Cytotoxic Synergy between Indomethacin and Hydrochloric Acid in Gastric Mucosal Cells

Ken-ichiro Tanaka,1) Wataru Tomisato, Shinji Tsutsumi,1) Tatsuya Hoshino,1) Tomofusa Tsuchiya, and Tohru Mizushima1,*)

Faculty of Pharmaceutical Sciences, Okayama University; 1–1–1 Tsushima-naka, Okayama 700–8530, Japan.
Received February 12, 2004; accepted April 8, 2004

Orally ingested non-steroidal anti-inflammatory drugs (NSAIDs) and acid in gastric secretions are gastric irritants that co-exist at the surface of the gastric mucosa. Here, we examined the individual and combined effects of indomethacin, a typical NSAID, and hydrochloric acid on cell death in primary cultures of guinea pig gastric mucosal cells. Indomethacin alone (at concentrations less than 200 μM) did not induce apoptosis; however, hydrochloric acid-induced apoptosis was stimulated in the presence of indomethacin (50—200 μM). Isobologram analysis confirmed the presence of a cytotoxic synergy between indomethacin and hydrochloric acid. The synergistic response between the two gastric irritants was also observed for necrosis. Given that the IC50 value of indomethacin for inhibition of prostaglandin synthesis is about 5 μM, the synergistic response between indomethacin and hydrochloric acid appears to be independent of the inhibition of cyclooxygenase activity by indomethacin.

Key words cytotoxic synergy; hydrochloric acid; indomethacin; gastric mucosal cell; apoptosis; necrosis

The gastric mucosa is frequently exposed to different types of irritants (such as alcohol, acid, pharmaceutical drugs and bacteria). These irritants can cause gastric mucosal cell death, resulting in the production of gastric lesions in vivo. Using primary cultures of guinea pig gastric mucosal cells, we recently reproduced this gastric irritant-induced cell death in vitro and revealed that all of the gastric irritants tested (ethanol, hydrogen peroxide, hydrochloric acid and non-steroidal anti-inflammatory drugs (NSAIDs) such as indomethacin) induced both necrosis and apoptosis of cells depending on the treatment conditions used. Short-term treatment of cells with relatively high concentrations of these irritants and long-term treatment of cells with relatively low concentrations of them induce necrosis and apoptosis, respectively.2,3) We have also previously shown that endogenous factors that protect the gastric mucosa from such irritants (heat shock proteins (HSPs) and prostaglandins (PGs)) or anti-ulcer drugs suppress apoptosis and necrosis in this cell model.4—9) This suggests that this in vitro system is useful for understanding the mechanisms underlying irritant-induced gastric lesions in vivo.

Most previous studies (including ours) pertaining to irritant-induced cell death in vitro have examined the effects of a single irritant on gastric mucosal cells. However, in vivo, multiple irritants co-exist simultaneously at the gastric mucosal surface. For example, when an NSAID is orally administered, the gastric mucosa is exposed not only to the NSAID but also to gastric acid (hydrochloric acid). Such an exposure to multiple irritants can give rise to gastric lesions. On this basis, in order to understand the mechanism of production of gastric lesions in vivo, it is important to examine what takes place in gastric mucosal cells in vitro when a combination of several irritants is used. In this study, we identified a synergistic response between indomethacin and hydrochloric acid in the induction of both apoptosis and necrosis in guinea pig gastric mucosal cells under primary culture. Furthermore, from the results obtained it is suggested that this synergistic response cannot be explained by an inhibitory effect of indomethacin on cyclooxygenase (COX) activity with a subsequent down regulation of PG synthesis.

MATERIALS AND METHODS

Chemicals and Media Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, New York, U.S.A.). RPMI 1640 was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Pronase E and type I collagenase were purchased from Kaken Pharmaceutical Co. (Kyoto, Japan) and Nitta Gelatin Co. (Osaka, Japan), respectively. Hydrochloric acid, indomethacin, PD98059, SP600125 and SB203580 were from Wako Co. (Tokyo, Japan). Hoechst 33342 (Ho 342), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and propidium iodide (PI) were from Sigma Co. (Tokyo, Japan). Male guinea pigs (4 weeks of age) were purchased from Shimizu Co., LTD (Kyoto, Japan). ELISA kit used for PGE2 analysis was from Cayman Chemical Co (Ann Arbor, Michigan, U.S.A.).

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.

Cell Culture Gastric mucosal cells were isolated from guinea pig fundic glands as described previously.9) Isolated gastric mucosal cells (6×105 cells/dish) were cultured for 12 h in RPMI 1640 containing 0.3% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in type-I collagen-coated plastic culture plates (Iwaki) under the conditions of 5% CO2/95% air and 37°C. After removing non-adherent cells by washing with RPMI 1640, cells that were attached to plates at about 50% confluence were used. Guinea pig gastric mucosal cell preparations cultured under these conditions have been previously characterized, with the majority (about 90%) of cells being identified as pit cells.10,11)

Treatment of Cells with Hydrochloric Acid and Indomethacin Cells were exposed to hydrochloric acid and/or indomethacin by replacement of the entire bathing medium with fresh medium containing these irritants at con-
centrations as noted in the text. Under the conditions of our culture medium, 10, 15, 18, 20 and 22 mM hydrochloric acid (final concentrations) caused the pH values of 7.8, 7.3, 6.8, 6.3, 5.8 and 4.8, respectively. Since we pre-warmed the medium under the conditions of 5% CO₂/95% air and 37 °C before the use of cell culture, these pH values were maintained from start to the end of cell culture (after 24 h).

For monitoring cell viability, cells were incubated for 2 h with MTT solution at a final concentration of 0.5 mg/ml. Cells were exposed to isopropanol and hydrochloric acid used at final concentrations of 50% (v/v) and 20 mM, respectively. The optical density of each sample at 570 nm was determined by spectrophotometer using a reference wavelength of 630 nm.¹²)

PGE₂ levels in culture media in the presence of different concentrations of indomethacin were determined by ELISA as previously described.¹³)

DNA Fragmentation Assay Apoptotic DNA fragmentation was monitored as previously described.¹¹) Briefly, cells were collected with a rubber policeman and suspended in 20 μl of lysis buffer, consisting of 50 mM Tris–HCl (pH7.8), 10 mM EDTA, and 0.5% sodium-N-lauroylsarcosinate. Proteinase K was added to give a final concentration of 1 mg/ml, and the lysate was incubated at 50 °C for 2 h. RNaseA was then added to give a final concentration of 0.5 mg/ml and the solution was incubated again at 50 °C for a further 30 min. Samples were analyzed by 2% agarose gel electrophoresis in the presence of 0.5 μg/ml ethidium bromide.

Nuclear Staining Assay Cells were washed with PBS and incubated with 0.17 mM Ho 342 and 100 μg/ml PI for 20 min, following which they were analyzed using fluorescence microscopy as previously described.¹⁰)

Statistical Analysis All values are expressed as the mean±standard error (S.E.M.). A one-way analysis of variance (ANOVA) followed by Scheffe’s multiple comparison was used for the evaluation of differences between 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.

Isobologram analysis was used for analyzing the combined effects of hydrochloric acid and indomethacin on cell death. The synergy between these irritants was estimated by determining a combination index (CI) value. The combined effect is one of summation when CI=1, synergism when CI<1, and antagonism when CI>1.¹⁴,¹⁵)

RESULTS AND DISCUSSION

A Synergistic Response between Indomethacin and Hydrochloric Acid in the Induction of Apoptosis In a first set of experiments we measured the sensitivity of primary cultures of gastric mucosal cells to exposure to indomethacin. Treatment of cells for 24 h with indomethacin at concentrations lower than 200 μM did not significantly decrease cell viability (Fig. 1A). On this basis we used concentrations of indomethacin less than 200 μM when examining

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Fig. 1. Cell Death Induced by Exposure to Indomethacin
Cultured gastric mucosal cells were treated with indicated concentrations of indomethacin for 24 h (A) or for 1 h (B). Cell viability was determined by the MTT method. Values are mean±S.E.M. (n=3).

Fig. 2. Cell Death Induced by the Combined Use of Indomethacin and Hydrochloric Acid
Cultured gastric mucosal cells were treated with different concentrations of hydrochloric acid (0, 10, 15, 18, 20, 22 mM) in the presence of indicated concentrations of indomethacin for 24 h (A) or for 1 h (B). The pH value of the medium was determined and plotted. Cell viability was determined by the MTT method. Values are mean±S.E.M. (n=3). ***p<0.001; **p<0.01; *p<0.05.
the combined effects of indomethacin and hydrochloric acid on the induction of cell death in cultured gastric mucosal cells.

Cells were exposed for 24 h to a range of concentrations of hydrochloric acid in the presence of different concentrations of indomethacin (Fig. 2A). In the absence of indomethacin, cell viability in the presence of hydrochloric acid decreased in a dose-dependent manner. In the presence of indomethacin (50, 100, 200 μM), the decrease in cell viability was observed at lower concentrations of hydrochloric acid (higher pH values) than when acid alone was used. Indomethacin did not affect the degree of hydrochloric acid-induced cell death when it was employed at concentrations lower than 50 μM (data not shown). Based on previous reports from this laboratory, it was postulated that the acid-induced cell death observed here and its up-regulation by indomethacin were most likely to be mediated by apoptosis. In support of this, DNA fragmentation assays showed that apoptotic DNA fragmentation was induced in a dose-dependent manner in the presence of hydrochloric acid and up-regulated in the presence of indomethacin (Fig. 3A). Moreover, in double-staining experiments with PI and Ho 342, we tested for the presence of

![A] Indomethacin (μM) pH 7.8 6.3 5.8 6.3 5.8

![B] Control (pH 7.8) 200 μM Indomethacin (pH 7.8)

![pH 6.3] 200 μM Indomethacin (pH 6.3)

![pH 4.8]

Fig. 3. Apoptotic DNA Fragmentation and Chromatin Condensation by a Combination between Indomethacin and Hydrochloric Acid

Cultured gastric mucosal cells were treated with various concentrations of hydrochloric acid in the presence or absence of 200 μM indomethacin for 24 h. The pH value of the medium was determined and shown. Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (A). After staining with both PI and Ho 342, cells were observed under fluorescence microscope. For positive control of necrosis, cells were cultured under the conditions of pH 4.8 (B).
apoptosis on the basis that living cells and apoptotic cells would not stain pink with propidium iodide, but that necrotic cells would stain in this way due to a loss of membrane integrity. Furthermore, Ho 342 can visualize the chromatin condensation of apoptotic cells.\(^{19}\) As shown in Fig. 3B, cells treated both hydrochloric acid and indomethacin under the above conditions were not stained with propidium iodide and showed the chromatin condensation. Further confirmation of induction of apoptosis by measuring cytochrome c release from mitochondria may be necessary.

In order to address the issue of a synergistic relationship between indomethacin and hydrochloric acid in the induction of apoptosis, CI values were calculated from the results of experiments presented in Figs. 1A and 2A. The CI value was 0.39, showing that a synergistic response for apoptosis was obtained when indomethacin and hydrochloric acid were used in unison.

**A Synergistic Response between Indomethacin and Hydrochloric Acid for Necrosis** As described in the Introduction, short-term (1 h) treatment of cells with relatively high concentrations of gastric irritants induces necrosis.\(^{2,23}\) We next examined the synergy between indomethacin and hydrochloric acid in relation to the induction of necrosis. The sensitivity of gastric mucosal cells to short-term (1 h) indomethacin treatment is shown in Fig. 1B. As shown in Fig. 2B, in the presence of indomethacin, a decrease in cell viability was observed at lower concentrations of hydrochloric acid (higher pH values) than in the absence of indomethacin. It was concluded that the cell death induced in this manner was mainly mediated by necrosis, given that the cell death was not accompanied by apoptotic DNA fragmentation and that pink nuclear staining was observed in double-staining experiments with PI and Ho 342 (data not shown).

The CI values between indomethacin and hydrochloric acid for necrosis were calculated from the results of experiments presented in Figs. 1B and 2B. The CI value was 0.56, showing that indomethacin and hydrochloric acid used in a combined manner also act synergistically to produce necrosis in gastric mucosal cells. The overall response, however, was not as strong as that for apoptosis.

**Mechanism of the Synergistic Response between Indomethacin and Hydrochloric Acid** NSAIDs inhibit COX activity and as such they decrease the level of circulating PGs. In turn, PGs, and PGE\(_2\) in particular, have cytoprotective effects on the gastric mucosa.\(^{7,8,17,18}\) We previously reported that the primary culture of guinea pig gastric mucosal cells produced PGE\(_2\) continuously even without stressors.\(^{19}\) On this basis we considered whether or not the effect of indomethacin on hydrochloric acid-induced apoptosis such as that seen in Fig. 2A might be caused by an indomethacin-induced inhibition of COX activity. PGE\(_2\) levels in culture media after 1 h treatment with a range of concentrations of indomethacin were measured by ELISA and compared to the effect of indomethacin on the IC\(_{50}\) (concentration required for 50% reduction of cell viability) values of hydrochloric acid for apoptosis. As shown in Fig. 4, much higher concentrations of indomethacin were required to decrease the IC\(_{50}\) value than were required to decrease PGE\(_2\) levels. This inhibitory effect of indomethacin on PGE\(_2\) levels was basically same when the period of treatment was prolonged to 24 h (data not shown). It seems that the synergistic response between indomethacin and hydrochloric acid for apoptosis cannot be explained by an inhibitory effect of indomethacin on COX activity and subsequent PG synthesis. Recently, some NSAIDs were reported to activate mitogen-activated protein kinases (MAPKs), such as extracellular signal-regulated kinase (ERK), c-jun NH\(_2\)-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38).\(^{20,21}\) They were then treated for 24 h with different concentrations of hydrochloric acid in the presence 200 μM indomethacin. The pH value of the culture medium was determined and plotted. Cell viability was determined by the MTT method. Values are mean±S.E.M. (n=3).

![Fig. 4. Relationship between Inhibition of PG Synthesis and the Synergistic Response by Indomethacin](image)

**Fig. 4. Relationship between Inhibition of PG Synthesis and the Synergistic Response by Indomethacin**

Cultured gastric mucosal cells were treated for 24 h with different concentrations of hydrochloric acid in the presence of a range of indomethacin concentrations. Cell viability was determined by the MTT method. The IC\(_{50}\) values for hydrochloric acid (concentration of H\(^+\) required for 50% reduction of cell viability) were determined. PGE\(_2\) levels in culture media were determined by ELISA after 1 h treatment of indomethacin.

![Fig. 5. Effect of Inhibitors of MAPKs on the Cytotoxic Synergy between Indomethacin and Hydrochloric Acid](image)

**Fig. 5. Effect of Inhibitors of MAPKs on the Cytotoxic Synergy between Indomethacin and Hydrochloric Acid**

Cultured gastric mucosal cells were pre-treated for 1 h with 10 μM PD98059 (MEK1/ERK inhibitor), SP600125 (an inhibitor for JNK) and SB203580 (an inhibitor for p38). They were then treated for 24 h with different concentrations of hydrochloric acid in the presence 200 μM indomethacin. The pH value of the culture medium was determined and plotted. Cell viability was determined by the MTT method. Values are mean±S.E.M. (n=3).
In addition to activation of MAPKs, NSAIDs are known to affect cell physiology through various COX-independent mechanisms, such as activation of peroxisome proliferator-activated receptors (PPAR-γ) and inhibition of NF-κB. An antagonist of PPAR-γ (GW9662) did not affect the level of hydrochloric acid-induced apoptosis in the presence of indomethacin (data not shown), suggesting that PPAR-γ is not involved in the synergistic response between indomethacin and hydrochloric acid. It is possible that other mechanisms such as inhibition of NF-κB are involved in this synergistic response.

In this study, we have shown for the first time that hydrochloric acid-induced cell death (apoptosis and necrosis) can be observed at lower acid concentrations when experiments are performed in the presence of indomethacin. Isobologram analysis (CI values) showed that the combined effect between indomethacin and hydrochloric acid was synergistic. When gastric lesions are produced following the oral administration of NSAIDs, the gastric mucosa is exposed not only to the NSAID but also to gastric acid. Concentrations of indomethacin used in this study were much lower than our previous reports, however, it is still unclear whether or not these concentrations of indomethacin are clinically relevant, considering the low pH value at gastric mucosa. Detection of apoptosis at gastric mucosa in vivo is important to solve this question.

We recently proposed that both COX inhibition by NSAIDs together with the direct cytotoxicity of NSAIDs are involved in NSAID-induced gastric lesions. That is, intravenously administered indomethacin, which completely inhibited COX activity at the gastric mucosal surface, did not produce gastric lesions. Moreover, nor did orally administered selective COX-2 inhibitors, which have direct cytotoxic effects (apoptosis and necrosis). However, a combination of the oral administration of selective COX-2 inhibitors together with the intravenous administration of indomethacin clearly produced gastric lesions. The fact that parenterally administered NSAIDs cause gastric and duodenal lesions in effect worked against our model because the NSAID concentration at the gastric mucosal surface after parenteral NSAID administration is too low to cause direct cytotoxicity. However, based on the results of the present study, we consider that cell death (apoptosis and necrosis) could be induced in the gastric mucosa in the presence of such low concentrations of NSAID due to the cytotoxic synergy that exists between NSAID and gastric acid.

REFERENCES

1) Present address: Graduated School of Medical and Pharmaceutical Sciences, Kumamoto University, 5–1 Oe-honmachi, Kumamoto 862–0972, Japan.