Prevention of Gastric Ulcer Relapse Induced by Indomethacin in Rats by a Mutein of Basic Fibroblast Growth Factor

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ABSTRACT—We found indomethacin aggravates healed gastric ulcers (ulcer relapse) in rats. In the present study, we examined the effects of human basic fibroblast growth factor (bFGF) mutein CS23 (TGP-580) and histamine H₂-receptor antagonists (H₂-RAs) on ulcer relapse in this model. In male SD rats, gastric ulcers were induced in the antrum by injection of acetic acid. Indomethacin (1 mg/kg/day) given s.c. for 2 weeks starting 4 weeks after the operation aggravated the healed ulcer; the areas with and without indomethacin were 4.8±1.4 and 0.4±0.3 mm², respectively. Drugs were given orally once daily for 4 weeks starting 2 days after the operation or for the 2-week indomethacin administration period. Treatment with ranitidine (100 mg/kg), cimetidine (100 mg/kg) and TGP-580 (0.1 mg/kg) for 4 weeks accelerated the healing. The aggravation by indomethacin was significantly inhibited by pretreatment with TGP-580 and mildly inhibited by cimetidine but not ranitidine. When the drugs were co-administered with indomethacin for 2 weeks, the aggravation was significantly prevented by ranitidine and mildly inhibited by cimetidine and TGP-580. Both TGP-580 and H₂-RAs can prevent the ulcer relapse induced by indomethacin but via different modes of action: TGP-580 inhibits relapse mainly by acting on the process of healing, while H₂-RAs act mainly on the process of aggravation.

Keywords: Gastric ulcer relapse, Basic fibroblast growth factor (bFGF), bFGF mutein CS23, TGP-580, Histamine H₂-receptor antagonist

Thanks to the development of histamine H₂-receptor antagonists (H₂-RAs) and proton pump inhibitors, most peptic ulcers can now be cured rapidly and satisfactorily. However, relapse occurring after the cessation of treatment is still a big problem in ulcer therapy. There are many reports suggesting that Helicobacter pylori plays a role in the relapse of peptic ulcers, and many investigations about ulcer relapse have centered on the eradication of this organism using various antibiotics in the clinical setting (1, 2). The pathogenesis of ulcer relapse might be partially explained by this organism but is still not fully understood due to the lack of reliable animal models. It has been reported that nonsteroidal anti-inflammatory drugs such as indomethacin aggravate gastrointestinal ulcers in patients (3–5). Therefore, we examined the effect of indomethacin on healed gastric ulcers in rats and found that indomethacin obviously aggravates the healed ulcers (relapse). It has also been reported that both human basic fibroblast growth factor (bFGF) and its mutein CS23 (TGP-580) accelerate the healing of gastrointestinal ulcers not only in rats (6–12) but also in humans (13, 14) and that their mode of action is different from those of antisecretory agents (7–9, 11, 12). In the present study, we examined the effects of TGP-580 on the ulcer relapse induced by indomethacin in the rat, and the results were compared with those obtained with H₂-RAs.

MATERIALS AND METHODS

Formation, healing and relapse of gastric ulcers

Seven-week-old male Jcl:Sprague-Dawley rats weighing 200–240 g were used. Gastric ulcers were produced as described by Takagi et al. (15). Rats were anesthetized with ether, and a laparotomy was done via a midline incision. After exposing the stomach, 20 μl of 20% acetic acid solution was injected into the subserosal layer of the anterior wall of the antrum, and the abdomen was closed. To examine the spontaneous healing of gastric ulcers, animals were autopsied every week up to 12 weeks after the operation, and time-dependent changes in the ulcer-
ated area were observed.

To examine its effect on healed gastric ulcers, indomethacin (0.3–3 mg/kg/day) was subcutaneously administered continuously for 2 weeks with a mini-osmotic pump and starting 4, 8 or 12 weeks after ulcer formation. To examine the time dependency of the changes, indomethacin (1 mg/kg/day) administration was started 4 weeks after ulcer formation, and animals were sacrificed 1, 3, 5, 7 or 14 days later. In another study, indomethacin (1 mg/kg/day) was administered subcutaneously to normal rats for 2 weeks mainly to see its effect on gastrointestinal mucosa.

Measurement of gastric and intestinal ulcers

Each animal was sacrificed by CO₂ asphyxiation, and the stomach was removed, filled with 10 ml of a 1% formalin solution, immersed in the same formalin solution for 10 min and opened along the greater curvature. The stomach was then spread flat on a piece of paper. The small intestine was removed and opened along the longitudinal axis, and the contents were removed. The ulcerated area (mm²) was measured under a dissecting microscope with a 1-mm square grid eyepiece (×10).

For the histologic study, the stomach was embedded in paraffin after being fixed in 10% buffered formalin, and several consecutive thin sections from almost the center of the ulcer were prepared and stained with Hematoxylin-Eosin or Azan stain. Color micrographs of specimens stained with Azan stain were made, and histomorphometric measurement of the ulcer was carried out with an IBAS-2000 (Carl Zeiss, Frankfurt, Germany) according to the method of Tabayashi (16). The length or thickness of each of the following parameters depicted in Fig. 1 was measured under blind conditions: length of ulcer crater, regenerated mucosa, ruptured muscularis mucosa and ruptured muscularis propria and thickness of the ulcer bed and ulcer margin.

Measurement of myeloperoxidase (MPO) activity

Each animal was sacrificed by CO₂ asphyxiation, and its stomach was removed, filled with 10 ml of ice-cold saline and immersed in ice-cold saline for 10 min. The stomach was opened along the greater curvature, washed with saline and spread flat on a piece of paper. After excess moisture was absorbed with paper, samples of the ulcerated area of the gastric wall (80 mm²) were taken using a punch (diameter: 10 mm) and weighed. According to the methods described by Krawisz (17), MPO activity in the tissue was measured. The tissue was minced in a test tube containing 1 ml of 0.5% hexadecyltrimethylammonium bromide (Tokyo-Kasei, Tokyo) dissolved in 50 mM phosphate buffer (pH 6.0). The tissue was then homogenized with a Polytron® (Kinematica, Lucerne, Switzerland) on ice. The homogenate was sonicated for 10 sec and then frozen and thawed three times. The homogenate was then centrifuged for 2 min at 12,500 × g, and the supernatant was stored at −80 °C until it was assayed.

MPO activity was measured as follows: The supernatant was dissolved and a 10 µl of sample was combined with 290 µl of 50 mM phosphate buffer containing 0.167 mg/ml O-dianisidine hydrochloride (Tokyo-Kasei) and 24 µl of 0.062% H₂O₂. The change in absorbance at 460 nm was measured with a spectrophotometer (COBAS FRA II®; Roche, Basle, Switzerland) for 200 sec. One unit of MPO activity was defined as that degrading 1 µmol of peroxide per min at 25°C.

Distribution of MPO-positive cells in the ulcerated area

To examine the changes in the distribution of MPO-positive cells in the ulcerated area during indomethacin-induced ulcer relapse, the stomachs were removed just before or 1, 3, 5 or 7 days after starting the administration of indomethacin (1 mg/kg/day, s.c.) 4 weeks after ulcer formation. Ten rats were used in each group. Several consecutive thin sections from almost the center of the healed ulcer were prepared as described previously, and MPO was stained immunohistochemically by the ABC method using polyclonal rabbit anti-human myeloperoxidase antiserum (DAKO Japan, Kyoto).

Measurement of gastric acid secretion

In a study to examine the effect of TGP-580 and H₂-RAs on gastric acid secretion, each drug or vehicle was administered orally after a 24-hr fast. One hour later, the pylorus was ligated under light ether anesthesia, and the abdomen was closed by suturing. In a study to examine the effect of indomethacin on gastric acid secretion, indomethacin (0.3–3 mg/kg/day) was continuously administered s.c. beginning 24 hr before the pylorus was ligated.
Each animal was sacrificed by CO₂ asphyxiation 3 hr after the pylorus was ligated, and the stomach was removed. The gastric contents were collected and centrifuged at 1,500 x g for 15 min. The volume of supernatant of each sample was measured, and the acid concentration was determined by automatic titration (TTA81; Radiometer, Copenhagen, Denmark) to pH 7.0 with 0.1 N NaOH. Total acid output during the 3-hr period was then calculated.

Drugs

The following drugs were used: Indomethacin, cimetidine, ranitidine (Sigma, St. Louis, MO, USA). TGP-580 (recombinant human basic fibroblast growth factor (FGF-2) mutein CS23) was produced using gene technology (18) and purified in our division. Indomethacin was dissolved in 2% NaHCO₃ and continuously injected subcutaneously using a mini-osmotic pump (Type 2002 or 2ML2, Alzet®; Alza Co., Palo Alto, CA, USA). TGP-580 and other drugs were dissolved or suspended in 0.5% methylcellulose containing 1% NaHCO₃ (pH 8.55) and administered orally in a volume of 1 ml/100 g body weight. In the control group, the same volume of vehicle was administered. In the study to examine the effect of drugs on healing and relapse of gastric ulcers, each drug or vehicle was given orally once a day for 4 weeks starting 2 days after ulcer formation or for the 2-week indomethacin treatment period. The animals were autopsied 4 or 6 weeks after ulcer formation, and the area of gastric ulceration was measured.

Statistical analyses

Data are expressed as mean values and the standard errors. The statistical significance of the differences among the groups was determined by Dunnett’s test or by Student’s t-test.

Approval by animal welfare committee

This study was approved by the animal welfare committee of Takeda Chemical Ind., Ltd.

RESULTS

Healing and relapse of gastric ulcers

Time-dependent changes in gastric ulcers are shown in Fig. 2. The area of the gastric ulcer was 33.8±2.4 mm² (n=10) 1 week after ulcer formation. The area decreased rapidly after the first week and later decreased more gradually. The area was minimum both 4 and 6 weeks after ulcer formation, and thereafter, the area seemed to increase slightly up to 12 weeks after formation.

Indomethacin (0.3–3 mg/kg/day, s.c.) given for 2 weeks starting 4 weeks after ulcer formation increased the area of the ulcer in a dose-dependent manner (Fig. 3). The area of the ulcer 4 weeks after formation was 1.9±0.4 mm² (n=10). The area in animals given 3 mg/kg/day of indomethacin for 2 weeks was 5.2±1.1 mm² (n=10), and it was significantly (P<0.05) larger than that before indomethacin administration (at 4

Fig. 2. Time-dependent changes in the area of the gastric ulcers induced by acetic acid in rats. Twenty microliters of 20% acetic acid was injected into the subserosal layer of the gastric antrum, and the gastric ulcers were examined at the indicated times. Data represent the mean values and the S.E. for the 10 rats in each group.

Fig. 3. Effect of indomethacin on healed gastric ulcers in rats. Indomethacin or vehicle was given s.c. continuously for 2 weeks starting 4 weeks after ulcer formation. Aggravation (relapse) of gastric ulcers was observed with higher doses of indomethacin. Data represent the mean values and the S.E. for the 8 to 10 rats in each group. #: P<0.05 vs pre (just before treatment with indomethacin), **: P<0.01 vs vehicle. W: weeks.
weeks) and that in the vehicle group (Fig. 3). Indomethacin, however, at a dose of 3 mg/kg/day did not induce any new lesions in the normal areas of the gastric mucosa.

In the other experiment, indomethacin (3 mg/kg/day) was given for 2 weeks starting 4, 8 and 12 weeks after ulcer formation. As seen in Fig. 4, indomethacin administered at 4 or 8 weeks obviously aggravated the ulcer, but did not when administered at 12 weeks.

**MPO activity in ulcerated tissue**

The MPO activity in the gastric antrum in normal rats was $0.02 \pm 0.01$ U/80 mm$^2$ ($0.24 \pm 0.06$ U/g tissue, n=6). The area of and the MPO activity in the ulcerated tissue 1, 3 and 4 weeks after ulcer formation and after 1, 3, 7 and 14 days of indomethacin treatment (1 mg/kg/day) were examined (Fig. 5). The MPO activity was markedly increased 1 week after ulcer formation ($3.66 \pm 0.19$ U/80 mm$^2$, $17.96 \pm 3.24$ U/g tissue, n=12). The activity then decreased with ulcer healing, and it was $0.78 \pm 0.32$ U/80 mm$^2$ (n=10) 4 weeks after ulcer formation. The administration of indomethacin (1 mg/kg/day, s.c.) slightly increased both the area and MPO activity after one day, and significant increases in both parameters were seen after 3 days: $7.7 \pm 1.4$ mm$^2$ and $2.52 \pm 0.36$ U/80 mm$^2$ (n=12), respectively. This continued through day 14. The values in the group given vehicle instead of indomethacin for 14 days were $1.1 \pm 0.6$ mm$^2$ and $0.66 \pm 0.17$ U/80 mm$^2$ (n=10), respectively, and these values do not differ from those before indomethacin administration. A close relationship between the area and MPO activity in the ulcerated mucosa was observed (Fig. 6).

When 1 mg/kg/day of indomethacin was given subcutaneously in normal rats, it slightly increased the MPO activity in the gastric wall ($0.04 \pm 0.01$ on day 3, $0.04 \pm 0.01$ on day 7 and $0.11 \pm 0.01$ U/80 mm$^2$ on day 14, n=10) compared with that of normal rats ($0.02 \pm 0.01$ U/80 mm$^2$, n=6), but no visible damage was observed in the gastric mucosa.

**Changes in the distribution of MPO-positive cells during ulcer relapse**

The gastric ulcers were healed 4 weeks after ulcer for-
formation and the ulcerated area was almost entirely covered with regenerated mucosa in all 10 rats. Before indomethacin administration, MPO-positive cells were observed mainly in the lower part of the lamina propria near the muscularis mucosa in the regenerated mucosa (Fig. 7A), and most of them were eosinophils. Twenty-four hours after starting the administration of indomethacin, there was no obvious changes in the regenerated mucosa or the distribution of MPO-positive cells, but degranulation was observed in many eosinophils (Fig. 7B), and pyknosis was observed in some of the glandular epithelial cells near the degranulated eosinophils. Three days after starting the administration of indomethacin, regenerated mucosa was lost in 3 out of the 10 rats, and marked infiltration of MPO-positive cells (most of them eosinophils and neutrophils) was observed in the regenerated mucosa and granulation tissue. Eosinophils were frequently observed at the ulcer edge. In the non-ulcerated mucosa, there was no obvious increase in MPO-positive cells. These changes increased with time up to 7 days after starting the administration of indomethacin (Fig. 7C).

**Effects of drugs on ulcer healing**

Each drug or vehicle was given orally once a day for 4 weeks after ulcer formation, and the effects of drugs on ulcer healing were examined both macroscopically and histologically.

**Macroscopic observation:** The area of the ulcer in animals given vehicle for 4 weeks was 3.6±1.4 mm² (n=8). The administration of TGP-580 (0.1 mg/kg), cimetidine (100 mg/kg) and ranitidine (100 mg/kg) accelerated ulcer healing, but there were no significant differences among the groups (Table 1). The effect of ranitidine, however, appeared to be the best.

**Histological observation:** In the group given vehicle for 4 weeks, the ulcer base was covered with regenerated mucosa, and most of the collagen-like fibers in the granulation tissue were distributed parallel to the muscle layers.

### Table 1. Macroscopic and histological measurements of the effect of drugs on the healing of acetic acid-induced gastric ulcers in rats

|                     | Vehicle (1% NaHCO₃) | TGP-580 (0.1 mg/kg) | Cimetidine (100 mg/kg) | Ranitidine (100 mg/kg) |
|---------------------|---------------------|--------------------|------------------------|------------------------|
| **Macroscopy**      |                     |                    |                        |                        |
| Area of ulcer       | 3.6±1.4             | 1.8±0.7            | 2.4±0.7                | 1.0±0.4                |
| **Histology**       |                     |                    |                        |                        |
| Length of UC        | 2.02±0.44           | 1.34±0.29          | 1.64±0.22              | 1.03±0.26              |
| Length of RM        | 3.06±0.42           | 3.08±0.18          | 3.26±0.13              | 3.12±0.12              |
| Length of RMM       | 6.13±0.70           | 5.81±0.62          | 5.85±0.45              | 6.65±0.68              |
| Length of RMP       | 6.23±0.48           | 5.27±0.55          | 6.36±0.49              | 5.64±0.34              |
| Thickness of UB     | 2.34±0.27           | 1.95±0.20          | 1.97±0.19              | 1.99±0.27              |
| Thickness of UM     | 2.60±0.28           | 2.39±0.24          | 2.28±0.22              | 2.35±0.28              |

Each drug or vehicle was administered orally once daily for 4 weeks starting 2 days after ulcer formation, and the animals were autopsied 24 hr after the final dose. The area (mm²) of the ulcer and the length (mm) and the thickness (mm) of each parameter were measured. UC: ulcer crater, RM: regenerated mucosa, RMM: ruptured muscularis mucosa, RMP: ruptured muscularis propria, UB: ulcer bed, UM: ulcer margin, Data represent the mean±S.E. for 7 to 9 rats.
Fig. 7. Distribution of MPO-positive cells in the gastric wall during ulcer relapse in rats. Indomethacin (1 mg/kg/day) was given s.c. continuously starting 4 weeks after ulcer formation. A (Healed ulcer 4 weeks after formation; before indomethacin treatment, MPO was stained immunohistochemically using anti-MPO antiserum, ABC method, ×170): MPO-positive cells (shown by arrows) are observed in the lower part of the lamina propria of the regenerated mucosa, and most of them are eosinophils. B (Regenerated mucosa 24 hr after starting the administration of indomethacin, H.E. stain, ×340): many degranulated eosinophils (shown by arrows) and several pyknotic glandular cells (shown by arrow heads) near the degranulated eosinophils are observed. C (Relapsed ulcer after the administration of indomethacin for 5 days, MPO was stained by the ABC method, ×170): many MPO-positive cells (eosinophils and neutrophils) are observed in the granulation tissue. D (Non-ulcerated area from the same preparation as in Fig. C): Several MPO-positive cells are observed in the mucosa.
Regeneration of the muscle layer and muscularis mucosa were observed. There was slight infiltration of lymphocytes and eosinophils in the serosal layer. Results of histological measurements are shown in Table 1. Compared with the vehicle group, in the group given TGP-580, the length of regenerated mucosa was not changed, but the length of the ulcer crater and ruptured muscularis mucosa was increased. In the group given cimetidine, the lengths of the ulcer crater and ruptured muscularis mucosa were slightly decreased, but the length of the ruptured muscularis propria was rather increased. Ranitidine obviously decreased the length of the ulcer crater and slightly decreased the length of the ruptured muscularis propria but rather increased the length of the ruptured muscularis mucosa.

Effect of pretreatment with drugs on ulcer relapse

Each drug or vehicle was given once a day for 4 weeks after ulcer formation. Indomethacin (1 mg/kg/day) was then administered continuously for 2 weeks to examine the effect of pretreatment with drugs on ulcer relapse.

Macroscopic observation: Experiment 1: The effect of TGP-580 on ulcer relapse is shown in Table 2. The area of the ulcer in the group given vehicle for 4 weeks was 2.4 ± 1.0 mm² (n = 9), and the administration of indomethacin for 2 weeks obviously increased the area (5.6 ± 1.6 mm², n = 10). The increase in ulcerated area was prevented by pretreatment with TGP-580. The areas in the groups given TGP-580 (0.01 and 0.1 mg/kg/day) were 3.8 ± 1.3 mm² (n = 10) and 0.4 ± 0.4 mm² (n = 9), respectively. The area in the group given 0.1 mg/kg was significantly (P < 0.01) smaller than that in the vehicle group both before and after treatment with indomethacin.
Table 2. Macroscopic and histological measurements of the effect of TGP-580 on the indomethacin-induced relapse of gastric ulcers in rats

| Macroscopy                          | Vehicle (4 w) | Vehicle (4 w) + IM (2 w) | TGP-580 (4 w) + IM (2 w) |
|-------------------------------------|---------------|--------------------------|--------------------------|
| Area of ulcer                       | 2.4 ± 1.0     | 5.6 ± 1.6                | 0.4 ± 0.4d               |
| Histology                           |               |                          |                          |
| Length of UC                        | 0.89 ± 0.25   | 2.37 ± 0.50*             | 0.37 ± 0.26*             |
| Length of RM                        | 3.23 ± 0.33   | 3.31 ± 0.29              | 3.95 ± 0.46              |
| length of RMM                       | 5.28 ± 0.55   | 6.52 ± 0.52              | 5.02 ± 0.44              |
| Length of RMP                       | 4.46 ± 0.70   | 6.10 ± 2.39              | 2.19 ± 0.61*             |
| Thickness of UB                     | 1.31 ± 0.27   | 2.17 ± 0.35              | 1.09 ± 0.22*             |
| Thickness of UM                     | 1.78 ± 0.15   | 2.65 ± 0.36*             | 1.72 ± 0.20              |

TGP-580 (0.1 mg/kg) or vehicle was administered orally once daily for 4 weeks starting 2 days after ulcer formation. Indomethacin (1 mg/kg/day, s.c.) was then administered for 2 weeks. The area (mm²) of the ulcer and the length (mm) and the thickness (mm) of each parameter were measured. Abbreviations are the same as in Table 1. IM: indomethacin. Data represent the mean ± S.E. for 9 or 10 rats. *: P<0.05 vs vehicle (4 w), #: P<0.05, b#: P<0.01 vs vehicle + IM.

Fig. 9. Effects of TGP-580, cimetidine (Cim) and ranitidine (Ran) on the indomethacin-induced relapse of gastric ulcers in rats. Each drug or vehicle was administered orally once daily for 4 weeks starting 2 days after ulcer formation. Indomethacin was then administered for 2 weeks. Data represent the mean values and the S.E. for the 8 to 10 rats in each group. #: P<0.05 vs vehicle + vehicle, *: P<0.05 vs vehicle + indomethacin.

Experiment 2: The effect of TGP-580 (0.1 mg/kg) was compared with that of cimetidine (100 mg/kg) and ranitidine (100 mg/kg). The ulcerated area in the group given indomethacin for 2 weeks after 4-week pretreatment with vehicle was 4.8 ± 1.4 mm² (n=9), which was significantly larger than that in animals given the vehicle instead of indomethacin (0.4 ± 0.3 mm², n=8). The aggravation of ulcers by indomethacin was significantly inhibited by pretreatment with TGP-580, mildly inhibited by cimetidine and not inhibited by ranitidine (Fig. 9, Table 3).

Histological observation: As seen in Fig. 8B, administration of indomethacin for 2 weeks caused loss of regenerated mucosa and production of thick granulation tissue in the ulcer bed with many inflammatory cells such as lymphocytes and eosinophils, and many collagen-like fibers were distributed randomly in the granulation tissue. In the group treated with TGP-580, the ulcer bed was covered with regenerated mucosa in spite of the admin-

Table 3. Macroscopic and histological measurements of the effect of drugs on the indomethacin-induced relapse of gastric ulcers in rats

| Macroscopy                          | Vehicle (1% NaHCO₃) | TGP-580 (0.1 mg/kg) | Cimetidine (100 mg/kg) | Ranitidine (100 mg/kg) |
|-------------------------------------|---------------------|---------------------|------------------------|------------------------|
| Area of ulcer                       | 4.8 ± 1.4           | 0.8 ± 0.4*          | 2.0 ± 1.0              | 5.0 ± 0.9              |
| Histology                           |                     |                     |                        |                        |
| Length of UC                        | 2.57 ± 0.39         | 0.93 ± 0.30**       | 1.42 ± 0.46            | 2.63 ± 0.25            |
| Length of RM                        | 3.43 ± 0.26         | 4.28 ± 0.30         | 3.33 ± 0.28            | 3.13 ± 0.36            |
| length of RMM                       | 7.50 ± 0.46         | 6.76 ± 0.30         | 6.48 ± 1.00            | 7.12 ± 0.29            |
| Length of RMP                       | 6.54 ± 0.43         | 5.70 ± 0.55         | 7.18 ± 1.11            | 6.28 ± 0.41            |
| Thickness of UB                     | 1.78 ± 0.30         | 1.01 ± 0.18         | 1.11 ± 0.19            | 1.75 ± 0.19            |
| Thickness of UM                     | 1.94 ± 0.29         | 1.35 ± 0.15         | 1.63 ± 0.92            | 2.01 ± 0.23            |

Each drug or vehicle was administered orally once daily for 4 weeks starting 2 days after ulcer formation. Indomethacin (1 mg/kg/day, s.c.) was then administered for 2 weeks. The area (mm²) of the ulcer and the length (mm) and the thickness (mm) of each parameter were measured. Abbreviations are the same as in Table 1. Data represent the mean ± S.E. for 8 to 10 rats. *: P<0.05, **: P<0.01 vs vehicle.
istration of indomethacin, and the ulcer was not aggravated, but healing was rather accelerated (Fig. 8C). Prominent progress in repair of ruptured muscle layers was observed. In the animals pretreated with cimetidine, partial loss of regenerated mucosa and thick granulation tissue with inflammatory cells were observed (Fig. 8D). These changes were more obvious in the group pretreated with ranitidine.

Results of the histological measurements in Experiment 1 are shown in Table 2. The length of the ulcer crater in the group given vehicle (before indomethacin) was 0.89 ± 0.25 mm (n = 9), and the administration of indomethacin significantly (P < 0.05) increased the length to 2.37 ± 0.50 mm (n = 10). Although the length of the regenerated mucosa did not differ between the two groups, indomethacin increased the length of the ruptured muscularis mucosa and ruptured muscularis propria and the thickness of the ulcer bed and ulcer margin. These results suggest that indomethacin caused not only inhibition of ulcer healing but also aggravation of the ulcer. Pretreatment with TGP-580 obviously prevented the changes in each parameter caused by indomethacin. In addition, the lengths of the ulcer crater and ruptured muscularis propria were shorter than those before indomethacin.

Results of the histological measurements in Experiment 2 are shown in Table 3. Administration of indomethacin aggravated the ulcer as seen in Experiment 1. The length of the ulcer crater in animals given indomethacin was 2.57 ± 0.39 mm (n = 8), which was significantly longer than that in the vehicle group (0.81 ± 0.32 mm, n = 8). Pretreatment with TGP-580 (0.1 mg/kg) prevented the aggravation of the ulcer by indomethacin as seen in Experiment 1. The aggravation of the ulcer by indomethacin was mildly prevented by cimetidine (100 mg/kg) and not prevented by ranitidine (100 mg/kg). In the group treated with cimetidine, the length of the ulcer crater seemed to be decreased, but the length of the ruptured muscularis propria was rather increased.

Table 4. Effects of drugs on gastric acid secretion in pylorus-ligated rats

| Treatment | Dose (mg/kg, p.o.) | No. of rats | Volume (ml/3 hr) | Acidity (µEq/ml) | Acid output (µEq/3 hr) |
|-----------|-------------------|-------------|------------------|-----------------|------------------------|
| Vehicle   |                   | 7           | 2.7 ± 0.3        | 77.3 ± 7.9      | 214.6 ± 44.7           |
| TGP-580   | 0.001             | 7           | 3.6 ± 0.4        | 76.3 ± 5.7      | 275.6 ± 41.3           |
| TGP-580   | 0.01              | 7           | 3.4 ± 0.6        | 83.7 ± 5.3      | 299.4 ± 62.1           |
| TGP-580   | 0.1               | 7           | 3.4 ± 0.4        | 79.0 ± 5.4      | 271.7 ± 34.1           |
| Cimetidine| 10                | 7           | 2.3 ± 0.2        | 61.9 ± 6.3      | 145.1 ± 27.7           |
| Cimetidine| 30                | 7           | 2.1 ± 0.2        | 40.9 ± 6.1**    | 90.9 ± 19.4            |
| Cimetidine| 100               | 7           | 1.9 ± 0.3        | 26.9 ± 7.5**    | 52.6 ± 20.5**          |
| Ranitidine| 10                | 7           | 1.9 ± 0.4        | 39.0 ± 3.8**    | 81.0 ± 23.7*           |
| Ranitidine| 30                | 7           | 1.9 ± 0.3        | 26.2 ± 6.1**    | 48.9 ± 14.3**          |
| Ranitidine| 100               | 7           | 1.8 ± 0.3        | 12.5 ± 3.7**    | 24.7 ± 9.8**           |

Each drug or vehicle was given orally after a 24-hr fast, and 1 hr later, the pylorus was ligated under ether anesthesia. Gastric juice was collected 3 hr after pylorus ligation. Data represent the mean ± S.E. *: P < 0.05, **: P < 0.01 vs vehicle.
Effect of co-administration of drugs on ulcer relapse

The inhibitory effect of each drug on ulcer relapse was then examined by co-administration of drug and indomethacin for 2 weeks. The aggravation of the ulcer was significantly (P<0.05) prevented by ranitidine (100 mg/kg) and mildly prevented by TGP-580 (0.1 mg/kg) and cimetidine (100 mg/kg) (Fig. 10).

Effects of TGP-580 and H2-RAs on gastric acid secretion

Gastric acid secretion in the control group given vehicle was 214.6±44.7 μEq/3 hr (n=7). Both cimetidine (10-100 mg/kg) and ranitidine (10-100 mg/kg) inhibited the acid secretion in a dose-dependent manner, and the ID50 values were 23.6 and 3.1 mg/kg, respectively (Table 4). TGP-580 (0.001 -0.1 mg/kg) did not inhibit the acid secretion but rather increased it slightly (Table 4).

Effect of indomethacin on gastric acid secretion

Continuous administration of indomethacin (0.3–3 mg/kg/day, s.c.) increased the volume of gastric juice in a dose-dependent manner, and the effect at 3 mg/kg was significant (Table 5). The acidity of the gastric juice was not affected by treatment with indomethacin. Ranitidine (100 mg/kg) given orally 1 hr before the pylorus ligation markedly decreased both the volume and acidity of the gastric juice in rats given indomethacin (1 mg/kg/day) (Table 5).

DISCUSSION

Although relapse or recurrence of ulcers is a big problem in ulcer therapy, the pathogenesis of ulcer relapse is not well understood. This may be due to the lack of reliable animal models for ulcer relapse. It has been reported by Okabe and Pfeiffer (19) that gastric ulcers induced by acetic acid in rats often relapse spontaneously, and this was confirmed by Fukawa et al. (20) by endoscopic observation of these same animals. However, according to the reports, it takes 50–200 days for ulcer relapse after ulcer formation, and the time of relapse and severity differ from animal to animal. It is well known that non-steroidal anti-inflammatory drugs such as indomethacin can cause gastrointestinal disorders as a side effect and sometimes cause aggravation of healed ulcers (3–5). In the present study, we examined the effect of indomethacin on healed gastric ulcers in the rat and found that continuous injection of indomethacin subcutaneously after almost complete ulcer healing (4 weeks after formation) caused 1) an increase in the area of the ulcer upon macroscopic observation, 2) loss of regenerated mucosa, infiltration of many inflammatory cells and production of a large amount of granulation tissue with many collagen-like fibers upon histological observation and 3) an increase in MPO activity and MPO-positive cells such as eosinophils and neutrophils in the ulcerated area. These results indicate that the ulcers are aggravated (relapse) by injection of indomethacin. The gastric ulcer relapse in the present model is easily caused within a short time by injecting indomethacin subcutaneously with a mini-osmotic pump, and the results are reproducible. This model should be useful not only for pathological study of ulcer relapse but also for evaluating the effect of drugs on ulcer relapse.

The aggravation of ulcers was obviously observed at doses of 1 and 3 mg/kg/day of indomethacin, and a significant increase in the ulcerated area was obtained 3 days after starting the administration of indomethacin (1 mg/kg/day). The relapse of ulcers was observed when in-
domethacin was given to the rats starting 4 and 8 weeks but not 12 weeks after ulcer formation. As there was no difference in the ulcer area measured macroscopically at 8 and 12 weeks, the difference in ulcer relapse in these 2 groups may be due to the difference in the degree of healing in the inner layer (ulcer bed) of the ulcer. In these experiments, indomethacin did not induce any new lesions in the normal mucosa. The effect of indomethacin on normal mucosa was further examined in normal rats. When the gastric mucosa was examined 3, 7 and 14 days after starting the injection of indomethacin (1 mg/kg/day), no visible lesions were observed in the gastric mucosa, although MPO activity in the gastric wall was slightly increased. These results suggest that immature regenerated mucosa is more sensitive to the damaging action of indomethacin than normal mucosa and that an important consideration in ulcer therapy for the prevention of relapse is the degree of healing in the inner layer (ulcer bed).

It is not clear how indomethacin aggravates healed ulcers. It is well known that indomethacin decreases mucosal protective activities such as blood flow, mucus secretion and bicarbonate secretion and increases aggressive factors such as motility (21). In the present study, indomethacin increased secretion of gastric juice, and the aggravation of ulcers as well as the increase of gastric secretion by indomethacin was significantly inhibited by concomitant administration of ranitidine. These results suggest that gastric acid, as well as other factors, plays a role in the ulcer relapse by indomethacin.

It has been reported that there is a positive correlation between MPO activity and neutrophil infiltration in intestinal inflammation models (17). In the present study, the MPO activity in the ulcerated area of the gastric wall increased when an ulcer was formed, decreased with ulcer healing and increased again with aggravation of the ulcer by indomethacin. These results suggest a close relationship between the state of the ulcer and MPO activity. However, it is not clear whether the increase in MPO activity is a result of ulceration of the mucosa or a cause of the ulcer. Wallace et al. (22–24) have reported that indomethacin-induced gastric ulcer formation was prevented by pretreatment with an antibody against neutrophils or antibodies against endothelial adhesion molecules and suggested that the infiltration of neutrophils plays an important role in indomethacin-induced ulcer formation. In the present study, 4 weeks after ulcer formation, MPO-positive cells were observed in the lamina propria in the regenerated mucosa, and most of them were eosinophils. Twenty-four hours after starting the administration of indomethacin, there was no increase in MPO-positive cells, but many eosinophils degranulated as seen in Fig. 7B. These results suggest that indomethacin first stimulates the eosinophils to release bioactive substances such as major basic proteins, leukotrienes, PAF and cytokines, which are related to inflammation (25, 26). Neutrophil infiltration might be a secondary reaction following the release of these substances. To clarify the sequence of events, more detailed studies will be needed. It has been reported that indomethacin delays ulcer healing in a gastric ulcer model (27, 28) via reducing blood flow in the ulcer margin (29) or inhibiting contraction of the ulcer base (30). We also found that continuous administration of indomethacin (0.3–3 mg/kg/day, s.c.) starting 2 days after ulcer formation delayed healing of the ulcers and increased MPO activity in a dose-dependent manner (unpublished observations). Recently, Asaka et al. (31) found that PMN cell infiltration in the ulcer margin was markedly decreased by eradication of H. pylori in gastric and duodenal ulcer patients and have suggested that H. pylori may play an important role in the pathogenesis of gastric and duodenal ulcers by inducing infiltration of inflammatory cells in the mucosa. These facts and the results in the present study suggest that not only neutrophils but also eosinophils play an important role in ulcer relapse as well as ulcer formation and the delay in ulcer healing induced by indomethacin.

It has been reported that the gastric and duodenal ulcers healed by treatment with H2-RAs often relapse after treatment is stopped and that continuous treatment with H2-RAs after ulcer healing can effectively prevent ulcer relapse (32, 33). In the present study, H2-RAs, especially ranitidine, accelerated ulcer healing but could not prevent the ulcer relapse induced by indomethacin after the treatment was stopped. In addition, ranitidine obviously inhibited relapse when it was coadministered with indomethacin. These results indicate the similarities in the profiles of ulcer relapse in the present model and in ulcer patients. We examined the effect of TGP-580 on ulcer relapse using this animal model and found that TGP-580 could prevent ulcer relapse even after treatment was stopped, although the effect on ulcer healing was less potent than that of ranitidine upon macroscopic observation. These results were confirmed by histological observation; i.e., the histological changes in the ulcers caused by the administration of indomethacin such as the increase of ulcer crater were not affected by pretreatment with ranitidine, but were markedly inhibited by TGP-580. In the group treated with TGP-580, most of the ulcer base was covered with regenerated mucosa; both the ruptured muscularis mucosa and the muscularis propria were well repaired, and the ulcer bed was thin. Furthermore, most parameters of ulcer severity, such as the length of the ulcer crater and ruptured muscularis propria, were less than those before indomethacin administration, suggesting that the effect of TGP-580 on ulcer healing continued
even after treatment was stopped.

Upon histological observation after 4 weeks of treatment with each drug, the length of the ulcer crater was the shortest in the group treated with ranitidine, confirming the macroscopic observations. However, the lengths of the ruptured muscularis propria in this group was longer than that in the vehicle group, suggesting a delay in healing in the inner layer of the ulcer. On the other hand, in the group given TGP-580, the length of the ulcer crater was longer than that with ranitidine, but the lengths of the ruptured muscularis mucosa and ruptured muscularis propria were decreased compared with the values in the vehicle group and ranitidine-treated group, and the thicknesses of the ulcer bed and ulcer margin were also decreased. From these results, TGP-580 seems to accelerate the healing of the ulcer in the inner layer more than accelerating the regeneration of mucosa, differing from the action of H2-RAs. These results suggest that healing of the ulcer in the inner layer is more important than the regeneration of mucosa for prevention of ulcer relapse.

To elucidate the difference in the mode of action of TGP-580 and H2-RAs, the effect of TGP-580 on gastric acid secretion was examined in pylorus-ligated rats. Both cimetidine and ranitidine markedly inhibited acid secretion, but TGP-580 even at a high dose of 0.1 mg/kg did not inhibit acid secretion but rather increased it slightly. It has been reported that the angiogenic activity of TGP-580 plays an important role in the healing effect of this peptide on duodenal ulcers in the rat (7, 12). We examined the effect of TGP-580 and cimetidine on angiogenesis in the ulcer bed of gastric ulcers and found that TGP-580 significantly increased the number of microvessels in the ulcer bed, while cimetidine decreased the number (11). Tsuchida et al. (34) also have reported that cimetidine inhibited angiogenesis in the ulcer bed of gastric ulcers in the rat. It has been reported that bFGF stimulates proliferation of many cells including fibroblasts, endothelial cells and smooth muscle cells (35) and also stimulates migration of gastrointestinal epithelial cells (36, 37), which are involved in the healing of damaged tissue. In the present study, proliferation of smooth muscle cells was one of the prominent effects of TGP-580, suggesting that repair of the ruptured muscularis mucosa and muscularis propria is important for preventing ulcer relapse. Recently, Nakamura et al. (38) reported that TGP-580 stimulates the growth of cholinergic neurons in the ulcer bed. These facts suggest that TGP-580 stimulates the proliferation of many cells in the ulcer bed in addition to angiogenesis and that it actively heals ulcers from the inner layer, while H2-RAs may passively heal the ulcers by eliminating one of the aggressive factors, gastric acid. These differences in the mode of action of TGP-580 and H2-RAs may explain why ulcers healed by treatment with TGP-580 are more resistant to the relapse induced by indomethacin.

Both TGP-580 and H2-RAs can prevent the ulcer relapse induced by indomethacin but do so via different modes of action; i.e., TGP-580 inhibits ulcer relapse mainly by acting on the process of ulcer healing (such as stimulation of angiogenesis in the ulcer bed), while H2-RAs act mainly on the process of ulcer aggravation by inhibiting acid secretion. The results of this study further indicate that TGP-580 may provide a better quality of ulcer healing than H2-RAs. Recently, it was reported that TGP-580 was effective in treatment of both peptic ulcers (13) and NSAID-induced ulcers (14) in patients. In the near future it will be revealed whether this new approach to ulcer therapy produces a better quality of ulcer healing as compared with antisecretory agents.

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REFERENCES
1 Marshall BJ, Goodwin CS, Warren JR, Murray R, Blincow ED, Blackbourn SJ, Phillips M, Waters TE and Sanderson CR: Prospective double-blind trial of duodenal ulcer relapse after eradication of Campylobacter pylori. Lancet ii, 1437–1442 (1988)
2 Hentschel E, Brandstatter G, Dragosics B, Hirschl AM, Nemec H, Schutze K, Tauer M and Wrurzer H: Effect of ranitidine and amoxicillin plus metronidazole on the eradication of Helicobacter pylori and the recurrence of duodenal ulcer. N Engl J Med 328, 308–12 (1993)
3 Kaufman HJ and Taubin HL: Nonsteroidal anti-inflammatory drugs activate quiescent inflammatory bowel disease. Ann Intern Med 107, 513–516 (1987)
4 Soli AH, Kurata J and McGuigan JE: Ulcers, nonsteroidal anti-inflammatory drugs, and related matters. Gastroenterology 96, 561–568 (1989)
5 Freston JW: Mechanism of relapse in peptic ulcer disease. J Clin Gastroenterol 11, Supp 1, S34–S38 (1989)
6 Szabo S, Vattay P, Morales RE, Johnson B, Katk K and Folkman J: Orally administered bFGF mutein: Effect on healing of chronic duodenal ulcers in rats. Dig Dis Sci 34, 1323 (1989)
7 Folkman J, Szabo S, Stovroff M, McNeil P, Li W and Shing Y: Duodenal ulcer: Discovery of a new mechanism and development of angiogenic therapy that accelerates healing. Ann Surg 214, 414–427 (1991)
8 Konturek SJ, Brzozowski T, Majka J, Szlachcic A, Biełanski W, Stachura J and Otto W: Fibroblast growth factor in gastro-protection and ulcer healing: interaction with sulcrateil. Gut 34, 881–887 (1993)
9 Sato H, Shino A, Inatomi N, Nagaya H, Sato F, Szabo S and Folkman J: Effect of rhbFGF mutein CS23 (TGP-580) on the healing of gastric ulcers induced by acetic acid in rats Gastro-
enterology 100, A155 (1991)

10 Satoh H, Takami K, Katao K, Folkman J and Szabo S: Effect of bFGF and its muretein on healing of colonic ulcers induced by N-ethylmaleimide in rats. Gastroenterology 98, A203 (1990)

11 Satoh H, Shinoh T, Sato T, Asano S, Murakami I, Inatomi N, Nagaya H, Katao K, Szabo S and Folkman J: Role of endogenous basic fibroblast growth factor in the healing of gastric ulcers in rats. Jpn J Pharmacol 73, 59–71 (1997)

12 Szabo S, Folkman J, Vattay P, Morales RE, Pinkus GS and Katao K: Accelerated healing of duodenal ulcers by oral administration of a muretein of basic fibroblast growth factor in rats. Gastroenterology 106, 1106–1111 (1994)

13 Wolfe MM, Bynum TE, Parsons WG, Malone KM, Szabo S and Folkman J: Safety and efficacy of an angiogenic peptide, basic fibroblast growth factor (bFGF) in the treatment of gastro-duodenal ulcers: A preliminary report. Gastroenterology 106, A212 (1994)

14 Hull MA, Cullen DJE, Hudson N and Hawkey CJ: Basic fibroblast growth factor treatment for non-steroidal anti-inflammatory drug associated gastric ulceration. Gut 37, 610–612 (1995)

15 Takagi K, Okabe S and Saziki R: A new method for the production of chronic gastric ulcer in rats and the effect of several drugs on its healing. Jpn J Pharmacol 19, 418–426 (1969)

16 Tabayashi T: A new clamping-cortisone method for experimental chronic gastric ulcer in rats-Studies on the histological findings. Jpn J Gastroenterol 62, 1533–1549 (1965)

17 Krawisz JE, Sharon P and Stenson WF: Quantitative assay for acute intestinal inflammation based on myeloperoxidase activity. Gastroenterology 87, 1344–1350 (1984)

18 Seno M, Sasada R, Iwane M, Sudo K, Kurokawa T, Ito K and Igarashi K: Stabilizing basic fibroblast growth factor using protein engineering. Biochem Biophys Res Commun 151, 701–708 (1988)

19 Okabe S and Pfeiffer CJ: Chronicity of acetic acid ulcer in the rat stomach. Dig Dis Sci 17, 619–629 (1972)

20 Fukawa K, Kawana O, Misaki N, Uchida M and Irino O: Experimental studies on gastric ulcer (6). Endoscopical evaluation of healing processes of acetic acid ulcer in rats (2): Healing, recurrence and relapse of ulcer. Folia Pharmacol Jpn 83, 69–77 (1984) (Abstr in English)

21 Takeuchi K, Okada M, Niida H and Okabe S: Possible mechanisms involved in gastric hypermotility caused by indomethacin in the rat: Role of glycoprivic response. Dig Dis Sci 35, 984–992 (1990)

22 Wallace JL, Keenan CM and Granger DN: Gastric ulceration induced by nonsteroidal anti-inflammatory drugs is a neutrophil-dependent process. Am J Physiol 259, G462–G467 (1990)

23 Wallace JL, Arfors K-E and McKnight W: A monoclonal anti-body against the CD18 leukocyte adhesion molecule prevents indomethacin-induced gastric damage in the rabbit. Gastroenterology 100, 878–883 (1991)

24 Wallace JL, McKnight W, Miyasaka M, Tamatani T, Paulson J, Anderson DC, Granger DN and Kubes P: Role of endothelial adhesion molecules in NSAID-induced gastric mucosal injury. Am J Physiol 265, G993–G998 (1993)

25 Gleich GJ and Adolphson CR: The eosinophilic leukocyte: Structure and function. Adv Immunol 39, 177–253 (1986)

26 Moqbel R, Levi-Schaffer F and Kay AB: Cytokine generation by eosinophils. J Allergy Clin Immunol 94, 1183–1188 (1994)

27 Tanaka H, Shuto K and Nakamizo N: Exacerbation of acetic acid ulcer induced by nonsteroidal anti-inflammatory drugs in rats. Jpn J Pharmacol 33, 447–454 (1983)

28 Wang JY, Yamasaki S, Takeuchi K and Okabe S: Delayed healing of acetic acid-induced gastric ulcers in rats by indomethacin. Gastroenterology 96, 393–402 (1989)

29 Hirose H, Takeuchi K and Okabe S: Effect of indomethacin on gastric mucosal blood flow around acetic acid-induced gastric ulcers in rats. Gastroenterology 100, 1259–1265 (1991)

30 Oigihara Y and Okabe S: Mechanism by which indomethacin delays gastric ulcer healing in the rat: Inhibited contraction of the ulcer base. Jpn J Pharmacol 61, 123–131 (1993)

31 Asaka M, Kato M, Kudo M, Meguro T, Kimura T, Miyazaki T and Inoue K: The role of Helicobacter pylori in peptic ulcer disease. Gastroenterol Jpn 28, Supp 5, 163–167 (1993)

32 Zell S, Carmichael JM and Reddy AN: Rational approach to long-term use of H2-antagonists. Am J Med 82, 796–802 (1987)

33 Chiverton SG and Hunt RH: Initial therapy and relapse of duodenal ulcer: Possible acid secretory mechanisms. Gastroenterology 96, 632–639 (1989)

34 Tsuchida T, Tsukamoto Y, Segawa K, Goto H and Hase S: Effects of cimetidine and omeprazole on angiogenesis in granulation tissue of acetic acid-induced gastric ulcers in rats. Digestion 47, 8–14 (1990)

35 Gospodarowicz D, Ferrara N, Schweigerer L and Neufeld G: Structural characterization and biological functions of fibroblast growth factor. Endocrine Rev 8, 95–114 (1987)

36 Paimela H, Goddard PJ, Carter K, Khakee R, McNeil PL, Ito S and Silen W: Restitution of frog gastric mucosa in vitro: Effect of basic fibroblast growth factor. Gastroenterology 104, 1337–1345 (1993)

37 Diagnass AU, Tsunekawa S and Podolsky DK: Fibroblast growth factors modulate intestinal epithelial cell growth and migration. Gastroenterology 106, 1254–1262 (1994)

38 Nakamura M, Oda M, Inoue J, Ito T, Akiba Y, Kitajima M and Tsuchiya M: Reinervation of microvessels from acetic acid-treated gastric ulcer by basic fibroblast growth factor. Gastroenterology 104, A155 (1993)