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Expression of claudin-3 in the esophagus and larynx of rat reflux model

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ABSTRACT

Objective: The aim of this study was to investigate the association between laryngeal expression of claudin-3 and laryngopharyngeal reflux (LPR) in a rat reflux model.
Methods: Eight Wistar rats were divided into two groups. Four rats underwent total esophageal myectomy to induce reflux, and the remainder underwent a sham operation as a control. All animals were sacrificed 12 weeks after surgery to perform tissue histology and Western blot analysis.
Results: Lymphocyte infiltration increased significantly in the study group in both esophageal and laryngeal samples ($P = 0.001, 0.002$, respectively). Both esophageal and laryngeal expressions of claudin-3 were significantly lower in the study group when compared with that in the control group ($P = 0.045, 0.037$, respectively).
Conclusion: The results of this study suggest that a decrease in claudin-3 could be a sensitive indicator of reflux laryngitis in rats.

1. Introduction

Retrograde reflux of gastric contents up to the level of the pharynx and larynx that produces a troublesome disorder: laryngopharyngeal reflux (LPR). LPR results in symptoms in the pharynx, larynx, paranasal sinuses and even the middle ear [1–3]. LPR and gastroesophageal reflux disease (GERD) are related disorders. An imbalance in offensive and defensive factors in the esophageal and laryngopharyngeal mucosa results in GERD and LPR, but the concrete details remain elusive.

Tight junctions (TJs) are responsible for paracellular sealing of the epithelium. It has been shown that expression of tight junction proteins acts as an important defensive factor in the pathogenesis of some disorders [4–6]. Claudins are a family of tight junction membrane proteins. Claudins bind to the occludin protein and thereby regulate paracellular permeability of epithelia to maintain epithelial defense [7].

A markedly dilated intercellular space (DIS) and reduction in the number of TJs were observed in several studies on GERD and LPR [8–10]. It has been suggested that dissociation and dispersion of claudin-3 from the tight junction could be one of the most sensitive indicators of reflux esophagitis in rats [11]. Because of the relationship between GERD and LPR, we hypothesized that claudin-3 may also play a crucial role in the pathogenesis of LPR. In this study, we used a rat model to investigate the association between the expression of claudin-3 and LPR.

2. Materials and methods

2.1. Outline of the study

Eight male Wistar rats (weight 250–300 g) were divided into two groups. Four rats underwent total esophageal myectomy to induce reflux, and the other four rats, acting as controls, underwent a sham operation. All the animals were sacrificed to obtain histological and Western blot results 12 weeks after surgery. The study was approved by the Research Ethics Committee of Fu Dan University.

2.2. Surgical procedure

We used the surgical procedure to achieve experimental reflux in the literature before [12]. The animals were fastened for 12 h prior to surgery. Intraperitoneal anesthesia was attained with 20 mg/ml xylazine chloride and 100 mg/ml ketamine chloride in a 1:1 ratio, and 0.1 ml of the solution was administered for every 100 g in weight. After removing hair from the abdomen, the skin was cleaned with 10% polyvinylpyrrolidone iodine. A 3-cm midline incision was made starting at the xiphoid process, and the peritoneal cavity was inspected. In the study group animals, the

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muscle layer of the abdominal esophagus was resected starting at the gastroesophageal junction. A segment 1.5 cm in length was resected circumferentially around the esophagus (total myectomy). The control rats received only a midline incision. The animals were allowed to drink water on the first postoperative day and a regular daily diet on the second postoperative day.

2.3. Histology

The animals were sacrificed 12 weeks after surgery. Tissue samples of the proximal esophagus (1 cm from the gastroesophageal junction) and the larynx (corresponding to the position of human vocal cords) were obtained for histology and Western blot. The samples for histological examination were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. Sections of 4-μm thickness were mounted on glass slides and stained with hematoxylin and eosin (H&E). Light microscopy was used to compare lymphocyte infiltration in the esophageal and laryngeal samples between groups. Lymphocytes were counted in three randomly selected high-power fields (HPFs, 400×), and the results were expressed as the mean number of cells/HPF (400×). All the sections were coded and the observers were blinded during the examination.

2.4. Western blot analysis

Western blot analysis was performed to measure the expression level of claudin-3. Each esophageal and laryngeal mucosa sample was homogenized in ice-cold lysis buffer containing 50 mmol/l Tris–HCl pH 8.0, 150 mmol/l NaCl, 1 mmol/l ethylene diamine tetraacetic acid (EDTA), 1% Triton X-100, and Complete Mini, according to manufacturer recommendations (EDTA-free, protein inhibitor cocktail tablets) (Roche Mannheim, Germany). The homogenates were centrifuged at 12,000 × *g* for 5 min at 4 °C and the supernatant was used for analysis.

Protein concentrations were measured at 660 nm, using bovine serum albumin as a standard. Equal amounts of total protein (7.5 μg/lane) were subjected to 12% sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The protein was then transferred electrophoretically onto a polyvinylidene difluoride (PVDF) membrane, which was incubated for 1 h with a buffer containing T-TBS (20 mM Tris–HCl pH 7.5, 0.15 M NaCl, 0.05% Tween 20) and 5% nonfat dried milk. The membrane was then incubated with rabbit anti-claudin-3 antibody (Abcam, 1:200) and anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) antibody (Boster, 1:2000) overnight at 4 °C. The membrane was washed with T-TBS and then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson, 1:2000) for 2 h. After extensive washing with T-TBS, the membrane was probed for antibodies using enhanced chemiluminescence. The intensity of each band was scanned with Gel-pro Analyzer (Media Cybernetics).

2.5. Statistical analysis

Statistical analysis was performed using Student's *t*-test; *P*-values <0.05 were considered to be statistically significant. Data are shown as the means ± standard deviation (SD).

3. Results

3.1. Histology

There were significant differences between the study group and the control group in both the esophagus and the larynx ($P = 0.001$ and 0.002, respectively). Lymphocyte infiltration increased from 15.0 ± 6.6/HPF to 42.8 ± 23.2/HPF in esophageal samples and from 11.4 ± 5.2/HPF to 38.9 ± 26.4/HPF in laryngeal tissues (Fig. 1a and b).

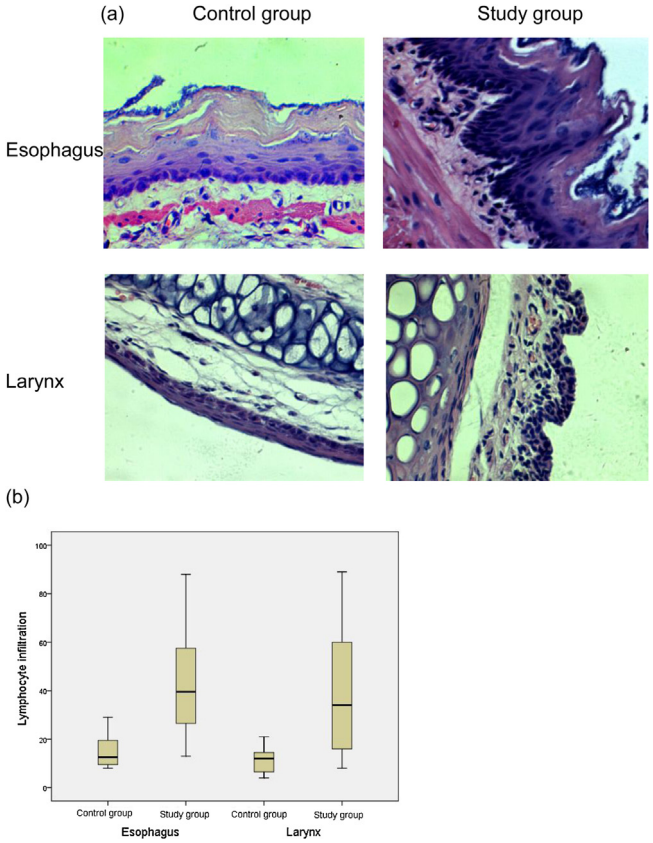

Fig. 1. (a) Lymphocyte infiltration of esophageal and laryngeal mucosa in the study and control group (H&E, 400×). (b) Boxplot chart showing the distribution in the values of esophageal and laryngeal lymphocyte infiltration in the study and control group ($P = 0.001$ and 0.002, respectively).

3.2. Western blot analysis

Both esophageal and laryngeal expressions of claudin-3 were significantly lower in the study group when compared with the control group (Fig. 2a and b). The esophageal mean value of claudin-3/GAPDH was 0.43 ± 0.29 in the study group compared with 1.39 ± 0.71 in the control group ($P = 0.045$). In the laryngeal samples, the mean value of claudin-3/GAPDH was 0.58 ± 0.39 compared with 1.56 ± 0.62 in the control group ($P = 0.037$).

4. Discussion

Previous reflux researches focused on factors such as acid, pepsin, and biomarker expression in LPR. Failure of intrinsic defense in the larynx may cause changes in laryngeal epithelia [13]. DIS arises as a morphological feature of acid-induced damage to the squamous epithelium [14,15].

Abnormalities in TJs and regulation of claudins are closely related to the integrity of epithelial surfaces. TJs provide a selective barrier, establish cellular polarity [16] and play a critical role in the epithelial defense system. The claudin proteins are a family of transmembrane proteins essential in the formation and maintenance of TJs [17].

It has been suggested that variation in claudin-3 expression could be a sensitive indicator of reflux esophagitis in a rat model [11]. In LPR, there are several studies which have focused on DIS in laryngeal mucosa [8–10], but research on claudins in LPR is absent.