Cyclooxygenase-2/prostaglandin E$_2$ accelerates the healing of gastric ulcers via EP$_4$ receptors

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It is well known that nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin and indomethacin, not only damage the gastrointestinal mucosa but impair the healing of preexisting ulcers as well (24, 32, 43, 46, 48). These effects of NSAIDs are considered to be brought about by a deficiency of prostaglandins (PGs) because of the inhibition of cyclooxygenase (COX). COX exists in two isoforms; COX-1 is found constitutively expressed in various tissues, including the stomach, whereas COX-2 does not appear to be expressed or is expressed at very low levels in most tissues and is rapidly upregulated in response to growth factors and cytokines (11, 21, 30, 35). Recently, a third isoform of this enzyme family, COX-3, has been proposed to have implications for the prescription of both existing and new-generation anti-inflammatory drugs (6, 49). This isoform was later found to be identical in sequence to COX-1, and like its counterpart COX-1, it does not generally appear to be induced by acute inflammatory stimulation (6).

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/PG 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, Schmassmann 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, PGE$_2$ exerts its diverse effects by binding to four different EP receptor subtypes, EP$_1$ through EP$_4$, resulting in the activation of different intracellular signal-transduction pathways (16a, 29). EP$_1$ receptors lead to increases in intracellular calcium through a G$_q$-independent mechanism; EP$_2$ and EP$_4$ receptors couple to G$_i$, leading to the elevation of cAMP; EP$_3$ receptor exists in multiple splice variants generated by alternative splicing of the COOH-terminal tail and is coupled to G$_q$, G$_i$, and G$_s$ proteins. We recently found that endogenous PGE$_2$ plays a role in the healing of NSAID-induced intestinal ulcers through EP$_4$ receptors (16). However, it still remains unknown how PGE$_2$ 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 PGE2 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 mm² for rat and 5 mm² 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), 11-deoxy-PGE1 (EP3/EP4 agonist; 1 mg/kg), or 4-{1-[5-chloro-2-(4-fluorophenyoxy)benzamido]ethyl}benzoic acid; a selective EP4 antagonist; 3 and 10 mg/kg for rats; 10 mg/kg for mice) were given periorally once daily for 7 days starting 3 days after ulceration. Indomethacin (2 mg/kg), rofecoxib (5 mg/kg), or CJ-42794 (3 and 10 mg/kg for rats; 5 mg/kg for mice), were given subcutaneously twice daily for 14 days starting 3 days after ulceration. Control animals were given subcutaneous saline twice daily for 14 days. The doses of EP agonists were chosen to induce the respective pharmacological action, according to the previous studies (1, 13, 22, 40). In addition, the spontaneous healing of gastric ulcers was also compared in wild-type, COX-1(−/−), and COX-2(−/−) mice.

On various days (3, 10, and 17 days) after ulceration, the animals were killed under deep ether anesthesia. The stomach was removed, inflated by injecting 10 ml (rats) or 0.8 ml (mice) of 1% formalin for 10 min to fix the inner wall, and opened along the greater curvature. The ulcer area (mm²) of the stomach was measured under a dissecting microscope with a square grid (×10). The person measuring the ulcer area did not know the treatments given to the animals.

**Histological observations and evaluation of angiogenesis.** Angiogenesis was examined in the ulcerated mucosa of mice 10 days after ulceration. Indomethacin (2 mg/kg), rofecoxib (5 mg/kg), or CJ-42794 (10 mg/kg) was given periorally once daily for 7 days starting 3 days after ulceration. At the time of autopsy, 12-µm frozen sections were prepared. For the evaluation of angiogenesis, sections were incubated with an antibody for von Willebrand factor (factor VIII-related endothelial antigen; Dako, Glostrup, Denmark) after the deactivation of endogenous peroxidase with 0.3% H2O2 and the blockade of nonspecific binding sites was performed. The microvasculature was visualized by the avidin-biotin-peroxidase complex method by using 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 dithiothreitol, 32 mM sucrose, 10 µg/ml soybean trypsin inhibitor, 10 µg/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 biocinchoninic 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-com
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-H9262m 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 g/ml streptomycin, and 100 U/ml penicillin in a humidified atmosphere of

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).

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).
5% CO₂ at 37°C. The obtained cells were vimetin positive. After reaching confluence, gastric fibroblasts were incubated for 24 h without serum in 100-mm dishes and were stimulated with PGE₂ or AE1-329 (EP₄ agonist) (1) for 6 h in the absence or presence of CJ-42794 (EP₄ antagonist) (41). The cells were collected and sonicated, and then they were subjected to Western blotting of VEGF as described. In some cases, the effects of forskolin (0.1 and 1 μM) and dibutyryl (db) cAMP (10 and 100 μM) on VEGF protein expression in gastric fibroblasts were also examined.

Preparation of drug. The drugs used were indomethacin (Sigma), SC-560, butaprost (Cayman Chemicals, Ann Arbor, MI), rofecoxib (synthesized in our laboratory), PGE₂, 17-phenyl-PGE₂, NT-012, 11-deoxy-PGE₁, AE1-329 (EP₄ agonist; Ono Pharmaceutical, Osaka, Japan), forskolin, dbcAMP (Nacalai, Kyoto, Japan), and CJ-42794 (EP₄ antagonist; Pfizer, Aichi, Japan). All COX inhibitors and CJ-42794 were suspended in a hydroxypropylcellulose solution (Wako Pure Chemicals, Osaka, Japan). Prostanoids were dissolved in absolute ethanol and were diluted with saline to the desired concentrations. Other drugs were dissolved in saline. All drugs were prepared immediately before use and were administered periorally or subcutaneously in a volume of 0.5 ml/100 g body wt.

Statistics. Data are presented as means ± SE of 3–10 animals or experiments per group. Statistical analyses were performed by using the two-tailed Dunnett’s multiple-comparison test, and values of P < 0.05 were considered significant.

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. Data are presented as means ± SE from 6 rats.

*Significant difference from control at P < 0.05. B: gross appearances of gastric ulcers on day 17 after ulceration in rats.

Fig. 4. Effects of rofecoxib (COX-2 inhibitor) and CJ-42794 (EP₄ antagonist) on healing of gastric ulcers in rats (A and B) and mice (C). Gastric ulcers were produced by thermocauterization (70°C, 20 s), and animals were killed 17 days after ulceration. Rofecoxib (3 and 10 mg/kg for rat and 5 mg/kg for mouse) or CJ-42794 (3 and 10 mg/kg) was given periorally once daily for 14 days, starting 3 days after ulceration. Data are presented as means ± SE from 6–8 mice. Significant difference at P < 0.05.

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Fig. 3: A: effects of various EP agonists on delayed healing of gastric ulcers induced in mice with indomethacin. Gastric ulcers were produced by thermocauterization (70°C, 20 s), and animals were killed 17 days after ulceration. Indomethacin (2 mg/kg) was given periorally once daily for 14 days starting 3 days after ulceration, whereas 17-phenyl-PGE₂ (1 mg/kg), butaprost (3 mg/kg), NT-012 (3 mg/kg), or 11-deoxy-PGE₁ (1 mg/kg) was given subcutaneously twice daily, 30 min before and 9 h after indomethacin treatment for 14 days. Data are presented as means ± SE from 6–8 mice. Significant difference at P < 0.05 [*from normal (vehicle); #from control (saline)]. B: mucosal expression of EP₄ receptor mRNA in mouse gastric mucosa at various time points after ulceration. Animals were killed on 0, 3, and 7 days after ulceration. Expression of EP₄ receptor mRNA was analyzed by RT-PCR.
PGE₂ 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 (5 mg/kg) had no effect on the increased PGE₂ content. Similar results were obtained in mice concerning the changes in PGE₂ content in the ulcerated mucosa and the effects of various COX inhibitors, although absolute values of PGE₂ content were slightly smaller than those in rat stomachs. As shown in Fig. 1D, the PGE₂ 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 mm² to 2.4 ± 0.3 mm², followed by a gradual healing, the ulcer area on day 17 being 0.9 ± 0.2 mm² (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 mm², 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 PGE₂ 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 PGE₂ 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 PGE₂ 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,

Fig. 5. Immunohistochemical localization of COX-2 and VEGF in gastric ulcer tissue. Gastric ulcers were produced in rats by thermocauterization (70°C, 20 s), and animals were killed 5 days after ulceration. A: COX-2 in gastric ulcer tissue (×20). B: VEGF in gastric ulcer tissue. C and D: COX-2 in the ulcerated mucosa (higher magnification of A; ×200). E and F: VEGF in ulcerated mucosa (higher magnification of B; ×200). Note colocalization of COX-2 and VEGF protein in gastric ulcer tissue, especially at ulcer base.
the healing of the gastric ulcer was markedly impaired by indomethacin (2 mg/kg) given periocularly 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 in 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 (EP₃ 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 significantly decreased in these animals. Gastric ulcers were produced by thermoautoerization (70°C, 20 s), and animals were killed 10 days after ulceration. Indomethacin (2 mg/kg), SC-560 (5 mg/kg), rofecoxib (5 mg/kg), or CJ-42794 (10 mg/kg) was given periocularly once daily for 7 days, starting 3 days after ulceration. A: expression of VEGF was determined by Western blotting. B: densitometric quantification was determined by Quantity One software, and results are expressed as %control. Significant difference at P < 0.05 (* from normal; # from control).

*Fig. 6. Effects of various COX inhibitors and CJ-42794 (EP₄ antagonist) on expression of VEGF in ulcerated mucosa in mouse stomachs. Gastric ulcers were produced by thermoautoerization (70°C, 20 s), and animals were killed 10 days after ulceration. Indomethacin (2 mg/kg), SC-560 (5 mg/kg), rofecoxib (5 mg/kg), or CJ-42794 (10 mg/kg) was given periocularly once daily for 7 days, starting 3 days after ulceration. A: expression of VEGF was determined by Western blotting. B: densitometric quantification was determined by Quantity One software, and results are expressed as %control. Significant difference at P < 0.05 (* from normal; # from control).*

*ever, 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 expression 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 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.

**Fig. 7.** Effect of PGE2 and AE1-329 (EP4 agonist) on VEGF expression in primary rat gastric fibroblasts. After 24 h starvation, gastric fibroblasts were incubated with PGE2 (10 µM) for 3–12 h (A) or PGE2 (0.1–10 µM) for 6 h (B). Effect of CJ-42794 (EP4 antagonist) on increased expression of VEGF induced in primary rat gastric fibroblasts by PGE2 (10 µM) or AE1-329 (10 µM) (C). Gastric fibroblasts were incubated with PGE2 or AE1-329 for 6 h, in absence or presence of EP4 antagonist (0.5 mM). Expression of VEGF was determined by Western blotting, and densitometric quantification was determined by Quantity One software. Data are expressed as %control and represent means ± SE from 3–4 experiments. Significant difference at P < 0.05 (*from control; #from corresponding saline group).
PGE$_2$ exerts its diverse effects by binding to four different EP receptor subtypes, named EP$_1$ through EP$_4$ (16a, 29, 40). For example, PGE$_2$ exhibits a gastroprotective action against necrotizing agents or indomethacin through EP$_1$ receptors (39, 40); stimulates HCO$_3^-$ secretion in the stomach or the duodenum, mediated by EP$_1$ or EP$_3$/EP$_4$ receptors, respectively (1); causes a dual effect on acid secretion, inhibition by the activation of EP$_4$ receptors and stimulation mediated by EP$_3$ receptors (22); and also protects gastric mucosal cells from apoptosis via EP$_2$ and EP$_4$ receptors (17). However, it remains unexplored which EP receptor subtype is responsible for the healing-promoting action of PGE$_2$ 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-PGE$_1$, the EP$_3$/EP$_4$ agonist, but not of other EP receptor agonists, including 17-phenyl-PGE$_2$ (EP$_1$ agonist), butaprost (EP$_2$ agonist), and NT-012 (EP$_3$ agonist). These results are in agreement with our recent observation that endogenous PGE$_2$ contributes to the healing of indomethacin-induced intestinal ulcers via the activation of EP$_4$ receptors (16) and suggest the involvement of EP$_4$ receptors in the healing-promoting action of PGE$_2$ 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 EP$_4$ 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 PGE$_2$ production in the gastric mucosa after ulceration (data not shown). Accordingly, it is assumed to be highly likely that the PGE$_2$ produced by COX-2 accelerates the healing of gastric ulcers by the activation of EP$_4$ 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...
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/PGE2 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 PGE2 from COX-2 upregulates the expression of VEGF via EP4 receptors, we used the effect of the EP4 antagonist on VEGF expression in the stomach after ulceration both in vivo and in vitro. As expected, we found that the EP4 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 PGE2 stimulates both VEGF expression and angiogenesis in the ulcerated mucosa through the activation of EP4 receptors.

VEGF is expressed in various kinds of cells upon stimulation by PGE2 or cytokines (3, 5, 7, 8, 14, 44). Miura et al. (26) reported that PGE2 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 PGE2 in rat gastric fibroblasts in a dose-dependent manner and further showed that the action was mimicked by AII1-329, the EP4 agonist, but was completely inhibited by CJ-42794, the EP4 antagonist. These results demonstrated for the first time that PGE2 stimulates VEGF release in gastric fibroblasts via the activation of EP4 receptors. At present, the exact mechanism by which PGE2 stimulates VEGF expression via EP4 receptors in gastric fibroblasts is unknown. Ding et al. (9) reported that PGE2 upregulates VEGF expression in gastric cancer cells via the transactivation of EGF receptors. Spinella et al. (36) reported that PGE2 regulated VEGF production and ovarian carcinoma cell invasion via EP2/EP4 signaling. Other studies showed that PGE2 induces MAP kinase activation with or without the involvement of EGF receptor transactivation (23, 25). Furthermore, PGE2 has been reported to induce VEGF via SP-1 binding sites on the VEGF promoter via EP2/EP4 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 PGE2 derived from COX-2 plays an important part in the spontaneous healing of gastric ulcers and that COX-1/PGE2 does not contribute greatly to the ulcer healing. Our data also indicate that the healing-promoting action of PGE2 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 EP4 receptors. Finally, because VEGF stimulates cell proliferation (14, 43), it is possible that PGE2 via EP4 receptors promotes not only angiogenesis but also 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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