Localization of cyclooxygenase-2 and regulation of its mRNA expression in gastric ulcers in rats

SATORU TAKAHASHI,1 JUN-ICHI SHIGETA,1 HIROYASU INOUE,2 TADASHI TANABE,2 AND SUSUMU OKABE1
1Department of Applied Pharmacology, Kyoto Pharmaceutical University, Misasagi, Yamashina, Kyoto 607-8414; and 2Department of Pharmacology, National Cardiovascular Center Research Institute, Suita, Osaka 565-8565, Japan

IT IS WELL ESTABLISHED THAT PGs play a physiological role in maintaining the integrity of the gastric mucosa. Nonsteroidal anti-inflammatory drugs (NSAIDs) induce gastric mucosal injury in rats and humans (1, 28). Exogenous PGs protect the gastric mucosa against various types of damage caused by necrotizers, including NSAIDs (17, 19). In addition, PGs are known to play an important role in the healing of gastric ulcers. PG production is elevated in ulcerated tissue and that COX-2 mRNA expression might be regulated positively by IL-1β and negatively by TGF-β1.

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Northern blot analysis. Gastric specimens were taken from both intact (posterior side) and ulcerated tissue (anterior side) of stomachs with ulcers and from stomachs without ulcers. Total RNAs were extracted from the specimens by means of the acid-guanidinium thiocyanate-phenol-chloroform method (2), using TRIzol (GIBCO BRL, Gaithersburg, MD). Poly(A)+ RNAs were isolated by using Oligo(dT)30 (TaKaRa, Kyoto, Japan). Poly(A)+ RNAs (0.1–0.5 µg) were separated by electrophoresis on 1% agarose gels, transferred onto nylon membranes (Gene Screen Plus; NEN, Boston, MA), and then hybridized with 32P-labeled cDNA probes (20). The detection and quantification of hybridized mRNAs were carried out with an imaging analyzer (BAS-5000 Mc; Fuji Film, Tokyo, Japan). Unless otherwise stated, the levels of the mRNAs were expressed as a ratio against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Western blot analysis. Gastric specimens were taken from both intact (posterior side) and ulcerated tissue (anterior side) of stomachs with ulcers and from stomachs without ulcers. COX proteins were partially purified from the specimens as described by Gierse et al. (4). After aliquots (60 µg) of the proteins eluted from a DEAE-Sepharose column and standard COX proteins (0.1 µg; Cayman Chemical) had been subjected to SDS-PAGE (10%), the separated proteins were electrophoretically transferred onto Immobilon-P membranes (Millipore, Bedford, MA) as described by Towbin et al. (26). The membranes were incubated with the antibody against COX-1 or COX-2 protein after nonspecific binding sites had been blocked. COX proteins were visualized by the avidin-biotin-peroxidase complex (ABC) method, using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and 3,3’-diaminobenzidine tetrahydrochloride (Dojindo Laboratories, Kumamoto, Japan).

Immunohistochemical analysis. Gastric specimens were taken from both intact (posterior side) and ulcerated tissue (anterior side) of stomachs with ulcers and from stomachs without ulcers. After they had been fixed with 4% paraformaldehyde in PBS, frozen sections (16 µm thick) were prepared. The sections were incubated with anti-COX-2 antibody after deactivation of endogenous peroxidase with 0.3% H2O2 and blockage of nonspecific binding sites. COX-2 protein was visualized by the ABC method as described above. The sections were successively stained with hematoxylin.

Tissue culture. On days 3 and 10, the ulcer base was excised and divided into six fragments. The fragments were placed in the wells of 48-well culture plates (Corning & Costar, Corning, NY) and then incubated in 0.5 ml of DMEM (GIBCO BRL) supplemented with 0.5% fetal bovine serum (GIBCO BRL), 100 U/ml penicillin, 100 U/ml streptomycin, and 0.25 µg/ml amphotericin B in the presence of the indicated agents at 37°C under 5% CO2 in air. Forty-eight hours later, poly(A)+ RNAs were isolated from the fragments and subjected to Northern blot analysis. In addition, PGE2 production assay was performed as described above. After a 48-h incubation with the reagents, the contents (µg/tissue) of DNA, total RNA, and poly(A)+ RNA were nearly similar, compared with those before culturing. Histologically, granulation tissue remained unchanged, and fibroblasts, epithelial cells, and immune cells were observed after the incubation.

Materials. NS-398 and FR-1767653 were kindly supplied by Taisho Pharmaceutical (Tokyo, Japan) and Fujisawa Pharmaceutical (Osaka, Japan), respectively. Polyclonal rabbit antiserum against ovine COX-1 (PGH synthase 1) and mouse COX-2 (PGH synthase 2) were purchased from Cayman Chemical. These antibodies have been known to cross-react with rat enzymes (8). Other agents and their sources were as follows: indomethacin (Sigma Chemical, St. Louis, MO); synthesized oligonucleotides, Moloney murine leukemia virus RT, and Taq DNA polymerase (GIBCO BRL); human GAPDH cDNA probe (Clontech, Palo Alto, CA); [α-32P]dCTP (NEN); IL-1 receptor antagonist (Pepro Tech, Rocky Hill, NJ); and anti-mouse TNF-α antibody, anti-human TGF-β1 antibody, and anti-human TGF-β receptor II antibody, which react with rat TNF-α, rat TGF-β1, and rat TGF-β receptor II, respectively (Santa Cruz Biotechnology, Santa Cruz, CA). All other chemicals used were of reagent grade.

Statistical analysis. Data are presented as means ± SE. Statistical differences in the dose-response studies were evaluated by Dunnett’s multiple comparison test. Student’s t-test was also applied to comparisons between two groups. P < 0.05 was regarded as significant.
RESULTS

PGE$_2$ production by gastric tissue with ulcers. On day 0, there were round, well-defined ulcers in all animals, with the ulcerated area being $41.0 \pm 4.8$ mm$^2$ (Fig. 1A). The ulcers spontaneously healed. The areas decreased to $14.9 \pm 2.4$ and $4.9 \pm 1.3$ mm$^2$ on days 7 and 14, respectively.

PGE$_2$ production in gastric tissue of the normal stomach amounted to $103.0 \pm 5.7$ pg·mg$^{-1}$·min$^{-1}$ (Fig. 1B). PGE$_2$ production in the ulcerated tissue significantly increased by about threefold during days 0–7, compared with that in the normal tissue. Thereafter, the production decreased and returned to the normal level on day 14. However, PGE$_2$ production in the intact tissue was not affected by the presence of ulcers in the stomach. The level was nearly the same as that in the normal tissue.

In addition, we examined the effects of COX inhibitors on PGE$_2$ production (Fig. 2). Indomethacin dose dependently inhibited PGE$_2$ production by gastric tissue with ulcers as well as that by normal gastric tissue. In contrast, NS-398, a selective COX-2 inhibitor (3), did not affect the production by the intact tissue with ulcers or that by normal tissue even at 50 µM. However, NS-398 dose dependently and significantly reduced the increased PGE$_2$ production by the ulcerated tissue. It was evident that NS-398 is selective over somewhat narrow concentrations. However, the effect of indomethacin on ulcerated tissue was more potent than that of NS-398, with the inhibition being $\sim 70\%$ by indomethacin and $\sim 50\%$ by NS-398 at 25 µM, and $\sim 80\%$ by indomethacin and $\sim 65\%$ by NS-398 at 50 µM.

COX-2 expression in ulcer base. We examined the expression of COX proteins in the rat gastric tissue by means of Western blotting (Fig. 3A). Anti-COX-1 and anti-COX-2 antibodies reacted with the respective standard proteins without any cross-reaction. In the normal gastric tissue, one protein (70 kDa) was recognized by anti-COX-1 antibody. No proteins were detected by anti-COX-2 antibody. The same results were obtained for the intact tissue of the stomach with ulcers. However, in the ulcerated tissue, anti-COX-2 antibody reacted with one protein, $\sim 70$ kDa in weight, whose relative mobility was apparently identical to that of the standard COX-2 protein. One immunoreactive protein corresponding to COX-1 was also detected.

We also determined the pattern of COX mRNA expression by Northern blotting. As shown in Fig. 3B, COX-1 mRNA (2.9 kb) was found in all tissue, i.e., not only in normal gastric tissue but also in ulcerated tissue and intact sections of gastric tissue with ulcers. The level of COX-1 mRNA in the ulcerated and intact sections of gastric tissue with ulcers did not change throughout the experiments, although the level in the ulcerated tissue was slightly lower than that in the other tissue. In contrast, COX-2 mRNA was not detected in the normal stomach or the intact section of gastric tissue with ulcers but was expressed in the ulcerated tissue. The expression of COX-2 mRNA (4.0 kb) had been induced on day 0, its level being retained until day 7. Thereafter, COX-2 mRNA expression decreased with time.

Furthermore, we defined the cellular localization of COX-2 protein by immunohistochemical staining (Fig. 4). In the gastric mucosa of the normal stomach, immunoreactivity for COX-2 protein was not detected. Similarly, there were no appreciable signals with anti-
COX-2 antibody in the gastric glands around ulcers. In contrast, in the ulcer base, strong COX-2 immunoreactivity appeared in the upper portion. Immunoreactive COX-2 protein was abundant in spindle-shaped cells (fibroblasts), mononuclear cells (monocytes/macrophages), and polymorphonuclear cells (granulocytes). The numbers of these cells decreased as the ulcers healed (degeneration of the ulcer base). The numbers and proportions of COX-2-expressing cells in the ulcer base also decreased with ulcer healing. However, epithelial cells that regenerated over the ulcer base after day 10 showed no COX-2 immunoreactivity. When the antibody preincubated with standard COX-2 protein was applied to the sections, no immunoreactive signals appeared.

Regulation of COX-2 mRNA expression. It is well known that IL-1β and TNF-α induce COX-2 expression and that TGF-β1 modulates COX-2 expression (6). Therefore, we first examined whether or not the expression of IL-1β, TNF-α, and TGF-β1 mRNAs was induced in the ulcer base (Fig. 5). Their mRNAs were not detected in either the mucosa of the normal stomach or the intact mucosa of the stomach with ulcers. However, all three mRNAs were expressed in the ulcerated tissue on day 0. The levels of mRNA expression remained nearly constant until day 7 and decreased thereafter. The rate of the decrease in TGF-β1 mRNA expression after day 7 was less than that of the decreases in IL-1β and TNF-α.

To examine whether or not IL-1β, TNF-α, and TGF-β1 are involved in COX-2 mRNA expression in the ulcer base, we antagonized the actions of IL-1β, TNF-α, and TGF-β1 in a culture of the isolated ulcer base. After the isolated base was incubated for 48 h in the presence of the indicated additives, the expression of COX mRNAs and PGE2 production was determined. We confirmed that the IL-1 receptor antagonist (2 µg/ml), anti-TNF-α antibody (5 µg/ml), anti-TGF-β1 antibody (5 µg/ml), and anti-TGF-β receptor II antibody (4 µg/ml) completely inhibit the cell responses to 2 ng/ml IL-1β, 5 ng/ml TNF-α, 10 ng/ml TGF-β1, and 10 ng/ml TGF-β1, respectively (data not shown).

In both the ulcer bases isolated on day 3 (in the early phase of ulcer healing) and day 10 (in the late phase), treatment with IL-1 receptor antagonist dose dependently caused decreases in COX-2 mRNA expression and PGE2 production (Fig. 6). The inhibitory effect of IL-1 receptor antagonist was more potent on day 3 than on day 10. IL-1 receptor antagonist at 4 µg/ml significantly reduced COX-2 mRNA expression and PGE2 production by 43.1 ± 6.6 and 33.2 ± 6.7% on day 0 and by 25.0 ± 7.9 and 22.7 ± 8.4% on day 10, respectively, compared with the corresponding control. However, IL-1 receptor antagonist failed to inhibit COX-1 mRNA expression on days 3 and 10.

In the case of anti-TNF-α antibody, similar results were obtained (Fig. 7). In the ulcer base isolated on day 3, COX-2 mRNA expression and PGE2 production were
inhibited by anti-TNF-α antibody in a dose-dependent manner, with the inhibition by the antibody at 10 μg/ml being 34.7 ± 7.4 and 30.6 ± 6.4%, respectively. In the base isolated on day 10, anti-TNF-α antibody dose dependently reduced COX-2 mRNA expression and PGE₂ production, but the significant effect was observed only in the inhibition (17.9 ± 6.1%) of COX-2 mRNA expression at 10 μg/ml anti-TNF-α antibody. COX-1 mRNA expression was not affected by anti-TNF-α antibody.

In contrast, treatment with anti-TGF-β1 antibody promoted COX-2 mRNA expression (Fig. 8). The antibody at 10 μg/ml significantly increased COX-2 mRNA expression by 27.4 ± 4.4 and 46.1 ± 13.1% above the corresponding control on days 3 and 10, respectively. Along with the increase in COX-2 mRNA expression, PGE₂ production was also stimulated. Anti-TGF-β1 antibody at 10 μg/ml increased the production by 23.7 ± 7.3 and 34.1 ± 9.6% above the control on days 3 and 10, respectively. In addition, anti-TGF-β receptor II antibody at 4 μg/ml also significantly enhanced COX-2 mRNA expression and PGE₂ production by 132.1 ± 7.9 and 130.6 ± 9.0%, respectively, in the base isolated on day 10. However, the expression of COX-1 mRNA was not affected by anti-TGF-β1 antibody or anti-TGF-β receptor II antibody.

In addition, we examined the effect of FR-167653 on COX-2 mRNA expression and PGE₂ production in the ulcer base isolated on day 3 (Fig. 9). FR-167653 is a dual specific inhibitor of IL-1 and TNF-α production but has no effect on the production of other inflammatory proteins, such as IL-6 (30, 31). In addition, 10 μM
FR-167653 did not affect COX-2 mRNA expression in phorbol ester-stimulated fibroblasts (data not shown). FR-167653 significantly suppressed the expression of both IL-1β and TNF-α mRNAs in a dose-dependent manner but had no effect on the expression of TGF-β1 or COX-1 mRNAs. COX-2 mRNA expression was significantly reduced by the compound, in association with the decreases in the expression of IL-1β and TNF-α mRNAs. The inhibition by 10 µM FR-167653 of the expression of IL-1β, TNF-α, and COX-2 mRNAs was 62.0 ± 8.8, 35.7 ± 5.7, and 55.9 ± 3.0%, respectively. Furthermore, FR-167653 also dose dependently inhibited PGE2 production, with the inhibition at 10 µM being 42.4 ± 3.9%.

**DISCUSSION**

These results clearly indicate that the expression of COX-2 mRNA and protein is induced only in the ulcerated gastric tissue in rats and that the level of COX-2 mRNA decreases with ulcer healing. These are consistent with the recent findings by Mizuno et al. (16) concerning mice. As reported previously by Szelenyi et al. (23) and us (29), PGE2 production was significantly elevated in the ulcerated tissue, compared with that in the intact tissue, and the increased production returned to the normal level with ulcer healing. The change in PGE2 production was well associated with COX-2 mRNA expression. In fact, the increased PGE2 production in the ulcerated tissue, but not the production in other tissue, was dose dependently inhibited by NS-398, a selective inhibitor of COX-2 (3). In addition, we (21) confirmed that indomethacin inhibits PGE2 production in both intact and ulcerated tissue, while NS-398 reduces production only in ulcerated tissue, after the drugs are administered to the rats with gastric ulcers. The inhibition of COX-2 mRNA expression in the isolated ulcer base also leads to the decrease in PGE2 production. Accordingly, these results indicate that COX-2 might contribute to the elevation of PGE2 in the ulcerated tissue. In addition, COX-1 might also be involved in PGE2 production in the ulcerated tissue, because COX-1 protein and mRNA were present in the ulcerated tissue and indomethacin more potently reduced PGE2 production in the ulcerated tissue than NS-398 did.
Moreover, we defined the cellular localization of COX-2 protein. There were no immunoreactive signals for COX-2 protein in the intact mucosa around the ulcer base or the mucosa of normal rats, but strong immunoreactivity for COX-2 protein was found in fibroblasts, monocytes/macrophages, and granulocytes in the upper portion of the ulcer base. These findings are strongly supported by the results of Western and Northern blot analyses. In vitro studies (6, 15) have revealed the induction of COX-2 in these cell types in response to various stimuli. However, it remains unknown why COX-2 protein is enriched only in the cells present in the upper portion of the base, although fibroblasts, monocytes/macrophages, and granulocytes also exist in other portions of it. It is known that conventional NSAIDs inhibit PGE2 production in the ulcerated tissue, thereby impairing ulcer healing (11, 13, 23, 29). Recently, Mizuno et al. (16) reported that NS-398 prevents ulcer healing in mice. Although PG production was not examined after the administration of NS-398, Mizuno et al. (16) claim that selective inhibition of COX-2 activity may cause a delay in the healing. Taken together with the present results, it is suggested that these COX-2-expressing cells in the ulcer base may play a key role in the healing of gastric ulcers. The increased PGs will most likely exert various effects only in and around the ulcer base, because PGs act within a

Fig. 7. Effect of anti-TNF-α antibody on COX mRNA expression and PGE2 production in cultured ulcer base. After ulcer base was isolated on day 3 (A) and day 10 (B), base was cultured for 48 h in the presence of anti-TNF-α antibody. Thereafter, levels of COX-1 (open bars) and COX-2 (solid bars) mRNAs were determined by Northern blotting. PGE2 production was also determined. Data are presented as means ± SE (n = 8). *Significantly different (P < 0.05) from corresponding control.

Fig. 8. Effect of anti-TGF-β1 antibody on COX mRNA expression and PGE2 production in cultured ulcer base. After ulcer base was isolated on day 3 (A) and day 10 (B), base was cultured for 48 h in the presence of anti-TGF-β1 antibody. Thereafter, levels of COX-1 (open bars) and COX-2 (solid bars) mRNAs were determined by Northern blotting. PGE2 production was also determined. Data are presented as means ± SE (n = 8). *Significantly different (P < 0.05) from corresponding control.
small area due to their short half-lives. One such effect is an increase in blood flow around gastric ulcers (7).

COX-2 mRNA expression was strongly induced by gastric ulceration, and the increased expression decreased with ulcer healing (i.e., degeneration of the ulcer base). The COX-2-expressing cells are fibroblasts, monocytes/macrophages, and granulocytes, which are infiltrated into the ulcerated tissue to form granulation. The numbers and proportions of such cells in the ulcer base decreased with ulcer healing. Accordingly, it is considered that growth factors and cytokines stimulating the migration and proliferation of these cells, such as fibroblast growth factor and IL-8, may be involved in COX-2 expression.

We report here that COX-2 mRNA expression is locally regulated in the ulcer base. Our results indicate that IL-1β and TNF-α might stimulate COX-2 mRNA expression in the base. IL-1β and TNF-α are typical COX-2 inducers in a variety of cell types, including fibroblasts, monocytes/macrophages, and granulocytes (6, 15). In fact, we also confirmed that IL-1β and TNF-α mRNAs are expressed only in the ulcerated tissue. Similarly, Kinoshita et al. (9) reported that IL-1α is induced by gastric ulceration. In addition, the IL-1 type I receptor might be implicated in the action of IL-1 on COX-2 mRNA expression, because IL-1 receptor antagonist is known to bind more preferably to the type II receptor than the type I receptor (22). In contrast, TGF-β1 might negatively regulate COX-2 mRNA expression in the ulcer base, through the partial mediation of the TGF-β type II receptor. In this study, TGF-β1 mRNA expression was found only in ulcerated tissue, as reported by Tominaga et al. (25). However, TGF-β is reported to exert differential effects on COX-2 mRNA expression in vitro. In endotoxin-activated macrophages, TGF-β attenuates COX-2 mRNA expression (18), while it enhances expression in mitogen-stimulated fibroblasts (5). At present, the reason why TGF-β reduces COX-2 mRNA expression in the base remains unclear. Because it is well known that TGF-β serves as a strong immunosuppressor (14), the inhibitory effect of TGF-β toward inflammatory cells may be more potent than its stimulatory effect toward fibroblasts in the ulcer base. Our results also suggest that, in the local regulation of COX-2 mRNA expression, the contribution of IL-1 and TNF-α to the induction is greater in the early phase of ulcer healing than in the late phase, while the inhibitory action of TGF-β1 is more potent in the late phase than in the early phase. The levels of IL-1β and TNF-α mRNA expression on day 10 were reduced to ~50% of those on day 3. Because it is suspected that factors other than IL-1β, TNF-α, and TGF-β1 might also be involved in COX-2 mRNA expression in ulcerated gastric tissue, functional interaction between them should be considered. Among the factors stimulating COX-2 mRNA expression, the proportion of IL-1β and TNF-α may be high in the early phase but low in the late phase. In contrast, the expression of TGF-β1 mRNA was not largely different between days 3 and 10. The ratio of stimulatory factors, including IL-1β and TNF-α, to TGF-β1 may be crucial in the expression of COX-2 mRNA. In general, the effects of inhibitors and antagonists are known to be attenuated with increases in stimulating activities. Namely, when the ratio is large in the early phase, the effects of the stimulating factors may be strong, so that expression of the inhibitory effect of TGF-β1 may be slight. In contrast, TGF-β1 may reduce COX-2 mRNA expression more strongly, as the stimulating factors decrease with ulcer healing.

In conclusion, these results indicate that COX-2 protein is highly localized in fibroblasts, monocytes/macrophages, and granulocytes in the base of gastric ulcers in rats and that COX-2 mRNA expression might be regulated positively by IL-1β and TNF-α and negatively by TGF-β1.

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Address for reprint requests: S. Takahashi, Dept. of Applied Pharmacology, Kyoto Pharmaceutical Univ., Misasagi, Yamashina, Kyoto 607-8414, Japan.

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