Role of Thromboxane A_2 in Healing of Gastric Ulcers in Rats

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ABSTRACT—We investigated the role of thromboxane (TX) A_2 in gastric ulcer healing in rats. Acetic acid ulcers were produced in male Donryu rats. TXA_2 synthesis in the stomachs with ulcers was significantly elevated in ulcerated tissue, but not in intact tissue, compared with that in the gastric mucosa of normal rats. Indomethacin inhibited both TXA_2 and prostaglandin E_2 (PGE_2) synthesis in ulcerated tissue, while NS-398 (selective cyclooxygenase-2 inhibitor) reduced only PGE_2 synthesis. OKY-046 (TXA_2 synthase inhibitor) dose-relatedly inhibited only TXA_2 synthesis. The maximal effect of OKY-046 (80% inhibition) was found at more than 30 mg/kg. When OKY-046 was administered for 14 days, the drug at more than 30 mg/kg significantly accelerated ulcer healing without affecting acid secretion. The maximal reduction of ulcerated area by OKY-046 was about 30%, compared with the area in the control. Histological studies revealed that regeneration of the mucosa was significantly promoted by OKY-046, but neither maturation of the ulcer base nor angiogenesis in the base were affected. OKY-046 and TXB_2 had no effect on proliferation of cultured rat gastric epithelial cells, but U-46619 (TXA_2 mimetic) dose-relatedly prevented the proliferation without reducing cell viability. These results indicate that the increased TXA_2, probably derived from cyclooxygenase-1 in ulcerated tissue, exerts a weak inhibitory effect on ulcer healing in rats. The effect of TXA_2 might be due partly to prevention of gastric epithelial cell proliferation at the ulcer margin.

Keywords: Acetic acid ulcer, Healing, Thromboxane A_2, OKY-046, Mucosal regeneration

Thromboxane (TX) A_2 is generated from arachidonic acid by the sequential actions of cyclooxygenase (COX) and TXA_2 synthase. TXA_2 is reported to worsen HCl /taurocholate-induced lesions in the gastric mucosa of dogs (1). In addition, Ogletree et al. (2) reported that TXA_2 might play a crucial role in formation of acute gastric lesions caused by various necrotizing agents in rats. The mechanism underlying the damaging actions of TXA_2 has been attributed partly to the vasoconstrictive effect of TXA_2 on the gastric mucosal and submucosal microvasculature (1, 3). Furthermore, cytotoxic effects of TXs on gastric mucosal cells have been suspected. In vitro studies showed that TXB_2 exerts direct cytotoxicity toward rabbit gastric mucosal cells (4), and inhibition of TXA_2 synthesis is associated with protection of gastric mucosal cells against taurocholate-induced damage (5). These results suggest that TXA_2 may mitigate the healing of gastric ulcers. In fact, TXA_2 synthesis in ulcerated gastric tissue was found (6). Therefore, in the present study, we examined the effect of OKY-046 (TXA_2 synthase inhibitor) on the healing of acetic acid-induced ulcers in rats and investigated the role of TXA_2 in gastric ulcer healing.

MATERIALS AND METHODS

Production of gastric ulcers

Male Donryu rats (Nihon SLC, Hamamatsu), weighing 280–300 g, were used. Under ether anesthesia, gastric ulcers were induced by submucosal injection of 20% acetic acid (0.04 ml) at the border between the antrum and the fundus on the anterior wall of the stomach (7). After closure of the abdomen, the rats were maintained in the usual manner. Since deep and well-defined ulcers were observed 5 days after the acid injection, we defined the 5th day as the day of ulceration (day 0). On day 14, rats were sacrificed and their stomachs were excised. Subsequently, the stomachs were incised along the greater curvature and the ulcerated area (mm^2) was determined under a dissecting microscope ($\times 10$; Olympus, Tokyo). The person (S.O.) determining the ulcer size was unaware of the treatment given the animals.

Determination of TXA_2 and PGE_2 syntheses in gastric tissues

The prostanoid synthesis assay was performed according to the method of Lee and Feldman (8). On the indi-

cated days, the animals were sacrificed, and then gastric specimens were taken from both intact and ulcerated tissues of stomachs with ulcers and from normal stomachs. For evaluation of the effects of drugs on TXA_2 and prostaglandin E_2 (PGE_2) syntheses, specimens were taken from ulcerated tissue 3 hr after the administration on day 3 (bolus administration) and the additional dose on day 14 (repeated administration). The specimens were placed in 50 mM Tris-HCl (pH 8.4) buffer and then finely minced with scissors. After the tissues had been washed and resuspended in 1 ml of buffer, they were subjected to vortex mixing at room temperature for 1 min to stimulate prostanoid synthesis, followed by centrifugation at $10,000 \times g$ for 15 sec. The prostanoid syntheses proceeded until 3-min stimulation with vortex mixing. Because TXA_2 is rapidly metabolized to the stable substance TXB_2, the amount of TXB_2 was measured as produced TXA_2. TXB_2 and PGE_2 contents in the resulting supernatants were determined by enzyme-immunoassay (TXB_2 EIA kit and PGE_2 EIA kit; Cayman Chemicals, Ann Arbor, MI, USA). TXA_2 and PGE_2 syntheses were expressed as pg TXB_2 and pg PGE_2/mg tissue/min, respectively.

Histological evaluation of ulcer healing

On day 14, gastric specimens were taken from ulcerated tissues and then fixed in 10% formalin and embedded in paraffin. Four-micron sections were prepared and then stained with hematoxylin and eosin. The length of the regenerated mucosa on the ulcer base and the thickness of the base were measured under a light microscope (Olympus), as described previously (9, 10). These parameters are presented as regeneration of the mucosa and maturation of the ulcer base, respectively.

For evaluation of angiogenesis in the ulcer base, gastric specimens were fixed in 4% paraformaldehyde and 14-μm frozen sections were prepared. The sections were incubated with the antibody against von Willebrand factor (factor VIII-related endothelial antigen) (DAKO, Glostrop, Denmark) after deactivation of endogenous peroxidase with 0.3% H_2O_2 and blockage of nonspecific binding sites. Microvessels were visualized by the avidin-biotin-peroxidase complex method using a Vectastain ABC-peroxidase kit (Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto). The number of microvessels in the ulcer base was determined in three randomly chosen fields ($1 \text{ mm}^2 \times 3$) under a light microscope. The microvessel density was expressed as the number of microvessels per mm^2.

Determination of gastric acid secretion

Gastric acid secretion of the animals treated with drugs for 14 days was determined by the pylorus ligation method. The pylorus was ligated 1 hr after the additional administration of drugs on day 14. Three hours later, the animals were sacrificed, and then their stomachs were excised. The gastric contents were collected and analyzed as to volume and acidity. Acidity was determined by automatic titration of the contents against 0.1 M NaOH to pH 7.0 (Comtite 5; Hiranuma, Tokyo). Total acid output was calculated as volume × acidity and expressed as μEq/hr.

Determination of proliferation and viability of gastric epithelial RGM1 cells

The normal gastric epithelial cell line RGM1, established from the stomach of Wistar rats by H. Matsui (11), was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO BRL, Gaithersburg, MD, USA), 100 units/ml penicillin, 100 units/ml streptomycin and 0.25 μg/ml amphotericin B. Cells (3×10^4 cells/0.2 ml of medium) were inoculated in 48-well plates, cultured for 48 hr, and then starved for 48 hr in DMEM supplemented with 1 mg/ml bovine serum albumin (BSA) at 37℃ under 5% CO_2 in air.

Cell proliferation was assessed as DNA synthesis, which was determined as the incorporation of [^{3}H]thymidine into DNA. After starvation, the medium was replaced with 0.2 ml of the medium containing 1 mg/ml BSA and [^{3}H]thymidine (7.4 kBq, 2.22–3.2 TBq/mmol; Amersham, Buckinghamshire, England), and then the cells were further incubated with the indicated drugs at 37℃ for 24 hr. At the end of the incubation, the cells were washed with 0.2 ml of ice-cold 10% trichloroacetic acid twice and then solubilized with 0.2 ml of 0.3 M NaOH. The radioactivity (25 μl) in the lysate was measured with a liquid scintillation counter (Beckman, Fullerton, CA, USA).

Cell viability was determined by the membrane permeability assay. The membrane permeability was assessed by the dye exclusion method (12). After starvation, the cells were incubated in 0.2 ml of DMEM containing 1 mg/ml BSA and the indicated drugs at 37℃ for 24 hr. Cells were washed with phosphate-buffered saline, and then 0.1 ml of 0.1% trypan blue solution was added to each well. Three minutes later, the number of stained and nonstained cells were determined in four randomly chosen fields in each well under a light microscope. Cell viability was expressed as follows:

$$\text{Viability (\%)} = [\text{Nonstained cells} / (\text{Stained cells} + \text{Nonstained cells})] \times 100$$

For the effect of OKY-046 on TXA_2 synthesis by RGM1 cells, TXB_2 in the medium was determined after incubation of the cells with OKY-046 for 24 hr, as described above. Data are expressed as pg TXB_2/ml/hr.

Drugs

OKY-046 ((*E*)-3-[*p*-(1*H*-imidazol-1-ylmethyl)phenyl]-2-propenoic acid; TXA_2 synthase inhibitor) (13) and NS-398 (*N*-[2-(cyclohexyloxy)-4-nitrophenyl]methanesulfonamide; selective COX-2 inhibitor) (14, 15) were kindly provided by Ono Pharmaceutical Co. (Osaka) and Taisho Pharmaceutical Co. (Tokyo), respectively. OKY-046 was dissolved in saline. Indomethacin (Sigma Chemicals, St. Louis, MO, USA) and NS-398 were suspended in a trace of Tween 80 and saline. For evaluation of the effect of OKY-046 on ulcer healing, it was administered twice daily for 14 days starting from day 0 or for 7 days starting from day 0 or day 7. Alternatively, the drugs were administered once on day 3. The drugs were subcutaneously administered in a volume of 5 ml/kg body weight. Control animals received saline alone.

For in vitro study, OKY-046 was dissolved in DMEM containing 1 mg/ml BSA. U-46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$; TXA_2 mimetic) (16, 17) and TXB_2 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). U-46619 and TXB_2 were dissolved in ethanol, followed by dilution with DMEM containing 1 mg/ml BSA. The final concentration of ethanol was 0.1%, at which concentration cell growth and viability were not influenced. Recombinant rat transforming growth factor-α (TGF-α) (Bachem Feinchemikalien AG, Bubendorf, Switzerland) was dissolved in DMEM containing 1 mg/ml BSA.

All other chemicals used here were of reagent grade.

Statistical analyses

The data are presented as means±S.E.M. Statistical differences in the dose-response studies were evaluated by Dunnett's multiple comparison test. Student's *t*-test was also applied to the comparison between two groups. A P value of <0.05 was regarded as significant.

RESULTS

Elevation of TXA_2 synthesis in ulcerated tissue

We examined TXA_2 synthesis in gastric tissues during the healing of acetic acid ulcers (Fig. 1). TXA_2 synthesis in the gastric mucosa of normal stomachs amounted to 9.0±1.8 pg TXA_2/mg/min. TXA_2 synthesis was markedly elevated by gastric ulceration. The synthesis significantly increased by around sixfold during days 3–7, compared with that in the normal stomachs. Thereafter, the synthesis decreased with time. In spite of the presence of ulcers, TXA_2 synthesis did not increase in the intact tissue of stomachs.

We examined the effects of indomethacin (nonselective COX inhibitor), NS-398 (selective COX-2 inhibitor) and OKY-046 (TXA_2 synthase inhibitor) on the increased TXA_2 synthesis in the ulcerated gastric tissue when the drugs were administered once on day 3 (Fig. 2). Indomethacin at 2 mg/kg potently inhibited TXA_2 and PGE_2 syntheses, the inhibition being around 80%. NS-398 at 6 mg/kg had no effect on TXA_2 synthesis, although it significantly reduced PGE_2 synthesis. In contrast, OKY-046 inhibited only TXA_2 synthesis in a dose-related manner. OKY-046 at 10 mg/kg slightly reduced the synthesis, while the drug at 30 and 60 mg/kg significantly inhibited TXA_2 synthesis by around 80%. The maximal effect of OKY-046 was observed even at 30 mg/kg.

Effect of OKY-046 on gastric ulcer healing

We examined the effect of OKY-046 on the healing of gastric ulcers, when administered for 14 days (Fig. 3). OKY-046 at 10 mg/kg did not affect ulcer healing, while the drug at 30 and 60 mg/kg significantly enhanced the healing. The effect of OKY-046 did not differ between 30 and 60 mg/kg, the reduction of ulcerated area being about 30% compared with that in the control. Similarly, TXA_2 synthesis was markedly inhibited by OKY-046 at 30 and 60 mg/kg. The inhibition was around 80%. However, 30 mg/kg OKY-046 did not suppress gastric acid secretion. Acid output was 118.7±18.9 and 134.9±13.5 μEq/hr in the control and OKY-046-treated rats, respectively (n=8). In addition, bleeding in the stomach was not observed in control or 30 mg/kg OKY-046-treated rats throughout the experiment.

Regeneration of the gastric mucosa on the ulcer base was significantly promoted by 30 mg/kg OKY-046 (Table

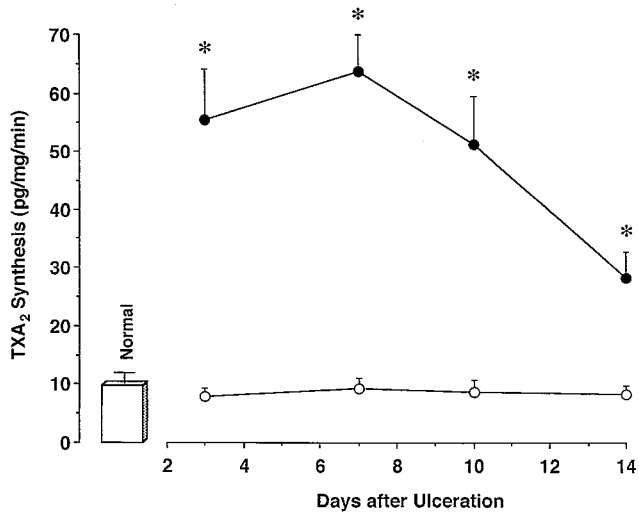

Fig. 1. TXA_2 synthesis in the gastric tissues of rats. On the indicated days after gastric ulceration, TXA_2 synthesis in the intact (○) and ulcerated (●) gastric tissues were determined. The synthesis in the gastric mucosa of normal rats was also determined. The data are presented as means±S.E.M. (n=6). *Significantly different from the normal tissue, $P<0.05$.

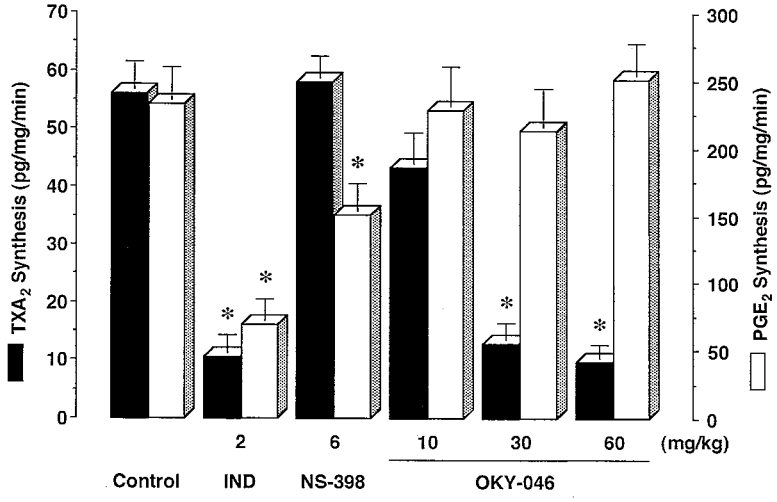

Fig. 2. Effects of bolus administration of indomethacin (IND), NS-398 and OKY-046 on TXA_2 and PGE_2 syntheses in ulcerated gastric tissue of rats. On day 3, TXA_2 and PGE_2 syntheses in ulcerated gastric tissue were determined 3 hr after the administration of indomethacin, NS-398 and OKY-046. The data are presented as means±S.E.M. (n=6). *Significantly different from the corresponding control, $P<0.05$.

1). In contrast, OKY-046 did not affect thickness of the base or density of microvessels in the base.

Furthermore, ulcer healing was also examined when 30 mg/kg OKY-046 was administered for 7 days (from day 0 to day 7, and from day 7 to day 14). The short-term treatments with OKY-046 did not cause an acceleration of ulcer healing. The ulcerated area was 7.9±1.5 and 10.5±0.7 mm^2 for the early and late treatments, respectively.

The administration of 30 mg/kg OKY-046 for 14 days to normal rats did not cause any lesions or ulcers in their stomachs. The height of gastric epithelium in OKY-046-treated rats was not different from that in the control.

Effects of OKY-046, U-46619 and TXB_2 on proliferation of cultured gastric epithelial cells

Furthermore, we examined the direct effects of OKY-046, U-46619 (TXA_2 mimetic) and TXB_2 toward gastric epithelial cells. When gastric epithelial RGM1 cells were incubated with OKY-046, U-46619 and TXB_2, prolifera-

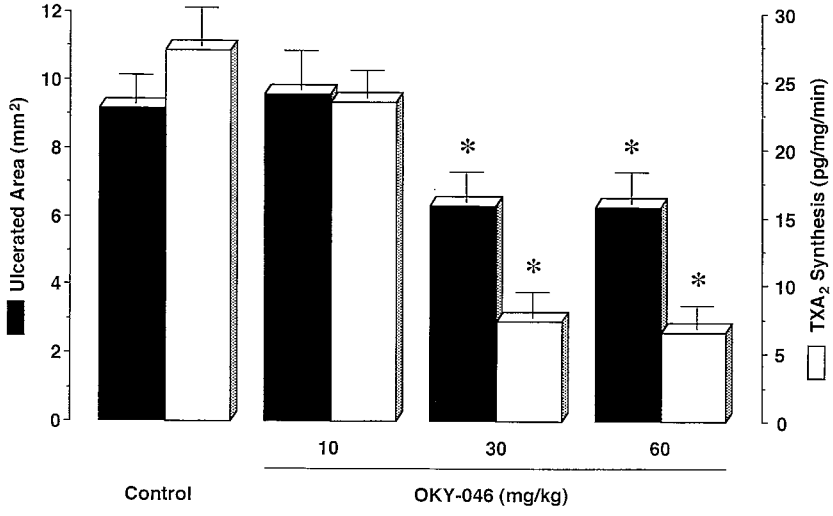

Fig. 3. Effect of repeated administration of OKY-046 on the healing of gastric ulcers and TXA_2 synthesis in ulcerated tissue. OKY-046 was administered twice daily for 14 days starting from day 0, and then the ulcerated area and TXA_2 synthesis in ulcerated tissue were determined. The data are presented as means±S.E.M. (n=8). *Significantly different from the corresponding control, $P<0.05$.

Table 1. Histological evaluation of gastric ulcer healing after repeated administration of OKY-046

	Control	OKY-046 (30 mg/kg)
Length of regenerated mucosa (mm)	1.84±0.08	2.13±0.11*
Thickness of ulcer base (mm)	1.00±0.12	0.89±0.09
Density of microvessels (counts/mm^2)	58.5±4.8	57.3±3.2

OKY-046 was administered twice daily for 14 days starting from day 0, and then the length of the regenerated mucosa, thickness of the ulcer base and microvessel density in the base were histologically determined (n=8). *Significantly different from the control, $P<0.05$.

tion and viability of the cells were determined. OKY-046 even at 100 μM failed to affect proliferation of RGM1 cells (96.5±2.6% in the absence of TGF-α and 101.5±3.0% in the presence of TGF-α, compared with the corresponding control, n=6). At that time, 100 μM OKY-046 significantly inhibited TXA_2 synthesis by RGM1 cells (control, 12.7±3.6 pg TXB_2/ml/hr and OKY-046, 4.7±1.1 pg TXB_2/ml/hr, n=6). In contrast, U-46619 dose-relatedly inhibited RGM1 cell proliferation without any loss of viability (Fig. 4). The significant effect of U-46619 on the basal proliferation was observed at more than 1 nM. Similarly, U-46619 at more than 1 nM significantly inhibited the proliferation stimulated with 10 ng/ml TGF-α. In both cases, the inhibitory effect of U-46619 reached its maximum at the dose of 10 nM. The maximal inhibition by U-46619 was about 30% and about 25% in the basal and TGF-α-stimulated proliferation, respectively. However, 100 nM TXB_2 had no effect on proliferation of RGM1 cells (98.2±3.0% in the absence of TGF-α and 100.0±3.6% in the presence of TGF-α, compared with the corresponding control, n=6).

DISCUSSION

We confirmed that TXA_2 synthesis is elevated in ulcerated tissue, but remains unaffected in intact tissue. Indomethacin inhibited both TXA_2 and PGE_2 syntheses in ulcerated tissue, but NS-398 reduced only PGE_2 synthesis. These results suggest that the increased TXA_2 might be derived from COX-1 in ulcerated tissue, although TXA_2-producing cells in the tissue are unidentified.

We found that inhibition of TXA_2 synthesis causes a significant acceleration of gastric ulcer healing. Repeated administration of OKY-046 for 14 days overall was required for acceleration of ulcer healing, suggesting that TXA_2 persistently exerts an inhibitory effect throughout

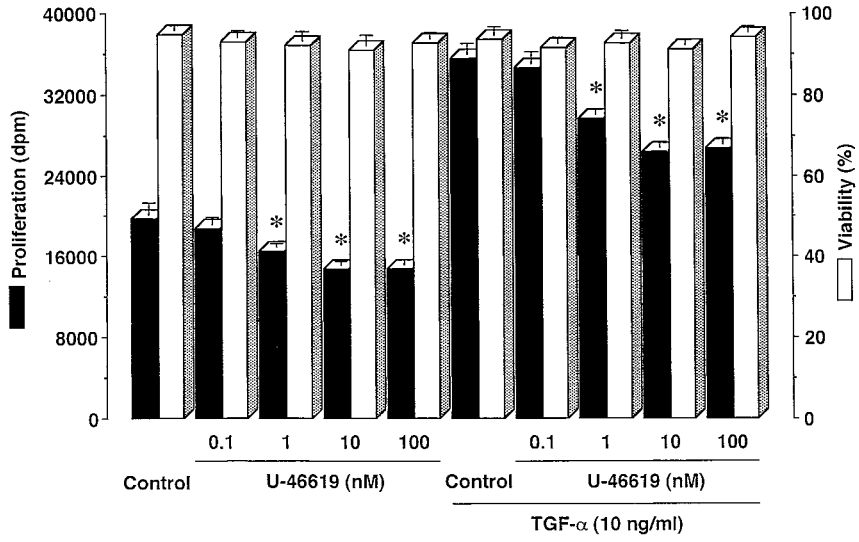

Fig. 4. Effect of U-46619 on proliferation and viability of gastric epithelial RGM1 cells. RGM1 cells were incubated with U-46619 in the absence and presence of TGF-α. Twenty-four hours later, proliferation and viability of RGM1 cells were determined. The data are presented as means±S.E.M. (n=6). *Significantly different from the corresponding control, $P<0.05$.

ulcer healing. Upon histological analysis, regeneration of the gastric mucosa on the ulcer base was significantly promoted by blockade of TXA_2 synthesis. In contrast, neither maturation of the base nor angiogenesis in the base were affected. These results indicate that increased TXA_2 might exert an inhibitory effect on mucosal regeneration, leading to prevention of ulcer healing. It was ruled out that the increased TXA_2 stimulates acid secretion, leading to inhibition of the regeneration, because OKY-046 had no effect on acid secretion. Nonsteroidal anti-inflammatory drugs such as indomethacin are known to impair gastric ulcer healing in rats (18, 19). As described here, indomethacin inhibited both TXA_2 and PGE_2 syntheses, and exogenous PGE_2 prevents the indomethacin-induced delay in ulcer healing (19). Accordingly, the healing-promoting effect of PGE_2 in ulcerated tissue is suggested to be more profound than the inhibitory effect of TXA_2. In fact, the inhibitory effect of TXA_2 was regarded as weak. The maximal reduction of ulcerated area by OKY-046 was about 30%, although OKY-046 potently inhibited TXA_2 synthesis by around 80%. Similarly, the increase in length of regenerated mucosa caused by OKY-046 was around 20%. Certainly, it is possible that $PGF_{2\alpha}$ and PGI_2 syntheses may be elevated by inhibition of TXA_2 synthesis, thereby affecting ulcer healing. However, it is generally accepted that PGE_2 has a potent activity toward gastric ulcer healing among prostanoids, and the amount of synthesized TXA_2 in the ulcerated tissue was substantially smaller than that of PGE_2. It seems that even if $PGF_{2\alpha}$ and PGI_2 syntheses increased, these prostanoids may not largely contribute to ulcer healing.

Furthermore, we showed that U-46619 significantly inhibits proliferation of rat gastric epithelial RGM1 cells without reducing viability. The effect of U-46619 was similarly observed even in the case of TGF-α-stimulated proliferation. On the other hand, TXB_2 had no effect on RGM1 proliferation. These results suggest that TXA_2 itself serves as a growth inhibitor on gastric epithelial cells. Wong et al. (4) reported that exposure of rabbit gastric mucosal cells to TXB_2 causes a significant loss of viability. In their study, gastric mucosal cells were cultured as a suspension, whereas our epithelial cells were grown as a monolayer sheet. Since gastric mucosal cells adhere to the extracellular matrix under physiological conditions, breakdown of the cell-matrix interaction may increase susceptibility to the damaging action of TXB_2. Alternatively, the species difference between rat and rabbit may account for the difference of TXB_2 effects. It is known that TXA_2 stimulates proliferation of vascular smooth muscle cells and endothelial cells (20, 21). Similar to transforming growth factor-β (22), TXA_2 is also considered to be a bifunctional growth regulator. It is likely that the action of TXA_2 (stimulatory or inhibitory) may depend on the cell types.

It was reported that exogenous TXA_2 exerts a vasoconstrictive effect on the gastric mucosal and submucosal microvasculature, thereby aggravating the effect of HCl /taurocholate in rats (1, 3). Our group proposed that an increase in mucosal blood flow around gastric ulcers is important for ulcer healing in rats (23). Consequently, it is likely that reduction of the blood flow by TXA_2 is also involved in the inhibitory effect of TXA_2 on ulcer healing.

TXA_2 is well known to aggregate platelets, contributing to thrombogenesis. However, inhibition of TXA_2 synthesis did not cause bleeding in the ulcerated stomach. Other factors such as platelet-activating factor and thrombin may be activated enough to induce thrombogenesis in gastric ulcers.

There have been several reports that TXA_2 is produced in the normal gastric mucosa (24, 25) and in gastric epithelial cells (26). We also confirmed TXA_2 synthesis in the gastric mucosa of rats and in RGM1 cells. In the present study, the administration of OKY-046 for 14 days to normal rats did not cause any histological change, and OKY-046 did not affect proliferation of RGM1 cells. At present, the physiological significance of TXA_2 in the normal gastric mucosa is unknown.

Overall, we conclude that the increased TXA_2, probably derived from COX-1 in ulcerated tissue, exerts a weak inhibitory effect on ulcer healing in rats. The effect of TXA_2 might be due partly to prevention of gastric epithelial cell proliferation at the ulcer margin.

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