Role of salivary mucin in the protection of rat esophageal mucosa from acid and pepsin-induced injury

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Kinoshita, Mine, EisuKe Kume, Shigeki Igarashi, Nobuko Saito, and Hiroshi Narita. Role of salivary mucin in the protection of rat esophageal mucosa from acid and pepsin-induced injury. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G796–G800, 1999.—The mucosal defensive mechanisms of the esophagus against acid and pepsin remain to be elucidated. In the present study, we investigated the contribution of the salivary mucin in maintaining the integrity of the esophageal mucosa. When an everted esophageal sac, isolated from normal rat, was treated with N-acetyl-L-cysteine, a mucolytic agent, the amount of glycoprotein in the gel layer adherent to the epithelium was completely depleted and the susceptibility of the mucosa against acidified pepsin-induced digestion increased. In sialoadenectomized rats, 7 days after extirpation, the amount of glycoprotein adherent to the esophageal epithelium was definitely reduced, and the esophageal mucosa was significantly vulnerable to acidified pepsin-induced digestion compared with the sham-operated rats. Induction of regurgitation of the gastric juices into the esophagus resulted in the development of severe hemorrhagic esophageal lesions only in the sialoadenectomized rats but not in the sham-operated rats. In conclusion, the glycoprotein in the adherent gel layer in rat esophagus, which mainly derives from salivary glands, plays an important role in the preepithelial defense to maintain the integrity of the esophageal mucosa against acid and pepsin.

reflux esophagitis; salivary glands; mucus glycoprotein; epithelial growth factor; sialoadenectomy

THE GASTRODUODENAL epithelium maintains its integrity under the attack of acid, pepsin, and ulcerojenic agents through defensive mechanisms, such as mucus and bicarbonate secretion and mucosal blood flow. The esophageal epithelium is also exposed to acid, pepsin, and noxious agents; however, the mechanisms of how the esophageal epithelium withstands these harmful agents have not been as well documented. Recent clinical studies suggested the involvement of salivary and esophageal submucosal glands in the esophageal defense system, since the secretion of alkaline, mucins, epidermal growth factor (EGF), and PGE2 from these glands was enhanced by esophageal luminal perfusion with acid and/or pepsin in healthy subjects (3, 11, 16). Furthermore, the salivary and esophageal secretion in response to esophageal chemical stimulation has been reported to be impaired in patients with reflux esophagitis (10, 15). There have been, however, few experimen-
tal data to confirm the protective role of these components from esophageal and salivary glands.

In the gastroduodenal tracts, the elastic mucus gel layer covering the epithelium serves as an unstrirred buffering barrier, with epithelium-secreted bicarbonate protecting against acid and a diffusion barrier for luminal pepsin (1). Thus, in the esophagus, mucin, which originates from salivary and esophageal submucosal glands, may play an important role in the mucosal protection as a preepithelial barrier. In the present study, we first estimated the role of the adherent mucus in the protection of the esophagus against acid and pepsin by in vitro studies using N-acetyl-L-cysteine (NAC), a mucolytic agent. Second, to elucidate the contribution of the salivary mucin in the defense mechanisms of esophageal mucosa, we assessed the effects of sialoadenectomy on both the esophageal adherent mucin and the mucosal resistance against attack by gastric juices.

MATERIALS AND METHODS

Animals and Drugs

Male Sprague-Dawley rats (Charles River Japan, Kana
gawa, J apan) weighing 160–230 g were used.

TAC and Alcian blue 8GX were purchased from Nacalai Tesque (Kyoto, J apan), EGF extracted from mouse submaxil-

gary gland was obtained from Toyobo (Osaka, J apan), and

purified porcine pepsin (P6887, 3400 U/mg protein) was purchased from Sigma Chemical (St. Louis, MO).

Effect of NAC

Under ether anesthesia, a 5-cm length of esophagus was excised and immediately everted, and both ends of the esophagus were ligated. This everted sac was incubated in a 0.2% NaCl solution with or without 1% NAC, a mucolytic agent, for 20 min at room temperature. After the incubation, the following three experiments were performed.

Experiment 1. After incubation with NAC, the everted esophagus was placed in ice-cold Carnoy’s solution for 2 h and subsequently in absolute ethanol (9). The circular ring strip of the esophagus was embedded in paraffin. Sections (4 μm) were stained with periodic acid-Schiff and examined under a light microscope.

Experiment 2. After incubation with NAC, the esophageal sac was immediately placed in 3 ml of ice-cold distilled water, and the adherent materials to the esophageal surface were gently scrapped with a spatula. The scraped samples from six sacs were pooled and lyophilized. Mucus glycoprotein was isolated from the lyophilized sample by gel filtration and measured according to the method of Ohara et al. (12). The sample was dissolved by homogenization in 3 ml of 0.05 M Tris-HCl buffer (pH 7.2), Triton X-100 (100 μl) was added, and the sample was incubated for 1 h at 37°C. After centrifugu-

ation (8,000 g, 4°C, 30 min), the supernatant was collected. Two milliliters from the collected supernatant were applied to
a Sephacryl S-300HR column (1.6 × 60 cm, Pharmacia Biotech, Uppsala, Sweden) that was eluted with the Tris-HCl buffer containing 2% Triton X-100. The eluted fractions (5 ml) were assayed for hexose by the method of Dubois et al. (4) using galactose as a standard (the minimum detectable concentration of galactose was 0.1 µg/ml). The amount of hexose in void fraction was determined as glycoprotein.

Experiment 3. After treatment with NAC, the esophageal sac was immediately incubated in 3 ml of 0.1 N HCl-0.2% NaCl solution containing porcine pepsin (1 mg/ml) for 30 min at 37°C. Subsequently, the sac was removed, and one volume of 7% TCA was added to the incubation solution. After centrifugation (1,500 g, 4°C, 10 min), the TCA-soluble peptide in the supernatant was determined by the method of Folin and Ciocalteau (5) using tyrosine as a standard.

Statistical Analysis

All data are expressed as means ± SE. Data, except the incidence of reflux hemorrhagic esophagitis, were statistically analyzed with Student's t-test or with ANOVA followed by Bonferroni’s method. Incidence of the esophagitis was analyzed by Fisher's exact probability test. A P value of <0.05 was considered to be statistically significant.

RESULTS

Effect of Sialoadenectomy

Under halothane anesthesia, the submandibular-sublingual salivary gland complexes were removed bilaterally after their ducts were ligated (17). Sham-operated rats served as controls. One week after the sialoadenectomy, the integrity of the esophageal mucosa was assessed with the following protocols.

Experiment 1. One week after the sialoadenectomy, under ether anesthesia, a 5-cm length of esophagus was excised. Mucus glycoprotein in the materials adherent to the epithelium was isolated and measured as mentioned above.

Experiment 2. One week after the sialoadenectomy, under ether anesthesia, a 5-cm length of esophagus was excised and immediately everted, and both ends of the esophagus were ligated. The everted sac was incubated in 0.1 N HCl-0.2% NaCl solution containing porcine pepsin (1 mg/ml) for 30 min at 37°C, and the TCA-soluble released peptide from the sac was measured as mentioned above.

Experiment 3. Six days after the sialoadenectomy, the rats were fasted for 24 h with free access to water. After the fasting, under ether anesthesia, the pylorus and limiting ridge (transitional region between forestomach and glandular portion) were ligated to induce the reflux of gastric contents into the esophagus (13); 2.5 h later, the esophagus was excised and fixed with 10% neutral formalin solution, and the circular ring strip was embedded in paraffin. Sections (4 µm) were stained with hematoxylin and eosin and examined under a light microscope.

Fig. 1. Light micrographs of esophageal mucosa treated with N-acetyl-L-cysteine (NAC). A: control, without NAC. B: with NAC. The epithelial cell, the cornified layer, and the adherent periodic acid-Schiff (PAS)-positive materials covering the esophageal surface (debris derived from the cornified layer) were intact in both control and NAC-treated groups. Magnification = ×500. Sections were PAS stained.

Effect of NAC

NAC treatment did not affect the microscopic morphology of the esophageal epithelium, which consisted of the epithelial cells, the cornified layer, and the debris derived from the cornified layer (Fig. 1). After the NAC treatment, the glycoprotein adherent to the esophageal mucosa completely diminished and the acidified pepsin-induced release of TCA-soluble peptide from the everted esophageal sac remarkably increased (Table 1).

Effect of Sialoadenectomy

Seven days after extirpation of salivary glands, the amount of glycoprotein adherent to the esophageal mucosa remarkably increased (Table 1).

Table 1. Effects of NAC on the amount of adherent glycoprotein and the vulnerability to acidified pepsin-induced digestion in the isolated rat esophagus

<table>
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<th>Glycoprotein, µg hexose/g wet tissue</th>
<th>TCA-soluble Peptide, µg tyrosine/g wet tissue</th>
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<tr>
<td>Control</td>
<td>22.8 ± 7.3</td>
<td>1,010.3 ± 93.7</td>
</tr>
<tr>
<td>NAC</td>
<td>294.1*</td>
<td>2,788.5 ± 294.1*</td>
</tr>
</tbody>
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Values are means ± SE for 4 and 6 preparations in the glycoprotein determination and in the pepsin-induced digestion, respectively. NAC, N-acetyl-L-cysteine; ND, not detectable. *P < 0.01 compared with control.
mucosa was significantly reduced (Fig. 2, top), whereas the epithelium remained histologically intact without any degenerative changes (data not shown). The esophageal mucosa, isolated from sialoadenectomized rats, was more vulnerable to acidified pepsin-induced digestion compared with that from the sham-operated rats (Fig. 2, bottom).

The induction of regurgitation of the gastric contents into the esophagus did not affect the microscopic appearance of the esophageal mucosa in the sham-operated rats 2.5 h after the operation (Fig. 3A). In contrast, the reflux of the gastric juice disrupted the esophageal epithelium and vast severe hemorrhagic lesions developed that reached to the muscle layer in one-half of the sialoadenectomized rats (Fig. 3B). To assess the effect of removal of salivary glands on gastric secretory activity, the acid concentration and the pepsin activity were measured. The sialoadenectomy did not affect the acid concentration but increased the pepsin activity in gastric juices (Fig. 4). The EGF treatment did not prevent the sialoadenectomy-induced increase in vulnerability of the esophagus against the gastric juices; nevertheless, it suppressed the sialoadenectomy-induced increase in pepsin activity (Fig. 4).

**DISCUSSION**

The gastrointestinal tract is covered by a viscoelastic and lubricant layer of highly glycosylated proteins, termed mucins. This adherent mucus gel layer protects the gastrointestinal epithelium from acid, pepsin, nox-

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**Fig. 2.** Effect of sialoadenectomy (SALX) on the esophageal-adherent mucus glycoprotein and mucosal vulnerability against acidified pepsin. Results are means ± SE of 4 and 6 preparations in glycoprotein determination (top) and the pepsin-induced digestion (bottom), respectively. *P < 0.05 and **P < 0.01 compared with sham-operated rats (SHAM).

**Fig. 3.** Light micrographs of esophagus 2.5 h after induction of gastric juice reflux. A: sham-operated rats. Esophageal epithelium was intact in all 6 sham-operated rats. B: sialoadenectomized rats. Esophageal epithelium was disrupted, and vast severe hemorrhagic lesions developed that reached the muscle layer. These changes were observed in 3 of 6 sialoadenectomized rats. Magnification = ×60. Sections were hematoxylin and eosin stained.

**Fig. 4.** Effect of regurgitation of gastric juices on the esophageal mucosa and gastric secretion in the sialoadenectomized rats. SHAM, sham-operated rats treated with water; SALX, sialoadenectomized rats treated with water; SALX + EGF, sialoadenectomized rats treated with epidermal growth factor (EGF). Results are means ± SE of 11–12 rats. Numbers in parentheses indicate the incidence of esophageal lesion formation. *P < 0.05 compared with the sham-operated rats; #P < 0.05 compared with sialoadenectomized rats without EGF supplement.
ious agents, and microorganisms (1). In the present study, the elastic materials adherent to the surface of the rat esophageal epithelium contained the high-molecular-weight glycoprotein. Mucolytic treatment with NAC depleted this adherent glycoprotein without any microscopic damage, resulting in a marked increase in the susceptibility of the esophageal epithelium to acid- and pepsin-induced digestion. These findings indicate that the rat esophageal mucosa is covered with mucus glycoprotein, and this adherent mucus layer serves to buffer against the attack of acid and pepsin in the esophagus as well as in the stomach.

The rat esophagus is anatomically devoid of submucosal glands. The sialoadenectomy apparently decreased the amount of mucus glycoprotein in the gel layer adherent to the esophagus. Thus the major part of the rat esophageal-adherent mucus glycoprotein derives from salivary glands. The esophageal epithelium in sialoadenectomized rats was more susceptible to both acidified pepsin-induced digestion and the development of hemorrhagic lesions induced by gastric contents than that in the sham-operated rats. These results suggest that salivary mucin covers the esophageal epithelium and serves an important role in maintaining the integrity of the esophageal mucosa.

Salivary glands secrete several organic components such as mucin, EGF, and PGE$_2$. Salivary EGF has been reported to have potent mitogenic and protective effects on the alimentary tracts (14, 17). In a preliminary experiment, we confirmed that the EGF supplementary protocol used in the present study improved the aggravation of ethanol-induced gastric lesions in sialoadenectomized rats. However, in the present study, the EGF supplement did not exert any effect on the sialoadenectomy-induced increase in esophageal vulnerability to gastric juices. Therefore, the depletion of salivary EGF may hardly influence the functional integrity of the esophageal epithelium in short-term experiments such as those of the present study.

Although exogenous EGF has been reported to inhibit gastric acid and pepsin secretion (7), in the present study, the sialoadenectomy increased the pepsin activity in gastric juices without any effect on the acid concentration. This increase in pepsin activity was counteracted by the EGF supplement, indicating that depletion of salivary EGF induced the augmentation of pepsin secretion. Several reports demonstrate that pepsin plays an important role in the development of esophageal injury (6, 8). In the sialoadenectomized rats, whereas the EGF supplement reversed the increase in pepsin activity, it did not show any effect on both the incidence and severity of esophageal lesions. Thus the increase in pepsin activity may be less involved in the aggravation of the esophagitis after the depletion of salivary glands.

Reflex of gastric contents is considered to cause esophagitis; however, the severity or the symptoms of reflux esophagitis cannot be predicted on the basis of acid and pepsin exposure (18). Therefore, other damaging factors, such as impaired mucosal resistance, are possibly involved in the pathogenic mechanisms of reflux esophagitis. Some clinical reports suggest a contribution of organic (mucins and EGF) and inorganic secretions (alkaline) from salivary and esophageal submucosal glands to the preepithelial defense in esophagus. In healthy subjects, the secretion of alkaline, mucins, EGF, and PGE$_2$ from these glands was enhanced by esophageal luminal perfusion with acid and/or pepsin (3, 11, 16). The salivary and esophageal secretion in response to esophageal chemical stimulation was impaired in patients with reflux esophagitis (10, 15). These reports are not able to clarify the role of each component of the salivary and esophageal glands in the esophageal defense, despite indication of a comprehensive participation of these components in the maintenance of esophageal integrity. In the present study, we clearly demonstrated that salivary-derived mucin covered the rat esophageal epithelium, and the depletion of the adherent mucus layer worsened the acid- and pepsin-induced esophageal damage. In conclusion, the adherent mucin derived from salivary glands withstands the deleterious impact of the reflux of gastric contents as a preepithelial barrier.

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