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Role of direct cytotoxic effects of NSAIDs in the induction of gastric lesions

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Abstract

A major clinical problem encountered with the use of non-steroidal anti-inflammatory drugs (NSAIDs), is gastrointestinal complications. We previously reported that NSAIDs induce both necrosis and apoptosis *in vitro*. We here examined the cyclooxygenase (COX) dependency of this cytotoxic effect of NSAIDs and its involvement in NSAID-induced gastric lesions. Necrosis and apoptosis by NSAIDs was observed with all selective COX-2 inhibitors except rofecoxib and was not inhibited by exogenously added prostaglandin E_2, suggesting that cytotoxicity of NSAIDs seems to be independent of the inhibition of COX. Intravenously administered indomethacin, which completely inhibited COX activity at gastric mucosa, did not produce gastric lesions. Orally administered selective COX-2 inhibitors, which did not inhibit COX at gastric mucosa, also did not produce gastric lesions. Interestingly, a combination of the oral administration of each of all selective COX-2 inhibitors except rofecoxib with the intravenous administration of indomethacin clearly produced gastric lesions. These results suggest that in addition to COX inhibition by NSAIDs, direct cytotoxicity of NSAIDs may be involved in NSAID-induced gastric lesions.

Keywords: Direct cytotoxicity; Cyclooxygenase; NSAIDs; Gastric lesions; Prostaglandin E_2; Selective cyclooxygenase-2 inhibitors

1. Introduction

NSAIDs are one of the most frequently used classes of medicines in the world and account for nearly 5% of all prescribed medications [1]. However, NSAID administration is associated with gastrointestinal complications, such as gastric ulcers and bleeding, which sometimes become life-threatening diseases [2]. About 15–30% of chronic users of NSAIDs have gastrointestinal ulcers and bleeding [3–6]. In the United States, about 16,500 people per year die as a result of NSAID-associated gastrointestinal complications [7]. Therefore, the molecular mechanism governing NSAID-induced gastrointestinal damage needs to be elucidated in order to develop new NSAIDs that do not have these side effects.

Inhibition of COX by NSAIDs, which is responsible for their anti-inflammatory activity was previously thought to be fully responsible for their gastrointestinal side effects [8]. This is because COX is an enzyme essential for the synthesis of prostaglandins (PGs), which have a strong cytoprotective effect on the gastrointestinal mucosa [9]. There are at least two subtypes of COX, COX-1 and COX-2, which are responsible for the majority of COX activity at the gastric mucosa and tissues with inflammation, respectively [10,11]. Therefore, it is reasonable to speculate that selective COX-2 inhibitors have anti-inflammatory activity without gastrointestinal side effects [10]. In fact, a greatly reduced incidence of gastroduodenal lesions was reported for selective COX-2 inhibitors (such as celecoxib and rofecoxib) both in animal and clinical data [12–15], however, recently published paper showed no difference in serious gastrointestinal complications between celecoxib and two non-selective NSAIDs [16]. Two lines of evidence have worked against the idea that the gastrointestinal side

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Abbreviations: NSAID, non-steroidal anti-inflammatory drug; COX, cyclooxygenase; PG, prostaglandin; FBS, fetal bovine serum; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PGI_2, prostacyclin.

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effects of NSAIDs are caused only by the inhibition of COX-1. The first is that a selective COX-1 inhibitor (SC-560) induced no gastric injury even at high dosages, and COX-1 knockout mice showed no detectable gastric ulcers [17–20]. The second line of evidence is that the increased incidence of gastrointestinal lesions and the decrease in PG levels induced by NSAIDs are not always linked with each other. For example, higher doses of NSAIDs were required for producing gastric lesions than were required for inhibiting COX at the gastric mucosa [21,22]. The former contradiction can be explained by the recent proposal that inhibition of both COX-1 and COX-2 was necessary for NSAID-induced gastrointestinal lesions [17]. However, the latter contradiction cannot be explained by this idea, suggesting therefore that NSAID-induced gastrointestinal lesions involve additional mechanisms [23]. Understanding the additional mechanisms is necessary in order to establish an alternative method for development of gastrointestinally safe NSAIDs other than simply increasing their COX-2 selectivity. This new class of NSAIDs may be clinically beneficial because clinical disadvantages (i.e. risk of cardiovascular thrombotic disease) of selective COX-2 inhibitors were recently suggested [24,25].

In addition to various possibilities proposed for this additional mechanism (such as reduced blood flow, hypermotility, and activation of neutrophils) [26–28], a direct cytotoxic effect of NSAIDs (topical irritant property) on gastric mucosal cells was also proposed to be involved in NSAID-induced gastric lesions [23,29]. As for this direct cytotoxic effect, we previously reported that NSAIDs (indomethacin and aspirin) induced both necrosis and apoptosis in primary cultures of guinea pig gastric mucosal cells [30]. In this study, we suggested that the direct cytotoxic effect of NSAIDs is independent of the inhibition of COX and suggest that in addition to COX inhibition by NSAIDs, direct cytotoxic effect of NSAIDs is involved in NSAID-induced gastric lesions *in vivo* by use of a combination of the oral administration of selective COX-2 inhibitors with the intravenous administration of non-selective NSAIDs.

2. Materials and methods

2.1. Chemicals, media, and animals

FBS and trypan blue were from Gibco Co. Indomethacin, aspirin, and NS-398 were from Wako Co. Ibuprofen, diclofenac, and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma Co. Celecoxib was from LKT Laboratories Inc. Rofecoxib was synthesized in our laboratory. We confirmed that this rofecoxib has bioequivalence with that made by Merck, by measuring their inhibitory effect on inflammatory PG synthesis. Etodolac was gift kindly provided by Nippon Shinyaku Co. The ELISA kits for PGE_2 and 6-keto-$PGF_{1\alpha}$ quantitation were from Cayman Chemical Co. Male Wistar rats weighing 160–190 g and male guinea pigs weighing 200–300 g were purchased from Shimizu Co. The experiments and procedures described here were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health and were approved by the Animal Care Committee of Okayama University.

2.2. In vitro assay of cytotoxicity and DNA fragmentation

Gastric mucosal cells were isolated from guinea pig fundic glands, as described previously [31,32]. Isolated gastric mucosal cells were cultured for 12 hr in RPMI 1640 containing 0.3% (v/v) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in type-I collagen-coated plastic culture plates under the conditions of 5% CO_2/95% air and 37°. After removing non-adherent cells by washing with RPMI 1640, cells that were attached to the plate at about 50% confluence were used. Guinea pig gastric mucosal cells prepared under these conditions were previously characterized, with the majority (about 90%) of cells being identified as pit cells [31]. NSAIDs were dissolved in DMSO and control experiments (without NSAIDs) were performed in the presence of same concentrations of DMSO. Cells were exposed to NSAIDs by changing the entire bathing medium. Cell viability was determined by the trypan blue exclusion test or the MTT method as previously described [33].

Apoptotic DNA fragmentation was monitored as previously described [34]. Cells were collected using a rubber policeman and suspended in 70 μL of lysis buffer, consisting of 50 mM Tris–HCl (pH 7.8), 10 mM EDTA, and 0.5% sodium-*N*-lauroylsarcosinate. Proteinase K was added to a final concentration of 1 mg/mL, and the lysate was incubated at 50° for 2 hr. RNase A was then added to a final concentration of 0.5 mg/mL and incubated at 50° for 30 min. These samples were analyzed by 2% agarose gel electrophoresis in the presence of 0.5 μg/mL ethidium bromide.

2.3. Gastric damage assay

Rats (24 hr fasted) were orally administered with selective COX-2 inhibitors or non-selective NSAIDs with 1% methylcellulose in a volume of 5 mL/kg. Control rats received an equal volume of the vehicle (1% methylcellulose). In some experiments, 1 hr before the oral administration, indomethacin (dissolved in PBS), aspirin (dissolved in PBS), or vehicle (PBS) was administered intravenously *via* the tail vein. Six hours after the oral administration, the rats were anesthetized and the stomach was removed and scored for hemorrhagic damage by an observer unaware of the treatment that the rats had received. The score involved measuring the area of all

lesions in millimeters and summing the values to give an overall gastric lesion index. Determination of PGE_2 levels at the gastric mucosa was done by ELISA as previously described [35].

2.4. Caspase activity assay

The activity of caspase 3 was determined as described previously [30]. Briefly, cells were collected by centrifugation and suspended in extraction buffer (50 mM PIPES (pH 7.0), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, and 1 mM DTT). Suspensions were sonicated and centrifuged, after which the supernatants were incubated with fluorogenic peptide substrate (Ac-DEVD-MCA) in reaction buffer (100 mM HEPES–KOH (pH 7.5), 10% sucrose, 0.1% CHAPS, and 1 mg/mL BSA) for 15 min at 37°. The release of AMC was determined using a fluorescence spectrophotometer. One unit of protease activity was defined, as the amount of enzyme required to release 1 pmol AMC/min.

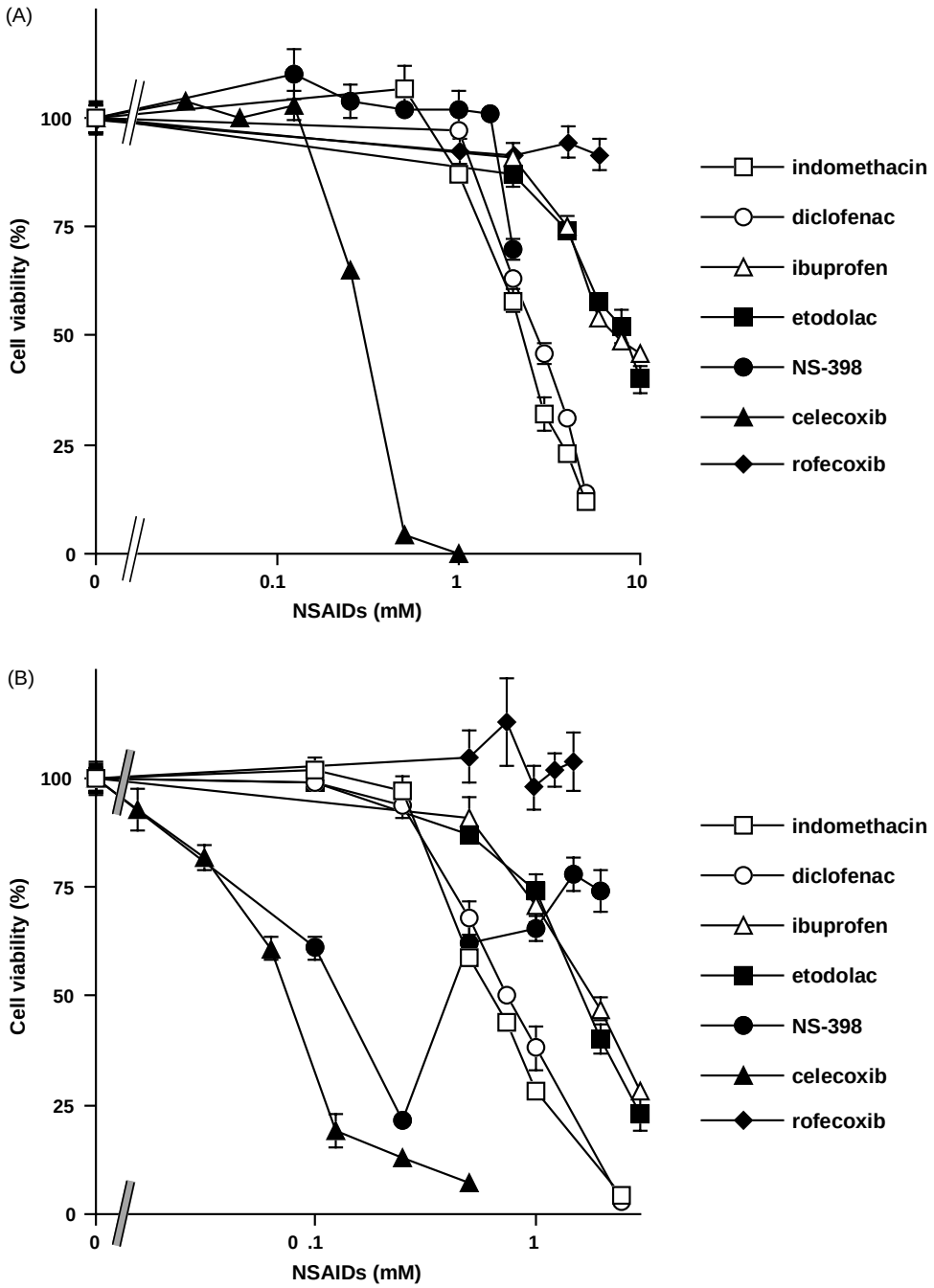

Fig. 1. Necrosis and apoptosis induced by various NSAIDs. Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of various NSAIDs for 1 hr (A, C) or 16 hr (B, C). Cell viability was determined by the MTT method (A, B). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (C). Values are mean ± SEM (N = 3).

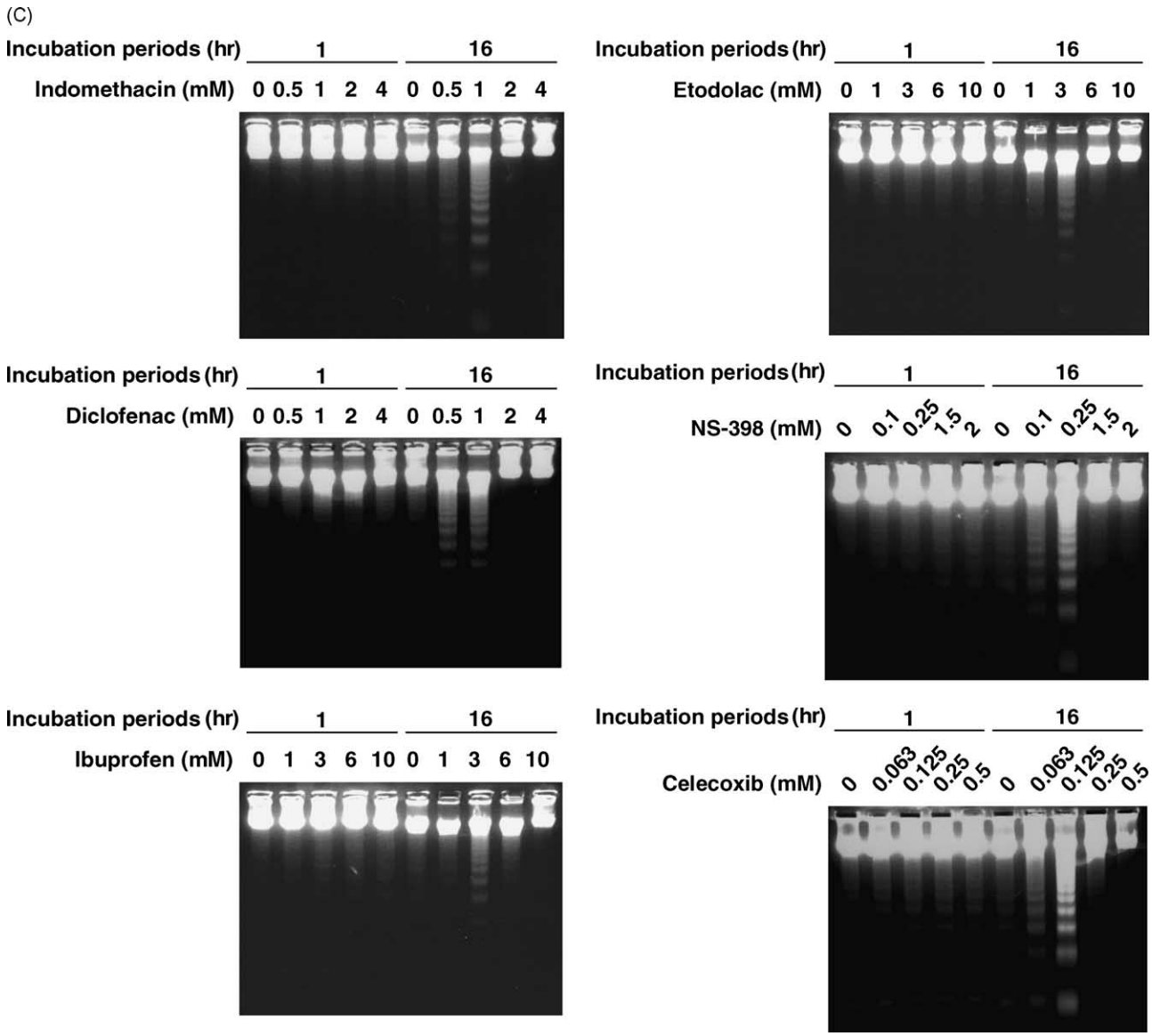

Fig. 1. (*Continued*).

2.5. Statistical analysis

All values are expressed as the mean ± standard error (SEM). One-way ANOVA followed by Scheffe's multiple comparison was used for evaluation of differences between the groups. A Student's *t* test for unpaired results was performed for the evaluation of differences between two groups. Differences were considered to be significant for values of $P < 0.05$.

3. Results

3.1. In vitro necrosis and apoptosis induced by various NSAIDs, and their relationship with COX inhibition

We previously reported that short-term (1 hr) treatment of primary cultures of guinea pig gastric mucosal cells with high concentrations of NSAIDs (indomethacin, 2.5 mM) and long-term (16 hr) treatment of these cells with low concentrations of NSAIDs (indomethacin, 1 mM) induced necrosis and apoptosis, respectively [30]. In the present study, selective COX-2 inhibitors (etodolac, NS-398, celecoxib, and rofecoxib) and non-selective NSAIDs (or slightly selective NSAIDs for COX-1 or COX-2) (indomethacin, diclofenac, and ibuprofen) were tested for their ability to induce necrosis and apoptosis *in vitro*. Necrosis and apoptosis were assessed on the basis of the presence and absence, respectively, of apoptotic DNA fragmentation and caspase 3 activation. The decrease in cell viability with short-term (1 hr) NSAIDs treatment (Fig. 1A) is not associated with apoptotic DNA fragmentation (Fig. 1C) and caspase 3 activation (Table 1), suggesting that it is mediated by necrosis. To confirm this finding, we carried out double-staining experiments with propidium iodide and Hoechst 33342. Since necrotic cells lose

Table 1
Activation of caspase 3 by NSAIDs

Incubation (hr)	NSAIDs	Caspase 3-like activity (U/mg protein)
1	Control	10 ± 3
	4 mM indomethacin	11 ± 1
	4 mM diclofenac	13 ± 6
	10 mM ibuprofen	13 ± 2
	10 mM etodolac	10 ± 3
	2 mM NS-398	11 ± 5
	0.5 mM celecoxib	10 ± 5
	6 mM rofecoxib	10 ± 4
16	Control	14 ± 4
	1 mM indomethacin	525 ± 23***
	1 mM diclofenac	564 ± 50***
	3 mM ibuprofen	591 ± 32***
	3 mM etodolac	455 ± 29***
	0.25 mM NS-398	473 ± 33***
	0.125 mM celecoxib	637 ± 61***
	3 mM rofecoxib	29 ± 8

Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of various NSAIDs for 1 or 16 hr. Activities of caspase 3 were examined by the use of specific fluorogenic peptide substrates (Ac-DEVD-MCA). Values are mean ± SEM (N = 3).
***$P < 0.001$.

their membrane integrity, propidium iodide staining causes pink nuclear staining in necrotic cells, whereas living cells and apoptotic cells are not stained with propidium iodide. We previously reported that 1 hr treatment with 2.5 mM indomethacin caused pink nuclear staining [30]. We here performed the same type of experiment for other NSAIDs used in Fig. 1 and found that 1 hr treatment with 4 mM indomethacin, 4 mM diclofenac, 10 mM ibuprofen, 10 mM etodolac, 2 mM NS-398, or 0.5 mM celecoxib caused pink nuclear staining (data not shown), strongly suggesting that cell death shown in Fig. 1A is mediated through necrosis. In contrast, the decrease in cell viability with long-term (16 hr) NSAIDs treatment (Fig. 1B) is associated with apoptotic DNA fragmentation (Fig. 1C) and caspase 3 activation (Table 1), suggesting that it is mediated by apoptosis. Among all of NSAIDs tested here, only rofecoxib induced neither necrosis nor apoptosis (Fig. 1). Interestingly, apoptosis induced by NS-398 was greatest at a concentration of 0.25 mM and the higher concentrations of NS-398 caused the less induction. We have no explanation for this phenomenon at present. We consider that the concentrations of NSAIDs required for necrosis and apoptosis *in vitro* are possible *in vivo* associating with gastric ulceration in animal models, as discussed in our previous paper [30]. However, it is unclear whether gastric mucosal cells can be exposed to NSAIDs as long as 16 hr in animal models. Furthermore, it is also unclear whether these concentrations are relevant for clinical use of NSAIDs. There seemed to be no direct relationship between the cytotoxicity (concentrations of NSAIDs required for necrosis and apoptosis) and the selectivity for COX-2 of NSAIDs.

The level of PGE_2 in the medium upon treatment of cells with various NSAIDs was measured. Compared to non-selective NSAIDs, higher concentrations of selective COX-2 inhibitors were required for inhibiting PGE_2 synthesis, being consistent with the idea that the majority of COX activity is derived from COX-1 activity in gastric mucosal cells [10,11]. Comparing results (Figs. 1 and 2), it is clear that there is no relationship between them in terms of their cytotoxicity and their ability to inhibit PGE_2 synthesis. For example, celecoxib was the strongest compound in terms of cytotoxicity, but the weakest for inhibiting PGE_2 synthesis. Therefore, it seems that the cytotoxic

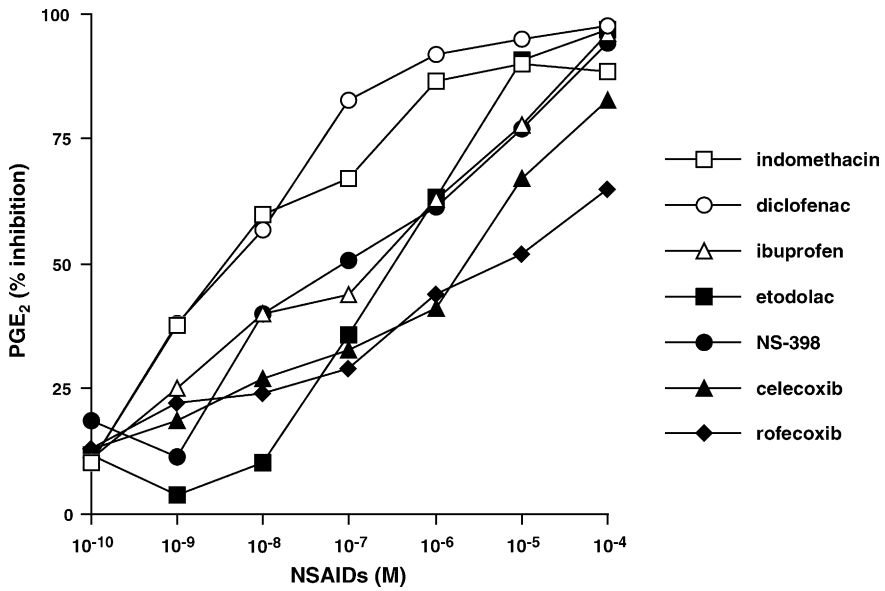

Fig. 2. Inhibition of PGE_2 synthesis by exposure to various NSAIDs in cultured cells. Cultured guinea pig gastric mucosal cells were incubated with indicated concentrations of various NSAIDs for 30 min, following which the levels of PGE_2 in the media were determined by ELISA. Values are expressed as relative to control (without NSAIDs) (2×10^{-9} M).

effects of NSAIDs (necrosis and apoptosis) are independent of their ability to inhibit COX. For further confirmation of this point, we examined the effect of exogenously added PGE_2 on necrosis and apoptosis induced by indomethacin. Exogenously added PGE_2 did not affect the extent of cell death by short-term or long-term treatment with indomethacin (necrosis or apoptosis, respectively) even at higher concentrations of PGE_2 than is present endogenously in medium (10^{-9} M) (Fig. 3).

It was recently reported that prostacyclin (PGI_2) protects cells from apoptosis [36,37]. Therefore, we also measured the level of PGI_2 in the medium. Since PGI_2 is very unstable in medium, we determined the level of 6-keto-$PGF_{1\alpha}$ (metabolite of PGI_2) instead of PGI_2. In the absence of NSAIDs, the concentration of 6-keto-$PGF_{1\alpha}$ in the medium was 0.7 nM. The IC_{50} value of indomethacin and celecoxib for inhibiting 6-keto-$PGF_{1\alpha}$ synthesis was about 5×10^{-9} M. We also examined the effect of exogenously added PGI_2 on necrosis and apoptosis induced by indomethacin. Due to the instability of PGI_2 in medium, we used carbaprostacyclin (stable analogue of PGI_2) instead of PGI_2. Exogenously added carbaprostacyclin did not affect the extent of cell death by short-term or long-term treatment with indomethacin (necrosis or apoptosis, respectively) (Fig. 3C and D). These results suggest that inhibition of PGI_2 synthesis by NSAIDs is not involved in NSAID-induced necrosis and apoptosis.

3.2. Development of gastric lesions by a combination of the oral administration of selective COX-2 inhibitors with the intravenous administration of non-selective NSAIDs

We considered that not only COX inhibition (inhibition of PG synthesis) but also the COX-independent direct cytotoxic effect of NSAIDs is involved in the development of gastrointestinal lesions *in vivo*. For testing this idea by pharmacological experiments, it is necessary to separate

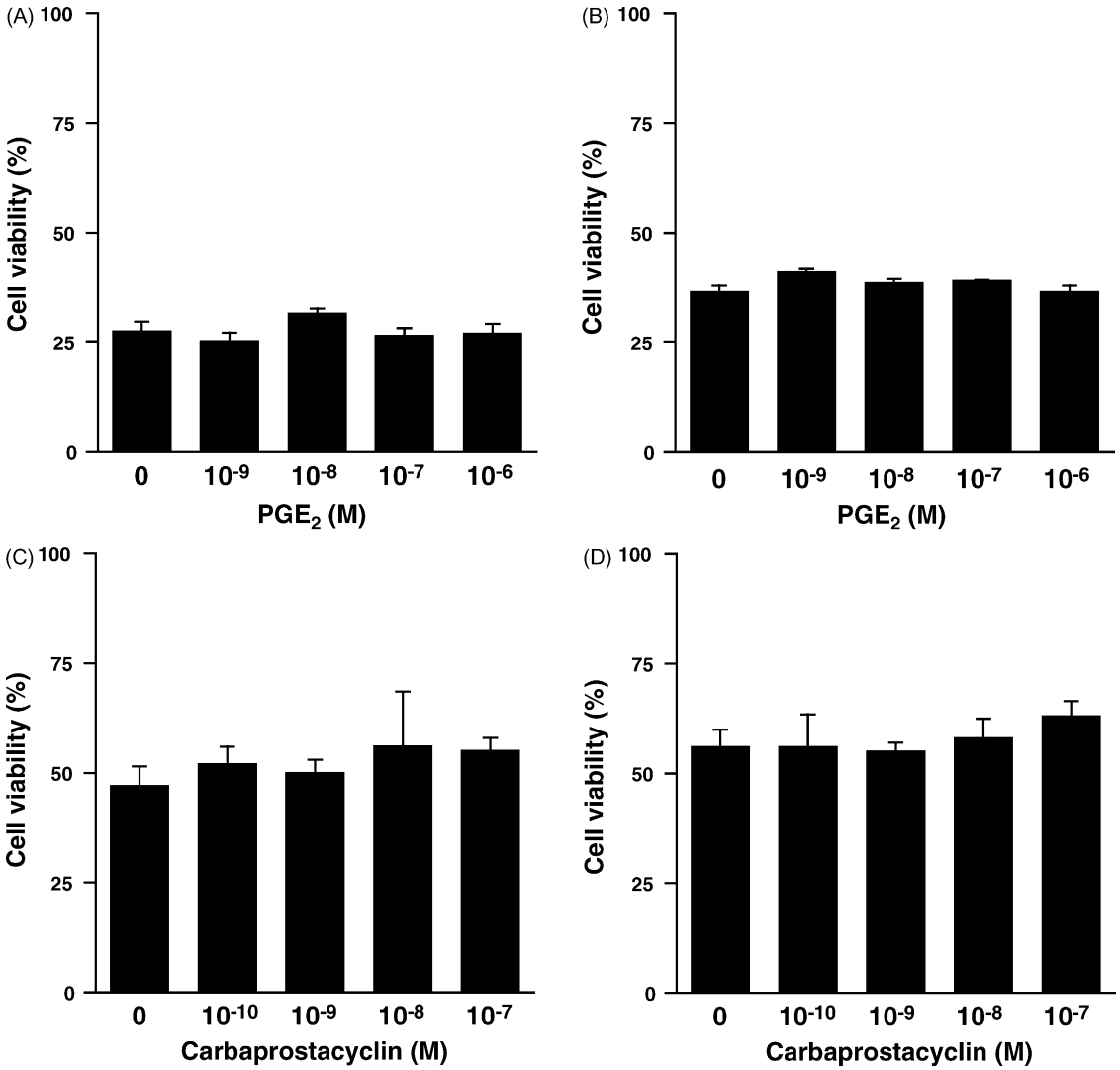

Fig. 3. Effect of PGE_2 on necrosis and apoptosis induced by NSAIDs. Cultured guinea pig gastric mucosal cells were incubated with 2.5 mM (A, C) or 0.9 mM (B, D) indomethacin for 1 hr (A, C) or 16 hr (B, D) in the presence of indicated concentrations of PGE_2 (A, B) or carbaprostacyclin (C, D). Cell viability was determined by the trypan blue exclusion test. Values are mean ± SEM (N = 3). Similar results were obtained by MTT assay.

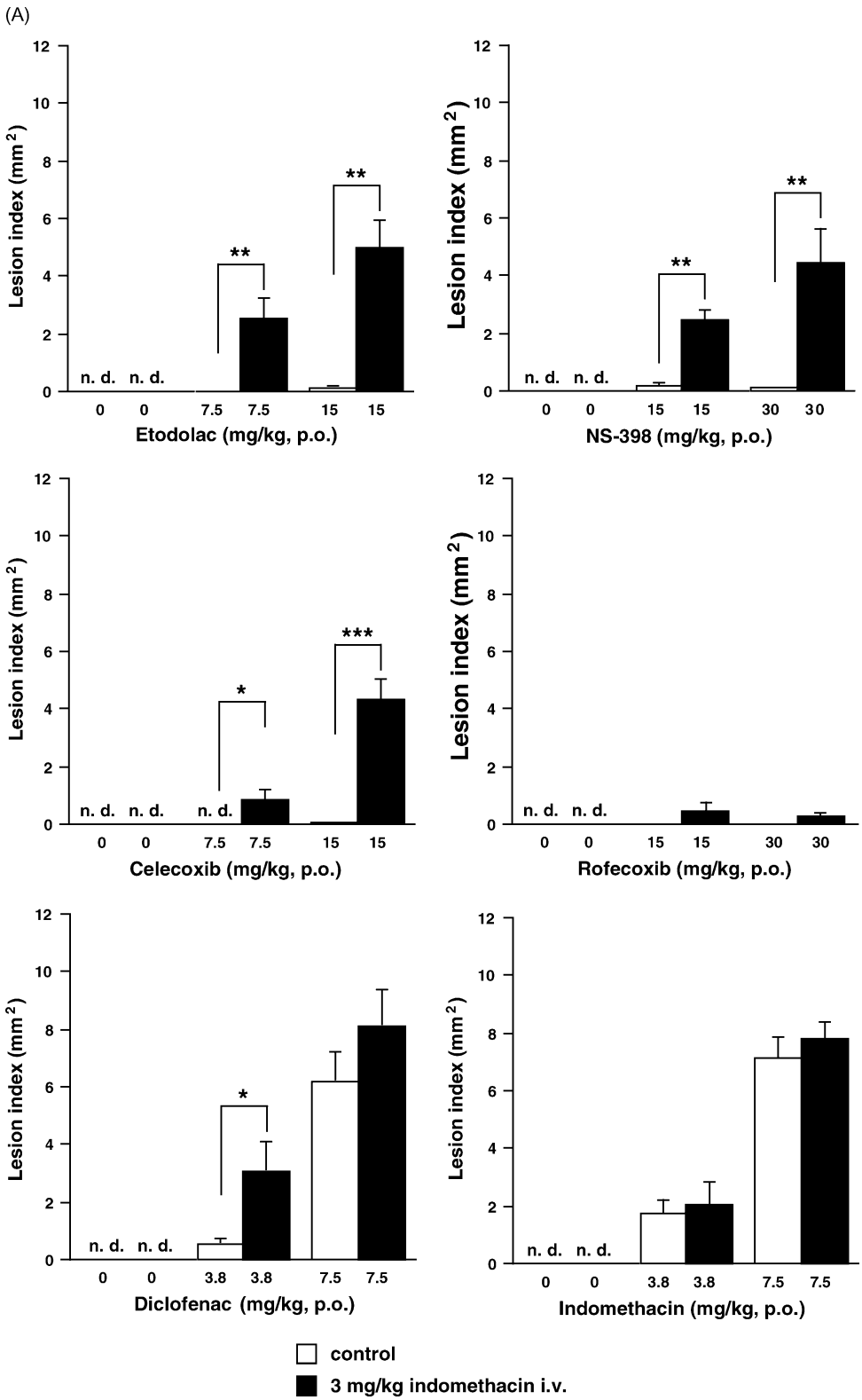

Fig. 4. Production of gastric lesions in rats. Rats were intravenously administered with 3 mg/kg indomethacin (A), both 3 mg/kg indomethacin and indicated dose of etodolac (B), 100 mg/kg aspirin (C), or vehicle. After 1 hr, animals were orally administered with NSAIDs as indicated or vehicle (A, C) (no oral administration (B)). After 6 hr, the stomach was removed and scored for hemorrhagic damage. Values are mean ± SEM (N = 6). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$ (both intravenously and orally administered groups vs. only orally administered groups). n.d.; not detected.

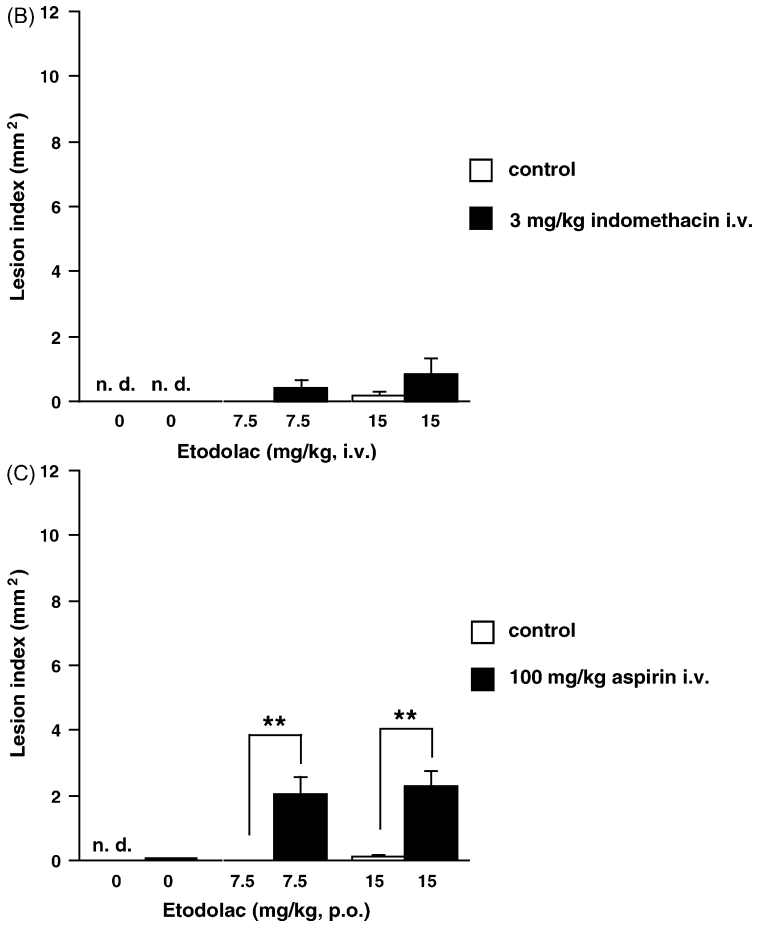

Fig. 4. (*Continued*).

these two properties of NSAIDs (i.e. COX inhibition and direct cytotoxicity) in the model of NSAID-induced gastric lesions *in vivo*. We tried to achieve this by employing intravenous administration of a non-selective NSAID (indomethacin) and oral administration of selective COX-2 inhibitors in rats. Intravenous administration of non-selective NSAIDs may cause inhibition of both COX-1 and COX-2 (thus inhibition of PG synthesis) at the gastric mucosa without any direct cytotoxicity to the gastric mucosa, because the concentration of NSAIDs at the gastric mucosa following intravenous administration is much lower compared to when NSAIDs are orally administered. On the other hand, oral administration of selective COX-2 inhibitors (except rofecoxib) may cause direct cytotoxicity to the gastric mucosa without inhibition of COX-1, and thus PG synthesis may be maintained.

Intravenous administration of indomethacin (3 mg/kg) in rats did not produce gastric lesions (Fig. 4A) even though the level of PGE_2 at the gastric mucosa was reduced by more than 90% (Table 2). These data suggest that inhibition of COX is not sufficient to produce gastric lesions. On the other hand, oral administration of selective COX-2 inhibitors (etodolac, NS-398, celecoxib, and rofecoxib) did not by themselves (i.e. without intravenous administration of indomethacin) produce gastric lesions (Fig. 4A). PGE_2 synthesis at the gastric mucosa was not inhibited by the oral administration of these selective COX-2 inhibitors (except for weak inhibition by 15 mg/kg etodolac) (Table 1). Therefore, the absence of gastric lesions only by oral administration of these selective COX-2 inhibitors can be explained by the fact that inhibition of PG synthesis is required for the development of gastric lesions by NSAIDs.

Table 2
Inhibition of gastric PGE_2 synthesis by NSAIDs *in vivo*

NSAIDs	Gastric PGE_2 (ng/g tissue)
Control	26.8 ± 1.0
3 mg/kg indomethacin i.v.	1.4 ± 0.4***
100 mg/kg aspirin i.v.	1.4 ± 0.2***
3.8 mg/kg indomethacin p.o.	2.3 ± 0.4***
3.8 mg/kg diclofenac p.o.	12.2 ± 1.1***
7.5 mg/kg diclofenac p.o.	3.5 ± 1.5***
7.5 mg/kg etodolac p.o.	18.2 ± 3.8
15 mg/kg etodolac p.o.	5.1 ± 1.4***
30 mg/kg NS-398 p.o.	20.2 ± 3.2
15 mg/kg celecoxib p.o.	25.3 ± 1.3
30 mg/kg rofecoxib p.o.	19.9 ± 4.1

Rats were intravenously (i.v.) or orally (p.o.) administered with indicated doses of NSAIDs. After 6 hr (p.o.) or 7 hr (i.v.), the level of PGE_2 in gastric mucosa was determined by ELISA. Values are mean ± SEM (N = 4–6).
***$P < 0.001$.

Interestingly, a combination of intravenous administration of indomethacin and oral administration of COX-2-selective inhibitors (except rofecoxib) clearly produced gastric lesions (Fig. 4A). On the other hand, a combination of intravenous administration of indomethacin and oral administration of rofecoxib did not significantly produce gastric lesions (Fig. 4A). We repeated experiments in Fig. 4A using piroxicam instead of indomethacin and obtained similar results (data not shown). Since among all of COX-2-selective inhibitors, only rofecoxib did not show direct cytotoxicity *in vitro* (Fig. 1), results in Fig. 4A suggest that direct cytotoxicity of NSAIDs is involved in production of gastric lesions. However, since it was recently proposed that inhibition of both COX-1 and COX-2 is required for the development of gastric lesions by NSAIDs [17,19,20], and indomethacin has a weak selectivity for COX-1 (1:0.3) [38], one can argue that the inhibition of COX-2 by intravenously administered indomethacin was not enough and that orally administered selective COX-2 inhibitors inhibited the remaining COX-2 activity, thereby resulting in the development of gastric lesions. However, this possibility was ruled out by an experiment which showed that intravenous administration of both indomethacin (3 mg/kg) and etodolac (15 mg/kg), which must inhibit both COX-1 and COX-2 [39], did not produce gastric lesions (Fig. 4B). We also showed that a combination of intravenous administration of indomethacin and oral administration of SC-560 (selective COX-1 inhibitor) produced gastric lesions (data not shown). Furthermore, we confirmed that the level of PGE_2 at the gastric mucosa with intravenous administration of indomethacin was not further decreased by oral administration of COX-2-selective inhibitors (data not shown). These combined results support our idea that not only COX inhibition (inhibition of PG synthesis) but also the COX-independent direct cytotoxic effect of NSAIDs is involved in the development of gastric lesions *in vivo*.

On the other hand, oral administration of indomethacin did produce gastric lesions without intravenous administration of indomethacin (Fig. 4A). Based on the hypothesis described above, these data can be explained by the fact that orally administered indomethacin had not only a direct cytotoxic effect but also resulted in COX inhibition (inhibition of PG synthesis) (Table 2), and thus produced gastric lesions without intravenous administration of indomethacin. Production of gastric lesions by oral administration of 3.8 but not 7.5 mg/kg diclofenac was increased by the prior intravenous administration of indomethacin (Fig. 4A), which may be related to the fact that orally administration of 7.5 mg/kg but not 3.8 mg/kg diclofenac inhibited PG synthesis completely (about 90%) (Table 2).

We also intravenously administered aspirin, a non-selective NSAID, instead of indomethacin (Fig. 4C). Administration of aspirin alone in this way did not produce gastric lesions, but lesions were produced when etodolac was administered orally in conjunction with intravenously administered aspirin (Fig. 4C). Similar results were obtained for NS-398 and celecoxib (data not shown). This result not only supports our hypothesis but also provides us with an important suggestion for the clinical use of selective COX-2 inhibitors (see Section 4).

4. Discussion

In this study, we have shown that the cytotoxic effects (necrosis and apoptosis) of NSAIDs on gastric mucosal cells *in vitro* are independent of COX inhibition by those NSAIDs. Furthermore, *in vivo* analysis using both oral and intravenous administration of NSAIDs suggested that not only COX inhibition but also the COX-independent direct cytotoxic effect of NSAIDs is involved in the development of gastric lesions. Both increase in aggressive factors and decrease in defensive factors cause gastropathy. As for NSAID-induced gastropathy, the decrease in defensive factors by NSAIDs (inhibition of PG synthesis) had been paid much attention. Results in this paper suggested that increase in aggressive factors by NSAIDs (direct cytotoxic effect of NSAIDs) is also involved in NSAID-induced gastropathy. This finding can be used to explain the previously unsolved issue that the decrease in PG levels and gastrointestinal lesions by NSAIDs are not always linked (see Section 1). Our findings can explain the fact that higher doses of NSAIDs were required for producing gastric lesions than those for inhibiting COX at the gastric mucosa [21,22], because higher concentrations were required for inducing the direct cytotoxic effect of NSAIDs than were required for inhibiting PG synthesis (Figs. 1 and 2). On the other hand, the fact that parenterally administered NSAIDs causes gastric and duodenal lesions [40–42] and that immunoneutralization of PGs causes gastric lesions [43] show that the direct cytotoxicity of NSAIDs is not essential for the development of gastric lesions by NSAIDs. Furthermore, at present, it is possible that mechanisms other than direct cytotoxicity and COX inhibition (such as increase in gastric motility by NSAIDs) are involved in results in Fig. 4. For example, the beneficial roles of COX-2 at gastric mucosa, such as stimulation of wound healing and resolution of inflammation, were reported [44]. Therefore, inhibition of these beneficial roles of COX-2 by oral administration of selective COX-2 inhibitors may be partly involved in results in Fig. 4. The mechanism of the direct cytotoxicity of NSAIDs and lack of the direct cytotoxicity in rofecoxib are also unclear at present. Furthermore, we have no direct evidence that necrosis and apoptosis are induced, accompanying with production of gastric lesions by NSAIDs *in vivo*.

A recently raised issue concerning the use of selective COX-2 inhibitors is their potential risk for cardiovascular thrombotic events [24,25], although there are still discussions on this point. PGI_2, a potent anti-aggregator of

platelets and a vasodilator, is mainly produced by COX-2 in vascular endothelial cells, while thromboxane A_2, a potent aggregator of platelets and a vasoconstrictor, is mainly produced by COX-1 in platelets [45–47]. Therefore, selective COX-2 inhibitors, but not non-selective NSAIDs, may lead to increased prothrombotic activity. Recent genetic studies using knockout mice for the receptor of PGI_2 or thromboxane A_2 supported this notion [48,49]. Furthermore, both animal and clinical data suggest that, compared to non-selective NSAIDs, selective COX-2 inhibitors increase cardiovascular thrombotic events [24,50,51]. Therefore, the method for decreasing the gastric side effects of NSAIDs other than increasing their selectivity for COX-2 may be useful in order to develop safer NSAIDs for both gastrointestine and cardiovascular. Considering our hypothesis described above, NSAIDs that do not exhibit direct cytotoxicity on gastric mucosal cells (i.e. NSAIDs that do not induce necrosis and apoptosis in gastric mucosal cells) may be safe for the gastrointestinal tract even if they do not have high selectivity for COX-2.

Low doses of aspirin are widely used for preventing thrombosis. Therefore, it is not unusual that patients who use aspirin chronically for preventing thrombosis are further administered with selective COX-2 inhibitors as anti-inflammatory drugs. Results (Fig. 4C) suggest that the oral administration of selective COX-2 inhibitors into chronic aspirin users causes gastric lesions, even though such administration into non-aspirin users is safe. Similar results were reported recently using simultaneous oral administration of aspirin and selective COX-2 inhibitors [52]. In fact, the Celecoxib Long-term Arthritis Safety Study (CLASS) showed that, compared to non-selective NSAIDs, celecoxib (at dosages greater than those indicated clinically) was clearly associated with a lower incidence of symptomatic lesions and lesion complication for patients not taking aspirin concomitantly. The difference in gastric side effects between them (non-selective NSAIDs and celecoxib) was not so clear for patients taking aspirin concomitantly [13]. Therefore, much attention should be paid to the concomitant use of both aspirin and selective COX-2 inhibitors.

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