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Dilated intercellular space in the larynx and esophagus of a rabbit reflux model

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ABSTRACT

Objective: In this study, we investigated histological and electron microscopic changes of the laryngeal and esophageal epithelium in an animal model of reflux to demonstrate: (1) the association between laryngopharyngeal reflux (LPR) and gastroesophageal reflux disease (GERD) and (2) the value of dilated intercellular space (DIS) as a marker of LPR.
Methods: Eight New Zealand albino rabbits were utilized. Four rabbits underwent total cardiomyectomy to induce reflux. The remains underwent a sham operation as controls. The animals were sacrificed 12 weeks after surgery to obtain histological and electron microscopic results.
Results: There were significant differences in the histological results between the study group and the control group in both the esophagus and the larynx ($P = 0.041$ and 0.014). Significant changes in the intercellular space (IS) were observed between the study group and the control group in the esophageal and laryngeal samples ($P < 0.001$).
Conclusion: The results of this study suggest that LPR and GERD have a common mechanism and DIS is a morphologic marker of LPR in rabbits.

1. Introduction

Laryngopharyngeal reflux (LPR) and gastroesophageal reflux disease (GERD) are both closely related to the excessive reflux of gastric contents, resulting in symptoms of the esophagus, pharynx, larynx, paranasal sinuses and even the middle ear [1–4]. Several studies attempt to explain LPR as an extra-esophageal manifestation of GERD [5–7]. However, it is revealed that although certain patients exhibit both LPR and GERD, most patients with LPR do not have GERD [8,9]. The cause-and-effect relationship between LPR and GERD remains elusive.

Based on measurements of the intercellular space (IS), several investigators recommend dilated intercellular space (DIS) observed using transmission electron microscopy (TEM) as a sensitive marker of GERD in patients with esophagitis [10,11]. However, it is controversial for LPR. Franchi et al. suggest that DIS of the laryngeal epithelium may be a morphologic marker of LPR [12]. But this assumption is questioned by other studies [13,14].

In a previous study using a rabbit gastroesophageal reflux model, we reported reflux laryngitis secondary to chronic lower esophageal sphincter dysfunction [15]. In the current study, in addition to histological findings, we evaluated esophageal and laryngeal ultramicroscopic damage by DIS measurement using TEM in the same animal model to clarify the relationship between LPR and GERD and whether DIS of the laryngeal epithelium is a sensitive marker of LPR.

2. Materials and methods

2.1. Outline of the study

Eight healthy, male New Zealand albino rabbits (2.5–3.5 kg) were used. Four rabbits underwent total cardiomyectomy to induce reflux secondary to lower esophageal sphincter dysfunction, and the remaining four rabbits underwent a sham operation as controls. The animals were sacrificed 12 weeks after surgery to obtain histological and electron microscopic results. The study was approved by the Research Ethics Committee of Fu Dan University.

2.2. Surgical procedure

After intramuscular anesthesia with diazepam (2.5 mg/kg) associated with ketamine (25 mg/kg), the animals were restrained on the surgical table by the four paws without the need for a tracheal cannula. An upper midline incision was created on the abdomen. The muscle fibers of the distal 2 cm of the esophagus and the proximal 1 cm of the stomach were excised. The full 360° of

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muscle fibers of the esophagus and stomach were excised, producing a total cardiomyectomy. The sham-operated rabbits, receiving only a midline incision, served as a control group. The animals were allowed 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 and vocal cords were obtained for TEM. The remains were fixed in 10% buffered formalin, dehydrated and embedded in paraffin. 4-μm thick sections were mounted on glass slides and stained with hematoxylin and eosin (H&E). Light microscopy was used to compare the lymphocyte infiltration of the esophageal and laryngeal samples among the groups. All of the sections were coded to avoid observers' bias during the examination and the observers were blinded.

For the esophagus and larynx, lymphocyte infiltration was assessed at a magnification of 400×, and the number of lymphocytes present in the submucosa was scored as follows: 0 (0–20 lymphocytes); 1 (21–50 lymphocytes); 2 (51–80 lymphocytes); 3 (81–120 lymphocytes); 4 (>120 lymphocytes).

2.4. Tissue preparation for electron microscopy

Tissue samples of the proximal esophagus and vocal cords were fixed in 2.5% glutaraldehyde in 0.1 ml phosphate-buffered saline (pH 7.2) and postfixed in 1% OsO_4. The samples were stained en bloc in 2% uranyl acetate in 50% ethanol, dehydrated in increasing concentrations of ethanol, cleared in propylene oxide, embedded in epoxy resin, and cut using a diamond knife. Ultrathin serial sections were mounted, stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope (Philips CM-120).

2.5. Intercellular space measurement with TEM

TEM digital photographs were taken of each sample with an identical magnification power of 5000×. The suprabasal region of the epithelium was selected for measuring the intercellular space. Ten photomicrographs were taken from each sample, and 10 measurements were obtained from every photomicrograph. Using the Image-Pro Plus 6.0 software (Media Cybernetics Inc.), the lines of the measurements were maintained at a distance of at least 1 μm to ensure the even distribution of the measurement sites. A total of 100 measurements per sample were obtained, and the mean values were calculated for comparison among the groups. The investigator measuring the intercellular space was blinded.

Table 1
The esophageal and laryngeal lymphocyte infiltration of the experimental and control groups.

	Esophagus		Larynx	
	Experimental group	Control group	Experimental group	Control group
1	3	0	2	0
2	3	0	3	1
3	2	1	1	0
4	1	0	1	0
Mean ± SD	2.25 ± 0.96	0.25 ± 0.50	1.75 ± 0.96	0.25 ± 0.50
P	0.041		0.014	

2.6. Statistical methods

The histopathological differences were evaluated by paired *t*-test. The Mann–Whitney *U*-test was performed to compare DIS results. The statistical significance level was established at $P < 0.05$, and the confidence interval was 95%.

3. Results

3.1. Histology

Significant differences were observed between the experimental group and the control group in both the esophagus and the larynx ($P = 0.041$ and 0.014, respectively. Table 1). Squamous epithelium with elongation of the papillae was observed in esophageal samples of 12-week group (Fig. 1).

3.2. TEM

Fig. 2 summarizes the measurement of the IS in the experimental and control groups (esophagus and vocal cords). In esophageal samples, the mean values of IS in the experimental and control groups were 0.317 ± 0.141 μm and 0.210 ± 0.038 μm. The vocal cord samples had a mean value of 0.298 ± 0.049 μm in the

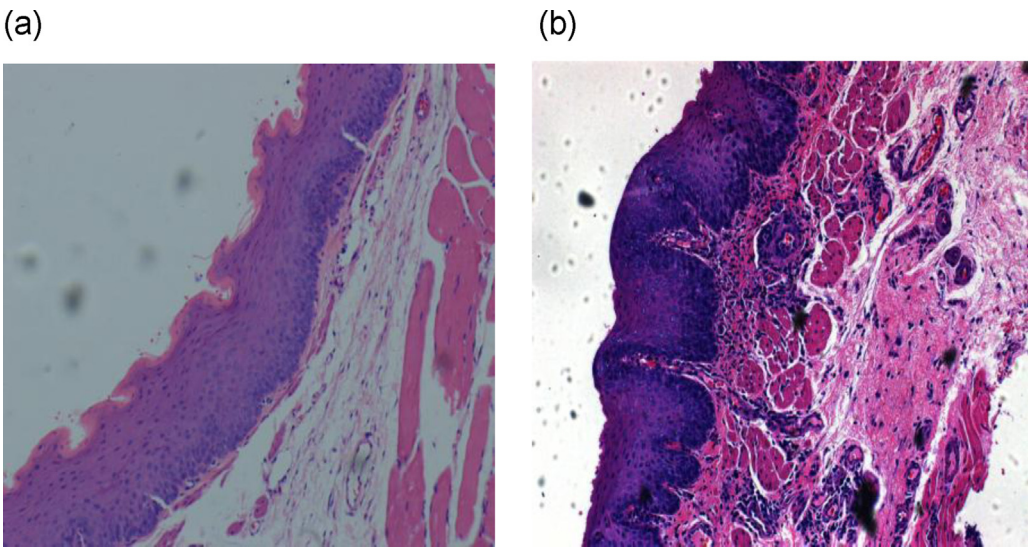

Fig. 1. (a) Esophageal samples of controls (H&E 100×). (b) Esophageal inflammation 12 weeks after surgery which showed an elongation of the papillae and lymphocyte infiltration (H&E 100×).