Different effects of two types of H₂-receptor antagonists, famotidine and roxatidine, on the mucus barrier of rat gastric mucosa

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ABSTRACT

Compared with the aggressive factors, little attention has been paid to the mucosal defensive factors in ulcer therapy, and the role of the H₂-receptor antagonists in gastric mucosal protection has not been well characterized. In the present study, the effects of different types of H₂-receptor antagonists (famotidine and roxatidine) on rat gastric mucus cells were investigated using both biochemical and histological methods. Each drug (famotidine, 3 mg/kg; roxatidine, 100 mg/kg) was orally administered to rats by gavage once daily for 7 days. The biosynthesis and tissue content of mucin were compared in the gastric mucosa treated with each drug. Using anti-mucin monoclonal antibodies, the mucin content and immunohistochemical localization were also compared. Both the biosynthesis and the accumulation of gastric mucin were significantly decreased in the famotidine-treated rats, but not in the roxatidine. Both the content and the immunoreactivity of surface mucus cell-derived mucin were reduced by famotidine, while they were maintained in roxatidine-treated rat stomachs. There was no difference between the groups in the content and immunoreactivity of mucous neck cell-derived mucin. H₂-receptor antagonists should be classified in relation to gastric surface mucus cell function, raising the possibility of more effective ulcer therapy.

While acid, pepsin, and *Helicobacter pylori* are thought to be major factors in the pathophysiology of peptic ulcer diseases, the importance of the mucosal defense system has also been emphasized (4, 11, 16, 18, 19, 25). On the basis of the belief that ulcers occur as a result of an imbalance between aggressive and defensive factors, such as mucus secretion and mucosal blood flow, they are often treated in Japan with a combination of an acid suppressant (e.g. H₂-receptor antagonist or proton-pump inhibitor) and a mucosal protectant (4, 11, 16, 18, 19, 25).

Recent prospective randomized trials indicated that the addition of a mucosal protectant significantly augmented gastric ulcer healing and symptom relief by cimetidine (18, 19). Compared with the aggressive factors, little attention has been paid to the mucosal defensive factors in ulcer therapy, and the role of the H₂-receptor antagonists in gastric mucosal protection has not been well characterized.

Mucin, a major component of mucus, is considered to be a principal factor in the physiological defense of the gastric mucosa (1), and we previously showed that stimulation of gastric mucin biosynthesis is closely related to mucosal protective activity in experimental animals (10). Moreover, we recently reported that alterations in human gastric mucin accumulation by certain drugs were consistent with the results from the rat model (8, 12), and we have also established several monoclonal antibodies (mAb) that react with mucin synthesized and secreted from

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specific mucus cells of the rat stomach (13).

The purpose of the present study was to use biochemical and histological methods to determine the effects of different types of H₂-receptor antagonists, famotidine and roxatidine, on gastric mucus cells in the rat. Famotidine is a well-known conventional H₂-receptor antagonist (7). Roxatidine belongs to the second-generation H₂-receptor antagonist group characterized by a six-membered aromatic ring (7) (Fig. 1).

MATERIALS AND METHODS

Experimental animals. Seven-week-old male Wistar rats were purchased from CLEA-Japan (Tokyo, Japan) and housed in an animal care facility for 1 week while their body weights stabilized. The animals were housed in individual cages with raised mesh bases, in a temperature- and humidity-controlled environment with a 12 h dark-light cycle (from 6 : 00 pm to 6 : 00 am, dark cycle).

The study was conducted according to the guidelines of the Animal Laboratory Center of Kitasato University School of Medicine.

Drug administration. Famotidine (Sigma-Aldrich Corp., St. Louis, MO, USA) and roxatidine (ASKA Pharmaceutical Co., Tokyo, Japan) were suspended in 0.5% carboxymethylcellulose solution (CMC) and prepared immediately before use. Each drug was administered orally by gavage once daily for 7 days at the dose level usually adopted in animal pharmacological studies (famotidine, 3 mg/kg (5, 28); roxatidine, 100 mg/kg (27)). Control animals received 0.5% CMC only. Animals were sacrificed after an 18 h fast following final administration of the drugs.

Biochemical examination. Biosynthetic activity of mucin was assessed by incorporation of radiolabeled glucosamine into newly synthesized mucin using organ culture methods (10). The stomachs were ex-

cised and cut along the greater curvature. The corpus was selected and cut into 2 × 2 mm sections for treatment with culture medium (90% Eagle's minimum essential medium, 10% dialyzed fetal calf serum with 370 kBq/mL D-[1,6-³H(N)]glucosamine hydrochloride (2220 GBq/mmol; New England Nuclear, Boston, MA, USA). After culture at 37°C for 5 h in 5% CO₂ and 95% air, the samples were homogenized in 50 mmol/L Tris-HCl, pH 7.2, containing 2% Triton X-100 (Triton-Tris buffer). The extraction and isolation of mucin were performed as previously described (10). The homogenate was centrifuged and the supernatant was applied onto a Bio-Gel A-1.5 m column. The fractions eluted with the void volume were collected and radioactivity was measured. To compare the synthetic activity of mucin, the total radioactivity of the fractions was divided by the protein content of each homogenate to give a value relative to that of the control. The protein content in the tissue homogenate was determined using a Pierce protein assay kit (Pierce Biotechnology, Rockford, IL, USA) with bovine serum albumin as the standard.

Mucin content was assessed by hexose measurement in the mucin of gastric mucosa. Specimens from the corpus of three animals were pooled, lyophilized, and powdered for extraction of mucin by a previously described method (2). Each sample was suspended in Triton-Tris buffer, homogenized, and then incubated at 37°C for 1 h. After centrifugation at 8000 × g for 30 min at 4°C, the supernatant was collected and an aliquot was applied to a Bio-Gel A-1.5 m column and eluted with the Triton-Tris buffer. The void volume fraction (Fr-1) monitored by hexose measurement was collected as mucin. The hexose content in this fraction was measured by the phenol-sulfuric acid method using galactose as the standard. Mucin content (Fr-1 hexose value) is expressed as micrograms of hexose per tissue.

An enzyme-linked immunosorbent assay (ELISA) was used to evaluate the involvement of certain mu-

Fig. 1 Structures of the H₂-receptor antagonists used in this study.

cus cells in the quantitative changes of the mucin. Tenfold serial-doubling dilutions of the partially purified mucin from each specimen were prepared from 100 ng of mucin hexose per well. The ELISA well of a microtiter plate was kept overnight at 4°C, followed by blocking with 2% (w/v) skimmed milk (13). After the well had been washed, a specific amount of anti-mucin mAb (RGM21 or HIK1083) was added, followed by incubation at ambient temperature for 1 h. The well was successively incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (Dako, Kyoto, Japan) and 2,2'-azino-bis-[3-ethylbenzthiazoline-6-sulfonate] (ABTS)/H₂O₂ solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA), and the color was allowed to develop. The optical density (OD) at 405 nm was measured using a Model 680 microplate reader (Bio-Rad Laboratories, K.K., Tokyo, Japan).

Histological examination. Corpus mucosa samples were immediately fixed for 3 h in freshly prepared Carnoy's solution following the method described previously (20). After fixation, the materials were dehydrated through ethanol, cleared in xylene, and embedded in paraffin, from which 3 µm-thick sections were prepared for immunostaining with anti-mucin mAb. Immunohistochemical staining was carried out using the avidin-biotin-peroxidase method and an LSAB2 Kit (Dako, Carpinteria, CA, USA). Briefly, endogenous peroxidase activity was blocked with 0.3% H₂O₂, and then the tissue was sequentially incubated with 10% (v/v) normal swine serum, the anti-mucin mAb (RGM21 or HIK1083), biotinylated anti-mouse immunoglobulins, streptavidin horseradish peroxidase, and 0.02% 3,3-diaminobenzidine in 50 mmol/L Tris-HCl, pH 7.6, containing 0.005% H₂O₂. Counterstaining was performed with hematoxylin. The immunoreactivity of each mAb was observed using light microscopy.

Statistical analysis. Results in Fig. 2 are expressed relative to the average value of the corresponding control, and represent mean ± SE. The values presented in Fig. 3 are mean ± SE. One-way analysis of variance (ANOVA) with Scheffé's test was used for statistical analysis. A difference of $P < 0.05$ was considered statistically significant.

RESULTS

Biosynthetic activity of mucin

Fig. 2 shows the biosynthetic activity of mucin in the corpus mucosa of the rat stomach, as measured

by [³H]glucosamine incorporation. The mucin biosynthesis of the controls was $26,403 \pm 1,639$ dpm/mg tissue protein and following oral administration of famotidine for 7 consecutive days, [³H]glucosamine incorporation into the gastric mucin significantly decreased by 10%. In contrast, it increased by approximately 20% in the roxatidine-treated rats.

Mucin content

As shown in Fig. 3, administration of famotidine significantly decreased the mucin content of the rat stomach. The mucin content from rats given roxatidine was significantly higher than in the famotidine group.

Fig. 2 Effect of 7-day treatment with the H₂-receptor antagonists on mucin biosynthesis in rat corpus mucosa. Values represent means ± SE from six different samples derived from four different rats, given as a percentage of the controls. * $P < 0.05$, ** $P < 0.01$. Mucin biosynthesis in the control, expressed as disintegrations per minute per milligram tissue protein, was $26,403 \pm 1,639$.

Fig. 3 Influence of the H₂-receptor antagonists on mucin content in gastric mucosa. Specimens from three animals were pooled as one sample. Each experimental group comprised four samples. Mucin was isolated from the specimens by gel filtration chromatography. The void volume fraction (Fr-1) monitored by hexose measurement was collected as mucin and expressed as micrograms hexose per tissue. Each value is mean ± SE. * $P < 0.05$, ** $P < 0.01$.

To evaluate the involvement of mucin derived from surface mucus cells and mucous neck cells in this quantitative change, an appropriate amount of purified mucin from each specimen was reacted with anti-mucin mAb using an ELISA system (Fig. 4). ELISA OD values for the HIK1083 mAb did not differ significantly among the groups (OD range: 0.164–0.188). In contrast to HIK1083, RGM21 mAb values differed significantly between groups. As shown in Fig. 4, the relative antigenic activities of

Fig. 4 Immunoreactivity of RGM21 in the corpus mucin from rat stomachs treated with or without H_2 -receptor antagonists. Specimens from three animals were pooled as one sample. Each experimental group comprised four samples. The immunological response towards RGM21 was determined by enzyme-linked immunosorbent assay. The relative antigenic activities of RGM21 were significantly suppressed by administration of famotidine. In contrast, the OD values in roxatidine group did not differ in the control. * $P < 0.05$.

this mAb were significantly suppressed by administration of famotidine for 7 days, but not in the samples from the roxatidine group.

Histological findings

In the control group, immunoreactivity with RGM21 was fully detected in the surface mucus cells of the corpus mucosa (Fig. 5A, D). As shown in Fig. 5B, immunoreactivity with RGM21 was considerably reduced in the corpus of rats treated with famotidine. Surface mucus cells were not stained with RGM21 in Fig. 5E. In the gastric mucosa samples from the roxatidine group, RGM21 immunoreactivity was found not only in the specific mucus cells, but also in the secreted mucus in the surface layer as adherent mucus gel (Fig. 5C, F). No marked change in HIK1083 immunoreactivity was detected in sections of corpus mucosa from each treated group compared with the control (data not shown).

DISCUSSION

We found important disparities between famotidine and roxatidine based on the mucus barrier of rat gastric mucosa. Significant alterations of both the biosynthesis and the content of mucin in the rat stomach were revealed after oral administration of these H_2 -receptor antagonists once daily for 7 consecutive days. Famotidine inhibits acid secretion in a dose-dependent manner, with an ED_{50} value of

Fig. 5 Immunostaining of the rat corpus mucosa with RGM21 anti-mucin monoclonal antibody. Gastric mucosa from control rats (A, D), rats treated with famotidine (B, E), and rats treated with roxatidine (C, F). Note that the surface mucus cells in the corpus show positive staining with RGM21. Original magnification $\times 60$ (A–C) and higher magnification (D–F).

0.80 mg/kg in the rat, when administered orally (23), and the therapeutic dose used in the present study was appropriate for assessing the drug's effect on gastric mucosa (5, 17, 23, 26). Comparison of average antagonist dissociation constant (K_B) values obtained using guinea-pig atrium indicates that famotidine is about 10 times more potent than roxatidine (7, 26, 27). Taken together, these findings indicate that the agents used in this study, at clinically appropriate dosages, may have an important effect on mucus cells in the gastric mucosa.

In this study, famotidine decreased both the biosynthetic activity and the accumulation of mucin in the rat stomach at a dose of 3 mg/kg once daily for 7 consecutive days, which is consistent with prior studies showing that long-term administration of other acid suppressants, such as cimetidine and omeprazole, reduced the production and secretion of rat gastric mucin (5, 28). Because short-term pharmacological studies have shown that acid suppressants do not reduce mucin biosynthesis in rat gastric mucosa (10), the long-term effects of these agents on mucus cells are probably a secondary consequence of acid suppression. Our most striking finding is that certain mucus cell functions were selectively affected by administration of famotidine. Specific types of mucin are expressed by distinct mucus cells of the mammalian gastrointestinal tract (20, 24). In rats, the surface mucus cells and the mucous neck cells produce different types of mucin molecules, recognized by the anti-mucin mAb RGM21 and HIK1083, respectively (13, 14). Kawakubo *et al.* recently reported a natural antimicrobial effect of HIK1083-positive mucin against *Helicobacter pylori*, indicating individual differences in mucin function (15). We found a significant reduction in RGM21-positive mucin, which indicates that the treatment with famotidine for 7 days influences surface mucus cell function in rat gastric mucosa. A previous study, using the novel technique of high-pressure freezing/freeze substitution, which preserves intact not only the mucous gel but also the fluid luminal phase, showed a triple lamination structure of different mucins within the secreted mucous gel layer at the orifice of the gastric pits in the rat, suggesting the importance of surface mucus cell-derived mucin for the rheological properties of mucous gel (22). In a human study, Guslandi *et al.* reported that 4-week treatment with famotidine impaired the mucus-bicarbonate barrier in subjects with endoscopically normal gastric mucosa (3). Although the species differences between humans and rats should be kept in mind, the suppression of surface mucus cell-derived

mucin may lead to adverse effects with long-term administration of famotidine.

We also documented the stimulatory effect of 7-day treatment with roxatidine on mucin biosynthesis in the rat gastric mucosa. Roxatidine is one of the second-generation H₂-receptor antagonists (7) with increased action on gastric mucosal defense (9, 10). The structures of the second-generation H₂-receptor antagonists differ from the conventional H₂-receptor antagonists such as famotidine characterized by a five-membered aromatic ring. In addition, the structural requirements for mucosal protective activity in the second-generation agents are shown to be their amide structure and six-membered aromatic ring, such as benzene derivative. Using tissue culture system of rat gastric mucosa, we have already reported that the addition of this agent to the culture medium stimulated mucin biosynthesis in the surface mucus cells (9). Moreover, this effect, independent of its H₂-receptor antagonistic property, seems to be mediated by nitric oxide (9). In contrast to famotidine, our findings showed the maintenance of the RGM21-positive mucin accumulation by roxatidine treatment, suggesting no suppression of the surface mucus cell function with this drug.

Considerable information has been accumulated about the beneficial effect of co-administration of a mucosal protective agent with an acid suppressant for treatment of gastric ulcer (16, 18, 19, 25), although the question of whether combination therapy provides any additional benefit over acid suppression alone in the treatment of gastric ulcer has been raised (6). Because treatment with an acid suppressant, such as H₂-receptor antagonist or proton pump inhibitor, is highly effective for peptic ulcer disease, it may be difficult to estimate the additive or synergistic effects of combination therapy on ulcer healing. Recent work in rats documents that a certain mucosal protectant contributes to reducing the adverse effects of long-term treatment with famotidine (11, 21), and the present study showed the suppression of gastric surface mucus cell function caused by administration of famotidine for 7 days. On the basis of these findings, it is important for more effective ulcer therapy to evaluate the function of surface mucus cells in gastric mucosal defense during long-term treatment with acid suppressant. Although further studies are needed to clarify the detailed mechanism of the second-generation H₂-receptor antagonist treatment, we suggest that roxatidine can be prescribed for long-term acid suppression treatment.

To summarize, the administration of famotidine for 7 days caused a significant decrease in the con-

tent of surface mucus cell-derived mucin in the rat gastric mucosa. In contrast to famotidine, roxatidine stimulated the mucin biosynthesis and maintained the surface mucus cell-derived mucin accumulation. The H₂-receptor antagonists should be classified in relation to gastric surface mucus cell function, raising the possibility of a more effective ulcer therapy.

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