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Prophylactic Effect of Rebamipide on Aspirin-Induced Gastric Lesions and Disruption of Tight Junctional Protein Zonula Occludens-1 Distribution

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Abstract. Aspirin and nonsteroidal anti-inflammatory agents are known to induce gastroduodenal complications such as ulcer, bleeding, and dyspepsia. In this study, we examined the prophylactic effect of rebamipide, an anti-ulcer agent with free-radical scavenging and anti-inflammatory effect, on acidified aspirin-induced gastric mucosal injury in rats. In addition, we investigated the mucosal barrier functions disrupted by aspirin. Oral administration of acidified aspirin resulted in linear hemorrhagic erosions with increasing myeloperoxidase activity and thiobarbituric acid-reactive substance concentrations in the gastric mucosa. Rebamipide suppressed these acidified aspirin-induced gastric lesions and inflammatory changes significantly, and its protective effect was more potent in the case of repeated (twice daily for 3 days) treatment than single treatment before aspirin administration. Immunostaining of zonula occludens (ZO)-1, one of the tight junctional proteins, was strengthened in rat gastric mucosa after repeated administration of rebamipide. In addition, aspirin induced the increasing transport of fluorescine isothiocyanate-labeled dextrans with localized disruption and decreased expression of ZO-1 protein on rat gastric mucosal cell line RGM-1. Rebamipide effectively prevented aspirin-induced permeability changes and disruption of ZO-1 distribution. These results suggest that rebamipide protects against aspirin-induced gastric mucosal lesions by preserving gastric epithelial cell-to cell integrity in addition to the anti-inflammatory effects.

Keywords: aspirin, gastric mucosal injury, tight junction, zonula occludens (ZO)-1, rebamipide

Introduction

Aspirin is widely used as an anti-inflammatory agent, analgesic drug, and recently for the secondary prevention of cardiovascular and cerebrovascular disease (1 – 3). Many animal and human studies have documented the gastrointestinal complications accompanying the use of aspirin. Although it was well known that standard doses of aspirin for pain-relief induced gastric ulcer and bleeding, the recent clinical studies with low-dose aspirin for cardiovascular disease also have showed gastroduodenal complications (4 – 6). The mechanisms of gastric damage induced by non-steroidal anti-inflammatory drugs including aspirin is not clarified fully, but it is considered that at least four pathogenic mechanisms are important for the injuries: suppression of prostaglandin synthesis due to inhibition of cyclooxygenase (7 – 9), oxygen radical-mediated lipid peroxidation (10 – 12), neutrophil recruitment and activation (13, 14), and direct irritant action causing alterations of mucosal permeability (15 – 17).

To keep cell-to cell integrity and barrier function, various epithelial cells have expressed a highly constructed junctional complex composed of tight junctions,

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adherens junctions, gap junctions, and desmosomes (18 – 23). A variety of proteins playing different roles have been identified within the junctional complex. They include integral membrane proteins such as occludin, claudins, and E-cadherin; signaling molecules like protein kinase C, Src tyrosine kinases, and small G proteins; and the potential scaffolding proteins zonula occludens (ZO)-1, -2, and -3. Tight junctions are a complex of proteins that create intercellular boundaries between plasma membrane domains of epithelial and endothelial cells. ZO family members regulate cell growth and differentiation in addition to their original roles in the organization of the tight junction protein complex (24, 25). Although ZO-1 is expressed in multiple cell types, in epithelial cells, it is localized only in the tight junction, the most apical component of the junctional complex. The functions of ZO-1 proteins in regulating the junctions is unknown, but based on their domain structure, they are proposed to be scaffolding proteins. Recently, it was reported that ZO-1 acts as a signaling molecule by connecting signaling proteins (26). In the intestinal tract, disruption of tight junctions may lead to increased permeability to allergens, toxins, and pathogens, which appears to be a crucial mechanism involved in the pathogenesis of digestive diseases such as inflammatory disease, celiac disease, and alcoholic liver disease (18, 26, 27).

Rebamipide, a mucosal protective agent, has various actions for ulcer healing and prevention of gastric damage induced by non-steroidal anti-inflammatory drugs and *Helicobacter* infections (28, 29). The mechanisms of anti-ulcer and cytoprotective actions of rebamipide are mainly divided into the following 3 types: 1) acceleration of ulcer healing based on the induction of prostaglandin synthesis via COX-2 expression (30 – 32) and its receptor (33) and up-regulation of growth factor and its receptors such as epidermal growth factor (EGF) (34), vascular endothelial growth factor (VEGF) (35), and hepatocyte growth factor (HGF) (36); 2) cytoprotective activities such as induction of mucus secretion (37); 3) anti-inflammatory activities such as free-radical scavenging effect (38 – 40), inhibition of neutrophil activation, and migration (41, 42) and inhibition of cytokines production from leukocytes (43).

Previous studies have demonstrated the protective effect of rebamipide on aspirin- and indomethacin-induced gastric injury in humans and animals (42, 44 – 51). In the present study, we investigated the effect of rebamipide on aspirin-induced gastric mucosal injury, especially whether rebamipide can prevent the induction of gastric mucosal lesions, the increase in lipid peroxidation, and the neutrophil recruitment in rat. We also examined whether aspirin can increase the gastric mucosal permeability based on the disruption of tight junctional protein, "ZO-1", and rebamipide can prevent these disturbances induced by aspirin.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 180 – 220 g were obtained from Keari Co., Ltd. (Osaka) and were housed at 22°C in a controlled environment with 12 h of artificial light per day. They were fasted for 20 – 24 h before the experiments but had free access to drinking water. All experimental procedures described below were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine (Kyoto, Japan).

Chemicals

Aspirin and Fluorescine isothiocyanate (FITC)-labeled dextran (3 kDa) were purchased from Sigma Chemical (St. Louis, MO, USA). Rebamipide was donated by Otsuka Pharmaceutical Co. (Tokyo). Anti-zonula occludens-1 antibody and anti-claudin-1 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Zymed (South San Francisco, CA, USA), respectively. Thiobarbituric acid (TBA) and 3,3',5,5'-tetramethylbenzidine were obtained from Wako-Pure Chemical (Osaka).

Aspirin-induced gastric mucosal injury

Gastric hemorrhagic lesions were induced by intragastric administration of aspirin (200 mg/kg) and 0.15 N HCl in a volume of 0.5 ml/100 g body weight. Three hours after administration of aspirin, the animals were killed by exsanguination via the abdominal aorta under urethane anesthesia (1.25 mg/kg, i.p.).

Treatment schedule of rebamipide on aspirin-induced gastric injury

To investigate the prophylactic effect of rebamipide on aspirin-induced gastric mucosal injury, 30 mg/kg of rebamipide was given to rats orally according to two schedules. In the first schedule, rebamipide was administered to rats 1 h before aspirin administration (co-administration study group). In the second, rebamipide was administered twice daily (at 9:00 AM and 5:00 PM) for 3 days and 1 h before aspirin administration (prophylactic study group). Rebamipide was suspended in 0.5% CMC solutions. At 3 h after aspirin administration, the total area of gastric erosions, TBA-reactive substances (TBA-RS) concentration, and myeloperoxidase (MPO) activity in gastric mucosa were measured as an index of macroscopic injury, lipid peroxidation, and

neutrophil accumulation, respectively.

Evaluation of gastric mucosal lesions

Stomachs were removed, opened along the greater curvature, and rinsed with physiologic saline. Macroscopic gastric damage was measured using a dissecting microscope (×10 magnification) with a square grid and was expressed as the total area (mm^2) of hemorrhagic erosions by a person unaware of the experimental procedure.

Measurement of TBA-reactive substances and MPO activity

The gastric mucosa was scraped off with two glass slides and homogenized with 1.5 ml of 10 mM potassium phosphate buffer (pH 7.8) in a Teflon Potter-Elvehjem homogenizer to measure concentrations of TBA-RS and MPO activity. The concentration of TBA-RS in the gastric mucosa was measured by the method of Ohkawa et al. (52) and was expressed as nmol of malondialdehyde per milligram of protein using 1,1,3,3-tetramethoxy propane as a standard. The protein concentration in the gastric mucosal homogenates was measured by the method of Lowry et al. (53). MPO activity in the gastric mucosa was determined by a modification of the method of Grisham et al. (54). Briefly, homogenized gastric mucosal samples were centrifuged at $20,000 \times g$ for 15 min at 4°C. The supernatant was collected, and MPO activity was assessed by measuring H_2O_2-dependent oxidation of 3,3',5,5'-tetramethylbendine. One unit of enzyme activity was defined as a change in absorbance of 1.0/min at 655 nm and 25°C.

Immunohistochemistry of tight junctional proteins, ZO-1 and claudin-1, in rats

To investigate the expression of tight junctional proteins in rat gastric mucosa, rebamipide was administered according to the same administration schedule as aspirin-induced gastric injury. At 4 h after final administration of rebamipide, the stomach was removed and fixed with 4% paraformaldehyde. Paraffin Sections were made using the Young-type sliding microtome (Sakura Finetek Japan, Co., Ltd., Tokyo) and the disposable microtome blade. Sections were about 3 μm in thickness and mounted on silane-coated slides. Immunohistochemical staining was performed according to the labeled streptavidin–biotin method. After deparaffinized sections had been autoclaved at 120°C for 10 min to block endogenous peroxidase activity, they were incubated sequentially at 4°C for 1 h with the following reagents: anti-ZO-1 or anti-claudin-1 antibody (Zymed), diluted to 1:100 in Tris-buffered saline, and then incubated in streptavidin-biotin-peroxidase solution according to the instructions for the LSAB-kit (Dako Japan Co., Ltd., Tokyo). The immunoreaction was visualized by peroxidase-diaminobenzidine (DAB) reaction. The sections were finally counterstained with hematoxylin. The stained sections were then observed with a light microscope (OLYMPUS DP70; Olympus, Tokyo).

Cell cultures

The rat gastric mucosal cell line RGM-1 (Riken Cell Bank, Tsukuba) (55), established by Matsui and Ohno, was used for the determination of permeability and ZO-1 expression study. RGM-1 cells were routinely maintained on collagen-1 coated Biocoat tissue culture dishes at 37°C in a 5% CO_2 humidified atmosphere in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 mg/ml amphotericin.

Monolayer permeability assay using FITC-conjugated dextrans

FITC-conjugated dextrans (3-kDa molecular weight) was used for determination of paracellular permeability change induced by aspirin. RGM-1 cells were grown on transwell which is permeable filter with monolayer membranes, with or without 1 mM rebamipide for 3 days. When cells were confluent, the medium of the upper and lower chamber compartment was exchanged with culture medium containing neither aspirin nor rebamipide, medium containing 1 mM aspirin, or medium containing both 1 mM aspirin and 1 mM rebamipide. After 3 h, medium of the upper and lower chamber compartment was exchanged with culture medium containing FITC-conjugated dextrans (3 mg/ml). After 60 min, 100-μl samples of the medium in the lower chamber compartment were collected and transferred to a 96-well plate (Nunc, Roskide, Denmark). The fluorescence intensities of the samples were determined using a fluorescence spectrophotometer (Fluoromark; BioRad, Hercules, CA, USA) with 518-nm emission wavelength excited by 494-nm wavelength. The permeability was expressed as percentage of passed FITC-dextrans volume as compared with total volume of added FITC-conjugated dextrans. Rebamipide and aspirin were diluted with culture medium and final 0.1% ethanol, respectively. In the group without aspirin, 0.1% ethanol only was added to the medium as a control group.

Immunofluorescence microscopy

RGM-1 cells were seeded on 16-well Lab-Tek tissue chamber slides (Nunc) at a density of 1×10^5 cells/well.

After 24-h cultivation, the medium was exchanged with fresh medium with or without 1 mM rebamipide, and then the cells were incubated for 3 days. Subsequently, the medium was exchanged again with 1 mM aspirin or both 1 mM aspirin and 1 mM rebamipide supplemented medium and further incubated for 3 h. After washing with PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$, the cells were fixed with 50 μl ice cold acetone for 2 min on ice. Then cells were washed with PBS, and non-specific binding sites were blocked with PBS with 5% BSA for 30 min at 37°C. After incubation with primary antibody ZO-1 (Santa Cruz Biotechnology) diluted at 1:50 for 1 h, a secondary antibody (fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody: Sigma) diluted 1:1000 was added and incubated for 30 min. ZO-1 protein expression was visualized under a fluorescent light microscope.

Statistical analyses

All values were expressed as the mean ± S.E.M. Data were compared using an analysis of variance (ANOVA) followed by Fisher's protected least significant difference test (Fisher's PLSD). A value of $P<0.05$ was considered statistically significant.

Results

Effect of rebamipide on aspirin-induced gastric injury

Administration of acidified aspirin (200 mg/kg) consistently induced gastric hemorrhagic lesions in the mucosa of the glandular stomach as shown in Fig. 1. Rebamipide at 30 mg/kg reduced the gastric lesions by 35% in the co-administration group (Fig. 1A) and by 65% in the prophylactic administration group (Fig. 1B). The differences were significant between the aspirin group and rebamipide group in the prophylactic administration group, but not in the co-administration group.

In addition, administration of aspirin increased the TBA-reactive substances significantly as shown in Fig. 2. Administration of rebamipide reduced TBA-reactive substances by 26% in the co-administration group (Fig. 2A) and by 43% in the prophylactic administration group (Fig. 2B). The differences were significant between the aspirin group and rebamipide group in the prophylactic administration group.

Aspirin caused the increase of MPO activities in the gastric mucosa, and rebamipide showed a tendency to reduce the activities in both study groups. But there were no significant differences between the aspirin group and rebamipide group in both treatment schedules as shown in Fig. 3.

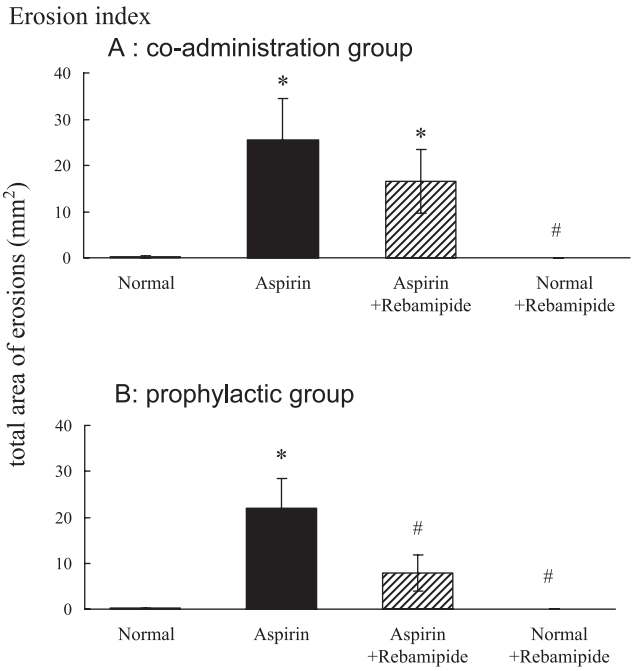

Fig. 1. Prophylactic effect of rebamipide on the total area of gastric hemorrhagic erosions induced by acidified-aspirin in rats. Rebamipide (30 mg/kg) was administered orally 1 h before aspirin administration (co-administration group, Fig. 1A) and twice daily 3 days and 1 h before aspirin administration (prophylactic group, Fig. 1B). Data are expressed as means ± S.E.M. of 8 rats in each group. $*P<0.05$, compared with normal group; $^{\#}P<0.05$, compared with aspirin group.

Effect of rebamipide on expression of tight junctional protein "ZO-1"

The expression of ZO-1 in the gastric mucosa was shown in Fig. 4. The repeated administration of rebamipide (Fig. 4C) have reinforced the expression of ZO-1 in the epithelial area of gastric mucosa as compared with normal (Fig. 4A) and single administration of rebamipide (Fig. 4B). The expression of claudin-1 was seen in the epithelial area of gastric mucosa as well as ZO-1, but there was no clear differences among the normal group, single administered group, and repeated administered group (data not shown).

Effect of rebamipide on aspirin-induced increase in paracellular permeability

The paracellular permeability using 3 kDa FITC-dextrans was significantly increased at 3 h after aspirin treatment (1 mM) to RGM-1 cells (Fig. 5). The addition of 1 mM rebamipide with aspirin showed a tendency to reduce the permeability of FICT-dextrans, by 7% and 35% in the co-administration group and prophylactic group, respectively. However, there were no significant differences between aspirin group and prophylactic group.

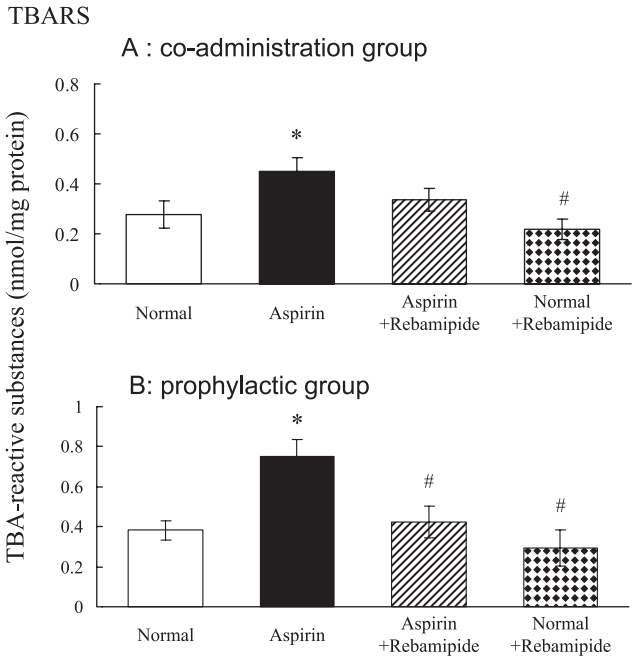

Fig. 2. Effect of rebamipide on the thiobarbituric-reactive substances (TBA-RS) of gastric mucosa induced by acidified-aspirin in rats. Rebamipide (30 mg/kg) was administered orally 1 h before aspirin administration (co-administration group, Fig. 1A) and twice daily 3 days and 1 h before aspirin administration(prophylactic group, Fig. 1B). Data are expressed as means ± S.E.M. of 8 rats in each group. **P*<0.05, compared with normal group; #*P*<0.05, compared with aspirin group.

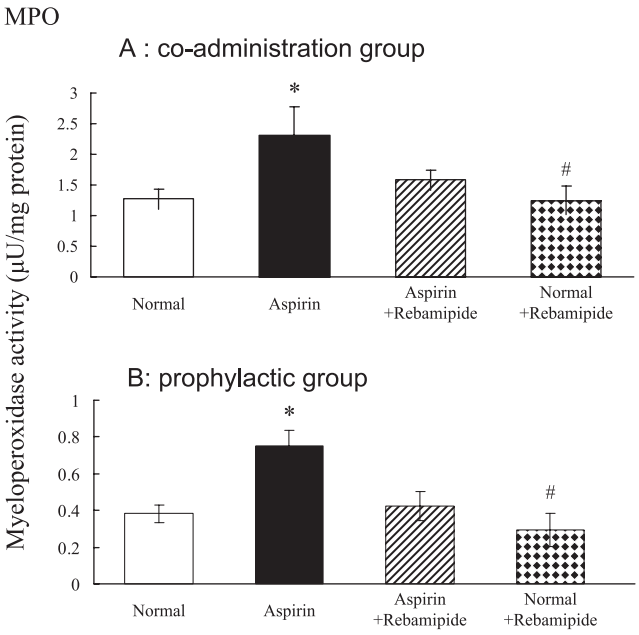

Fig. 3. Effect of rebamipide on the myeloperoxidase (MPO) activities of gastric mucosa induced by acidified-aspirin in rats. Rebamipide (30 mg/kg) was administered orally 1 h before aspirin administration (co-administration group, Fig. 1A) and twice daily 3 days and 1 h before aspirin administration (prophylactic group; Fig. 1B). Data are expressed as means ± S.E.M. of 8 rats in each group. **P*<0.05, compared with the normal group; #*P*<0.05, compared with the aspirin group.

Effect of aspirin with or without rebamipide on expression of the tight junctional protein ZO-1

The immunofluorescent staining of ZO-1 protein was seen in the surrounding area of untreated RGM-1 cells as shown in Fig. 6A. The exposure of 1 mM aspirin dramatically reduced the immunofluorescent staining of ZO-1 after 3 h (Fig. 6B). On the other hand, pre-treatment of RGM-1 cells with rebamipide for 3 days (Fig. 6C) protected against the decrease in the ZO-1 immunofluorescent staining induced by aspirin as shown in Fig. 6D.

Discussion

Aspirin is the one of the oldest drugs widely used in the world for the pain relief with high-dose and secondary prevention of cardiovascular disease with low-dose. It is well-known that high-dose aspirin caused gastrointestinal bleeding (6), and recently, it is reported that the risk is not reduced by decreasing the dose or using modified release formulations of aspirin (4, 5). So it is considered that prophylactic administration of an anti-ulcer agent is recommended to prevent gastrointestinal complications in a higher risk group with *Helicobacter* infection (56) or co-administration of non-steroidal anti-inflammatory drugs including selective COX-2 inhibitor (4, 57). It is well-known that the inhibition of cyclooxygenase-1 in the gastrointestinal mucosa and the consequent reduction in gastrointestinal mucosal prostaglandin levels have been proposed to be an important mechanism to induce gastrointestinal injury by aspirin (58), but it has recently been reported that low-dose aspirin fully induced gastric erosions without affecting cyclooxygenase expression or mucosal prostaglandin E_2 levels (59).

The prophylactic effect of rebamipide on aspirin-induced gastric lesions have been already evaluated by Damman et al. (44) in clinical study. However, the protective mechanisms of rebamipide on aspirin-induced gastric hemorrhagic lesions were not elucidated so far. In this study, we demonstrated the protective effect of rebamipide on acidified aspirin-induced gastric hemorrhagic lesions in rat. In the previous reports, we demonstrated that aspirin-induced gastric mucosal injury may be attributed to lipid peroxidation associated with oxygen-derived free radicals, neutrophil activation, and TNF-α (10, 14). The present results reveal that repeated administration of rebamipide more strongly

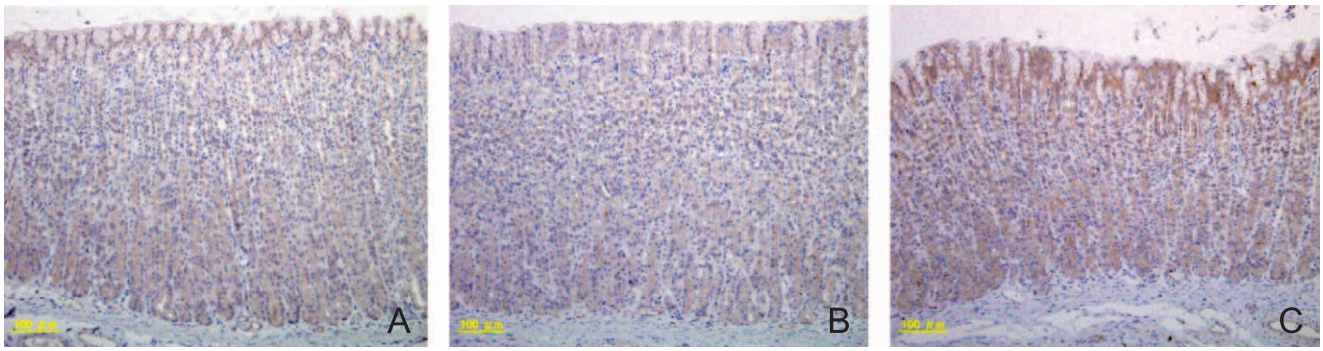

Fig. 4. Effect of rebamipide on expression of tight junctional protein "ZO-1" (a representative picture). Rebamipide (30 mg/kg) was orally administered to rats only once (n = 4) or twice daily for 3 days (n = 4). Normal rats (n = 4) were administered 0.5% CMC. After the stomach was removed and fixed with 4% paraformaldehyde., immunohistochemical staining for ZO-1 was performed according to the labeled streptavidin-biotin method. A: Normal, B: Single administration of rebamipide, C: Repeated administration of rebamipide. Scale bar = 100 μm.

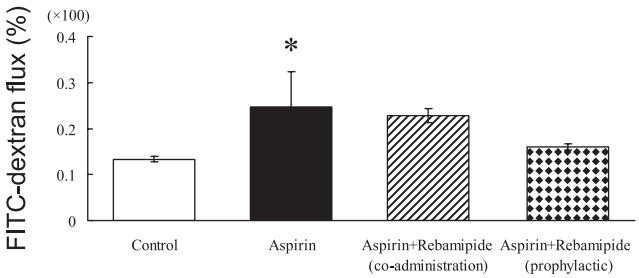

Fig. 5. Effect of rebamipide on aspirin-induced paracellular permeability in RGM-1 cells. RGM-1 cells were incubated with or without 1 mM rebamipide for 3 days. Subsequently, RGM-1 cells were stimulated with aspirin or both aspirin and rebamipide for 3 h. After incubation with aspirin and/or rebamipide, FITC-conjugated dextrans were added upper chamber. Sixty minutes after the addition of dextrans, fluorescence intensity was measured. Data are expressed as means ± S.E.M. of 4 experiments performed in triplicate in each group. *P<0.05, compared with the control group.

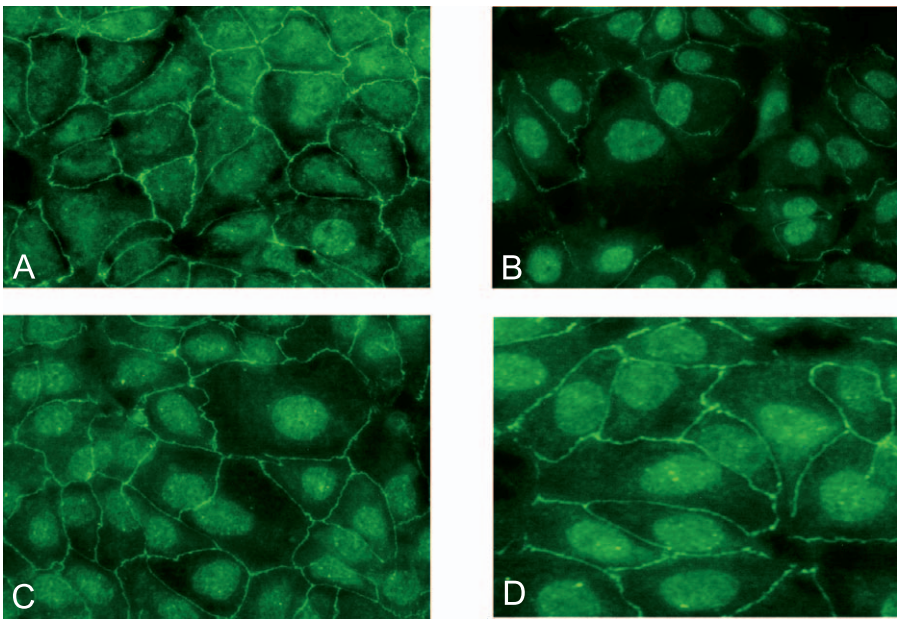

Fig. 6. Effect of aspirin and rebamipide on ZO-1 protein expression in RGM-1 cells (a representative picture). RGM-1 cells were incubated with or without 1 mM rebamipide for 3 days. Subsequently, RGM-1 cells were stimulated with 1 mM aspirin or aspirin and rebamipide for 3 h. After incubation with aspirin and/or rebamipide, ZO-1 protein was evaluated using immunostaining methods. A: untreated, B: 1 mM aspirin, C: 1 mM rebamipide (3 days), D: rebamipide + aspirin.

reduced macroscopic mucosal injury (Fig. 1), lipid peroxidation (Fig. 2), and neutrophil accumulation (Fig. 3) than co-administration of rebamipide. These findings suggest that the protective effect of rebamipide may be partly dependent on its anti-inflammatory activity, but the mechanisms for interaction between repeated administration and increase in anti-inflammatory effect need to be investigated in detail.

In this study, we also have aimed at investigating the direct noxious action of aspirin on the gastric mucosa because rebamipide exhibited a more potent protective effect when it was administered repetitively before aspirin administration as shown in Fig. 1B.

It has been reported that non-steroidal anti-inflammatory drugs including aspirin increase gastrointestinal permeability (17, 60, 61), and this permeability change is believed to result from the opening of tight junctions because of a reduction in prostaglandin synthesis and/or energy-depletion (60). In 1986, Meyer et al. reported the morphological change of tight junctions in the canine gastric epithelial cells after aspirin treatment by using light microscopy, freeze-fracture electron microscopy, and extracellular tracer transmission electron microscopy techniques. Interestingly, its tight junctional changes are induced within few minutes with no relation to dosage and exposure time to aspirin (62). So they concluded that the impairment of tight junction complexes between viable gastric mucosal epithelial cells may be a major contributing factor in the etiology of aspirin-induced gastric lesions.

ZO-1 proteins, a protein of the tight junctional complexes, is an important protein contributing to cell integrity, and a causal relationship between localization change of ZO-1 protein and permeability change has been proposed in several studies (63, 64). Recently, it was reported that aspirin also disrupted the distribution of ZO-1 proteins in HT-29 cells (65). It is reported that the effect of rebamipide on permeability increase induced by indomethacin is associated with the preservation of trans-epithelial electrical resistance (TEER) in RGM-1 cells (48), and recently, an increasing effect of TEER by rebamipide has been reported in T84 cells (66). However, these effects of rebamipide were not elucidated from the aspect of tight junctional complexes. So, we examined the effect of rebamipide on the localization of ZO-1 in rat gastric mucosa as shown in Fig. 4. The repeated administration of rebamipide reinforced the expression of ZO-1 in the area of gastric epithelia in all rats tested. Although we could not fully prove the suppressive effect of rebamipide on permeability changes induced by aspirin as shown in Fig. 5, its effect was more potent in the prophylactic group than the co-administration group. Furthermore, exposure of RGM-1 cells to aspirin clearly decreased the protein expression of ZO-1 time-dependently and decreased the immunofluorescent staining of ZO-1 proteins expressed on the peripheral area of RGM-1 cells (Fig. 6). Rebamipide obviously did not increase the immunofluorescent staining of ZO-1 protein, but preserved the immunofluorescent staining of ZO-1 protein against disruption of ZO-1 distribution. These results indicate that rebamipide also exhibited the protective effect on aspirin-induced gastric lesions via keeping tight junctional complexes. Although the mechanism of rebamipide to keep distribution of ZO-1 protein was not clarified in this study, Blikslager et al. reported that prostaglandins played an important role in keeping the barrier function by increasing intracellular cAMP and Ca^{2+} (67). In fact, Suestugu et al. reported (68) that rebamipide increased the cAMP production when it was co-cultured with prostaglandin E_2 in RGM-1 cells, although rebamipide itself did not increase cAMP-production. In addition, it is well known that rebamipide increases the gastric mucosal prostaglandins in rats (31). Taken together, these findings indicate that preservation of ZO-1 in the gastric mucosa may be related to the increase in cAMP via activation of prostaglandin cascade by rebamipide. On the other hand, Sheth et al. reported that EGF prevented the acetaldehyde-induced permeability, TEER change, and disruption of junctional proteins such as ZO-1 and occludin (63). It seems that signal transduction induced by growth factor is one of the crucial factors to keep junctional complexes intact. Interestingly, it is reported that rebamipide significantly accelerated the ulcer healing and increased the expression of EGF and its receptor in rat gastric mucosa (34). Further examinations need to be done to determine the mechanisms for preserving barrier function by rebamipide.

In conclusion, our results suggest that rebamipide has a protective effect against aspirin-induced gastric hemorrhagic lesions, not only by acting as a radical scavenger and suppressing neutrophil activation, but also by suppressing permeability change through maintaining the expression of the scaffolding protein "ZO-1". These data recommend rebamipide for the prevention of acute gastric injury induced by aspirin in humans.

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