

NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture

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Received 4 December 2000; accepted in final form 14 May 2001

Tomisato, Wataru, Shinji Tsutsumi, Kazuhito Rokutan, Tomofusa Tsuchiya, and Tohru Mizushima. NSAIDs induce both necrosis and apoptosis in guinea pig gastric mucosal cells in primary culture. *Am J Physiol Gastrointest Liver Physiol* 281: G1092–G1100, 2001.—A major clinical problem encountered with the use of nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin is gastropathy. In this study, we examined, using guinea pig gastric mucosal cells in primary culture, how NSAIDs damage gastric mucosal cells. The short-term treatment of cells with high concentrations of indomethacin decreased cell viability in the absence of apoptotic DNA fragmentation, chromatin condensation, or caspase activation. Cells lost membrane integrity with this short-term indomethacin treatment, suggesting that indomethacin induced necrosis under these conditions. In contrast, the long-term treatment of cells with low concentrations of indomethacin decreased cell viability and was accompanied by apoptotic DNA fragmentation, chromatin condensation, and caspase activation. Pretreatment of cells with inhibitors of caspases or protein synthesis suppressed cell death caused by long-term indomethacin treatment, suggesting that apoptosis was induced when the inhibitors were not present. These results imply that NSAIDs cause gastric mucosal damage through both necrosis and apoptosis of gastric mucosal cells.

gastric mucosal injury; indomethacin; aspirin; caspases; cell death

GASTROPATHY ASSOCIATED WITH the use of nonsteroidal anti-inflammatory drugs (NSAIDs) is one of the most frequent types of gastric mucosal injury (10, 22, 25). To establish a clinical protocol for overcoming this side effect, the molecular mechanism governing NSAID-induced gastric mucosal injury needs to be elucidated. Inhibition of the synthesis of PGs, which have cytoprotective effects on gastric mucosa, has been thought to be the major mechanism of the gastrototoxic effects of NSAIDs. However, evidence has suggested that the gastrototoxic effects of NSAIDs cannot be explained only by their inhibitory effects on PG synthesis. For example, administration of aspirin (25 mg/kg) inhibited ~90% of PG synthesis in rats, but it did not cause

gastric mucosal lesion (19). It has also been proposed that NSAID-dependent gastric hypermotility, disturbances in mucosal blood flow, and activation of neutrophils are involved in the gastrototoxic effects of NSAIDs (10, 22, 25). In addition to these indirect effects of NSAIDs, direct cytotoxic effects of NSAIDs on gastric mucosal cells are also important in relation to gastropathy associated with the use of NSAIDs in vivo (10, 22, 25).

Various stresses cause cell death through either apoptosis or necrosis. Several in vivo experiments (1, 7, 28) have suggested that NSAIDs induce apoptosis in gastric mucosal cells. Although there are no reports demonstrating that NSAIDs induce necrosis in gastric mucosal cells in vivo and in vitro, the activation by NSAIDs of neutrophils and macrophages accompanying gastropathy suggests that NSAID-induced necrosis may be also involved in the gastrototoxic effects of NSAIDs in vivo.

Studies (5, 26) on the mechanism of how NSAIDs prevent colon cancer showed that NSAIDs could induce apoptosis in colon cancer cells. Several experiments (9, 17) using cell lines derived from gastric mucosa suggested that NSAIDs could induce apoptosis in gastric mucosal cells. The use of cells in primary culture is thought to mimic cells in vivo better than the use of cell lines, in addition to which the primary culture of guinea pig gastric mucosal cells has been established and well characterized (13, 23). Thus an examination of the cytotoxicity of NSAIDs using primary culture cells may provide useful information about the mechanism of NSAID-induced gastropathy. We (32) recently reported, using primary cultures of guinea pig gastric mucosal cells, that the short-term exposure of cells to indomethacin rapidly decreased cell viability in vitro in a dose-dependent manner. However, the mechanism of this cell death is yet to be elucidated. In this study, we have examined the mechanism of indomethacin-dependent cell death in primary cultures of guinea pig gastric mucosal cells. The results clearly show that NSAIDs induce both necrosis and apoptosis depending

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on the treatment conditions; short-term treatment of cells with high concentrations of NSAIDs and long-term treatment of cells with low concentrations of NSAIDs induce necrosis and apoptosis, respectively.

MATERIALS AND METHODS

Chemicals and media. RPMI 1640 was obtained from Nissui Pharmaceutical (Tokyo, Japan). FCS, trypsin solution, and trypan blue were from GIBCO-BRL (Grand Island, NY). Pronase E and type I collagenase were obtained from Kaken Pharmaceutical (Kyoto, Japan) and Nitta Gelatin (Osaka, Japan), respectively. Indomethacin, aspirin, sodium-*N*-lauroylsarcosinate, and cycloheximide were from Wako (Tokyo, Japan). Proteinase K, RNase A, Hoechst 33342, and propidium iodide (PI) were from Sigma (Tokyo, Japan). Peptides for assays of caspases and an inhibitor of caspase activity [*N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk)] were from Peptide Institute (Osaka, Japan). [³H]glucosamine (18.7 Ci/mmol) was from Amersham Pharmacia Biotech (Tokyo, Japan).

Preparation and culture of gastric mucosal cells. Male guinea pigs (4 wk of age) were obtained from Shimizu (Kyoto, Japan). Gastric mucosal cells were isolated from guinea pig fundic glands, as described previously (13, 21, 23). Isolated gastric mucosal cells were cultured for 12 h in RPMI 1640, containing 0.3% vol/vol FCS, 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 nonadherent cells were removed by washing with RPMI 1640, cells attached to the plate at ~50% confluence were used. We (13, 21) previously characterized guinea pig gastric mucosal cell preparations under these conditions, with the majority (~90%) of cells being identified as pit cells.

Treatment of cells with indomethacin, aspirin, cycloheximide, or Z-VAD-fmk and trypan blue exclusion test. Cells were exposed to drugs by changing the entire bathing medium. Because indomethacin and aspirin were dissolved in 0.2 M Na₂CO₃ and DMSO, respectively, control experiments were carried out with final concentrations of 2 mM Na₂CO₃ or 0.3% vol/vol DMSO, which corresponded to the concentrations of each of the compounds in NSAID-treatment experiments. We confirmed that the concentrations of these compounds did not affect cell growth or viability or the pH value of the medium. For some experiments, cells were preincubated with 30 µM Z-VAD-fmk or 10 µg/ml cycloheximide for 1 h. After treatment with NSAIDs, cells were treated with 1% trypsin and collected by centrifugation. Cells were resuspended in PBS containing 0.2% trypan blue dye and observed under a microscope.

DNA fragmentation assay. Apoptotic DNA fragmentation was monitored, as described previously (20). Cells were collected with a rubber policeman and suspended with 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°C for 2 h. RNaseA was then added to a final concentration of 0.5 mg/ml and incubated at 50°C for 30 min. These samples were analyzed by 2% agarose gel electrophoresis in the presence of 0.5 µg/ml ethidium bromide.

Nuclear staining assay for necrosis and apoptosis. As previously described (12) for the determination of the extent of necrosis after NSAID treatment, cells were washed with PBS and incubated with 0.17 mM Hoechst 33342 and 100 µg/ml PI for 20 min. Cells were then analyzed using fluorescence microscopy.

To determine the level of apoptosis after NSAID treatment, cells were washed with PBS and fixed with 10% vol/vol formaldehyde for 4 h (12). Cells were then washed with PBS, stained with 0.17 mM Hoechst 33342, and observed under a fluorescence microscope as previously described (12).

Caspase activity assay. The activities of caspase-3, caspase-8, and caspase-9 were determined as described previously (11, 31). 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 MgCl₂, and 1 mM dithiothreitol). Suspensions were sonicated and centrifuged, after which the supernatants were incubated with fluorogenic peptide substrates [*N*-acetyl-DEVD-MCA (caspase-3), *N*-acetyl-IETD-MCA (caspase-8), and *N*-acetyl-LEHD-MCA (caspase-9)] in reaction buffer (100 mM HEPES-KOH, pH 7.5, 10% sucrose, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 1 mg/ml BSA) for 15 min at 37°C. The release of 7-amino-4-methylcoumarin (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.

Measurement of incorporation of [³H]glucosamine into cells. Measurement of incorporation of [³H]glucosamine into cells was done as described previously (23). Gastric mucosal cells were incubated with [³H]glucosamine (18.7 Ci/mmol) in the presence or absence of indomethacin for 16 h. Cells were washed with PBS three times and lysed by the solution containing Tris·HCl (pH 7.2) and 2% Triton X-100. Samples were incubated for 15 min on ice in the presence of 50% ethanol and washed with 70% ethanol three times. The precipitates were suspended with 1 N NaOH and neutralized by acetic acid. Radioactivity was calculated in a liquid scintillation counter.

Statistical analysis. All values are expressed as means ± SE. Student's *t*-test for paired results was performed for the evaluation of differences between the groups. Differences were considered to be significant for values of *P* < 0.05.

RESULTS

Effect of short- and long-term treatments with NSAIDs on cell viability and chromosomal DNA. We (32) previously reported that treatment of gastric mucosal cells in primary culture with indomethacin for 1 h decreased cell viability in a dose-dependent manner. To understand the mechanism of this cell death, we examined the state of chromosomal DNA under conditions of indomethacin treatment. Chromosomal DNA was extracted after the treatment of cells for 1 h with various concentrations of indomethacin, and the change in size of chromosomal DNA was analyzed by agarose gel electrophoresis in the presence of ethidium bromide. As shown in Fig. 1A, treatment of cells with indomethacin for 1 h decreased cell viability in a dose-dependent manner. The IC₅₀ of indomethacin for cell viability was estimated to be ~2 mM, which is much the same as that reported previously (32). Treatment of cells with indomethacin for 1 h did not affect the size of chromosomal DNA, even at high concentrations (2.5 mM) (Fig. 1B). These results suggest that the short-term treatment of gastric mucosal cells with indomethacin does not induce apoptosis, which would be accompanied with apoptotic DNA fragmentation. These indomethacin treatments may, however, induce necrosis in gastric mucosal cells.

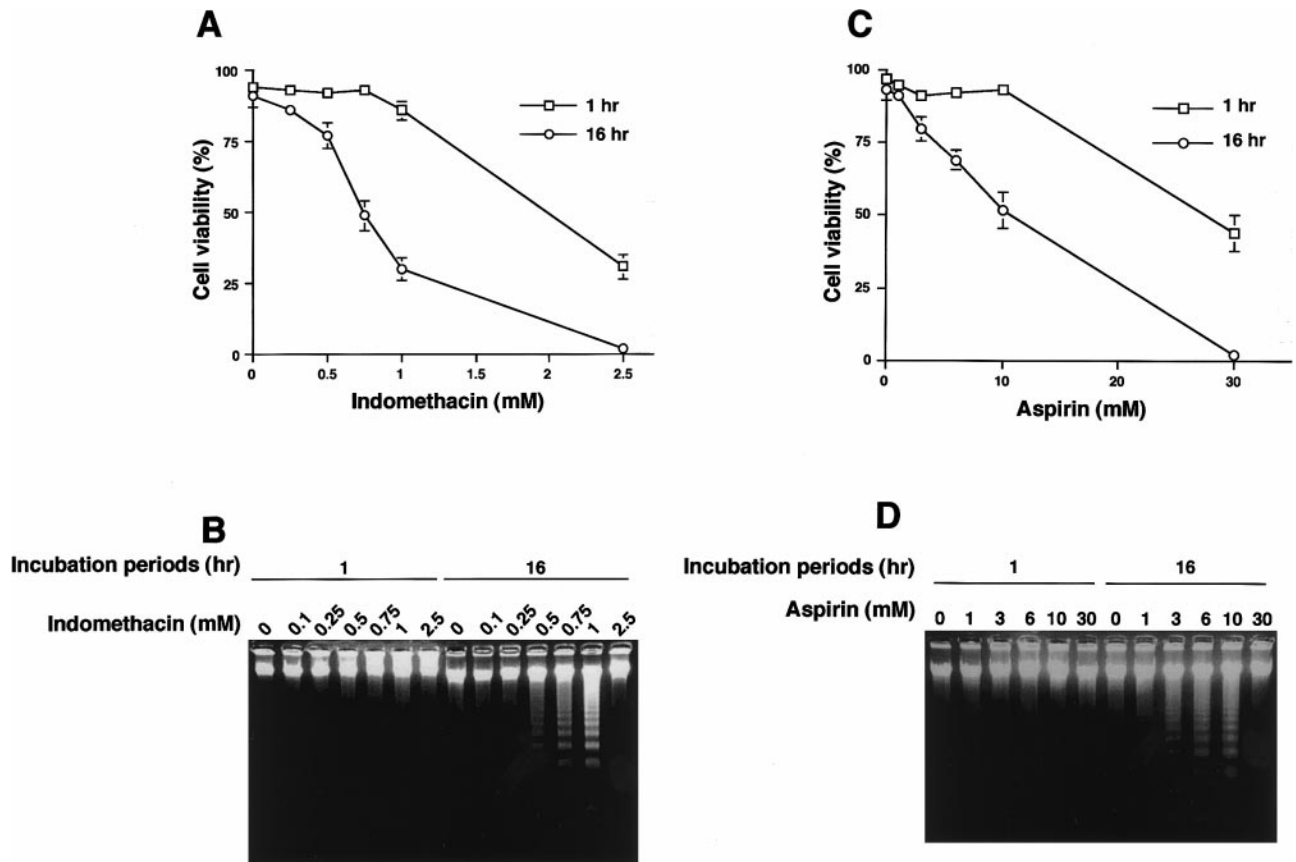


Fig. 1. Effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on cell viability and chromosomal DNA. Cultured gastric mucosal cells were incubated with indicated concentrations of indomethacin (A and B) or aspirin (C and D) for 1 or 16 h. Cell viability was determined by the trypan blue exclusion test (A and C). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (B and D). Values are mean \pm SD; $n = 3$.

Studies (5, 9, 17, 26) on cell lines derived from gastric mucosa and colon cancer cells showed that long-term treatment (6–48 h) of cells with indomethacin induced apoptosis. We therefore examined whether or not long-term treatment with indomethacin could induce apoptosis in gastric mucosal cells in primary culture. As shown in Fig. 1B, treatment of cells with 0.5–1 mM indomethacin for 16 h induced apoptotic DNA fragmentation. The IC_{50} of indomethacin for cell viability was estimated to be \sim 0.75 mM. Treatment of cells with a higher concentration (2.5 mM) of indomethacin did not induce apoptotic DNA fragmentation, although this treatment decreased cell viability to 0%. These results suggest that long-term treatment of gastric mucosal cells with low concentrations of indomethacin induce apoptosis.

Because the gastric mucosal cells are highly confluent in vivo, we performed the same experiments described above using 100% confluent cells. Results were basically the same as those with 50% confluent cells (Fig. 1, A and B) except for the concentrations required for cell death. The IC_{50} of indomethacin for cell viability after 1- and 16-h treatment was 3 and 1.5 mM, respectively. Apoptotic DNA fragmentation was observed in cells treated with 1 or 2 mM indomethacin for 16 h but not in cells treated with 2 or 4 mM indometh-

acin for 1 h (data not shown). Furthermore, very faint DNA fragmentation was observed in control cells (without indomethacin treatment) after 16 h of incubation (data not shown). This is because 100% confluent cells tend to undergo spontaneous apoptosis accompanied with maturation after long-period incubation in vitro (30). Thus we used 50% confluent cells in the following experiments.

It is possible that some products released into the medium during indomethacin treatment caused apoptosis and necrosis, i.e., indomethacin indirectly induced apoptosis and necrosis in cultured gastric mucosal cells. However, this may not be likely based on the following experiments. Culture medium after 1-h treatment of cells with 2.5 mM indomethacin (or 16-h treatment of cells with 1 mM indomethacin) was subjected to ultrafiltration (molecular weight limit, 5,000) to remove indomethacin. The resultant solution did not decrease cell viability when newly prepared gastric mucosal cells were incubated with the solution for 1 or 16 h. This is different from the culture medium before the ultrafiltration, which induced necrosis and apoptosis (data not shown). These results suggest that products (at least with high molecular weight) released to the medium from cells upon indomethacin treatment

were not involved in necrosis and apoptosis by indomethacin.

We also examined the effect of aspirin on cell viability and chromosomal DNA in the same way as for indomethacin to test whether the results for the time and concentration dependence of DNA fragmentation with indomethacin treatment are specific for indomethacin or are a general property of all NSAIDs. The results for aspirin treatment of gastric mucosal cells in primary culture were basically the same as for indomethacin; the treatment of cells for 1 h with aspirin decreased cell viability without apoptotic DNA fragmentation, whereas a 16-h treatment with aspirin decreased cell viability and induced apoptotic DNA fragmentation simultaneously (Fig. 1, *C* and *D*). We therefore find that NSAIDs can induce both necrosis and apoptosis in gastric mucosal cells in primary culture depending on the concentration strength and length of incubation period. The concentration of aspirin required for gastric mucosal cell death *in vitro* was ~10 times higher than that of indomethacin (Fig. 1, *A* and *C*), which is in agreement with the corresponding concentrations of these NSAIDs required for gastrotoxic effects *in vivo* (7, 18). Thus cell death induced by these NSAIDs *in vitro* may reflect their gastrotoxic effects on gastric mucosa *in vivo*.

Necrosis by short-term treatment with indomethacin. The results described above suggest that short-term treatments of gastric mucosal cells with indomethacin induce necrosis. To confirm this finding, we carried out double-staining experiments with PI and Hoechst 33342. Because necrotic cells lose their membrane integrity, PI staining causes pink nuclear staining in necrotic cells, whereas living cells and apoptotic cells

are not stained with PI (12). For these experiments, we used concentrations of indomethacin that caused an ~70% decrease in cell viability (1-h treatment, 2.5 mM; 16-h treatment, 1 mM). Control cells (for both 1- and 16-h incubations) and cells treated with indomethacin for 16 h did not stain with PI (Fig. 2). In contrast, cells treated with 2.5 mM indomethacin for 1 h showed pink nuclear staining (Fig. 2). These results strongly suggest that the short-term treatment of cells with indomethacin causes cell death through necrosis. Because cells were not fixed in these experiments, the staining of cells with Hoechst 33342 was weak.

Apoptosis by long-term treatment with indomethacin. One of characteristic features of apoptosis in cells is chromatin condensation. We therefore examined the state of chromatin in cells treated with indomethacin or aspirin by employing Hoechst 33342 staining. Cells were fixed with 10% formaldehyde and stained with 0.17 mM Hoechst 33342. We used concentrations of NSAIDs that caused an ~50–70% decrease in cell viability (1-h treatment, 2.5 mM indomethacin and 30 mM aspirin; 16-h treatment, 1 mM indomethacin and 10 mM aspirin). Observations using fluorescence microscopy showed that long-term treatment of cells with indomethacin or aspirin clearly induced chromatin condensation (Fig. 3). On the other hand, chromatin condensation was not apparent after the short-term treatment of cells with indomethacin or aspirin (Fig. 3). These results support the idea that long- and short-term treatments of gastric mucosal cells with NSAIDs induce apoptosis and necrosis, respectively.

Activation of caspases by indomethacin. Most apoptotic events are mediated by the sequential activation of caspases (4, 16). Among them, caspase-3 is located

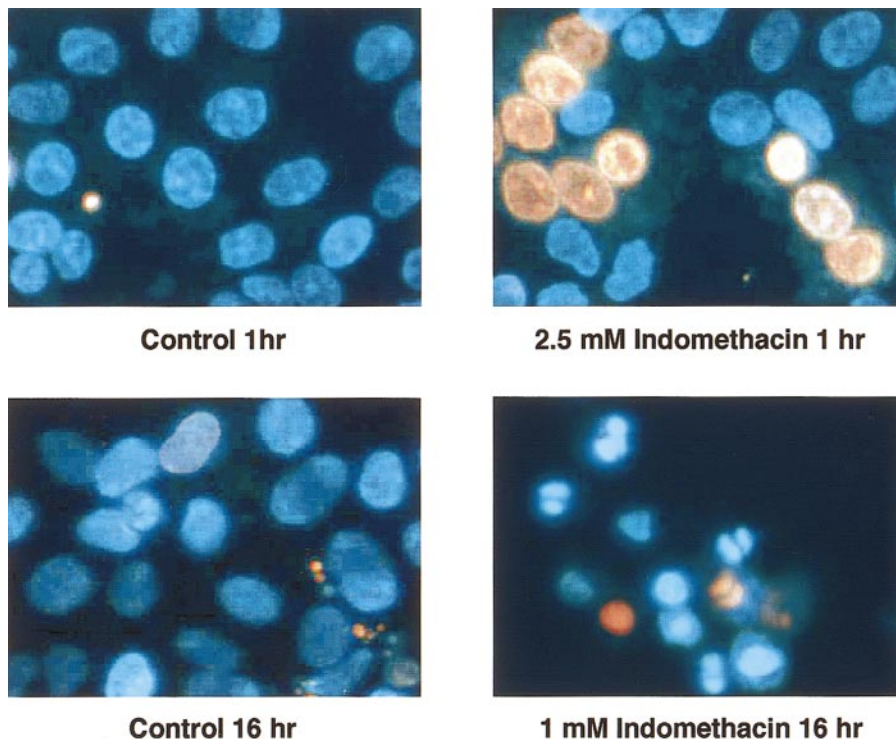


Fig. 2. Induction of necrosis by short-term treatment with indomethacin. Cultured gastric mucosal cells were incubated in the presence or absence of indomethacin for 1 or 16 h. After staining with both Hoechst 33342 and propidium iodide, cells were observed under a fluorescence microscope.

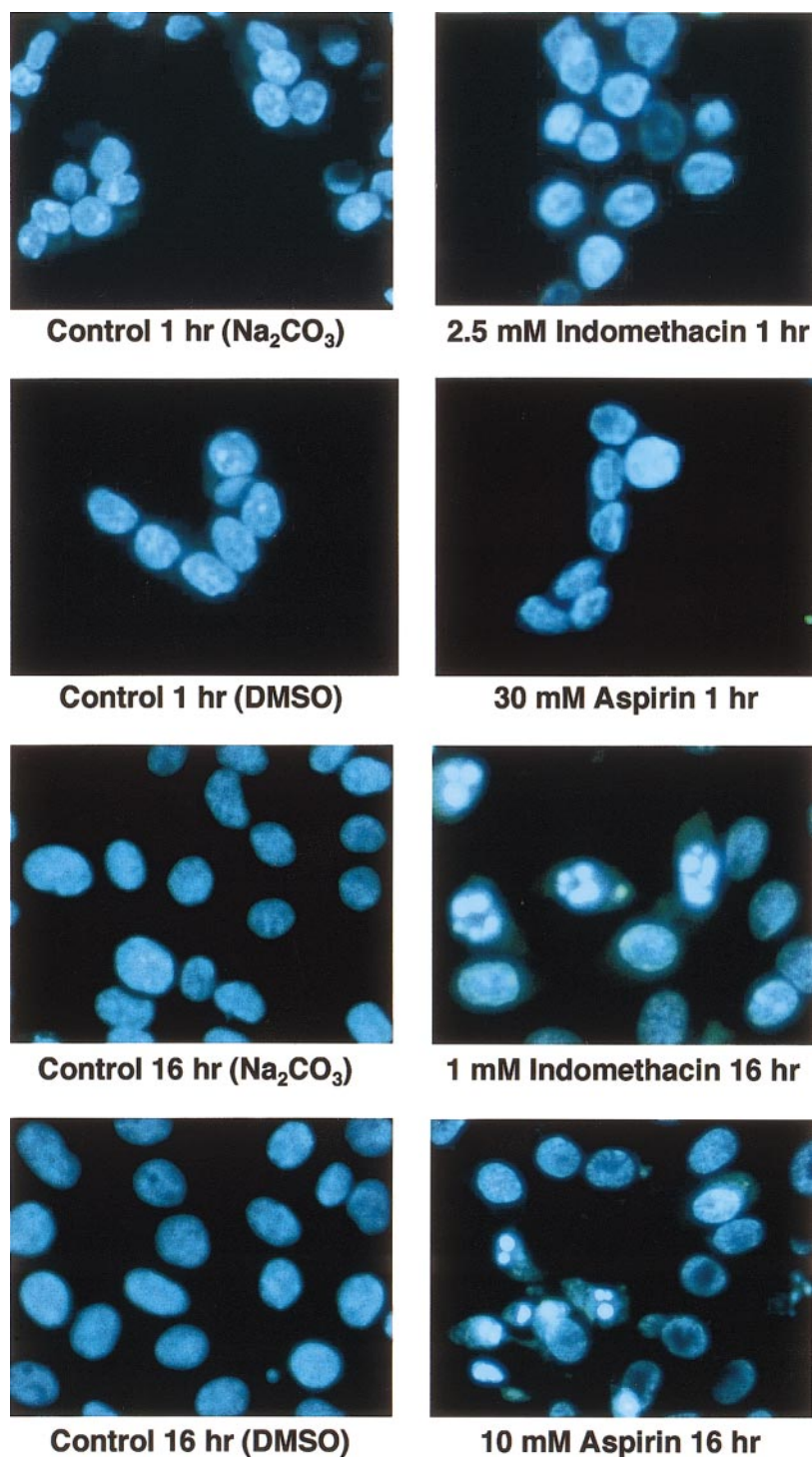


Fig. 3. Induction of apoptosis by long-term treatment with NSAIDs. Cultured gastric mucosal cells were incubated with indicated concentrations of indomethacin or aspirin for 1 or 16 h. Control experiments were performed with 2 mM Na_2CO_3 or 0.3% DMSO, corresponding to the concentrations of these chemicals in NSAID-treated experiments. Cells were fixed with formaldehyde, stained with Hoechst 33342, and observed under a fluorescence microscope.

downstream of the pathway and directly activates proteins responsible for DNA fragmentation and chromatin condensation (4). Because caspase-8 and caspase-9 activate caspase-3 (4), we subsequently examined whether or not NSAIDs were able to activate these caspases in gastric mucosal cells in primary culture. After incubation with indomethacin for 1 or 16 h, cells were extracted, and their caspase activities were examined by the use of specific fluorogenic peptide substrates [*N*-acetyl-DEVD-MCA (caspase-3), *N*-acetyl-IETD-MCA

(caspase-8), and *N*-acetyl-LEHD-MCA (caspase-9)]. We used concentrations of indomethacin that caused an ~70% decrease in cell viability (1-h treatment, 2.5 mM; 16-h treatment, 1 mM). As shown in Fig. 4, the long-term treatment of cells with indomethacin induced significant activation of all caspases tested. Treatment of cells with 1 mM indomethacin for 16 h caused 17-, 8-, and 11-fold increases in the activation of caspase-3, caspase-8, and caspase-9, respectively, suggesting that apoptosis by long-term treatment with indomethacin is mediated by

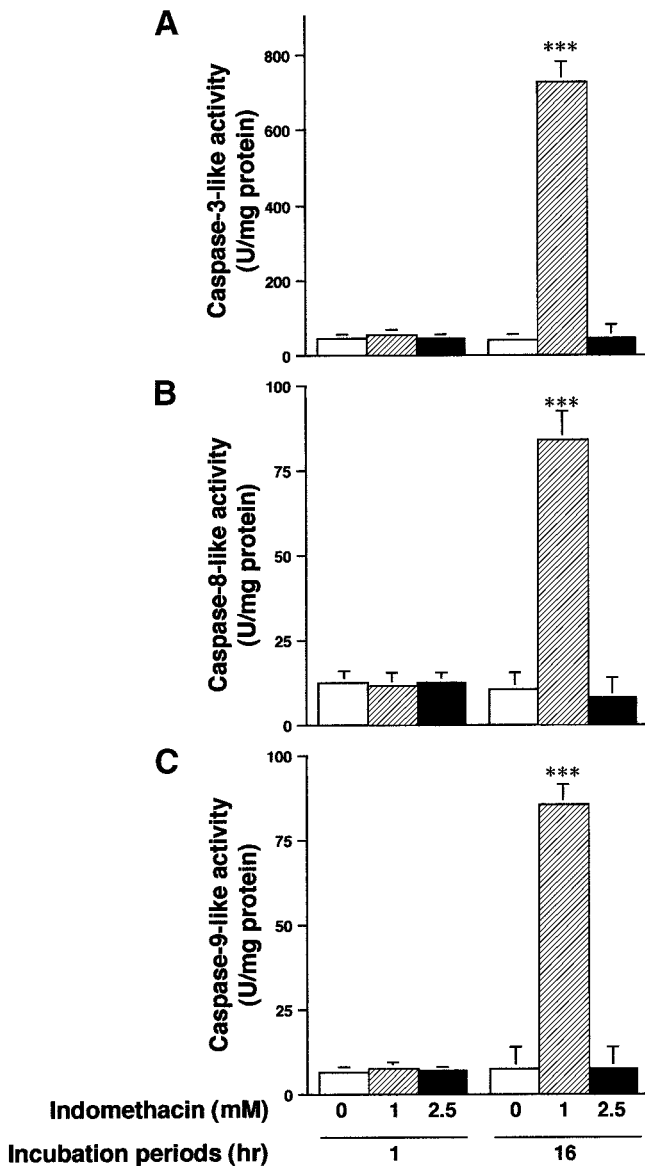


Fig. 4. Activation of caspases by indomethacin. Cultured gastric mucosal cells were incubated with indicated concentrations of indomethacin for 1 or 16 h. Cell lysates were prepared, and the activity of caspase-3 (A), caspase-8 (B), and caspase-9 (C) was measured by fluorometric assay using *N*-acetyl-DEVD-MCA, *N*-acetyl-IETD-MCA, and *N*-acetyl-LEHD-MCA. One unit of protease activity was defined as the amount of enzyme required to release 1 pmol 7-amino-4-methylcoumarin/min. Values are means \pm SD; $n = 3$. *** $P < 0.001$.

the activation of caspases. We also measured the activity of each caspase in the culture medium after indomethacin treatment of cells. Treatment of cells with 1 mM indomethacin for 16 h increased the activities of all these caspases. The activity of caspase-3, caspase-8, or caspase-9 in the culture medium was 0.528, 0.078, or 0.033 U/ml in the presence of indomethacin and 0.167, 0.033, or 0 U/ml in the absence of indomethacin. We calculated the total units of each caspase activity in both cells and culture medium and found that the majority of caspase activity was recovered in cells, suggesting that the apoptotic cells were not disrupted under the present conditions. On the other hand, 1-h treatment of cells with

2.5 mM indomethacin did not affect the activities of any of the caspases tested (Fig. 4). The results confirm that the short-term treatment of gastric mucosal cells in primary culture with indomethacin was mediated by necrosis. It should be noted that the background activities of caspases in control experiments were relatively high compared with published reports (11, 31) for other cells. This may be due to the fact that gastric mucosal cells in primary culture undergo maturation-dependent spontaneous apoptosis, which mimics the turnover of gastric mucosal cells in vivo (34).

Effect of inhibitors of caspases and protein synthesis on cell death induced by indomethacin. For further confirmation that long-term treatment of gastric mucosal cells in primary culture with indomethacin caused apoptosis, we examined whether or not the pretreatment of cells with an inhibitor of caspase activity was able to inhibit the cell death induced by indomethacin. We used a broad-spectrum inhibitor of caspases, Z-VAD-fmk, which inhibits the activities of most caspases, including caspase-3, caspase-8, and caspase-9 (35). Z-VAD-fmk (30 μ M) did not affect the cell viability or the state of chromosomal DNA in the absence of indomethacin (Fig. 5, A and C). As shown in Fig. 5A, pretreatment of gastric mucosal cells with 30 μ M Z-VAD-fmk clearly inhibited the dose-dependent cell death seen after treatment with indomethacin (1 mM) for 16 h. We subsequently confirmed that 30 μ M Z-VAD-fmk almost completely inhibited the activation of caspase-3, caspase-8, and caspase-9 by NSAIDs (data not shown). We also found that pretreatment of gastric mucosal cells with 30 μ M Z-VAD-fmk clearly inhibited the dose-dependent apoptotic DNA fragmentation seen after treatment with 1 mM indomethacin for 16 h (Fig. 5C). These results support the proposal that the long-term treatment of cells with indomethacin caused cell death through apoptosis, which is mediated by the activation of caspases. In contrast, the pretreatment of cells with 30 μ M Z-VAD-fmk did not prevent the cell death seen in response to treatment with 2.5 mM indomethacin for 1 h, which again suggests that the short-term treatment of cells with high concentrations of indomethacin caused cell death by necrosis.

We also examined the effect of an inhibitor of protein synthesis on indomethacin-induced cell death. We used 10 μ g/ml cycloheximide for this purpose, as this concentration is sufficient to inhibit protein synthesis in cultured gastric mucosal cells under these conditions (13). Treatment of cells with cycloheximide did not affect cell viability in the absence of indomethacin (Fig. 6). Pretreatment of cells with 10 μ g/ml cycloheximide made cells resistant to subsequent treatment with indomethacin for 16 h, suggesting that cell death by the long-term treatment with indomethacin requires protein synthesis. The cycloheximide treatment reduced the extent of the activation of caspase-3, caspase-8, and caspase-9 by indomethacin to 20%, 25%, and 20%, respectively (data not shown). In contrast, the decrease in cell viability seen with the short-term treatment of cells with indomethacin was not affected by pretreat-

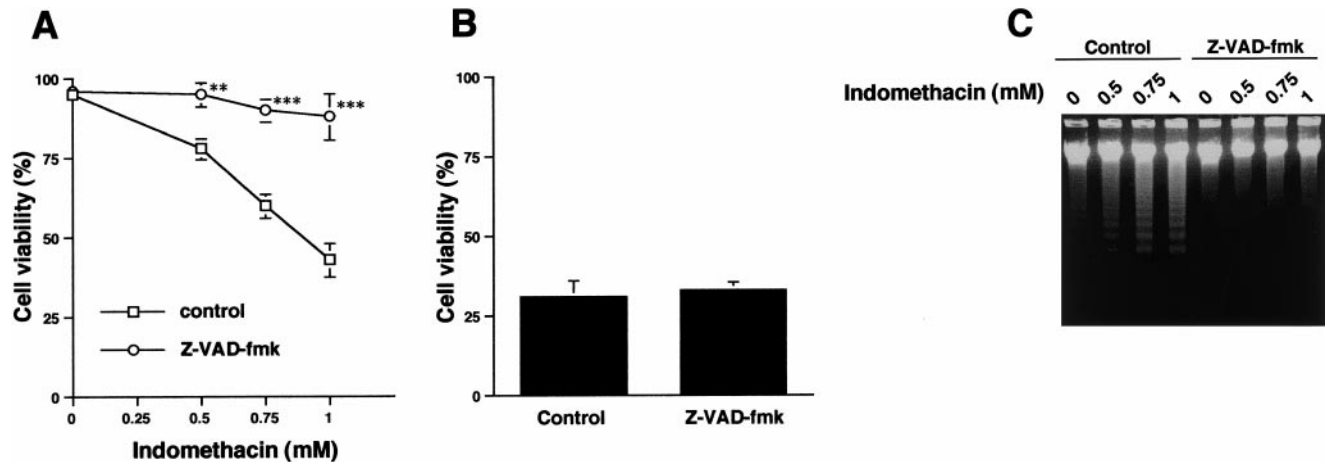


Fig. 5. Effect of caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) on indomethacin-induced cell death and apoptotic DNA fragmentation. Cultured gastric mucosal cells were preincubated with or without 30 μ M Z-VAD-fmk for 1 h. Cells were further incubated with 2.5 mM (B) or indicated concentrations (A and C) of indomethacin for 1 (B) or 16 h (A and C). Cell viability was determined by the trypan blue exclusion test (A and B). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (C). Values are means \pm SD; $n = 3$. ** $P < 0.01$; *** $P < 0.001$.

ment of cells with cycloheximide, supporting the idea that the short-term treatment of cells with high concentrations of indomethacin induces necrosis.

Effect of indomethacin on glucosamine uptake. Gastric mucosal cells are protected from various gastric irritants by secreted mucin *in vivo*. The guinea pig gastric mucosal cells in primary culture were shown to produce and secrete mucin (13, 21). We examined the effect of indomethacin on the incorporation of [3 H]glucosamine into cells. As shown in Fig. 7, indomethacin inhibited [3 H]glucosamine uptake in a dose-dependent manner. We also examined the amounts of mucin in cells after indomethacin treatment by the periodic acid-Schiff (PAS) staining method. Treatment of gastric mucosal cells with 1 or 2.5 mM indomethacin did not affect the pattern of PAS staining until 4 h (data

not shown), suggesting that indomethacin did not affect the retention of mucin in cells.

DISCUSSION

In this study, we have clearly shown that long- and short-term treatment with NSAIDs of gastric mucosal cells in primary culture caused cell death by apoptosis and necrosis, respectively. These findings are based on results obtained from experiments in which we examined apoptotic DNA fragmentation, PI staining, chromatin condensation, caspase activation, and the effects of inhibitors of caspase activity and protein synthesis. This study is the first to demonstrate that NSAIDs cause cell necrosis *in vitro*. Because necrotic cells activate neutrophils and macrophages and cause inflammation, we believe that much more attention should be paid not only to apoptosis but also to necrosis to understand NSAID-induced gastritis. As for apoptosis, our results were basically consistent with those previ-

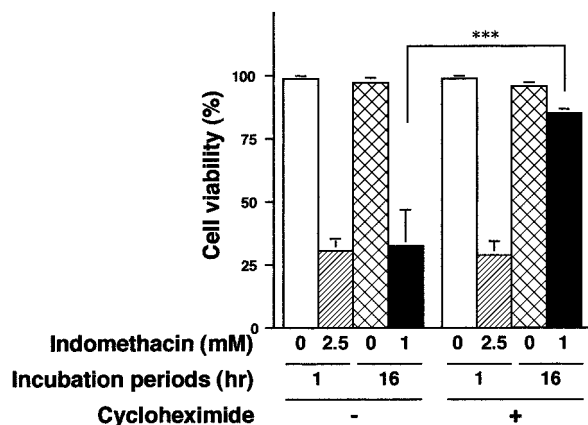


Fig. 6. Effect of cycloheximide on indomethacin-induced cell death. Cultured gastric mucosal cells were preincubated with or without 10 μ g/ml cycloheximide for 1 h. After removal of cycloheximide, cells were further incubated with indicated concentrations of indomethacin for 1 or 16 h. Cell viability was determined by the trypan blue exclusion test. Values are means \pm SD; $n = 3$. *** $P < 0.001$.

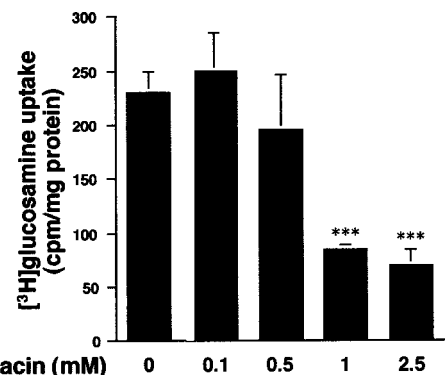


Fig. 7. Effect of indomethacin on glucosamine uptake. Cultured gastric mucosal cells were incubated with [3 H]glucosamine in the presence or absence of indomethacin for 16 h. The incorporated radioactivity was determined in a liquid scintillation counter. Values are means \pm SD; $n = 3$. cpm, Counts/min. *** $P < 0.001$.

ously reported (9, 17) using cell lines derived from gastric mucosa. Because primary culture of gastric mucosal cells is thought to effectively mimic gastric mucosal cells in vivo, our results suggest that NSAIDs may induce both necrosis and apoptosis in gastric mucosal cells in vivo.

The concentrations of indomethacin required for necrosis and apoptosis in vitro were 2.5 and 1 mM, respectively, whereas those for aspirin were 30 and 10 mM, respectively. Studies (7, 8, 18) on the oral administration of indomethacin and aspirin in rats showed that the concentrations of indomethacin and aspirin in the stomach were ~1–8 and ~10–100 mM, respectively, when these drugs caused gastric injury. Thus the in vitro concentrations of NSAIDs used in our study are physiologically significant in vivo. In other words, necrosis and apoptosis in vitro may mimic gastric mucosal injury after the single and repeated oral administration of NSAIDs in vivo. In fact, there are data (1, 7, 28) supporting the concept that indomethacin induces apoptosis in gastric mucosa in vivo. Serum concentration of NSAIDs after oral administration in rats is relatively low (7, 8, 18) compared with that required for apoptosis in vitro. Thus NSAIDs in serum may not be involved in apoptosis by NSAIDs in vivo. However, PGs, whose synthesis is inhibited by NSAIDs, have been reported (15) to be involved in cytoprotection, because PGs affect various physiological parameters such as blood flow. In other words, NSAIDs have a negative effect on the balance between aggressive and defensive factors for gastric mucosa by inhibiting the synthesis of PGs. It is therefore possible that reduction of PGs by NSAIDs increases the sensitivity of cells to NSAIDs and consequently low serum concentrations of NSAIDs induce apoptosis in vivo.

The identification of a pathway of NSAID-dependent apoptosis is important to understand the gastropathy associated with the use of NSAIDs and to establish a clinical protocol to protect gastric mucosal cells against the deleterious effects of NSAIDs. Recently (36), a novel pathway for stress-induced apoptosis was proposed, in which caspase-8 stimulates the release of cytochrome *c* from mitochondria through the degradation of Bid, resulting in the activation of caspase-9. Thus it is possible that apoptosis by NSAIDs is mediated by this pathway, because NSAIDs activated caspase-8 activity (Fig. 4). Because caspase-8 can be activated by caspase-3 (27), it is also possible that the activation of caspase-8 by NSAIDs is the result of caspase-3 activation by caspase-9.

Adaptation of gastric mucosal cells to NSAIDs has been reported (14, 29); after long-term NSAID exposure, gastric mucosal cells become resistant to further NSAID treatment in vivo. Recently (1), this adaptation was shown to be due to the increased resistance of gastric mucosal cells to apoptosis caused by NSAIDs. Combined with our current results, it is possible that treatment of gastric mucosal cells with NSAIDs induces proteins that inhibit the pathways of apoptosis discussed above. Heat shock proteins (HSPs) are apparent candidates for these proteins. Not only did some

HSPs directly interact with apoptotic protease-activating factor-1 and cytochrome *c* and inhibit apoptosis (2, 3, 6, 24) but also high concentrations of NSAIDs induced HSPs in gastric mucosal cells (32). However, HSPs were not induced in gastric mucosa when the adaptation to NSAIDs occurred in vivo (1). The identification of the proteins responsible for the adaptation upon chronic NSAID treatment may be important in establishing a clinical protocol for the prevention of the gastric mucosal injury induced by NSAIDs.

This work was supported by grants-in-aid for Scientific Research from the Japanese Ministry of Education, Science, Sports, and Culture and the Mochida Memorial Foundation for Medical and Pharmaceutical Research.

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