Original Article

The role of trefoil factor family in apparently healthy subjects administrated gastroprotective agents for the primary prevention of gastrointestinal injuries from low-dose acetylsalicylic acid: a preliminary study

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(Received 11 January, 2011; Accepted 9 February, 2011; Published online 25 August, 2011)

It is well-known that acetylsalicylic acid induces gastrointestinal complication. Recently, trefoil factor family has been reported as a mucosal protective factor. We focused on trefoil factor family as one of defensive system for gastrointestinal injuries. The aim of this trial was to evaluate trefoil factor family levels in the serum of healthy subjects with low-dose acetylsalicylic acid. Low-dose acetylsalicylic acid with placebo or proton pump inhibitor or rebamipide were administered in 30 healthy subjects. Transnasal endoscopy was performed at 0, 24 h, 3 and 7 day. Changing of trefoil factor family (1,2,3) and numbers of gastric injuries were evaluated. The numbers of gastric injuries were significantly increased in the placebo group at 3 and 7 days. Injuries in the proton pump inhibitor group were not induced, in the rebamipide group were slightly induced. Trefoil factor family level in the placebo group were decreased in 3 and 7 days compared with prior to starting the trial. Trefoil factor family may have an important association with acetylsalicylic acid-induced gastrointestinal damage. Proton pump inhibitor and rebamipide prevented low-dose acetylsalicylic acid-induced gastrointestinal complications compared with the placebo group.

***Key Words*:** **Low-dose ASA, transnasal endoscopy, rebamipide, proton pump inhibitor, trefoil factor**

Atherosclerotic diseases including coronary heart disease and cardiovascular events are associated with high frequencies of morbidity and mortality in developed populations.[1] Administration of low-dose acetylsalicylic acid (ASA) has been recommended for the primary and secondary prevention of these diseases.[2–3] Indeed, observational registry demonstrated that approximately 70–80% of patients with a high risk of atherothrombosis were given low-dose ASA for the prevention of future vascular events.[4]

On the other hand, it is well-known that low-dose ASA induces gastrointestinal (GI) complications. In a multinational prospective study of 187 patients receiving low-dose ASA for cardiovascular disease prophylaxis, the prevalence of endoscopically-detected peptic ulcers was 10.7% [95% confidence interval (CI); 6.3–15.1] in the US 5). There was also a report on low-dose ASA-induced GI complications for a Japanese population. Nema *et al.*[6] demonstrated that mucosal injuries were found in 61.4% of low-dose ASA users; the low-dose ASA users had more mucosal injuries than the non-users ($p<0.0001$). The frequency of mucosal injuries was not associated with the duration of ASA treatment. Also, there were several reports on the preventive effect of anti-acid agents for low-dose ASA-induced GI complications.

Proton pump inhibitor (PPI) was effective at reducing the risk of endoscopic duodenal ulcer (RR = 0.44, 95% CI; 0.32–0.74) and gastric ulcer (RR = 0.40, 95% CI; 0.32–0.51).[7] Shiotani *et al.*[8] reported that taking PPI [adjusted odds ratio (OR): 0.09; 95% CI: 0.02–0.39] was significantly associated with peptic ulcer. Prostaglandin (PG) analogue and inducer include misoprostol and rebamipide, and they are cytoprotective agents. Misoprostol is well-known reducing serious GI complications in patients with rheumatoid arthritis taking NSAIDs.[9] There is a randomized controlled trial compared with misoprostol and rebamipide for patients with NSAIDs-induced GI complications. It was resulted that the incidence of peptic ulcer was 4.4% in the misoprostol group and 4.5% in the rebamipide group.[10] Ono *et al.*[11] reported that rebamipide prevent low-dose aspirin-induced gastric injury in healthy subject.

In Europe it was reported that PPI was prescribed in 99.4% cases for prevention of low-dose ASA-induced GI complications.[12] On the other hand, in Japan cytoprotective agents (ex. rebamipide) were widely used for prophylaxis of gastrointestinal complication induced by low-dose ASA. Actually PPI and cytoprotective agents were prescribed equally for prophylaxis of gastrointestinal complication induced by low-dose ASA.[13]

In our previous report, low-dose ASA-induced GI complications for healthy subjects were strongly induced at 3 days and were reduced at 7 days.[14] This phenomenon was concluded as adaptation. The mechanism of adaptation is not yet well understood, therefore we conducted this study to clarify the phenomenon by measuring trefoil factor family (TFF).

Recently, TFF has been reported to be a mucosal protective factor. TFF is a mucin-associated protein involved in the maintenance of mucosal barrier and restitution of lining epithelial cells.[15] TFF were categorized into three family members, TFF1, 2, and 3. TFF are a family of short peptides, rich in disulfide bonds that form intramolecular loops.[16] TFF are up-regulated in the surface epithelial cells at the margin of gastric ulcer.[17] We focused on TFF as one candidate defensive system for ASA-induced GI complications.

The aim of this trial was to evaluate the level of TFF in the

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doi: 10.3164/jcbn.11-10

serum for healthy subjects on low-dose ASA, ASA plus PPI, and ASA plus rebamipide, and the preventive effect of PPI and rebamipide.

Materials and Methods

A randomized, double-blind, placebo-controlled trial comparing placebo, PPI and rebamipide were performed in 30 healthy subjects. Ten were assigned as the PPI group, ten were as the placebo group, and ten were as the rebamipide group. The study protocol was approved by the Ethics Committees of Tokyo Medical University Hospital, and written informed consent was obtained from all subjects.

Inclusion and exclusion criteria. Inclusion criteria were i) lack of gastric conditions such as bleeding or ulcer on endoscopy, and ii) *H. pylori* negativity on ^{13}C urea breath test. Subjects who were taking other medication were excluded.

Study design. The study design was as shown in Fig. 1. The thirty healthy subjects were divided into three groups: those taking ASA 100 mg with placebo, omeprazole 20 mg, or rebamipide 300 mg. The groups with drug administration received their dose three times daily for 7 consecutive days. Transnasal endoscopy was performed at 0 and 24 h on the first day, and again on the third and seventh days.

Randomization. Subjects were recruited for the treatment sequences in a random fashion according to a randomization schedule for the treatment period. A randomization number that was associated with a specific treatment, either rebamipide, omeprazole, or placebo, was assigned to each subject in the study. Randomized numbers were generated by SAS program.

Endpoints. The primary endpoint was to evaluate short-term changes of gastric mucosa in healthy subjects who took low-dose ASA with placebo, omeprazole, and rebamipide. The secondary endpoint was to evaluate short-term changes of TFF (TFF1, 2, and 3) levels in serum.

Evaluation criteria. The categories of injury were erythema, erosions, petechia, and ulcers. Erythema was defined as a region clearly redder than surrounding mucosa, erosion as a region with mucosal deficit, petechia as a bleeding region without mucosal deficit, and ulcer was defined as greater than 5 mm. Gastric mucosal injuries detected on endoscopy were calculated among numbers of erosions, erythema, petechia, and ulcers. All GI injuries were counted in each time course.

The number of erythema, erosions, petechia and ulcers were counted to evaluate low-dose ASA-induced GI injuries compared among the placebo, omeprazole, and rebamipide group. The number of erythema, erosions, petechia were evaluated as changing their numbers compared with before starting study and after (after had three points, 24 h, 3 days, and 7 days).

Endoscopic evaluation was done by two endoscopists blinded for subject number and groups. If the result was different, re-evaluation and discussion were done.

Measurement of serum and TFF1, 2, and 3 levels in the serum. Serum samples were obtained for all subjects at 0, 24 h on the first day, 3rd and 7th days after starting this trial. TFF1, TFF2, and TFF3 levels in the gastric juice and serum were measured by ELISA as described recently.[18,19] All measurements were performed in duplicate within the same assay run.

Evaluation of adverse events. The following GI symptoms and complications were to be recorded in the symptom diary for all subjects throughout the study period.

Statistical analysis. Difference of time course in TFF levels were shown as absolute different and 95% confidence index. Findings of $p<0.05$ were considered significant. Statistical analyses were performed using SAS® version 8.2 (SAS Institute, Cary, NC). Details of the statistical analysis were analyzed by intention-to-treat analysis.

Results

Thirty healthy subjects were enrolled. The mean age of the subjects was 28 ± 4 years. Nineteen were male and eleven were female. In addition, the backgrounds of all subjects who had smoking, drinking, and histology data before starting trial were shown in Table 1. No subjects had *H. pylori* infection. Thirty healthy subjects were divided into three groups: low-dose ASA plus placebo group, omeprazole, and rebamipide groups.

Short-term change of gastric mucosa by administration of low-dose ASA among the control, placebo, omeprazole, and rebamipide groups. Changing of erosion, erythema, and petechiae among three groups from before starting the trial were shown in Table 2. The number of erosion and erythema were in-

Table 1. Demographic in healthy subjects

	Omeprazole	Rebamipide	Placebo
	(n = 10)	(n = 10)	(n = 10)
Age (mean ± SD)	28 ± 4	28 ± 5	27 ± 3
Gender (male/female)	6 : 4	7 : 3	6 : 4
Smoking	2 : 10	3 : 10	2 : 10
Alcohol in take	9 : 10	9 : 10	9 : 10
Number of injury			
erosion	4.4 ± 10.8	0.5 ± 1.2	0.9 ± 1.5
erythema	4.0 ± 6.4	9.1 ± 13.9	3.5 ± 3.8
petechiae	8.2 ± 22.4	2.7 ± 4.2	2.4 ± 3.3

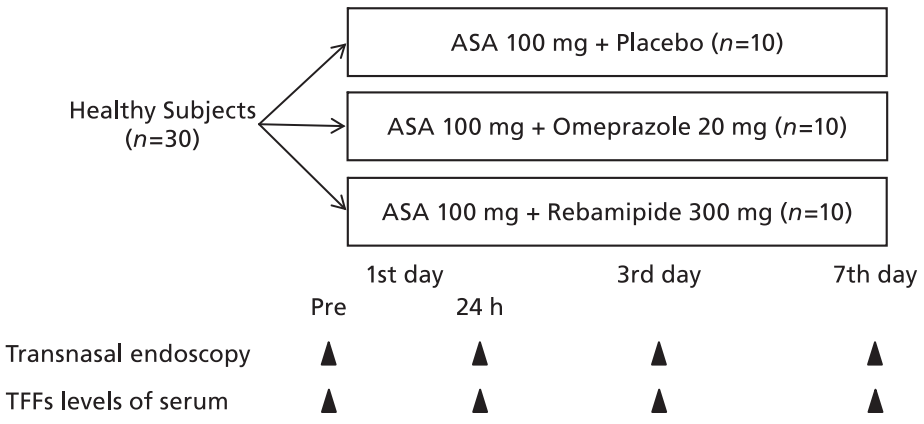

Fig. 1. Study design.

Table 2. Changing numbers of injuries from before treatment

	24 h	3 days	7 days
Erosion			
Placebo	0 ± 1.4	2.4 ± 3.1	1.4 ± 2.8
Omeprazole	2.9 ± 3.7	1.9 ± 4.5	–2.3 ± 8.5
Rebamipide	–0.1 ± 1.5	0.9 ± 1.8	1.2 ± 3.4
Erythema			
Placebo	1.8 ± 3.6	9.6 ± 10.5 ($p = 0.0327$)	2.2 ± 6.6
Omeprazole	–0.1 ± 4.7	1.4 ± 6.8 ($p = 0.0611$)	–1.2 ± 6.3
Rebamipide	–0.7 ± 3.6	0.3 ± 4.2	1.5 ± 5.8
Petechiae			
Placebo	2.4 ± 4.3	1.4 ± 5.1	8.3 ± 8.8 ($p = 0.0335$)
Omeprazole	–2.1 ± 18.4	0 ± 11.2	–5.6 ± 23.6 ($p = 0.0213$)
Rebamipide	0.1 ± 6.4	–0.4 ± 4.8	–1.6 ± 5.2

mean ± SD.

Table 3. Circadian variation of TFF1, 2, and 3 serum levels

	pre	24 h	3 days	7 days
TFF1				
Placebo	0.473 ± 0.225	0.449 ± 0.227 (–5.1)	0.379 ± 0.111 (–19.9)	0.482 ± 0.216 (1.9)
Omeprazole	0.432 ± 0.168	0.452 ± 0.170 (4.6)	0.421 ± 0.167 (–2.5)	0.495 ± 0.188 (14.6)
Rebamipide	0.560 ± 0.228	0.534 ± 0.189 (–4.6)	0.393 ± 0.090 (–29.8)*	0.489 ± 0.153 (–12.7)
TFF2				
Placebo	4.100 ± 1.500	3.449 ± 0.620 (–15.9)	3.093 ± 0.521 (–24.6)	3.093 ± 0.521 (–11.3)
Omeprazole	3.193 ± 0.499	3.449 ± 1.132 (8.0)	3.330 ± 1.222 (4.3)	3.431 ± 1.027 (7.5)
Rebamipide	3.732 ± 1.016	3.525 ± 1.188 (–5.5)	3.294 ± 1.176 (–11.7)*	3.918 ± 1.220 (5.0)
TFF3				
Placebo	3.140 ± 0.976	3.140 ± 0.976 (–12.6)	2.496 ± 0.943 (–20.5)*	3.000 ± 1.015 (–4.5)
Omeprazole	3.169 ± 1.228	3.140 ± 0.976 (–7.8)	3.279 ± 1.204 (3.5)	2.946 ± 1.337 (–7.0)
Rebamipide	2.509 ± 1.247	2.404 ± 1.207 (–4.2)	2.322 ± 1.144 (–7.5)	2.732 ± 1.225 (8.9)

* compared with before TFF levels $p<0.01$.

creased in the placebo group at 3 days compared with before starting the trial. Afterward these changes were recovered at 7 days. The number of petechiae was increased in the placebo group at 7 days compared with before starting the trial. The number of erythema was increased in the placebo group at 3 days compared with the omeprazole and the rebamipide groups, 9.6 ± 10.5 vs 1.4 ± 6.8 ($p = 0.0611$) vs 0.3 ± 4.2 ($p = 0.0327$). The number of petechiae was increased in the placebo group at 7 days compared with the omeprazole and the rebamipide groups: 8.3 ± 8.8 vs –5.6 ± 23.6 ($p = 0.0213$) vs –1.6 ± 5.2 ($p = 0.0335$), respectively.

Evaluation of TFF1, 2, and 3 levels in the serum. TFF levels at 3 day in the placebo group were decreased compared with before taking ASA, and were recovered at 7 days. On the other hand, TFF levels in PPI group were not changed compared with before starting the trial and 3, 7 days. TFF1, 2 levels at 3 day in rebamipide were significantly decreased compared with before taking ASA, and were also recovered at 7 days. (shown in Table 3)

Adverse events. The number of gastrointestinal symptoms or complications was recorded in the symptom diary by all subjects throughout the study period. There were no serious side-effects in three treatment groups.

Discussion

The action of ASA is derived from irreversible inhibition of the cyclooxygenase-1 enzyme, resulting in the inhibition of thromboxane A2 formation, however the novel effects of ASA mediated by its salicylate moiety complement the role of the acetyl moiety in terms of its platelet inhibitory effect. The action of ASA resulted from an interaction among platelet activation, endothelial inflammation/activation, and oxidative stress.[20] However, they may have contrary effects for GI mucosa.

In this study, it is interesting to note that the peak of erythema and erosion was induced on the 3rd day, and restored on the 7th day in spite of continued administration of ASA (Table 2). Konturek *et al.*[21] reported about adaptation of aspirin-induced gastric damage. Gastric injury may repeatedly occur and heal as a natural process, but it is not clear what kind of cycle is being repeated. In this study, the healing effect of placebo at 7 days may be resulted by adaptation, and also this action by adaptation may have a close relation to TFF.

TFF is relatively new family peptides which bear the three-loop trefoil domain. They are assumed to play important role in the protection of gastrointestinal mucosa.[22,23] TFF1 is produced mainly in the stomach, in superficial cells of the body and antral mucosa.[22,24] TFF2 is abundant in the mucous neck cells in the body and in the antral gland of the stomach.[22,25] TFF3 is expressed in the goblet cells of lower intestine and its expression in not found in normal gastric mucosa.[17] We also planned the measurement of TFF in gastric mucosa, but this procedure requires the use of biopsy forceps. We must take measurements many times (pre, 24 h, 3, 7 day), so constant use of the forceps cause injury to gastric mucosa. Such injuries could also conceivably be caused by low-dose aspirin as well. We cannot tell if the injury is caused by the forceps or low-dose aspirin. Therefore we used serum TFF in this study.

doi: 10.3164/jcbn.11-10

The relationship between TFF and NSAIDs is still somewhat controversial. Koitabashi *et al.*[26] reported Indometacin up-regulates TFF2 expression in gastric epithelial cell *in vitro*. Alderman *et al.*[27] investigated relationship between TFF1 & TFF2 and adaptation to aspirin in gastric mucosa *in vivo*. They reported that quantification of TFF1 and TFF2 protein level in adapted group slightly were decreased (but no significant change) than that of control group. Moreover there are no report related serum TFF and mucosal TFF, but transvenous administration of TFF inhibited ethanol induced injury.[28] It is possible that serum TFF is strongly related with mucosal TFF. TFF may possibly play an important role in gastric mucosal damage.

In addition, we investigated the relationship between TFF levels and mucosal damage by low-dose ASA with PPI or rebamipide. Low-dose ASA-induced GI injuries have been prevented in the PPI group, the level of TFF in the PPI group were not significant changed compared with before starting the trial. This was based on the acid secretion inhibitory action of PPI. On the other hand, rebamipide prevented GI injuries in spite of decrease in TFF levels. These results may show that protective effect of rebamipide was not influenced by TFF levels. Suzuki *et al.*[29] reported that rebamipide significantly suppressed ASA-induced gastric damages by protection of tight-junction. Iijima *et al.*[30] reported on the output of total gastric mucin was significantly increased by rebamipide. These actions may be one of rebamipide's GI mucosal protective mechanisms.

Although PPI is the most effective drug for patients with ASA-induced GI complications, it also has limitations, such as poor metabolizer as well as ASA and/or clopidogrel resistance. Some patients are known to have PPI resistance.[31,32] PPI can be classified as having either the phenotype of an extensive metabolizer or that of a poor metabolizer based on the level of CYP2C19 activity.[33,34] Manifestation of a PPI-independent preventive system for GI complications would be useful because there are numerous patients with PPI resistance. A candidate drug with a mechanism different from that of PPI should be developed. More recent interesting report demonstrated that concomitant use of PPI and gastroprotective effects of rebamipide, suggesting that it may be a good choice in aspirin users with gastroduodenal toxicity that is not suppressed by acid suppressants alone.[35]

Total care between cardiovascular events and GI complications are important for patients with atherosclerotic diseases who are taking anti-platelet agents. Moreover, various patients often have different circumstances; therefore it is necessary to prepare various clinical options. Recently, ASA-related small intestinal complication was well discussed. Niwa *et al.*[36] demonstrated that rebamipide has the preventive effect for NSAID-induced small-intestinal injuries. This result showed the possibility for management of the entire gastrointestinal tract including the small intestinal mucosa by concomitant therapy of PPI + rebamipide.[37] In addition, most patients who take ASA are asymptomatic, even if GI ulcer and bleeding occurs, and there is no option for these patients, except endoscopy testing. However, periodic diagnosis is essential. Recently, transnasal upper-gastrointestinal endoscopy has become popular; we too used this modality in this trial, and no subjects withdrew. Transnasal endoscopy has been developed as an alternative to trans-oral endoscopy to reduce medical costs as well as the risks associated with conscious sedation.[38,39] In addition, transnasal upper GI endoscopy is a comfortable and conventional test that imposes less stress due to sedation, such as respiratory depression, hypotension, paradoxical agitation, and anaphylaxis,[40] and most importantly, imposes less load on the cardiovascular pulmonary system.[41] This modality will become the mainstream method for periodical diagnosis. In this study, not only PPI but also rebamipide showed a preventive effect for low-dose ASA-induced GI complications. It was clear that the action of PPI was by acid suppression without TFF. The role of TFF was reported to be site protective action. TFF is part of the gastric mucosal defensive system.

In conclusion, Administration of low-dose ASA decreased TFF levels in serum. TFF may have an association with ASA-induced GI damage. These relations between GI-mucosal damage and the expression of various TFF, and the search for drugs that have an action for TFF, deserve further investigation.

Disclosure

Prof. Goto has received honoraria and consulting fees from Astellas, AstraZeneca, Bayer, Bristol-Myers Squibb, Daiichi-Sankyo, Eisai, GlaxoSmithKline, Kowa, Novartis, Otsuka, sanofi-aventis, Schering-Plough, and Takeda. Prof. Goto also received research grants from Eisai, Ono, sanofi-aventis, Astra Zeneca, Kowa, and Pfizer within the last three years.

Abbreviations

ASA	acetylsalicylic acid
CI	confidence interval
GI	Gastrointestinal
PPI	Proton pump inhibitor
TFF	trefoil factor family

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doi: 10.3164/jcbn.11-10