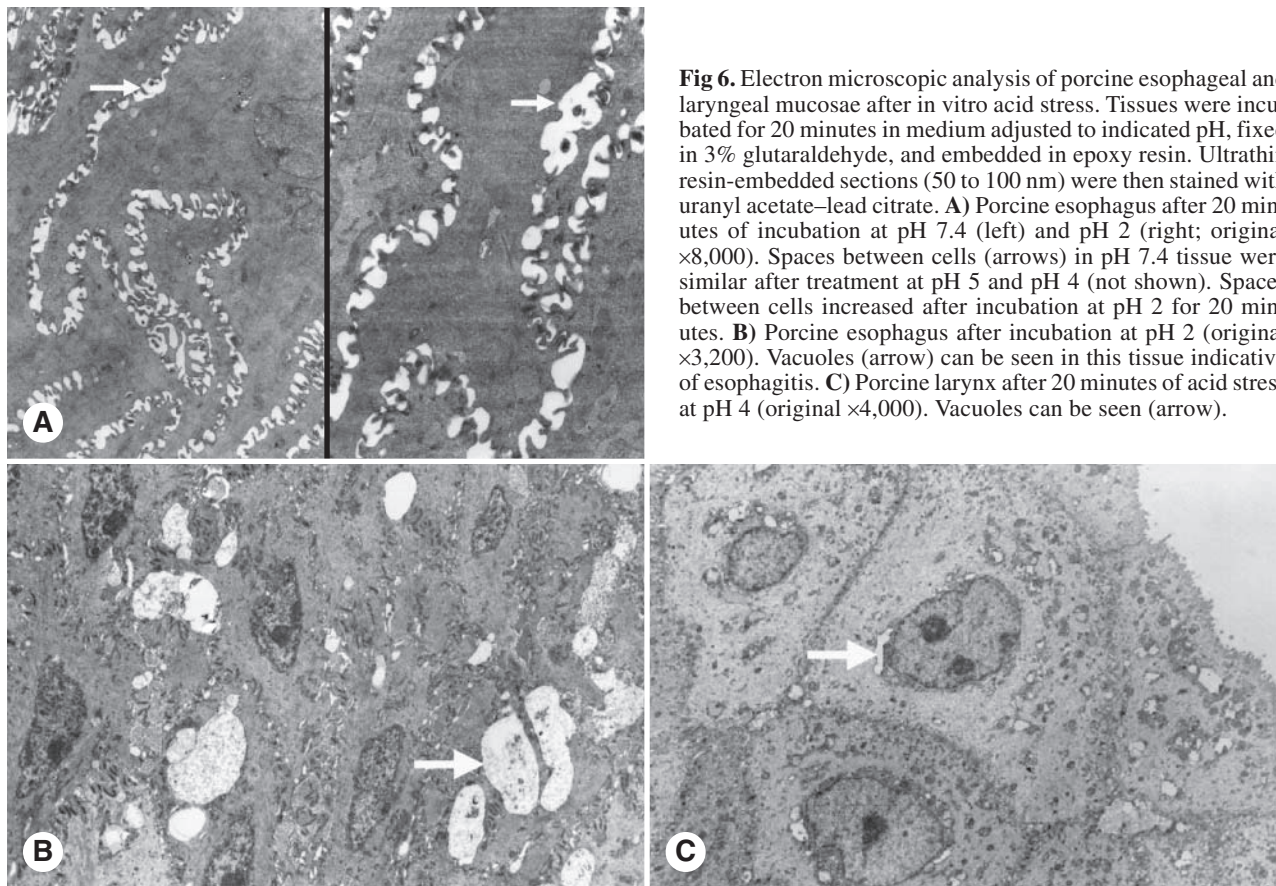
Fig 6. Electron microscopic analysis of porcine esophageal and laryngeal mucosae after in vitro acid stress. Tissues were incubated for 20 minutes in medium adjusted to indicated pH, fixed in 3% glutaraldehyde, and embedded in epoxy resin. Ultrathin resin-embedded sections (50 to 100 nm) were then stained with uranyl acetate–lead citrate. **A)** Porcine esophagus after 20 minutes of incubation at pH 7.4 (left) and pH 2 (right; original ×8,000). Spaces between cells (arrows) in pH 7.4 tissue were similar after treatment at pH 5 and pH 4 (not shown). Spaces between cells increased after incubation at pH 2 for 20 minutes. **B)** Porcine esophagus after incubation at pH 2 (original ×3,200). Vacuoles (arrow) can be seen in this tissue indicative of esophagitis. **C)** Porcine larynx after 20 minutes of acid stress at pH 4 (original ×4,000). Vacuoles can be seen (arrow).

development of many laryngeal diseases, including laryngeal cancer, it is important to examine the epithelial defenses of the laryngeal epithelia.

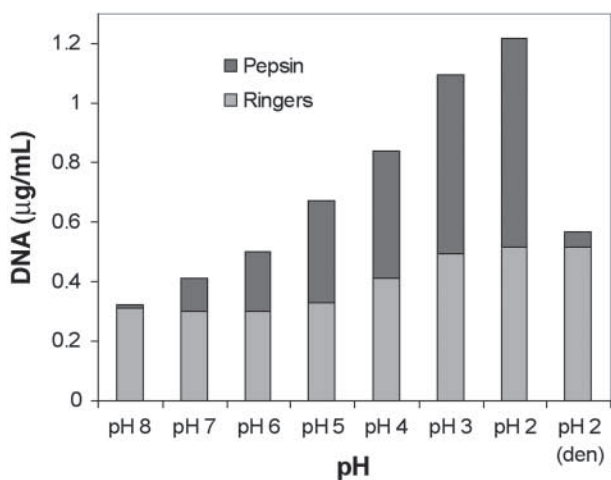These studies were designed to extend our initial

Fig 7. Effect of pH on pepsin activity in damage model. DNA release was measured by method of Hinegardner.[50] Lightly shaded bars represent DNA released by Krebs-Ringer solution (at pH indicated). Dark shading represents DNA released by 1 mg/mL porcine pepsin A solution, over and above that released by Krebs-Ringer solution alone (at pH indicated). den — Pepsin denatured by exposure to pH 12 for 30 minutes.

report[29] and to determine whether the laryngeal epithelium shows a similar response to such factors. Our findings[29] and those of Hopwood et al[57] in patients with GERD suggest that the expression of CA isoenzymes is modified and may be an important protective mechanism by increasing the cellular buffering capacity of inflamed esophageal tissue. The laryngeal mucosa does not show the same pattern of response as the esophagus.[29] In addition, CAIII protein expression was not detected in 63% (15/24) of vocal fold samples taken from LPR patients (Table 1). An additional 21% (5/24) of the laryngeal biopsy specimens analyzed had very low levels of CAIII protein expression.

In LPR patients, the finding of low levels of CAIII in vocal fold epithelium and of relatively high levels of CAIII in posterior commissure epithelium is interesting. Indeed, the data suggest the possibility of interesting relationships; ie, as LPR severity increases, vocal fold CAIII may be depleted, while CAIII in the posterior commissure may increase. One might speculate that increased levels of CAIII have a protective effect on posterior commissure epithelium. Primary laryngeal carcinoma arising in the posterior commissure, for example, almost never occurs.

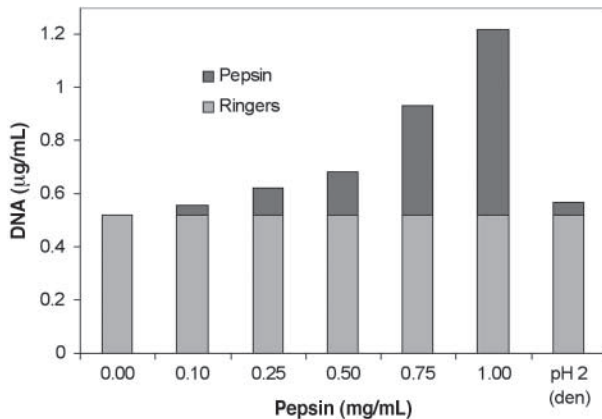To confirm that the low levels of vocal fold CAIII

Fig 8. Effect of pepsin concentration on damage in in vitro model. DNA release was measured by method of Hinegardner.[50] All incubations were carried out at pH 2 with porcine pepsin A concentrations varying from 0 to 1.0 mg/mL. Lightly shaded bars represent DNA released by pH 2 Krebs-Ringer solution alone. Dark shading represents DNA released by pH 2 Krebs-Ringer solution plus pepsin (at concentrations indicated). den — Pepsin denatured by exposure to pH 12 for 30 minutes.

TABLE 2. MUCIN GENE EXPRESSION IN NONSYMPTOMATIC SUBJECTS AND LPR PATIENTS

Position	Nonsymptomatic Subjects		LPR Patients (n = 10)	
	MUC4	MUC5AC	MUC4	MUC5AC
True vocal cord	+	+	7	0
False vocal cord	+	+	ND	ND
Ventricle	+	+	6	0
Posterior commissure	+	+	7	0

MUC4 is membrane-bound mucin; MUC5AC is secreted mucin gene. All normal samples tested positive for expression of both genes; for LPR group, number in Table indicates positive patients.

in LPR do not merely reflect normal variations, the CAIII expression was examined in normal laryngeal mucosal samples (and compared to the LPR patient data). Although a limited number of histologically normal laryngeal samples were obtained, preliminary results show that normal laryngeal epithelium expresses CAIII to high levels, in contrast to the majority of LPR patients, who show no detectable CAIII protein. This finding warrants further investigation. The effect of LPR on CA isoenzyme expression remains unclear and may be due to alterations in the translation of new CA protein or may be merely an effect on the existing protein present or even both. However, the down-regulation of CA isoenzymes in this tissue may result in a reduced ability of this epithelium to protect against LPR and may be a factor contributing to the development of laryngeal disease and dysfunction. In support of this idea, CAIII was found to be absent from laryngeal tumor samples (data not shown).

To ascertain whether the loss of CAIII protein expression is related to the damaging effects of gastric refluxate on the laryngeal epithelium, we used an in vitro organ culture system. The rationale for sampling posterior commissure mucosa from the porcine larynx was that this is the region that is thought to come into contact with refluxate in humans.[4,7,58] Using this model, we found that laryngeal CAIII protein levels decrease after 20 minutes of exposure to a pH of 4 or below. Thus, although the laryngeal epithelium has been shown to express some of the CA isoenzymes and has the potential to protect against the damaging effects of gastric refluxate, the different responses of the larynx in terms of CAIII to reflux damage sug-

gest that it may be more sensitive to injury than the esophageal epithelium.

Another observation is that the laryngeal epithelium is able to resume its normal buffering capacity after a recovery phase, suggesting that the effect of low acidic pH on epithelial CAIII expression is reversible. This reversal also occurs in the esophageal epithelium, in which a decrease in CAIII is observed after acid and pepsin treatment. However, if the laryngeal epithelium is exposed to pepsin at pH 4 or pH 2, full recovery is not observed. This finding implies that pepsin has an irreversible effect on CAIII expression in the laryngeal epithelium, but not in the esophageal epithelium. Furthermore, electron microscopy revealed morphological changes commonly associated with esophagitis[59] in esophageal tissue exposed to 20 minutes of acid stress at pH 2. Similar changes were observed at pH 4 in the larynx. These findings indicate that the laryngeal epithelium is more sensitive to the effects of acid and pepsin, and therefore to the gastric refluxate, than is the esophageal epithelium.

The in vitro porcine laryngeal damage model has provided clear evidence that the damaging activity of pepsin is retained up to pH 6. Therefore, reflux events at pH above 4 could still result in substantial damage to the laryngeal mucosa. Studies need to be performed to measure actual pepsin levels in the larynx in patients with LPR and the period of time pepsin remains there at pH 6 or below.

The in situ hybridization studies identifying mucin gene expression suggest that this expression may be altered in the inflammatory changes associated with LPR, as witnessed by a down-regulation of the secreted, columnar mucosa–associated mucin MUC-5AC. Further studies need to be undertaken to learn whether such changes in mucin gene expression affect overall mucosal protection and whether they predispose the larynx to the development of other diseases.

In this study we found that E-cadherin expression was down-regulated in the laryngeal mucosa from patients with LPR. The results indicate a defect in the integrity of the epithelial barrier of the larynx, which requires an intact E-cadherin molecule. However, it is not clear whether these changes in E-cadherin expression and also CA and MUC5AC expression are secondary to the inflammatory response associated with LPR, or whether they are the prime mediators of the laryngeal abnormalities seen in these patients. Further studies need to be undertaken to investigate the mechanisms underlying these changes.

CONCLUSIONS

Our findings from this study provide more evidence that the laryngeal epithelium does not show the same pattern of response to gastric reflux as the esophageal epithelium, and would appear to be more sensitive to its damaging effects. The absence of or decreased expression of CAIII protein observed in 64% of LPR patients could be attributed to the effects of acid and pepsin present in the refluxate. Our data suggest that pepsin remains active up to pH 6, and that pepsin has an irreversible effect on the laryngeal epithelium that is concentration-dependent, highlighting the need for investigating the presence of pepsin in the refluxate before and after symptom control. Additionally, down-regulation in the expression of the secreted mucin gene MUC5AC in LPR may be another factor contributing to impaired mucosal protection and the potential for further laryngeal damage.

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