Cyclooxygenase-2/prostaglandin E₂ accelerates the healing of gastric ulcers via EP₄ receptors

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Hatazawa R, Tanaka A, Tanigami M, Amagase K, Kato S, Ashida Y, Takeuchi K. Cyclooxygenase-2/prostaglandin E₂ accelerates the healing of gastric ulcers via EP₄ receptors. Am J Physiol Gastrointest Liver Physiol 293: G788–G797, 2007. First published August 2, 2007; doi:10.1152/ajpgi.00131.2007.—We examined the involvement of cyclooxygenase (COX)-1 as well as COX-2 in the healing of gastric ulcers and investigated which prostaglandin (PG) EP receptor subtype is responsible for the healing-promoting action of PGE₂. Male SD rats and C57BL/6 mice, including wild-type, COX-1(-/-), and COX-2(-/-), were used. Gastric ulcers were produced by thermocauterization under ether anesthesia. Gastric ulcer healing was significantly delayed in both rats and mice by indomethacin and rofecoxib but not SC-560 given for 14 days after ulceration. The impaired healing was also observed in COX-2(-/-) but not COX-1(-/-) mice. Mucosal PGE₂ content increased after ulceration, and this response was significantly suppressed by indomethacin and rofecoxib but not SC-560. The delayed healing in mice caused by indomethacin was significantly reversed by the coadministration of rofecoxib but not SC-560. The delayed healing in mice caused by indomethacin was significantly reversed by the coadministration of rofecoxib but not SC-560. The delayed healing in mice caused by indomethacin was significantly reversed by the coadministration of rofecoxib but not SC-560. The delayed healing in mice caused by indomethacin was significantly reversed by the coadministration of rofecoxib but not SC-560.

Many studies have reported that the healing-impairment effect of NSAIDs is shared by selective COX-2 inhibitors (2, 15, 27, 34, 45), suggesting an important role for COX-2/PGE₂ in the mechanism of ulcer healing. Mizuno et al. (27) first demonstrated that both COX-2 mRNA and protein were strongly expressed in mouse stomachs in which ulcers had been induced. Jones et al. (19) reported that both COX-1 and COX-2 are important for the regulation of angiogenesis and that selective COX-2 inhibitors inhibit angiogenesis through direct effects on endothelial cells, similar to conventional NSAIDs. Recently, Schmaassmann et al. (33) reported that gastric ulcer healing was unaffected in COX-1-deficient mice or those treated with SC-560, the selective COX-1 inhibitor. They further showed that inhibition of both COX-1 and COX-2 delayed the healing more markedly than inhibition of COX-2 alone and suggested that COX-1-derived PGs may be important in the healing mechanism when COX-2-derived PGs are deficient. Thus a definitive answer has not been obtained about the involvement of COX-1 in the mechanism of gastric ulcer healing.

On the other hand, PGE₂ exerts its diverse effects by binding to four different EP receptor subtypes, EP₁ through EP₄, resulting in the activation of different intracellular signal-transduction pathways (16a, 29). EP₁ receptors lead to increases in intracellular calcium through a Gₛ-independent mechanism; EP₂ and EP₃ receptors couple to Gₛₐ protein, leading to the elevation of cAMP; EP₃ receptor exists in multiple splice variants generated by alternative splicing of the COOH-terminal tail and is coupled to Gₛₐ and Gₛₐ proteins. We recently found that endogenous PGE₂ plays a role in the healing of NSAID-induced intestinal ulcers through EP₄ receptors (16). However, it still remains unknown how PGE₂ contributes to the mechanism of gastric ulcer healing, including the involvement of the EP receptor subtype.

In the present study, we examined the effects of various COX inhibitors on the healing of gastric ulcers in rats and mice and also compared the spontaneous healing of gastric ulcers in wild-type mice and those lacking COX-1 or COX-2. We also...
investigated which EP receptor subtype is responsible for the healing-promoting action of PGE\(_2\) by using various EP agonists and antagonists.

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley rats (200–260 g; Nippon Charles River, Shizuoka, Japan) and male C57BL/6 mice (20–25 g) of wild-type, COX-1(-/-), and COX-2(-/-) genotypes were used. Wild-type mice were purchased from SLC (Shizuoka, Japan), whereas those lacking COX-1 or COX-2 were purchased from Taconic (Hudson, NY). The distribution of the COX-1 or COX-2 genes was verified by Northern blot hybridization, which failed to detect messenger RNAs encoding the respective receptors in COX-1(-/-) or COX-2(-/-) mice. All experimental procedures described here were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University.

**Induction of gastric ulcers.** Chronic gastric ulcers were induced in rats and mice by thermal cauteryization, according to a method described previously (18). Under ether anesthesia, the stomach was exposed through a midline incision, the electric probe (Fuchigami, Kyoto, Japan; diameter: 8 mm\(^2\) for rat and 5 mm\(^2\) for mouse) was attached to the mid-corpus mucosa, and a gastric ulcer was induced by heating the tip at 70°C for 20 s. Various COX inhibitors (indomethacin (2 mg/kg), SC-560 (3 mg/kg for rats; 5 mg/kg for mice), rofecoxib (3 and 10 mg/kg for rats; 5 mg/kg for mice), CJ-42794 (2 mg/kg), SC-560 (3 mg/kg for rats; 5 mg/kg for mice), or rofecoxib (3 mg/kg for rats; 5 mg/kg for mice) once daily for the last 7 days. In a separate experiment, the PGE\(_2\) content was also measured in the gastric mucosa before and after ulceration in wild-type, COX-1(-/-), and COX-2(-/-) mice. Under deep ether anesthesia, the animals were killed, the stomach was removed, and the corpus mucosa was isolated, weighed, and put in a tube containing 100% methanol plus 0.1 mM indomethacin (13). The tissues were then minced with scissors, homogenized with a Polytron homogenizer (IKA, Tokyo, Japan), and centrifuged at 12,000 g for 10 min at 4°C. After the supernatant of each sample had been evaporated with N\(_2\) gas, the residue was resolved in assay buffer and used for the determination of PGE\(_2\). The concentration of PGE\(_2\) was measured using a PGE\(_2\) EIA kit (Amersham Biosciences, Little Chalfont, UK).

**Analysis for gene expression of EP\(_4\) receptors by RT-PCR.** Expression of EP\(_4\) receptor mRNA was examined in the rat gastric mucosa at various time points after ulceration. The animals were killed 0, 3, and 7 days after ulceration. The ulcerated mucosa was removed, frozen in liquid nitrogen, and stored at −80°C before use. The expression of EP\(_4\) receptor mRNA was analyzed by RT-PCR. Total RNA was extracted, primed by random hexadecoxynucleotide, and reverse transcribed with a Superscript preamplification system. The sequences of sense and antisense primers for rat EP\(_4\) receptor were 5'-CCCTGACGCGCTCAGT GACCTTT-3' and 5'-CTTGCTTCGAGGCCGCTTTAGTGT-3', respectively, giving rise to a 488-bp PCR product (38). For the rat GAPDH, a constitutively expressed gene, the sequences were 5'-GAACCAGGAAGCTCAGT GCATGGC-3' for the sense primer and 5'-TGAGGTCCACCACTCT GTTGGC G-3' for the antisense primer, giving rise to a 310-bp PCR product. An aliquot of the RT reaction product served as a template in 35 cycles of PCR with 1 min of denaturation at 94°C, 0.5 min of annealing at 58°C, and 1 min of extension at 72°C on a thermal cycler. A portion of the PCR mixture was electrophoresed in 1.8% agarose gel in TAE buffer (40 mM Tris buffer, 2 mM EDTA, and 20 mM acetic acid; pH 8.1), and the gel was stained with ethidium bromide and photographed.

**Immunostaining of COX-2 and VEGF.** Expression of COX-2 and VEGF was immunohistochemically examined in the rat gastric mucosa 5 days after ulceration. The stomachs were excised and placed into 4% buffered formalin. Then, small pieces of tissue containing ulcer area were embedded in paraffin and sectioned at a thickness of 4 μm. For evaluation of COX-2 or VEGF, sections were incubated with the antibody for COX-2 or VEGF (Santa Cruz Biotechnology). The immune complex was visualized by using the avidin-biotin-peroxidase complex method with the use of a Vectastain ABC-peroxidase kit (Vector, Burlingame, CA). Sections were successively stained with hematoxylin.

**Western blotting.** The mouse stomachs were homogenized in ice-cold 50 mM Tris·HCl buffer (pH 7.4.), containing 1 mM phenylmethylsulfonyl fluoride, 1 mM diithiothreitol, 32 mM sucrose, 10 μg/ml soybean trypsin inhibitor, 10 mg/ml leupeptin, and 2 μg/ml aprotinin. The homogenized samples or cell samples were sonicated and centrifuged at 100,000 g for 1 h at 4°C. The supernatant was removed, and the pellet was resuspended in the homogenized buffer. The protein concentration of the suspension was determined by using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). The samples (10 μg/lane) were then electrophoresed on 14% SDS-polyacrylamide gels and were transferred electrophoretically to nitrocellulose membranes (Protran; Schleicher & Schuell, Dassel, Germany). The membranes were incubated with anti-VEGF antibody (Santa Cruz Biotechnology) and were treated with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology). The immune complex was visualized by using the avidin-biotin-peroxidase complex method with the use of a Vectastain ABC-peroxidase kit (Vector, Burlingame, CA). Sections were successively stained with hematoxylin.
plexes were visualized by using the enhanced chemiluminescence detection system (Western Blot Chemiluminescence Reagent Plus; NEN, Boston, MA).

VEGF production in primary rat gastric fibroblasts. Primary gastric fibroblasts were isolated from the rat stomach and were cultured according to a previous study (3). In brief, the corpus mucosa was separated from the rat stomachs, minced into 2- to 3-mm² pieces, and then incubated in phosphate-buffered saline containing collagenase (0.35 mg/ml; Sigma, St. Louis, MO) at 37°C. After being passed through a 300-μm metal filter, the cell suspension was transferred into collagen-coated tissue plates. The cells were cultured in a mixture of Ham’s F-12 medium and Dulbecco’s modified minimum essential medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin in a humidified atmosphere of

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**Fig. 1.** Effects of various cyclooxygenase (COX) inhibitors on healing of gastric ulcers (A and C) and prostaglandin (PG) E₂ content in stomach with or without ulcers (B and D) in rats and mice. Gastric ulcers were produced by thermocauterization (70°C, 20 s), and animals were killed 17 days after ulceration. Indomethacin (2 mg/kg), SC-560 (3 mg/kg for rat and 5 mg/kg for mouse), or rofecoxib (3 mg/kg for rat and 5 mg/kg for mouse) was given periorally once daily for 14 days starting 3 days after ulceration. Mucosal PGE₂ content was measured by EIA. Data are presented as means ± SE from 6 rats. Significant difference at P < 0.05 (*from normal; #from control).

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**Fig. 2.** A: spontaneous healing of gastric ulcers in wild-type, COX-1(−/−), or COX-2(−/−) mice. Gastric ulcers were produced by thermocauterization (70°C, 20 s), and animals were killed 3, 10, or 17 days after ulceration. Data are presented as means ± SE from 6–7 mice. *Significant difference from corresponding control at P < 0.05. B: microscopic observation of gastric ulcers 10 days after ulceration in control (wild-type) and COX-2(−/−) mice. C: changes in PGE₂ content in gastric mucosa of wild-type, COX-1(−/−), and COX-2(−/−) mice with or without ulceration. Gastric ulcers were produced by thermocauterization (70°C, 20 s), and animals were killed 10 days after ulceration. Mucosal PGE₂ content was measured by EIA. Data are presented as means ± SE from 9–10 mice. Significant difference at P < 0.05 (*from corresponding values in intact mucosa; #from values in ulcerated mucosa of wild-type mice).
Expression of EP4 receptor mRNA was analyzed by RT-PCR.

RESULTS

Effects of various COX inhibitors on the healing of gastric ulcers and the PGE2 content of ulcerated mucosa in rats and mice.

Gastric ulcers produced in rats healed quite rapidly within the first 7 days after ulceration, followed by a gradual healing; the ulcer area decreased to 1/5 of the initial size within 17 days (data not shown). The healing of gastric ulcers was markedly delayed by indomethacin (2 mg/kg) given periorally once daily for 14 days from 3 days after ulceration, the ulcer area being 8.2 ± 1.6 mm², which was significantly greater than that (1.8 ± 0.2 mm²) of the control rats (Fig. 1A). Likewise, the healing was also significantly impaired by the selective COX-2 inhibitor rofecoxib (3 mg/kg) given once daily for 14 days, whereas the selective COX-1 inhibitor SC-560 (3 mg/kg) had no effect. The importance of COX-2 in the healing of gastric ulcers was confirmed in mice. Gastric ulcers produced in mice also started to heal from 3 days after ulceration and became quiescent within 10 days (data not shown). Similar to rats, the healing of gastric ulcers was significantly impaired by indomethacin (2 mg/kg) and rofecoxib (5 mg/kg) but not SC-560 (5 mg/kg) when these agents were given periorally once daily for 14 days starting 3 days after ulceration (Fig. 1C).

Levels of PGE2 markedly increased in the mucosa on day 7 after ulceration and reached a value of 40.3 ± 7.0 pg/mg tissue, about eight times that in normal rats (Fig. 1B).
PGE$_2$ content was significantly suppressed by the repeated perioral administration of indomethacin (2 mg/kg) and rofecoxib (3 mg/kg) for 7 days, the values approaching normal levels, whereas SC-560 (3 mg/kg) had no effect on the increased PGE$_2$ content. Similar results were obtained in mice concerning the changes in PGE$_2$ content in the ulcerated mucosa and the effects of various COX inhibitors, although absolute values of PGE$_2$ content were slightly smaller than those in rat stomachs. As shown in Fig. 1D, the PGE$_2$ content was increased on day 7 after ulceration and reached a value of 27.7 ± 5.0 pg/mg tissue, more than six times as much as that in normal animals, and this response was significantly inhibited by indomethacin (2 mg/kg) and rofecoxib (5 mg/kg) but not SC-560 (5 mg/kg) given periorally once daily for 7 days.

Spontaneous healing of gastric ulcers in wild-type and COX-1 or COX-2 knockout mice. To further investigate the involvement of COX isozymes in ulcer healing, we compared the healing of gastric ulcers in wild-type, COX-1(−/−), and COX-2(−/−) mice. Gastric ulcers in wild-type mice healed rapidly within 7 days, from 11.2 ± 0.8 mm$^2$ to 2.4 ± 0.3 mm$^2$, followed by a gradual healing, the ulcer area on day 17 being 0.9 ± 0.2 mm$^2$ (Fig. 2A). Similarly, gastric ulcers in COX-1(−/−) mice healed quite rapidly within 7 days after ulceration, just as seen in wild-type mice, and the ulcer area on day 10 or 17 was not significantly different from those in controls (wild-type mice). However, in COX-2(−/−) mice, the spontaneous healing was significantly impaired compared with the control animals; the ulcer area on day 10 was 6.5 ± 2.2 mm$^2$, which was about three times as large as that in wild-type or COX-1(−/−) mice. On day 17 after ulceration, the ulcer area in COX-2(−/−) mice was still significantly larger compared with both control and COX-1(−/−) animals, although the rapidity of healing from day 10 to day 17 was somewhat greater in COX-2(−/−) animals compared with control animals. The histological observation of gastric ulcers on day 10 clearly showed that the ulcer area in a COX-2(−/−) mouse was much greater than that in a control (wild-type) animal (Fig. 2B).

In wild-type mice, the PGE$_2$ content was significantly increased in the ulcerated mucosa on day 10 after ulceration, about five times greater than that observed in the stomach without ulceration (Fig. 2C). Likewise, COX-1(−/−) mice also showed a significant increase in the PGE$_2$ production in the gastric mucosa after ulceration, although the basal levels were significantly lower compared with those in wild-type animals. On the other hand, ulceration did not significantly increase the PG production in the stomachs of COX-2(−/−) mice, and the mucosal PGE$_2$ content remained in the same range of intact stomach even 10 days after ulceration.

Effects of various EP agonists on delayed healing of gastric ulcers induced by indomethacin in mice. To investigate which EP receptor subtype is involved in the healing of gastric ulcers, we examined the effects of various EP agonists on the delayed healing induced by indomethacin in mice. As shown in Fig. 3A,
the healing of the gastric ulcer was markedly impaired by indomethacin (2 mg/kg) given periorally once daily for 14 days starting 3 days after ulceration, the ulcer area on day 17 being 8.4 ± 0.9 mm², which is about four times as large as that (1.4 ± 1.2 mm²) of controls. The healing-impairment effect of indomethacin was significantly antagonized by cotreatment with 11-deoxy-PGE₁ (1 mg/kg), the EP₃/EP₄ agonist, given intraperitoneally twice daily for 14 days, and the ulcer area on day 17 was 2.0 ± 0.9 mm², almost equivalent to that in controls. In contrast, other prostanoids, such as 17-phenyl-PGE₂ (EP₂ agonist), butaprost (EP₂ agonist), and NT-012 (EP₃ agonist), did not affect the delayed healing caused by indomethacin.

The gene expression of EP₄ receptor was observed in the mouse gastric mucosa, without or with ulcers (Fig. 3B). In addition, the expression of EP₄ receptor mRNA remained unchanged on days 0, 3, and 7 after ulceration.

Effect of EP₄ antagonist on the healing of gastric ulcers in rats and mice. To further confirm the involvement of EP₄ receptors in the healing-promoting action of PGE₂, we examined the effect of CJ-42794 (EP₄ antagonist) on the ulcer healing in both rats and mice compared with that of rofecoxib.

The daily administration of rofecoxib (3 and 10 mg/kg) in rats for 14 days significantly impaired the healing of gastric ulcers in a dose-dependent manner, the ulcer area at 10 mg/kg being 5.8 ± 1.5 mm², about three times greater than that in control (Fig. 4, A and B). Likewise, CJ-42794 (3 and 10 mg/kg) given once daily for 14 days also impaired the healing of gastric ulcers in a dose-dependent manner, and a significant effect was observed at 10 mg/kg; the ulcer area was 6.8 ± 1.7 mm², almost equivalent to that observed in the animals treated with 10 mg/kg of rofecoxib. The same results were obtained in mice, and CJ-42794 (10 mg/kg) as well as rofecoxib (5 mg/kg) given for 14 days markedly delayed the ulcer healing, the ulcer area on day 17 being 0.80 ± 0.19 mm² and 0.86 ± 0.08 mm², respectively, both of which are significantly greater than that (0.28 ± 0.03 mm²) in the control mice (Fig. 4C).

Immunostaining of COX-2 and VEGF in ulcerated mucosa of the rat stomach. VEGF is a fundamental regulator of angiogenesis, the essential component of wound healing, in addition to COX-2/PGs (44). To confirm VEGF expression in gastric ulcer tissue, we examined the distribution of VEGF as well as COX-2 in the ulcerated mucosa of rat stomachs on day 5 after ulceration. As expected, the expression of COX-2 was apparently observed in gastric ulcer tissue, especially in the base of the ulcer (Fig. 5, A, C, and E). Likewise, the immunostaining of VEGF was also observed in the same area of the ulcerated mucosa, suggesting the colocalization of these proteins (Fig. 5, B, D, and F).

Effect of COX inhibitors and EP₄ antagonist on the expression of VEGF in ulcerated mucosa of the mouse stomach. Many studies demonstrated that the COX-2/VEGF pathway is involved in gastric ulcer healing (3, 7, 14), yet it remains unknown which EP receptor subtype is involved in the stimulatory effect of PGE₂ on VEGF expression. We examined the effects of various COX inhibitors and the EP₄ antagonist on VEGF expression in the ulcerated mucosa of the mouse stomach.

On conventional Western blot analysis, VEGF protein was constitutively expressed in both the normal mucosa and the ulcerated mucosa on day 10 after ulceration, although the expression was clearly upregulated in the latter (Fig. 6). However, the expression of VEGF in the ulcerated mucosa was apparently downregulated when the animals were treated with indomethacin (2 mg/kg) and rofecoxib (5 mg/kg) but not SC-560 (5 mg/kg) once daily for 7 days, and the immunoreactivity of VEGF was significantly reduced in these animals. Likewise, CJ-42794 (10 mg/kg po) also significantly suppressed the increase of VEGF expression, similar to rofecoxib.

Effects of a selective COX-2 inhibitor or an EP₄ antagonist on angiogenesis in the mouse stomach. VEGF is a highly specific stimulator of endothelial cells and may play an important role in angiogenesis in the process of ulcer healing (38, 44). Therefore, we examined the effects of rofecoxib and CJ-42794 on the angiogenic response in the mouse gastric mucosa after ulceration.

On day 10 after ulceration, the ulcer base was spontaneously reconstructed by the growth of granulation tissue and newly formed microvasculature (angiogenesis), as represented by factor VIII-positive cells (not shown). One-week treatment with indomethacin (2 mg/kg) and rofecoxib (5 mg/kg) apparently prevented the growth of granulation in the ulcer base; the degree of revascularization was 8.1 ± 0.6 microvessels/mm² and 7.8 ± 0.5 microvessels/mm², respectively, both of which were significantly less than that (20.0 ± 0.3 microvessels/mm²) in control mice. Likewise, CJ-42794 (10 mg/kg) also significantly decreased the angiogenic response, the degree of revascularization being 6.5 ± 0.4 microvessels/mm².
Effect of PGE₂ on VEGF expression in rat gastric fibroblasts.

Because Miura et al. (26) reported that COX-2 plays a key role in VEGF production in gastric fibroblasts stimulated by interleukin-1 in vitro, we examined the effects of PGE₂ and AE1-329 (EP₄ agonist) on VEGF protein expression in primary rat gastric fibroblasts in the absence or presence of CJ-42794 (EP₄ antagonist).

When PGE₂ (10 µM) was coincubated with gastric fibroblasts for ~3–6 h, this prostanoid increased the expression of VEGF in a time-dependent manner, a maximal response (162.7 ± 21.5%) being observed after 6 h incubation (Fig. 7A).

The coincubation of PGE₂ (0.1–10 µM) with the fibroblasts for 6 h dose-dependently increased the VEGF expression, the degree of increase at 10 µM being 164.2 ± 24.3% (Fig. 7B).

The increase of VEGF expression caused by PGE₂ (10 µM) or AE1-329 (10 µM) was totally inhibited by the coincubation of CJ-42794 (0.5 mM) (Fig. 7C).

Because the activation of EP₄ receptors is coupled to the Gₛ protein, resulting in elevation of cAMP (29), we further confirm the relationship between adenylate cyclase/cAMP and VEGF expression in gastric fibroblasts. The coincubation of forskolin (0.1 and 1 µM), a stimulator of adenylate cyclase, with the fibroblasts for 6 h increased the VEGF expression in a dose-dependent manner, the degree of increase at 1 µM being 56.3 ± 14.1% (Fig. 8). Likewise, the increase of VEGF expression in the fibroblasts was also observed by the coincubation of dbcAMP (10 and 100 µM) for 6 h, the degree of increase being 43.8 ± 11.2% and 84.4 ± 27.4%, respectively.

**DISCUSSION**

Many studies have demonstrated the upregulation of COX-2 expression in the ulcerated gastric mucosa, concomitant with an increase in endogenous PG production, and suggested that COX-2/PGs play a pivotal role in promoting the healing of gastric ulcers (2, 15, 27, 34, 45). Concerning the involvement of COX-1 in the ulcer healing, however, only very few studies have so far been reported, despite the fact that this enzyme is constitutively expressed in various tissues, including the stomach. Schmassmann et al. (33) recently reported that, although COX-1 has no significant role in healing when COX-2 is unimpaired, it becomes important when COX-2 is impaired. We also showed that endogenous PGE₂ plays a role in the healing of intestinal ulcers through EP₄ receptors, yet the COX isozyme involved differs depending on the stage of healing, COX-2 in the early stage and COX-1 in the late stage (16). Thus the involvement of COX-1 in gastric ulcer healing remains uncertain, as does the EP receptor subtype responsible for the healing-promoting action of PGE₂.
PGE2 exerts its diverse effects by binding to four different EP receptor subtypes, named EP1 through EP4 (16a, 29, 40). For example, PGE2 exhibits a gastroprotective action against necrotizing agents or indomethacin through EP1 receptors (39, 40); stimulates HCO3 secretion in the stomach or the duodenum, mediated by EP1 or EP3/EP4 receptors, respectively (1); causes a dual effect on acid secretion, inhibition by the activation of EP3 receptors and stimulation mediated by EP4 receptors (22); and also protects gastric mucosal cells from apoptosis via EP2 and EP4 receptors (17). However, it remains unexplored which EP receptor subtype is responsible for the healing-promoting action of PGE2 for gastric ulcers. To investigate which EP receptor subtype is involved in the healing of gastric ulcers, we examined in the present study the effect of various EP agonists on the delayed healing caused by indomethacin. As a result, the healing-impairment effect of indomethacin was significantly reversed only by the coadministration of 11-deoxy-PGE1, the EP3 agonist, but not of other EP receptor agonists, including 17-phenyl-PGE2 (EP1 agonist), butaprost (EP2 agonist), and NT-012 (EP3 agonist). These results are in agreement with our recent observation that endogenous PGE2 contributes to the healing of indomethacin-induced intestinal ulcers via the activation of EP2 receptors (16) and suggest the involvement of EP4 receptors in the healing-promoting action of PGE2 on gastric ulcers. This contention is further supported by the fact that the healing of gastric ulcers in rats and mice was markedly delayed by the daily administration of CJ-42794, the EP4 antagonist, in addition to indomethacin or rofecoxib. In a preliminary study, we confirmed that CJ-42794 did not affect the upregulation of COX-2 expression and the increase in PGE2 production in the gastric mucosa after ulceration (data not shown). Accordingly, it is assumed to be highly likely that the PGE2 produced by COX-2 accelerates the healing of gastric ulcers by the activation of EP4 receptors.

The healing mechanism in wounded tissues involves multiple steps, such as the formation of granulation tissue, the contraction of the ulcerated tissue, and reepithelialization (44), and these processes are regulated by growth factors, such as VEGF, EGF, basic FGF (bFGF), and other cytokines produced locally by regenerating cells (12, 38, 46). Angiogenesis, the essential component of wound healing (44), is induced by VEGF, which is known as a fundamental regulator of angiogenesis (10, 37). Szabo et al. (37) reported that exogenous VEGF enhanced the healing of duodenal ulcers. Ernst et al. (10) demonstrated that the local injection of bFGF to the base of gastric ulcers significantly accelerated the healing, in association with the increase in the amount of microvasculature and mucosal blood flow at the ulcerated area. Furthermore, they also showed that neutralization of endogenous bFGF by using a specific antibody caused a marked delay in gastric ulcer healing with less angiogenesis (10). More recently, Johnes et al. (20) reported that the gene therapy of VEGF significantly promoted the healing of gastric ulcers and that inhibition of accelerated healing by a neutralizing anti-VEGF antibody indicates an essential role for VEGF and enhanced angiogenesis in ulcer healing, strongly supporting the relationship between VEGF and ulcer healing. As expected, we found in the present study that rofecoxib, but not SC-560, significantly mitigated the angiogenic response in the ulcerated mucosa, similar to indomethacin, as evidenced by the immunohistochemical

Fig. 8. Effects of forskolin and dbcAMP on VEGF expression in primary rat gastric fibroblasts. After 24 h starvation, gastric fibroblasts were incubated with forskolin (0.1 and 1 μM) or dbcAMP (10 and 100 μM) for 6 h. A: expression of VEGF was determined by Western blotting. B: densitometric quantification was determined by Quantity One software. Results are expressed as %control and represent means ± SE from 4 experiments. *Significant difference from control at P < 0.05.
staining with factor VIII, as well as the determination of the number of microvessels. In addition, rofecoxib downregulated the expression of VEGF protein in the gastric mucosa after ulceration, similar to indomethacin. It is understandable that both indomethacin and rofecoxib suppressed the expression of VEGF through the inhibition of COX-2/PGE
production.

Miura et al. (26) found that the expression of COX-2 and VEGF was colocalized in fibroblast-like cells in the ulcer bed of human gastric tissues. In the present study, we also observed the colocalization of COX-2 and VEGF in the ulcerated mucosa, confirming a close relationship between COX-2 expression and VEGF production. To further investigate whether PGE
from COX-2 upregulates the expression of VEGF via EP
receptors, we used the effect of the EP
antagonist on VEGF expression in the stomach after ulceration both in vivo and in vitro. As expected, we found that the EP
antagonist CJ-42794 almost completely inhibited the increase of VEGF expression in the stomach after ulceration, similar to indomethacin. Furthermore, CJ-42794 also inhibited the angiogenic response in the ulcerated mucosa to decrease the number of microvessels. These results suggest that endogenous PGE
stimulates both VEGF expression and angiogenesis in the ulcerated mucosa through the activation of EP
receptors.

VEGF is expressed in various kinds of cells upon stimulation by PGE
or cytokines (3, 5, 7, 8, 14, 44). Miura et al. (26) reported that PGE
derived from COX-2 stimulated the release of VEGF in fibroblasts isolated from human gastric ulcers. In the present study, we confirmed the increase of VEGF release due to PGE
in rat gastric fibroblasts in a dose-dependent manner and further showed that the action was mimicked by AE1-329, the EP
agonist, but was completely inhibited by CJ-42794, the EP
antagonist. These results demonstrated for the first time that PGE
stimulates VEGF release in gastric fibroblasts via the activation of EP
receptors. At present, the exact mechanism by which PGE
stimulates VEGF expression via EP
receptors in gastric fibroblasts is unknown. Ding et al. (9) reported that PGE
upregulates VEGF expression in gastric cancer cells via the transactivation of EGF receptors. Spinella et al. (36) reported that PGE
regulated VEGF production and ovarian carcinoma cell invasion via EP
signaling. Other studies showed that PGE
induces MAP kinase activation with or without the involvement of EGF receptor transactivation (23, 25). Furthermore, PGE
has been reported to induce VEGF via SP-1 binding sites on the VEGF promoter via EP
receptors in a CAMP- and PKA-dependent mechanism (4). Indeed, we confirmed in the present study that the release of VEGF from the rat gastric fibroblast in vitro was significantly increased by forskolin as well as dbcAMP. Further study is certainly needed to elucidate this point.

On the basis of all the results of the present study, we conclude that endogenous PGE
derived from COX-2 plays an important part in the spontaneous healing of gastric ulcers and that COX-1/PGE
does not contribute greatly to the ulcer healing. Our data also indicate that the healing-promoting action of PGE
is associated with the increase of angiogenesis through the upregulation of VEGF expression in the fibroblasts of the gastric ulcer bed or margin via the activation of EP
receptors. Finally, because VEGF stimulates cell proliferation and migration of epithelial cells and by so doing contributes to the reconstitution of the ulcerated mucosa in the stomach.

GRANTS

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