# Role of Endogenous Basic Fibroblast Growth Factor in the Healing of Gastric Ulcers in Rats

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ABSTRACT—Recently, it has been pointed out that growth factors play an important role in the healing of gastrointestinal ulcers. In the present study, we examined the role of endogenous basic fibroblast growth factor (bFGF) in the healing of gastric ulcers in the rat. In male SD rats, gastric ulcers were induced in the antrum by injection of acetic acid. Time-dependent changes in the area and bFGF content in the ulcerated area and distribution of bFGF in the ulcerated mucosa were examined. Effects of bFGF mutein CS23 (TGP-580) and a monoclonal antibody for bFGF (MAb 3H3) on the healing of the gastric ulcers and angiogenesis in the ulcer bed were also examined. The content of bFGF in the ulcerated area increased with time as the ulcer healed and reached a maximum 7 days after ulcer formation. In the gastric ulcer bed, many cells such as fibroblasts and macrophages were positively stained immunohistochemically by anti-bFGF antiserum. MAb 3H3 (0.1 mg/rat/day, i.v.) inhibited angiogenesis in the ulcer bed and significantly delayed ulcer healing, while TGP-580 (0.001-0.1 mg/kg × 2/day, p.o.) increased the number of microvessels in the ulcer bed and accelerated the healing. These results suggest that endogenous bFGF may play an important role in the healing of gastric ulcers in the rat and that the angiogenic properties of bFGF (TGP-580) may be involved in its effect on ulcer healing.

*Keywords*: Basic fibroblast growth factor (bFGF), bFGF mutein CS23 (TGP-580), Angiogenesis, Gastric ulcer

Most of the studies on the healing of peptic ulcers in the past have focused on gastric acid secretion and mucosal protection. However, recently, from the viewpoint of wound healing they have focused on the role of growth factors such as epidermal growth factor (1-3), transforming growth factors (4, 5), fibroblast growth factors (6-11) and platelet derived growth factor (12) in the process of ulcer healing. Among them, basic fibroblast growth factor (bFGF) is the most active angiogenic peptide (13), and it has been found to play an important role in wound healing (14-16). We have reported that bFGF is present in the stomach and duodenum both in rats and humans, and the content of bFGF was found to increase in the ulcer bed of experimentally induced duodenal ulcers in rats (8). In addition, we found that oral administration of bFGF or its mutein CS23 (TGP-580) increased the number of microvessels in the ulcer bed and accelerated the healing of duodenal ulcers in rats (7-9). Konturek et al. (10) obtained similar results using bFGF in a rat model of gastric ulcers. These results strongly suggest that bFGF plays an important role in the healing of gastroduodenal ulcers. However, the role of endogenous bFGF in the healing of ulcers is not yet fully understood.

The purpose of the present study was to elucidate the role of endogenous bFGF in the healing of ulcers; and for this, we examined 1) the distribution of bFGF both in intact and ulcerated gastric wall, 2) time-dependent changes in the content of bFGF in ulcerated gastric wall, and 3) effects of a monoclonal anti-bFGF antibody (MAb 3H3) and TGP-580 on angiogenesis in the ulcer bed and healing of gastric ulcers in rats.

## MATERIALS AND METHODS

### *Formation of gastric ulcers*

Seven-week-old male Jcl:Sprague-Dawley rats weigh-

ing 200–240 g were used. Gastric ulcers were produced as described by Takagi et al. (17). Rats were anesthetized with ether, and 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 by suturing. The animals were sacrificed by $CO_2$ asphyxiation according to the schedule after the operation; and the stomach was removed, filled with 10 ml of a 1% formalin solution, immersed in the same formalin solution for 10 min, and then opened along the greater curvature. The stomach was then spread flat on a piece of paper. The ulcerated area ($mm^2$) and severity of the ulcer (Grade 0: almost normal, 1: erosion, 2: moderate ulcer with inflammatory products in the bottom of the ulcer, 3: deep ulcer or perforation) were measured under a dissecting microscope with a 1-mm square grid eyepiece (×10). We considered ulcers healed when the severity was superficial erosion or scar. An ulcer index was obtained as the product of the area and the severity grade. Those ulcers that adhered to the liver markedly or had many hairs in the bottom were eliminated from the calculations.

*Measurement of bFGF content in the gastric wall*

The animals were sacrificed by $CO_2$ asphyxiation, and the stomach was removed, filled with 10 ml of ice-cold saline and then immersed in ice-cold saline for 10 min. The stomach was opened along the greater curvature, washed with ice-cold 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 $mm^2$) were taken using a punch (diameter: 10 mm) and weighed. A sample of the posterior area of the antral wall was taken as a reference control. The tissue was minced in a test tube containing modified 50 mM Tris-HCl buffer (pH 7.6) in a volume 19 times the wet weight of the tissue. The Tris-HCl buffer contained 1.6 M NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA; Sigma, St. Louis, MO, USA), 1 mM phenylmethylsulfonyl fluoride (PMSF; Wako Pure Chemical, Osaka), 1 mM (*p*-amidinophenyl)-methanesulfonyl fluoride HCl (*p*-APMSF, Wako Pure Chemical) and 0.05 mM *N*-ethylmaleimide (NEM, Sigma). The tissue was then homogenized with a Polytron® (Kinematica, Lucerne, Switzerland) on ice. The homogenate was centrifuged for 2 min at 12,500 × *g*, and the supernatant was stored at −80℃ until it was assayed.

bFGF content in the supernatant was measured by a sandwich enzyme immunoassay (EIA) according to the method by Watanabe et al. (18) using 3 monoclonal antibodies (MAb 52, MAb 98 and MAb 3H3) for human bFGF. Rat bFGF (19) was used as the standard. This method allows a bFGF concentration of more than 100 pg/ml to be measured.

*Effects of monoclonal antibodies on ulcer healing*

Each monoclonal antibody (0.1 mg protein/rat) or vehicle (saline) was administered intravenously via a tail vein once daily for 14 days starting the day of ulcer formation. The animals were sacrificed 24 hr after the final dose, and the ulcer was observed macroscopically and histologically. Two types of MAbs were used in this experiment, i.e., MAb 3H3 which neutralizes bFGF activity (50 ng of MAb 3H3 antagonizes the proliferating activity of 1 ng of bFGF on endothelial cells of human umbilical vein origin) (20) and MAb 78 which binds to bFGF without affecting the activity (21).

*Effects of drugs on ulcer healing*

Each drug or vehicle was administered orally once daily for 14 days starting 2 days after ulcer formation. The animals were sacrificed 24 hr after the final dose, and the ulcer was observed macroscopically as described in the previous section. For the histologic study, the ulcerated area was removed and embedded in paraffin after being fixed with 10% buffered formalin. Several consecutive thin sections from almost the center of the ulcer were prepared and then stained with Hematoxylin-Eosin and Azan stains.

*Distribution of endogenous bFGF in the stomach*

The ulcerated gastric wall 7 days after the operation was taken and frozen with dry ice-acetone after being embedded in OTC Compound® (LAB-TEK Products, Elkhart, IN, USA). Several consecutive thin sections of 12 μm from almost the center of the ulcer were prepared using a cryostat microtome and mounted on gelatin-coated glass slides. The sections were fixed in ice-cold acetone for 10 min and treated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature to inactivate endogenous peroxidase. They were then immersed in 10% non-immune goat serum for 30 min, washed in 0.01 M phosphate buffer saline (pH 7.2) and stained with polyclonal rabbit anti-human bFGF antiserum for 2 hr at room temperature. Primary antibody preabsorbed with excess human bFGF (50 μg/ml) for 3 hr at room temperature or phosphate-buffered saline were substituted for the primary antibody to carry out a control staining. After washing in phosphate-buffered saline, bound antibody was detected by a Vectastain ABC Kit (Vector Labs., Burlingame, CA, USA). The sections were incubated for 30 min at room temperature in biotinylated goat anti-rabbit IgG and then in ABC reagent (avidin-biotin complex coupled to peroxidase) for 60 min at room temperature. To visualize the antigen-antibody complexes, the sections were then incubated for 6 min at room tem-

perature in 3-amino-9-ethyl-carbazole (Biomeda Corp., Foster, CA, USA). The antiserum used in this experiment has crossreactivity with rat bFGF but not with bovine acidic FGF, mouse FGF, mouse EGF (epidermal growth factor), mouse NGF (nerve growth factor), porcine PDGF (platelet-derived growth factor), bovine insulin, human TGF (transforming growth factor)-$\beta$ or human IL (interleukin)-2 (unpublished observation; M. Yamaoka et al., Takeda Chemical Ind., Ltd.). In addition, when the serum reacted with a sufficient amount of human bFGF, the section was not stained immunohistochemically.

In another study, the distribution of endogenous bFGF in the corpus and antral wall of the stomach was examined in normal rats.

*Histological measurement of angiogenesis in the ulcer bed*

The ulcerated gastric wall 16 days after the operation was taken and fixed in ice cold-acetone. After dehydration using a graded alcohol series, tissues were embedded in paraffin (Tissue Prep®; Fisher Labs., Inc., Fair Lawn, NJ, USA). Several consecutive thin sections of 4 $\mu$m from almost the center of the ulcer were prepared. The section showing the maximal length of the ulcer crater was chosen, and the endothelial cells of the microvessels were stained immunohistochemically using rabbit polyclonal antibodies for human factor VIII (Dako Japan Co., Kyoto). As microvessels in the ulcer bed originated from the ulcer margin and extended to the surface of the ulcer bed, photographs of two areas of the middle part of both ulcer edges were taken for each animal. The numbers of microvessels positively stained for factor VIII in the two areas (total of 2.3 $mm^2$) were measured under blind conditions.

*Distribution of TGP-580 in the ulcerated area*

Distribution of TGP-580 in the ulcer bed was determined by methods similar to those used to determine the distribution of endogenous bFGF in the ulcer bed. Briefly, TGP-580 (0.1 mg/kg) was administered to rats orally before or 3 or 6 hr or 1, 3, 5, 7, 10, 14, 21 or 28 days after ulcer formation, and the animals were killed by $CO_2$ asphyxiation 1 hr later. The 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 ice-cold saline and spread flat on a piece of paper. The ulcerated area and severity of the ulcer were measured as described previously. After excess moisture was absorbed with paper, samples of the ulcerated area of the gastric wall (80 $mm^2$) were taken using a punch (diameter: 10 mm) and weighed, and the content of TGP-580 was measured by sandwich EIA as described in the previous section for the measurement of endogenous bFGF. In this study, however, MAb ATI-3 was used instead of MAb 52 and MAb 98, because MAb ATI-3 is at least 1000 times more specific for TGP-580 (22).

The distribution of TGP-580 in the ulcer bed was also examined immunohistochemically. In this study, TGP-580 (0.1 mg/kg) was administered orally 7 days after ulcer formation, and the stomach was removed 1 hr later under ether anesthesia. Samples of the ulcerated and intact area were taken, and the distribution of TGP-580 was examined immunohistochemically using monoclonal anti-bFGF antibody MAb 3H3. This antibody responds to TGP-580 as well as bFGF (20).

*Measurement of gastric secretion*

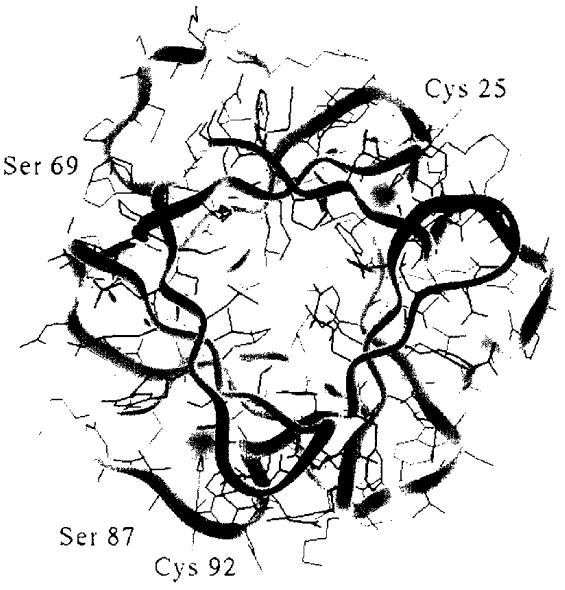
Each drug or vehicle was administered orally after a 24-hr fast. One hour later, the pylorus was ligated under light ether anesthesia and the abdomen was closed by suturing. Each animal was sacrificed by $CO_2$ asphyxiation 3 hr after the pylorus was ligated, and the stomach was removed. The gastric contents were collected and centrifuged at $1,500 \times g$ for 15 min. The volume of the 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 calculated.

*Drugs*

Cimetidine was obtained from Sigma (St. Louis, MO, USA). TGP-580 {recombinant human basic fibroblast growth factor (FGF-2) mutein CS23 in which Cys 69 and Cys 87 are both replaced by serine residues, Fig. 1} (23) and rat bFGF (19) were produced using gene technology and purified at our Division. MAbs (MAb 3H3, 52, 78, 98 and ATI-3) for bFGF and anti-bFGF antiserum were provided by Drs. Igarashi, Kozai, Hori, Ichimori and Sudo of Takeda Chemical Ind., Ltd. Each drug was dissolved or suspended in a 0.5% methylcellulose solution containing 1% $NaHCO_3$ (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. MAbs were stored at $-80$℃ until use. For intravenous injection, MAbs were thawed just before use and diluted with saline. Each MAb was administered intravenously via a tail vein in a volume of 0.5 ml/rat (0.1 mg/rat). In the vehicle group, the same volume of saline was injected.

*Statistical analyses*

Data are expressed as mean values with standard error. The statistical significance of the differences among the groups was determined by Dunnett's test or by Student's $t$-test for unpaired values. The significance of the differences in curative ratio was determined by the $\chi^2$-test.




*Approval by animal welfare committee*

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

## RESULTS

*Distribution of endogenous bFGF in the stomach*

Distribution of bFGF containing cells was determined immunohistochemically using anti-bFGF antiserum (Fig. 2 and Table 1). In the corpus, the mucosal epithelial cells, basal gland cells, lamina muscularis mucosae and

**Fig. 1.** Three dimensional structure of TGP-580. Two cysteine residues (Cys 69 and Cys 87) on the surface of human bFGF are replaced by serine residues in TGP-580. Since the amount of molecules with an amino-terminal Met was less than 0.1% in the TGP-580 preparation, the amino acid residues are numbered starting from the amino-terminal Pro of the mature molecule in this paper. (This photograph was furnished by A. Fujishima, Takeda Chemical Ind., Ltd.; for more detail, refer to J Biochem **110**, 360–363 (1991).)

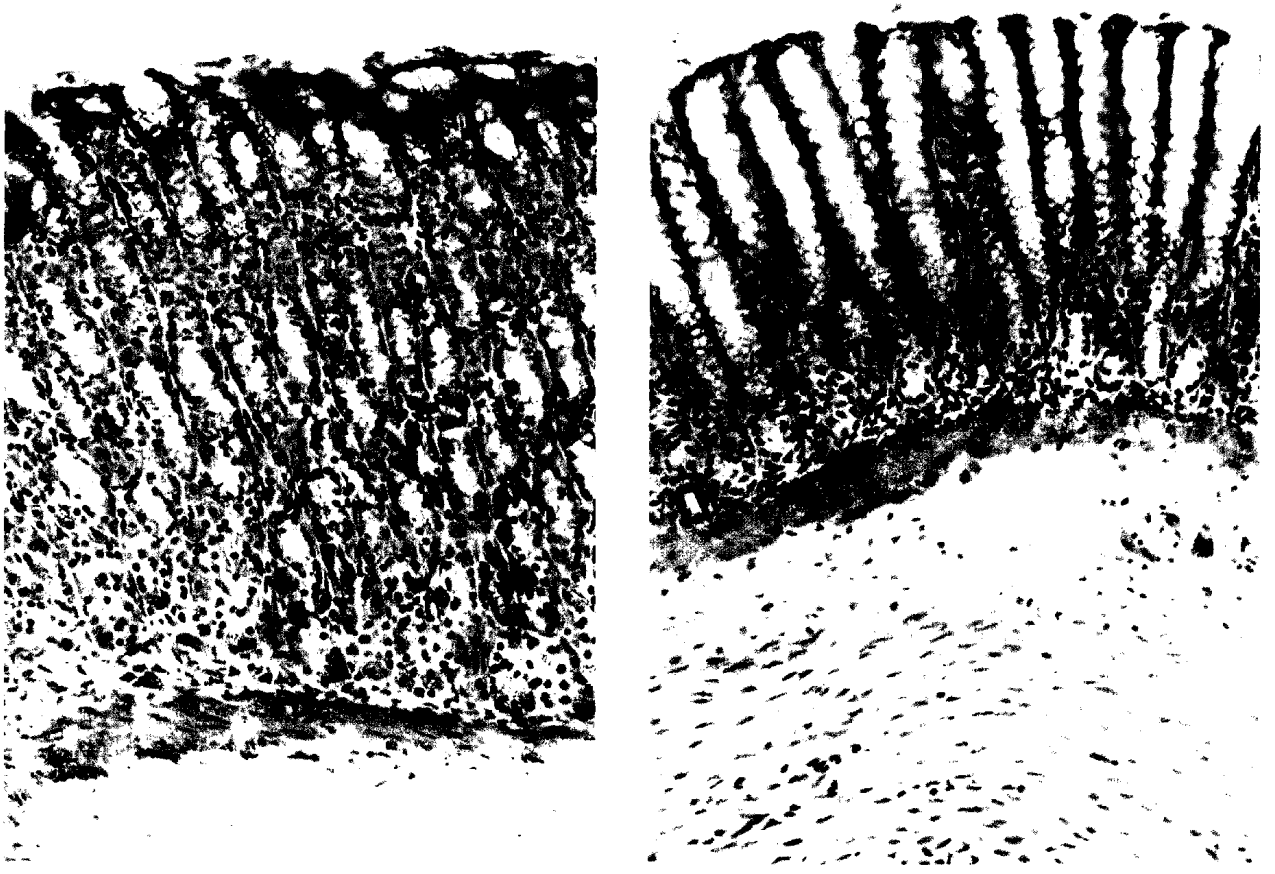


**Fig. 2.** Distribution of bFGF in the corpus (left) and antrum (right) of the rat gastric wall. bFGF was stained immunohistochemically (reddish area) using anti-bFGF antiserum. Mucosal epithelium, parietal cells, basal gland cells and lamina muscularis mucosae in the corpus and basement membrane of mucosal epithelial cells, lamina muscularis mucosae, blood vessels and muscle layers in the antrum are positively stained. (ABC method, ×170).

**Table 1.** Distribution of bFGF in the gastric wall of rats

| Tissue | Response to anti-bFGF antiserum | |
|---|---|---|
| | Corpus | Antrum |
| Mucosal layer | | |
| Mucosal epithelium | ++ | ++ |
| Goblet cell | + | + |
| Mucous neck cell | + | N.D. |
| Parietal cell | + | N.D. |
| Chief cell | + | N.D. |
| Basal gland cell | ++ | + |
| Tunica propria mucosae | | |
| Vascular wall | + | + |
| Lamina muscularis mucosae | ++ | ++ |
| Tunica submucosa | | |
| Vascular wall | + | + |
| Tunica muscularis | | |
| Smooth muscle cell | + | + |
| Perineurium | +~+ | ++ |

+, positive; ~ +, intensively positive; N.D., not determined.

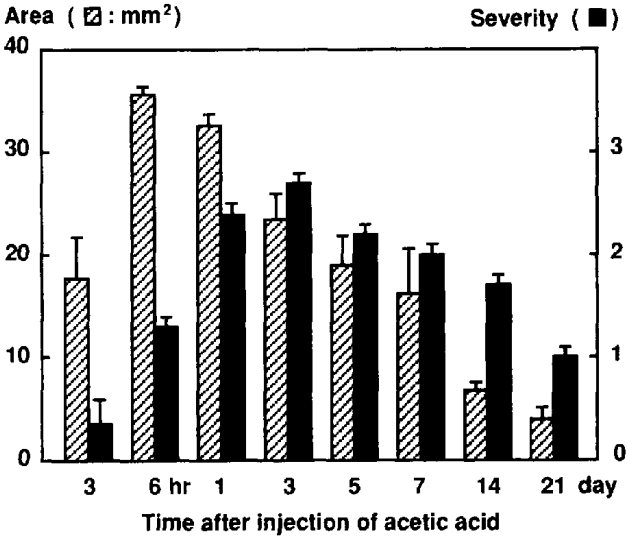



**Fig. 3.** Time-dependent changes in the area and severity of the gastric ulcers induced by acetic acid in rats. Twenty microliters of 20% acetic acid was injected into the subserosal layer of the gastric antrum, and the gastric ulcers were examined at the times indicated. Data show the mean values and the S.E. of the 7 rats in each group.

perineurium showed strongly positive staining for bFGF; and the Goblet cells, mucous neck cells, parietal cells, chief cells, vascular endothelial cells and smooth muscle cells showed weak staining. In these cells, the nuclear membrane and basement membrane were stained markedly as compared to the cytoplasm. In the antrum, a similar pattern of staining was observed.

*Time dependent changes in ulcers*

The animals were sacrificed 3 hr to 21 days after the injection of acetic acid, and time-dependent changes in the gastric mucosa were examined. Changes in the area and severity of the ulcers and ulcer index are shown in Figs. 3 and 4. At 3 hr, hyperemia and hemorrhage were observed in the gastric mucosa; in 2 out of the 7 rats, the mucosa was almost completely lost, and blood vessels were observed in the bottom of the lesion. At 6 hr, the mucosa was detached in all seven rats; and in most of the rats, a clot of aggregated blood was observed in the ulcer bed. At 24 hr, obvious ulcers were observed in all animals, and the ulcer bed was covered with white or yellowish materials consisting of a mixture of necrotized mucosa and inflammatory products. At 3 days, more severe ulceration of the mucosa was observed, and perforation was seen in 2 out of the 7 rats. Maximum ulcer area was seen 6 hr after the injection of acetic acid ($35.6 \pm 0.8$ mm$^2$, Fig. 3), but the ulcer severity continued to increase, and maximum severity was not seen until 3 days after injection (the grade was $2.7 \pm 0.1$, Fig. 3). The ulcer index obtained from the area and severity reached a maximum 24 hr after ulcer formation ($77.1 \pm 5.7$) and then gradually decreased (Fig. 4).

*Time-dependent changes in bFGF content*

The bFGF content in the anterior and posterior gastric antrum was $25.9 \pm 1.5$ ng/80 mm$^2$ ($395 \pm 17$ ng/g w.w.,

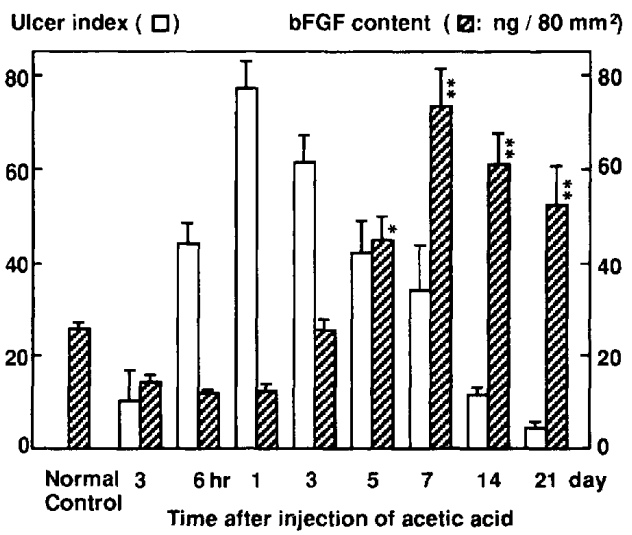



**Fig. 4.** Time-dependent changes in the ulcer index and the content of bFGF in the gastric ulcers induced by acetic acid in rats. The ulcerated area (80 mm$^2$) was sampled at the times indicated. The ulcer index was obtained as the product of the area and the severity grade of the ulcer. Data show the mean values and the S.E. of the 6 rats in each group. *: $P<0.05$, **: $P<0.01$ vs normal control.

n=6) and 25.2±1.4 ng/80 mm$^2$ (353±10 ng/g w.w., n=6), respectively. The bFGF content in the ulcerated area was decreased 3, 6 and 24 hr after the injection of acetic acid, but within 3 days after the injection, the level had returned to normal (Fig. 4). The content then increased with time, reaching a maximum at 7 days (73.5±7.6 ng/80 mm$^2$, n=6) and thereafter, decreased gradually. The level was though still high even at 21 days (52.7±8.0 ng/80 mm$^2$, n=6) (Fig. 4). The content in the intact posterior antrum did not change until 24 hr after ulcer formation. It was slightly high at 3 and 5 days, but the level was normal on day 7.

The wet weights of the normal anterior and posterior antrum were 66±4 and 72±4 mg/80 mm$^2$ (n=6), respectively. The weight of the ulcerated mucosa did not change until 24 hr after ulcer formation. It increased with time and reached a maximum at 7 days (244±31 mg/80 mm$^2$, n=6). It then gradually decreased. The weight of the intact anterior antrum did not change during the 21-day observation periods.

*Distribution of endogenous bFGF in the ulcer bed*

Seven days after ulcer formation, many cells including smooth muscle cells of the muscle layer, fibroblasts, macrophages, newly formed vascular endothelial cells and vascular wall cells near the margin of the ulcer bed were stained positively for bFGF (Fig. 5). In fibroblasts of granulation tissue, the extracellular matrix was also weekly stained in addition to the nucleus.

*Effects of monoclonal anti-bFGF antibodies on ulcer healing and angiogenesis in the ulcer bed*

Effects of MAb 3H3 and MAb 78 given i.v. for 14 days on ulcer healing and angiogenesis in the ulcer bed were examined, and the results are shown in Fig. 6. The area and severity of the ulcer and the ulcer index in the group given saline were 5.3±0.9 mm$^2$, 1.7±0.1 and 9.0±1.5 (n=12), respectively; and the number of microvessels in

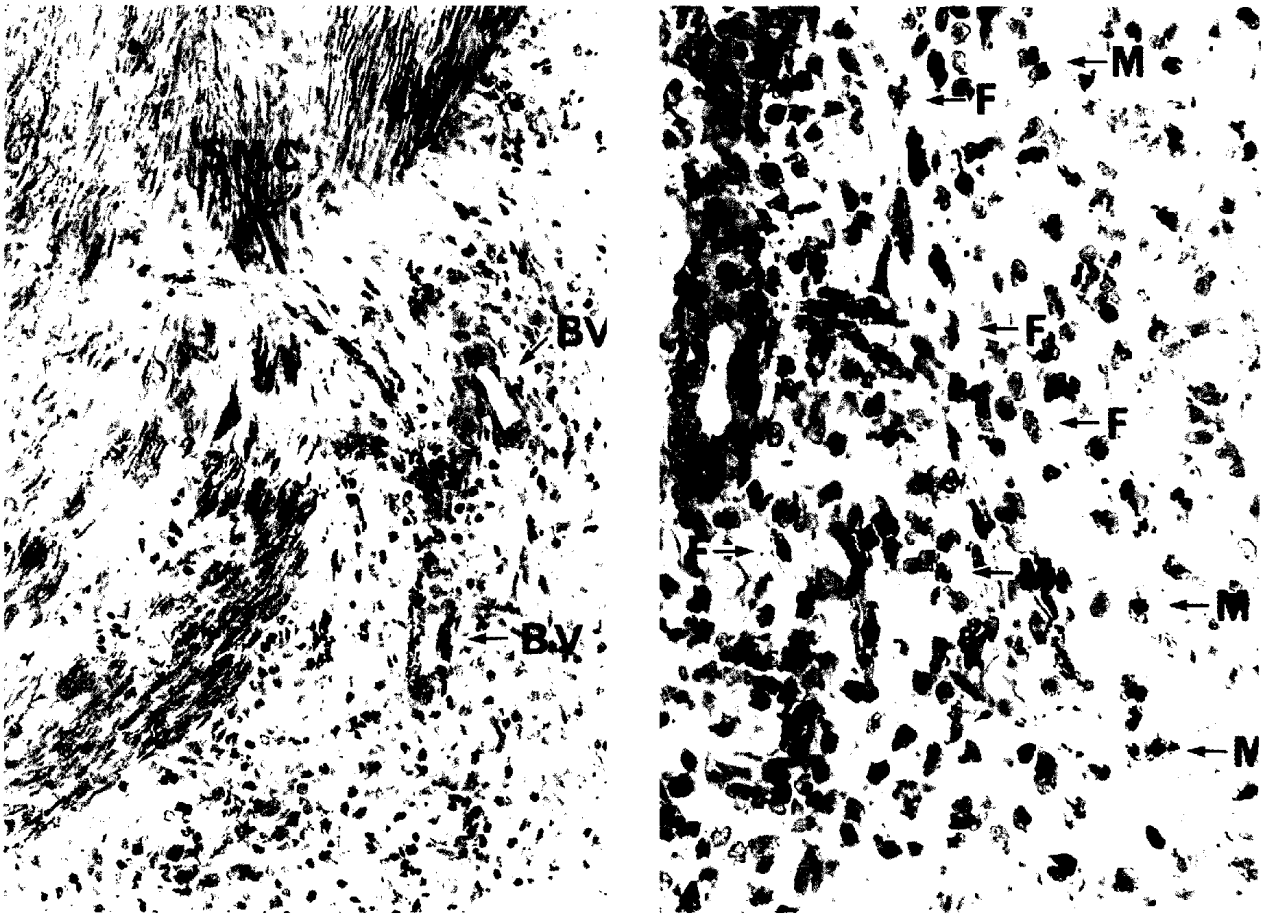



**Fig. 5.** Distribution of bFGF in the gastric ulcer bed 7 days after ulcer formation in rats. bFGF was stained immunohistochemically (reddish area) using anti-bFGF antiserum (ABC method, left: ×170, right: ×340). Smooth muscle cells (SMC) and blood vessels (BV) (on the left) and fibroblasts (F) and macrophages (M) (on the right) are stained intensively. In addition, the extracellular matrix of the fibroblasts is stained weakly (right).

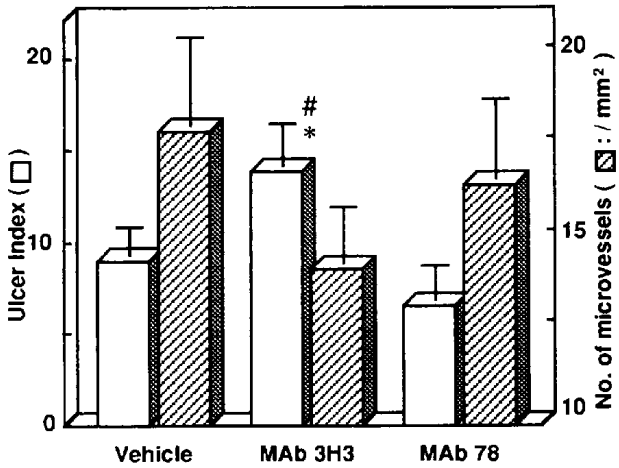



**Fig. 6.** Effect of anti-bFGF monoclonal antibodies (MAb 3H3 and MAb 78) on the healing of gastric ulcers and angiogenesis in the ulcer bed in rats. Monoclonal antibodies or vehicle were administered i.v. once a day for 14 days starting on the day of ulcer formation. Data show the mean values and the S.E. of the 10 to 12 rats in each group. *: $P<0.05$ vs vehicle, #: $P<0.05$ vs MAb 78.

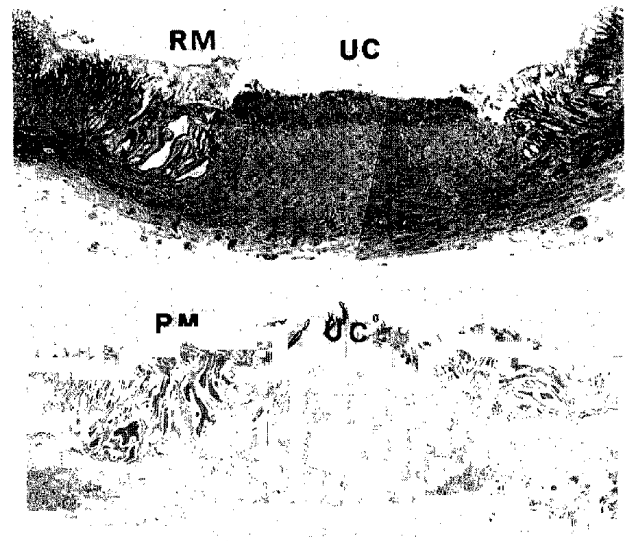



**Fig. 7.** Light micrographs of rat gastric ulcers treated with vehicle (upper) or TGP-580 (lower) (Azan stain, × 34). Vehicle or TGP-580 (100 μg/kg) was given orally twice a day for 14 days starting 2 days after ulcer formation, and the rats were autopsied the day after the final dosing day. In the group treated with TGP-580, many mature collagen fibers are arranged transversely in the ulcer bed; part of the ulcerated mucosa is covered with regenerated mucosa (RM); and the maximal length of the ulcer crater (UC) has decreased.

the ulcer bed was $18.0\pm2.4$ vessels/mm$^2$ ($n=12$). The administration of MAb 3H3 obviously inhibited the healing of the ulcer, i.e., the ulcer index was $13.9\pm2.2$ ($n=12$), which was significantly larger than that in the groups given saline or MAb 78. MAb 3H3 showed a tendency to decrease the number of microvessels in the ulcer bed ($14.3\pm1.5$ vessels/mm$^2$, $n=12$). The administration of MAb 78 did not inhibit the healing of the ulcer and rather slightly accelerated it, but did not affect the number of microvessels.

*Effect of TGP-580 on ulcer healing and angiogenesis in the ulcer bed*

Effects of TGP-580 and cimetidine given orally twice a day for 14 days on ulcer healing were examined, and the results are shown in Table 2. Cure rate, area and severity of the ulcer and ulcer index in the group given the vehicle were 8% (2 out of 25 rats healed), $4.4\pm0.8$ mm$^2$, $1.4\pm0.1$ and $7.2\pm1.8$ ($n=25$), respectively. TGP-580 at doses of 0.001, 0.01 and 0.1 mg/kg obviously accelerated the healing of the ulcer and increased the cure rate to 35%, 30% and 52%, respectively. TGP-580 decreased the area and severity of the ulcer in a dose-dependent manner. Cimetidine at a dose of 100 mg/kg also accelerated the ulcer

**Table 2.** Effects of drugs on the healing of gastric ulcers in rats

| Treatment | Dose (mg/kg, p.o.) | No. of rats | Cure rate | Gross appearance of the ulcer | | |
|---|---|---|---|---|---|---|
| | | | No. of rats healed (%) | Area (A) (mm$^2$) | Severity grade (G) (0-3) | Ulcer index (A × G) |
| Vehicle | | 25 | 2 ( 8 ) | 4.4±0.8 | 1.4±0.1 | 7.2±1.8 |
| TGP-580 | 0.001 | 23 | 8 (35* ) | 3.0±0.7 | 1.0±0.1* | 4.4±1.2 |
| TGP-580 | 0.01 | 23 | 7 (30* ) | 2.9±0.6 | 1.0±0.1 | 3.2±0.7 |
| TGP-580 | 0.1 | 21 | 11 (52**) | 2.0±0.4* | 0.7±0.1** | 1.8±0.4** |
| Cimetidine | 100 | 19 | 6 (32* ) | 2.5±0.7* | 1.1±0.1 | 4.1±1.7* |

Each drug or vehicle was administered orally twice daily for 14 days starting 2 days after ulcer formation, and the animals were autopsied 24 hr after the final dose. The area and severity of the ulcer were measured, and the ulcer index was obtained as the product of the area (A) and the severity grade (G). Ulcers with superficial erosion or scar were considered cured ulcers. Data show the mean values and the S.E. *: $P<0.05$, **: $P<0.01$ vs vehicle (by Dunnett's test, nonparametric).

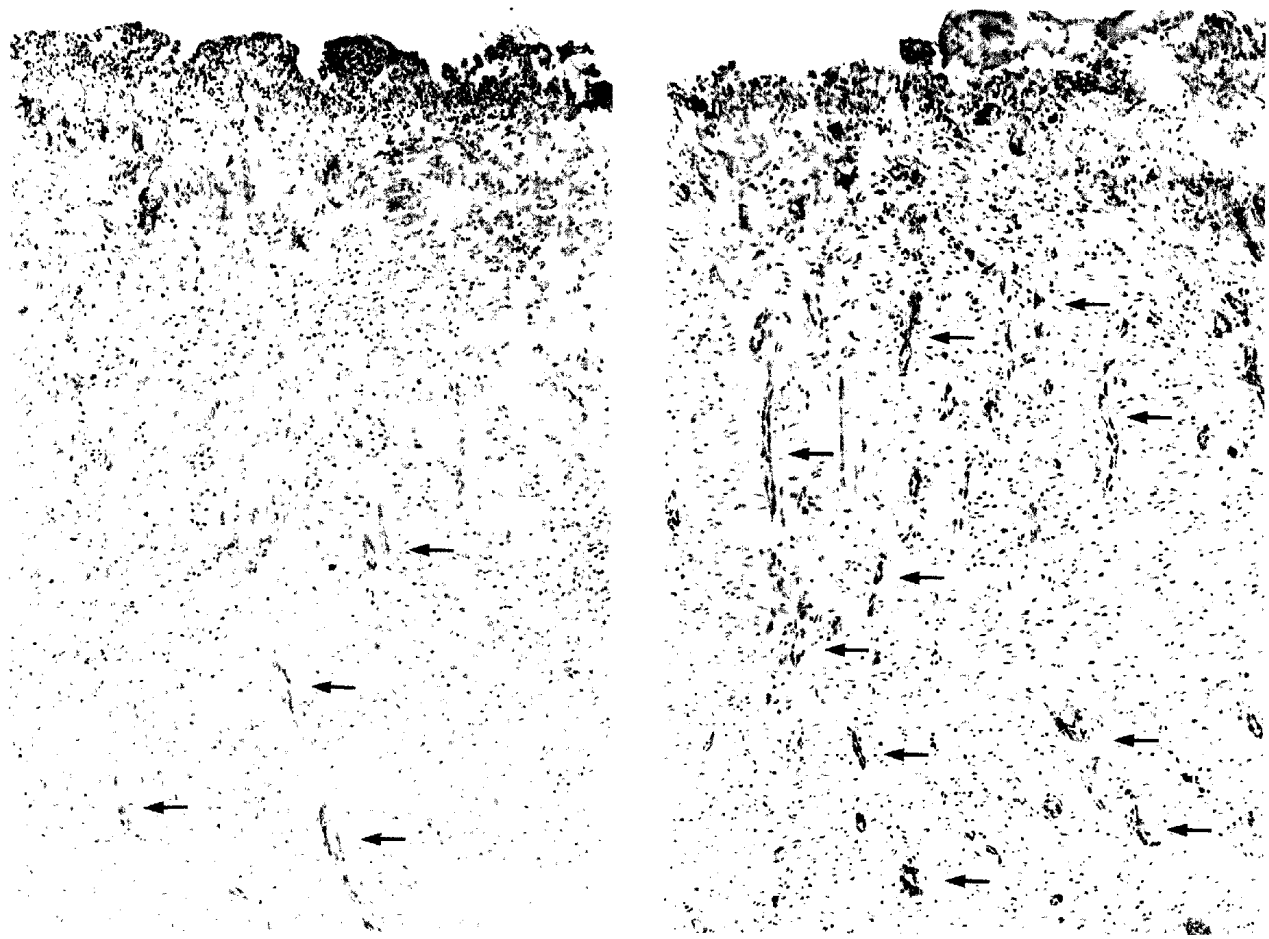


**Fig. 8.** Angiogenesis in the gastric ulcer bed in rats treated with vehicle (left) or TGP-580 (right). Microvessels were stained immunohistochemically (reddish area) using anti-Factor VIII antiserum (ABC method, ×85). The number of microvessels (shown by the arrow) is increased in the group treated with TGP-580.

healing; it increased the cure rate to 32% and decreased the area and severity.

As seen in Fig. 7, in the group treated with TGP-580, the ruptured muscle layers were well repaired, granulation tissue was well replaced with many matured collagen fibers, the thickness of the ulcer bed was thin and part of the ulcerated mucosa was covered with regenerated mucosa.

In the other study, the effects of TGP-580 and cimetidine on angiogenesis in the ulcer bed were examined. The results are shown in Figs. 8 and 9 with the effects on ulcer healing. In the group given the vehicle, the area and severity of the ulcer and ulcer index were $5.5 \pm 1.2$ mm$^2$, $1.8 \pm 0.1$ and $10.9 \pm 1.8$ ($n=11$), respectively; and the number of microvessels in the ulcer bed was $19.4 \pm 3.2$ vessels/mm$^2$ ($n=11$). The administration of TGP-580 (0.1 mg/kg, p.o.) accelerated the healing and decreased the ulcer index to $5.0 \pm 1.4$ ($P<0.05$) and increased the number of microvessels to $36.2 \pm 4.7$/mm$^2$ ($P<0.05$).

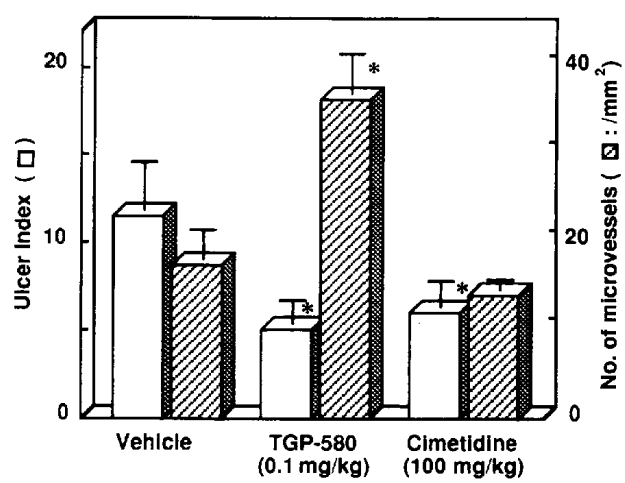



**Fig. 9.** Effect of TGP-580 and cimetidine on the healing of gastric ulcers and angiogenesis in the ulcer bed in rats. Each drug or vehicle was administered p.o. twice a day for 14 days starting 2 days after ulcer formation. Data show the mean values and the S.E. of the 9 to 11 rats in each group. *: $P<0.05$ vs vehicle.

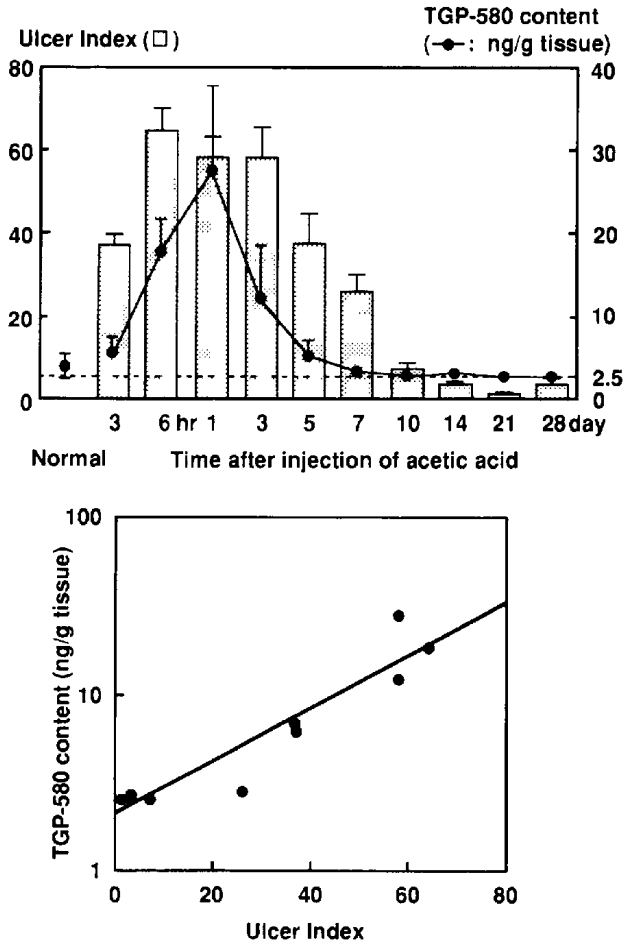



**Fig. 10.** Distribution of TGP-580 in the gastric ulcer bed in rats. Upper: Time dependent changes in the ulcer index and the concentration of TGP-580 in the ulcerated area. TGP-580 (0.1 mg/kg) was administered orally before or after ulcer formation, and samples of the ulcerated area (80 $mm^2$) were taken 1 hr later. Data show the mean values and the S.E. for the 7 rats in each group. Lower: Relationship between the ulcer index and the concentration of TGP-580 in the ulcerated area. Data are taken from the results shown above. Points represent the mean values for the 7 rats in each group. The concentration of TGP-580 is shown following logarithmic transformation. The correlation coefficient is 0.93 ($P<0.01$).

Cimetidine (100 mg/kg, p.o.) also accelerated healing of the ulcer, but did not increase the number of microvessels and rather decreased it to $13.9\pm1.1/mm^2$ (not significant).

*Distribution of TGP-580 in the ulcer bed*

TGP-580 (0.1 mg/kg) was administered orally before or up to 28 days after ulcer formation, and the content of TGP-580 in the ulcerated area was measured 1 hr later (Fig. 10). The concentration of TGP-580 in normal mucosa 1 hr after the administration of TGP-580 was $3.1\pm0.5$ ng/g tissue ($n=7$). The concentration in the ulcerated area increased with time after ulcer formation, reached a maximum when TGP-580 was administered 24 hr after formation ($27.9\pm10.4$ ng/g tissue, $n=7$) and then decreased with time. Ten days after ulcer formation or later, the concentration of TGP-580 was close to or less than 2.5 ng/g tissue (minimum detectable concentration by EIA). A close relationship between the ulcer index and TGP-580 concentration in the ulcerated mucosa was observed (Fig. 10).

In the other study, TGP-580 (0.1 mg/kg) was administered orally 7 days after the ulcer formation, and the distribution of TGP-580 in the ulcer bed and intact mucosa was examined histologically by immunohistochemical staining for TGP-580. As seen in Fig. 11, intensive staining derived from TGP-580 was observed in the surface layer of the ulcer bed but not in the intact mucosa (data not shown).

*Effect of TGP-580 on gastric acid secretion*

Gastric acid secretion in the vehicle group was $156.3\pm20.5$ $\mu$Eq/3 hr ($n=8$). Cimetidine (100 mg/kg, p.o.) significantly decreased the acid secretion by 86%, but TGP-580 (0.001, 0.01 and 0.1 mg/kg, p.o.) did not affect the secretion (Table 3).

**Table 3.** Effects of drugs on gastric acid secretion in pylorus-ligated rats

| Treatment | Dose (mg/kg, p.o.) | No. of rats | Gastric acid secretion | | |
|---|---|---|---|---|---|
| | | | Volume (ml/3 hr) | Acidity ($\mu$Eq/ml) | Acid output ($\mu$Eq/3 hr) |
| Vehicle | | 8 | $2.4\pm0.2$ | $65.6\pm4.5$ | $156.3\pm20.5$ |
| TGP-580 | 0.001 | 8 | $2.0\pm0.3$ | $55.4\pm8.6$ | $113.9\pm26.6$ |
| TGP-580 | 0.01 | 8 | $2.0\pm0.2$ | $59.7\pm6.5$ | $124.8\pm22.3$ |
| TGP-580 | 0.1 | 8 | $2.3\pm0.3$ | $62.8\pm8.8$ | $155.8\pm34.0$ |
| Cimetidine | 100 | 8 | $1.3\pm0.3$* | $19.8\pm4.5$** | $21.8\pm 6.6$** |

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 show the mean values and the S.E. *: $P<0.05$, **: $P<0.01$ vs vehicle.

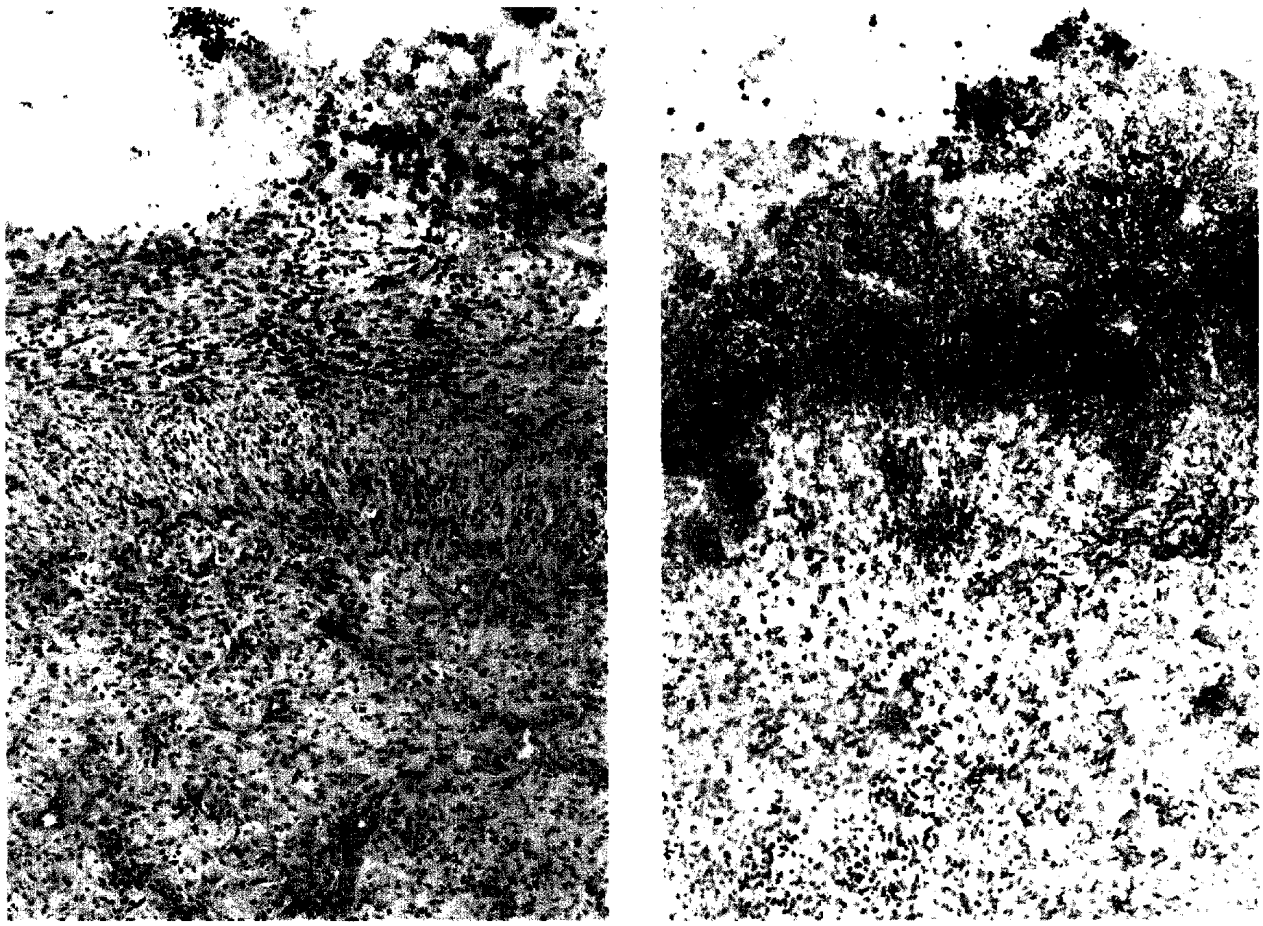


**Fig. 11.** Distribution of TGP-580 in the gastric ulcer bed 7 days after ulcer formation in rats. TGP-580 was stained immunohistochemically (reddish area) using MAb 3H3 (ABC method, ×85). TGP-580 (0.1 mg/kg) was given orally 7 days after ulcer formation, and 1 hr later, the stomach was removed. Compared with the group treated with vehicle (left), intense staining is observed in the surface layer of the ulcer bed in the group treated with TGP-580 (right).

## DISCUSSION

Recent reports have indicated that many growth factors play an important role in the healing of gastrointestinal ulcers (1–12). In the present study, we examined the role of endogenous bFGF in the healing of gastric ulcers induced by acetic acid in the rat and obtained the following results: 1) the content of bFGF in the ulcerated area increased during the period of ulcer healing; 2) upon histological examination, many cells responding to anti-bFGF antiserum were seen in the ulcer bed 7 days after ulcer formation, and most of them were fibroblasts, macrophages and vascular endothelial cells; 3) ulcer healing was significantly inhibited by the administration of anti-bFGF monoclonal antibody 3H3; and 4) ulcer healing was obviously accelerated by the administration of TGP-580, recombinant human bFGF mutein CS23. All of these results indicate that endogenous bFGF plays an important role in the process of gastric ulcer healing.

In cysteamine-induced duodenal ulcers in rats, we found that bFGF levels in the duodenal mucosa decreased in the pre-ulcer stage and the early stage of duodenal ulceration, suggesting that the depletion of mucosal bFGF plays a role in the pathogenesis of duodenal ulceration (24). In the present study, the content of bFGF in the ulcerated area decreased in the early phase of ulcer formation up to 24 hr after the injection of acetic acid. However, we think that the decrease in bFGF content in the present model may not be the cause of ulcer formation but rather a result of ulcer formation; i.e., 1) the amount of bFGF in the ulcerated area is decreased as a result of the release of bFGF from the damaged cells, 2) the response of bFGF to the antibodies used in the EIA decreased in the ulcerated area due to the degeneration of bFGF by acetic acid or 3) simply, the mucosa containing bFGF became detached during ulcer formation.

We found that bFGF content in the duodenal ulcer bed was significantly higher than that in normal mucosa 7

days after ulcer formation in rats (8). In the present experiment, the bFGF content in the ulcerated area began to increase in association with the start of ulcer healing (3 days after ulcer formation) and reached a maximum 7 days after the injection of acetic acid. Thereafter, the content was maintained at a high level until the end of the 3-week observation period. The results were consistent with the previous findings in duodenal ulcers and suggested that endogenous bFGF is involved in the process of ulcer healing. To confirm this possibility, we examined the effect of treatment with monoclonal antibodies for bFGF on the healing of gastric ulcers and found that the healing was significantly inhibited by administration of MAb 3H3 which can neutralize the activity of bFGF but was not affected by treatment with MAb 78 which binds to bFGF without inhibiting its activity. The content of bFGF in the ulcerated area 7 days after ulcer formation was $73.5 \pm 7.6$ ng/80 mm$^2$. Fifty nanograms of MAb 3H3 can neutralize the activity of 1 ng of bFGF (20), indicating that 4 $\mu$g of MAb 3H3 can neutralize 80 ng of bFGF. Therefore, the dose (100 $\mu$g/day) of MAb 3H3 should be sufficient to inhibit the activity of endogenous bFGF in the ulcerated area. Recently, we reported that MAb 3H3 inhibits the healing of duodenal ulcers in rats (25). We also found that the healing of colonic ulcers induced by NEM were prevented by the administration of MAb 3H3 (unpublished observation). These results indicate that bFGF plays an important role in the healing of ulcerated mucosa not only in the stomach but also in other areas of the gastrointestinal tract.

The results that anti-bFGF antibody (MAb 3H3) inhibited ulcer healing suggest that endogenous bFGF released from some cells is involved in the process of ulcer healing. However, an important and basic question is from which cells bFGF is released. It has been reported that bFGF is widely distributed in most cells, especially in vascular endothelial cells, fibroblasts, smooth muscle cells and macrophages (26–29) and stored in the basement membrane (30) and extracellular matrix (31). We previously demonstrated that bFGF is widely observed in the mucosa and submucosa of the rat stomach by immunohistochemical studies (8). In the present study, we examined the distribution of bFGF determined by the response to anti-bFGF antiserum in detail and found that the cells stained by anti-bFGF antiserum were widely observed in the intact stomach, and intensive staining was seen in the mucosal epithelial cells, basal gland cells, lamina muscularis mucosa and perineurium. In the ulcer bed 7 days after ulcer formation, many cells such as fibroblasts, macrophages, smooth muscle cells and endothelial cells of new vessels responded to the antiserum. These results revealed that there are many cells containing bFGF in the normal gastric wall and the ulcer bed. From these results, it is postulated that bFGF may be released from the damaged cells of the gastric wall during ulcer formation and that bFGF may be released from inflammatory cells such as macrophages during ulcer healing more than 3 days after ulcer formation. This is partly supported by the findings that bFGF content decreased during ulcer formation. However, the mechanism for the release of bFGF from intact cells is not yet understood as bFGF does not have the signal peptides needed for release of a peptide through the Golgi apparatus in the cells in its molecule. Recently, Yu et al. (32) pointed out the possibility that bFGF is released by exocytosis in bovine endothelial cells. This releasing mechanism may explain the source of bFGF during the healing stage in the present study.

In the present study, it was revealed that TGP-580 accelerated the healing of gastric ulcers in rats. Konturek et al. (10) have reported that bFGF given orally or intraperitoneally enhances the healing of acetic acid-induced gastric ulcers in rats. We have reported that both bFGF and TGP-580 accelerate the healing of duodenal ulcers induced by cysteamine in rats (7–9). We also found that both bFGF and TGP-580 accelerate the healing of colonic ulcers induced by NEM in rats (33). These results suggest that both bFGF and TGP-580 can accelerate the healing of ulcers not only in the stomach but also in other areas of the gastrointestinal tract.

Angiogenesis in granulation tissue in the ulcer bed is thought to be critical in ulcer healing as well as wound healing (8). As bFGF is the most active angiogenic peptide among the growth factors (13), it was expected that bFGF promotes neovascularization in the ulcer bed. In fact, we have found that both bFGF and CS23 (TGP-580) increase the number of microvessels in the duodenal ulcer bed in rats (8, 9). A similar angiogenic effect of bFGF has been reported by Konturek et al. (10) in a rat model of gastric ulcers. It has been suggested that the angiogenic effects of these peptides play an important role in the healing of ulcers. In the present study, TGP-580 significantly increased the number of microvessels in the gastric ulcer bed. On the other hand, the angiogenesis in the gastric ulcer bed was inhibited by MAb 3H3 which can neutralize the activity of bFGF, but it was not inhibited by MAb 78. These results support the previous findings and suggest that the angiogenic activity of TGP-580, as well as bFGF, is involved in the healing effect of this peptide on gastric ulcers. Paimella et al. (34) and Dignass et al. (35) reported that bFGF promotes the migration of gastrointestinal mucosal cells and suggested that bFGF may mediate rapid epithelial repair after surface injury. Recently, Nakamura et al. (36) reported that CS23 (TGP-580) promotes the reinnervation of newly-formed microvessels through its effect on the Schwann

cells in the gastric ulcer bed in rats. These actions of bFGF (TGP-580) may explain, in part, the healing effect of TGP-580 on gastric ulcers.

When TGP-580 was given orally, the concentration of TGP-580 in the ulcerated area increased with time after ulcer formation, reaching a maximum when TGP-580 was administered 24 hr after formation and subsequently decreasing with ulcer healing. A close relationship was seen between the ulcer index and TGP-580 concentration in the ulcerated area. Immunohistochemical studies revealed that TGP-580 was distributed in the surface layer of the ulcer bed 1 hr after administration. These results suggest that TGP-580 given orally is distributed in the ulcer bed from the surface of the ulcer base and promotes ulcer healing by stimulating proliferation of many cells in the ulcer bed. Furthermore, it is suggested that the distribution of TGP-580 into the ulcerated area depends on the severity of the ulcer; i.e., intense distribution is seen when the ulcer is active, but the distribution of TGP-580 decreases with the progress of ulcer healing, indicating that TGP-580 is distributed into the ulcerated area only when it is needed.

Gastric acid plays an important role in both the formation and healing of gastroduodenal ulcers. To elucidate the mode of action of TGP-580, the effect of TGP-580 on gastric acid secretion was examined in pylorus-ligated rats. Cimetidine at a dose of 100 mg/kg significantly inhibited the acid secretion, but TGP-580 did not affect the secretion even at a dose of 0.1 mg/kg. These results are in agreement with the previous findings (7–10) that neither bFGF nor CS23 (TGP-580) inhibited gastric acid secretion in rats and suggested that TGP-580 accelerates ulcer healing by a mechanism other than an antisecretory action. This is supported by the finding that TGP-580 accelerates the healing of colonic ulcers in rats in which acid is not involved (33).

bFGF is very unstable in the presence of acid or pepsin, and we have suggested that some antiulcer drugs may accelerate ulcer healing, at least in part, via stabilization of endogenous bFGF by inhibiting acid secretion or by binding to bFGF (8). However, in the present study, cimetidine did not stimulate but rather inhibited angiogenesis, a typical biological effect of bFGF, in the ulcer bed. Tsuchida et al. (37) also reported that cimetidine decreased the number of microvessels in the ulcer bed of gastric ulcers in rats. These results do not always support the hypothesis, but more detailed studies will be needed to draw any precise conclusion.

The present study revealed that endogenous bFGF plays an important role in the healing of gastric ulcers and also revealed that a mutein of bFGF, TGP-580, accelerates gastric ulcer healing by a new mode of action such as stimulation of angiogenesis in the ulcer bed. Antisecretory drugs such as histamine $H_2$-receptor antagonists and proton pump inhibitors accelerate ulcer healing indirectly by eliminating a deleterious substance (acid), but bFGF (TGP-580) can accelerate ulcer healing by acting directly on the ulcer bed in the presence of acid. It is interesting to compare the quality of ulcer healing with TGP-580 and antisecretory drugs. Recently, we found that gastric ulcers healed by TGP-580 treatment were more resistant to indomethacin-induced relapse than those healed by treatment with histamine $H_2$-receptor antagonists (38). Clinical studies on TGP-580 are now underway (39, 40), and in the near future, we hope the usefulness of this new approach to ulcer therapy will be widely recognized.

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