Mitochondrial Cytochrome *c* Release and Caspase-3-Like Protease Activation During Indomethacin-Induced Apoptosis in Rat Gastric Mucosal Cells (44521)

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Abstract. **Indomethacin (IND), a nonsteroidal anti-inflammatory drug, has been known to cause gastric mucosal injury as a side effect. Using a rat gastric mucosal cell line, RGM1, we determined whether apoptosis is involved in IND-mediated gastropathy, and whether caspase activation and mitochondrial cytochrome *c* release play an important role in producing apoptosis of IND-treated RGM1 cells in the presence of serum. IND caused caspase-3-like protease activation followed by apoptosis in a dose- and time-dependent manner. Caspase-1-like protease activity did not change during IND-induced apoptosis. IND also increased mitochondrial cytochrome *c* release in a time-dependent fashion. Mitochondrial cytochrome *c* efflux occurred just before or at the same time as caspase-3-like protease activation, and preceded the increase in apoptotic cell numbers. Z-VAD-FMK, a caspase inhibitor, inhibited both the increase in caspase-3-like protease activity and apoptosis in IND-treated RGM1 cells but did not affect caspase-1-like protease activity or mitochondrial cytochrome *c* release. These observations suggest that the apoptosis of gastric mucosal cells could be involved in IND-induced gastropathy, that cytochrome *c* is released from mitochondria into the cytosol during the early phase of IND-mediated apoptosis, and that subsequent activation of caspase-3-like protease, but not caspase-1-like protease, is required for the execution of apoptosis.** [P.S.E.B.M. 2000, Vol 224:102–108]

Nonsteroidal anti-inflammatory drugs (NSAIDs) block the biosynthesis of inflammatory prostaglandins (PGs), especially PGE_2 (1, 2), and it is generally accepted that inhibition of PGs is the primary mechanism by which these agents exert their anti-inflammatory action. However, NSAIDs often cause gastric mucosal injury as a side effect. Recently, it has been reported that indomethacin (IND)-induced gastric mucosal damage in rats occurs *via* tumor necrosis factor-α-triggered gastric epithelial cell apoptosis (3). Furthermore, it is conceivable that reactive oxygen species (ROS) produced by leukocytes recruited after NSAID treatment may induce apoptosis in gastric mucosal cells since NSAIDs seem to increase leukocyte infiltration into the gastric mucosa (4) and promote ROS generation (5). We have already reported that hydrogen peroxide, a membrane-permeable ROS, induces apoptosis in HL-60 cells (6). More recently, it has been shown that NSAIDs cause DNA fragmentation in cultured rat gastric mucosal cells deprived of serum (7). However, there is little known about the mechanisms of NSAID-triggered apoptotic signaling in gastric mucosal cells. In particular, to date, there have been no reports of caspase activation or the release of cytochrome *c* from mitochondria into the cytosol during NSAID-induced apoptosis in gastric mucosal cells. Understanding the mechanism underlying the apoptosis might lead us to control NSAID-mediated gastropathy.

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In the present study, we determined whether an NSAID, indomethacin, was able to induce apoptosis in a nontransformed rat gastric mucosal cell line, RGM1, in the presence of serum (i. e., under more physiological conditions). In addition, to explore the mechanisms underlying IND-mediated apoptosis at the molecular level, we examined caspase activities and cytosolic cytochrome *c* levels in IND-treated RGM1 cells, since these have been reported to change in other types of cells undergoing apoptosis (6, 8, 9).

Materials and Methods

Reagents. IND, propidium iodide (PI), and arachidonic acid (sodium salt) were purchased from Sigma Chemical Co. (St. Louis, MO). A 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12) was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). Fetal calf serum (FCS) was purchased from Gibco BRL (Grand Island, NY). 7-Amino-4-methylcoumarin (AMC), acetyl-Tyr-Val-Ala-Asp-AMC (Ac-YVAD-AMC), acetyl-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC) and carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK) were purchased from Peptide Institute, Inc. (Osaka, Japan). Hoechst 33342 was purchased from Molecular Probes, Inc. (Eugene, OR). Dimethylsulfoxide (DMSO) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). NS-398 was purchased from Cayman Chemical Company (Ann Arbor, MI). All other chemicals used were of analytical grade.

Cell Culture. The rat gastric epithelial cell line RGM1, a nontransformed diploid epithelial line isolated from normal Wistar rat gastric mucosa (10, 11), was obtained from Riken Cell Bank (Tsukuba, Japan). The cells were grown in DMEM/F12 supplemented with 10% FCS, 2 m*M* glutamine and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin) at 37°C in a humidified incubator with an atmosphere of 5% CO_2/95% air. All assays were performed before passage 20.

IND-Induced Apoptosis and Treatment with a Caspase Inhibitor. RGM1 cells were grown to near confluency on 90-mm culture dishes. They were then immersed in fresh DMEM/F12 supplemented with 10% FCS and exposed to IND at concentrations ranging from 100 to 1,000 μ*M* for periods of 12, 24, 36, 48, or 72 hr at 37°C under an atmosphere of 5% CO_2/95% air. Additionally, we examined whether treatment with 100–500 μ*M* NS-398 for 48 hr caused apoptosis in RGM1 cells. In some experiments, a pan-caspase inhibitor, Z-VAD-FMK, was added to the medium 1 hr prior to IND administration.

Cytofluorometric Analysis of Apoptosis. The occurrence of apoptosis was analyzed by flow cytometry according to a previously described method (12). Untreated or IND-treated cells were resuspended by trypsinization, washed twice in phosphate-buffered saline (PBS), and fixed in 70% ethanol. They were then centrifuged at 800*g* for 5 min, washed once in PBS, resuspended in phosphate-citrate buffer, and left to stand at room temperature for at least 30 min. After washing in PBS, the cells were treated with 0.1 mg/ml RNase A for 30 min at 37°C, and stained with 50 μg/ml PI. In each experiment, 10,000 cells were analyzed on an Epics cytofluorometer (Beckman Coulter, Inc., Fullerton, CA). Apoptotic cells were detected as a hypodiploid DNA peak (sub-G1). Each experiment was repeated at least three times.

Nuclear Fragmentation Assay. Cells were incubated with IND at 37°C, washed with PBS, and fixed with 2% glutaraldehyde for 2 hr. Samples were centrifuged, resuspended in PBS, stained with 1 m*M* Hoechst 33342, mounted on a glass slide, and observed using fluorescence microscopy.

Determination of Caspase Activity. The activities of caspase-1-like protease and caspase-3-like protease, referred to hereafter as YVADase and DEVDase, respectively, were determined as described previously (6). Briefly, measurement of their activities was based on cleavage of the fluorogenic peptide substrates Ac-YVAD-AMC and Ac-DEVD-AMC during a 60-min incubation at 37°C. One unit of protease activity was defined as the amount of enzyme required to release 1 pmol AMC/min.

Preparation for Mitochondria and the Cytosol. Mitochondrial and cytosolic fractions were prepared by a previously described method (13). Briefly, cells were harvested, washed, resuspended in ice-cold buffer A (20 m*M* HEPES-KOH, pH 7.5, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 1 m*M* EDTA, 1 m*M* EGTA, and 1 m*M* phenylmethylsulfonyl fluoride) containing 250 m*M* sucrose, and homogenized with 10 strokes of a Teflon homogenizer. The homogenates were then centrifuged twice at 1,000*g* for 10 min at 4°C. The supernatants were centrifuged at 10,000*g* for 15 min at 4°C to obtain the mitochondria pellets. The pellets were resuspended in buffer A, and frozen at −80°C. The above supernatants were further centrifuged at 100,000*g* for 1 hr at 4°C, and the resulting supernatants (S-100) were used as the cytosolic fraction.

Western Blot Analysis. The protein content of each mitochondrial or S-100 fraction was quantitated by the Bradford method. After adding 10% glycerol, 2% sodium dodecyl sulfate (SDS), 6% β-mercaptoethanol, and 0.003% bromophenol blue, the samples were boiled for 5 min, then 10–20 μg of protein from each sample were separated by SDS-polyacrylamide gel electrophoresis using 12% polyacrylamide gel, and electroblotted onto a polyvinylidine difluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking nonspecific binding sites with washing buffer (10 m*M* Tris/HCl, pH 7.5, 100 m*M* NaCl, and 0.1% Tween 20) containing 5% skim milk, the PVDF membrane was incubated for 1 hr at room temperature with antihuman cytochrome-*c* monoclonal antibody (Pharmingen, San Diego, CA) (1:500). It was then washed with washing buffer, incubated further with horseradish peroxidase–conjugated sheep antimouse Ig antibody (Amersham, Buckinghamshire, England) for 1 hr at room temperature, and rewashed

with washing buffer. The immunoblot was revealed using an enhanced chemiluminescence detection kit (Amersham).

Statistical Analysis. All results are expressed as mean ± SEM. The data shown are mean values of at least three separate experiments. Student's *t* test was used to compare differences. A *P*-value of less than 0.05 was considered statistically significant.

Results

Dose-Dependent Apoptosis Induction and DEVDase Activation in RGM1 Cells by IND Treatment. To determine the concentration of IND required to induce apoptosis in RGM1 cells in the presence of serum, we treated the cells with IND at concentrations ranging from 100 to 1000 μM for 48 hr. IND induced apoptosis at concentrations greater than 250 μM in a dose-dependent manner (Fig. 1A). On the other hand, treating with IND induced apoptosis in RGM1 cells at a concentration of less than 100 μM when serum was removed (data not shown). However, the serum-free condition evidently increased the number of necrotic cells. DEVDase activity also increased in a dose-dependent manner when IND was added to RGM1 cell cultures for 48 hr at concentrations of 100–500 μM (Fig. 1B). In contrast, YVADase activity remained unchanged at all the concentrations of IND used during the study (Fig. 1B).

Time-Dependent Apoptosis Induction, DEVDase Activation, and Cytochrome *c* Release in RGM1 Cells During IND Treatment. We next examined the time required to induce apoptosis with 500 μM IND in RGM1 cells. As shown in Figure 2A, apoptotic cells became evident after 24 hr of IND treatment. Their numbers increased with time up to 48 hr, then reached a plateau. In RGM1 cells treated with 500 μM IND, DEVDase activity also increased

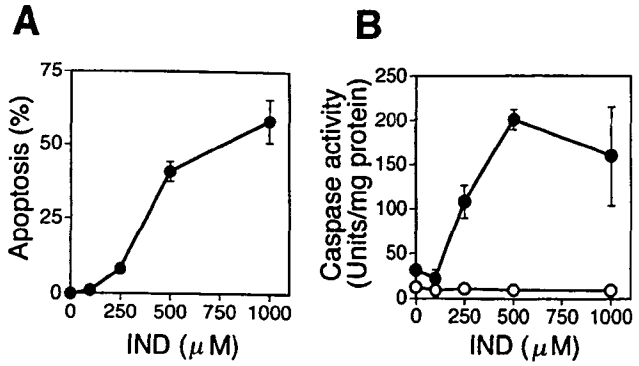

Figure 1. Apoptosis and caspase activities in RGM1 cells exposed to IND. (A) Dose-response of the ratio of apoptotic RGM1 cells in cultures exposed to IND. Cells were incubated with 0, 100, 250, 500, or 1,000 μM IND for 48 hr at 37°C under a 5% CO_2, 95% air atmosphere. Apoptotic cells were determined by flow cytometric analysis with PI staining. (B) Dose-response of DEVDase (solid circles) and YVADase (open circles) activities to IND. Caspase activities were measured using fluorescent substrates as described in Materials and Methods. Data points represent the mean ± standard error (SEM) of three separate experiments. Note: some error bars are too small to visualize.

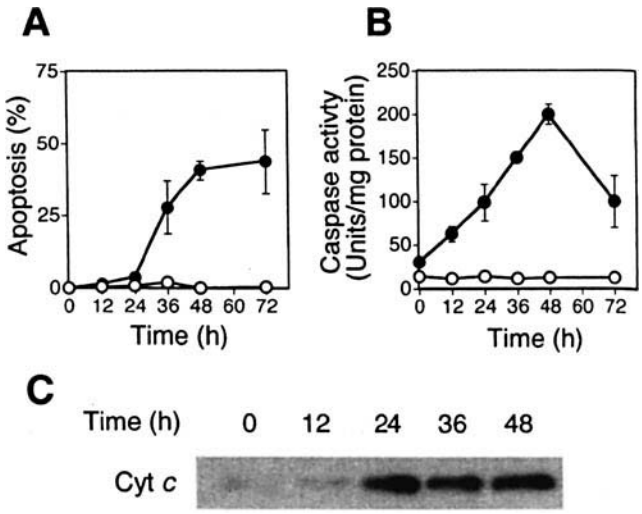

Figure 2. Apoptosis, caspase activities, and cytochrome *c* release in RGM1 cells exposed to IND as a function of time. (A) Time course of the appearance of apoptosis in RGM1 cells treated with 500 μM IND. Cells were incubated in the absence (open circles) or presence (solid circles) of 500 μM IND for 0, 12, 24, 36, 48, or 72 hr at 37°C under a 5% CO_2, 95% air atmosphere. (B) Time course of changes in DEVDase (solid circles) and YVADase (open circles) activities in cells treated with 500 μM IND. Apoptotic cells and caspase activities were determined as described in the legend to Figure 1. Data points represent the mean ± SEM of three separate experiments. Note: some error bars are too small to visualize. (C) Time-dependent release of cytochrome *c* (Cyt *c*) in RGM1 cells treated with 500 μM IND. At the indicated time points, refined cytosolic (S-100) fractions were prepared and analyzed for cytochrome *c* content by Western blotting. The results are representative of three separate experiments.

time-dependently and reached a maximum at 48 hr (Fig. 2B). By contrast, YVADase activity remained unchanged throughout the incubation period (Fig. 2B). In control cultures, vehicle (DMSO) affected neither DEVDase nor YVADase activities (data not shown). To investigate whether IND induces the release of cytochrome *c* from mitochondria to the cytosol, we determined cytochrome *c* levels in the cytosolic fractions of RGM1 cells that have been exposed to 500 μM IND using Western blot analysis. The amount of cytochrome *c* in the cytosol increased markedly from 24 hr onward during treatment with 500 μM IND (Fig. 2C). This increase occurred in parallel with DEVDase activation (Fig. 2B) and preceded the increase in the number of apoptotic cells (Fig. 2A).

Effect of a Caspase Inhibitor on IND-Induced Apoptosis in RGM1 Cells. To confirm whether caspase activation is required for the execution of IND-mediated apoptosis in RGM1 cells, 25 μM Z-VAD-FMK was added to the medium 1 hr before starting the 48-hr treatment with 500 μM IND. Apoptosis was assessed by fluorescence microscopy in cells stained with Hoechst 33342 (Fig. 3A) and flow cytometry in cells stained with PI (Fig. 3B). IND 500 μM induced obvious nuclear fragmentation in RGM1 cells (Fig. 3A (b)). However, addition of 25 μM Z-VAD-FMK to the culture produced a significant reduction in the number of fragmented nuclei (Fig. 3A (c)). Z-VAD-FMK 25 μM also significantly reduced the sub-G1 population from

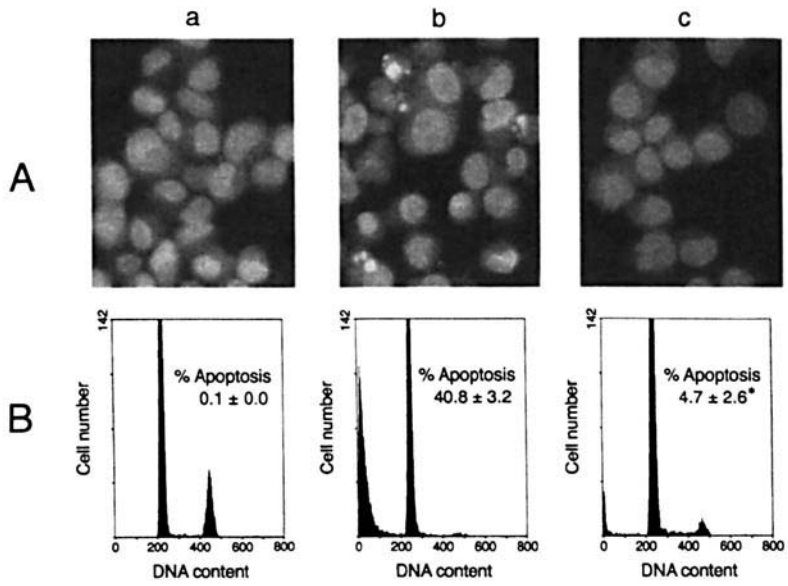

Figure 3. Inhibitory effect of a caspase inhibitor on IND-induced apoptosis in RGM1 cells. (A) Nuclear morphology was visualized by fluorescence microscopy after staining apoptotic nuclei with Hoechst 33342. (B) Flow cytometric patterns were analyzed by flow cytometry after PI staining. Cells were incubated in the absence (a) or presence (b) of 500 μ*M* IND for 48 hr. Z-VAD-FMK 25 μ*M* was added to the culture 1 hr before starting the 48-hr incubation with 500 μ*M* IND in some experiments (c). The data inserted within the flow cytometry histograms show the proportion of apoptotic cells and are mean ± SEM values for three separate experiments. **P* < 0.0005 versus IND-treated cells.

40.8% to 4.7% in the flow cytometric analysis (Fig. 3B (b, c)).

Effect of a Caspase Inhibitor on Cytochrome *c* Release and DEVDase Activity in IND-Treated RGM1 Cells. To determine the relationship between the release of cytochrome *c* and DEVDase activation during IND-mediated apoptosis in RGM1 cells, we treated the cells with Z-VAD-FMK 25 μ*M*. Z-VAD-FMK did not prevent the efflux of cytochrome *c* from mitochondria into the cytosol (Fig. 4). By contrast, this caspase inhibitor completely suppressed the IND-induced increase in DEVDase activity (Fig. 4). These results suggested that, during apoptosis, cytochrome *c* release occurs upstream of DEVDase activation.

Apoptosis and Caspase Activities in NS-398-Treated RGM1 Cells. IND is a nonselective cyclooxygenase (COX) inhibitor that inhibits both COX-1 and COX-2 (14). Since it has been reported that NS-398, a COX-2 selective inhibitor, increases the number of apoptotic cells in tumors (15), we determined whether NS-398 induced apoptosis and DEVDase activation in nontransformed RGM1 cells. NS-398 showed weak apoptosis-inducing activity but did not cause DEVDase activation in RGM1 cells at a concentration of 500 μ*M* (Fig. 5). In contrast, IND produced marked DEVDase activation and apoptosis in the cells at this dose (Fig. 5).

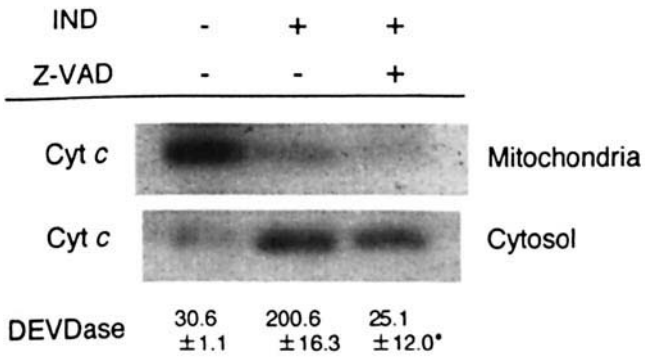

Figure 4. Inhibitory effect of a caspase inhibitor on cytochrome *c* (Cyt *c*) efflux and DEVDase activation in RGM1 cells treated with IND. Cells were incubated in the absence or presence of 500 μ*M* IND for 48 hr. Z-VAD-FMK 25 μ*M* was added to the culture 1 hr before starting the 48-hr incubation with 500 μ*M* IND in some cases. Mitochondrial and refined cytosolic (S-100) fractions were prepared and analyzed for cytochrome *c* content by Western blotting. DEVDase activities were determined as described in the legend to Figure 1. The values of DEVDase activity are expressed as the mean ± SEM (Units/mg protein) of three separate experiments. **P* < 0.0005 versus IND-treated cells.

Discussion

NSAIDs cause clinically important gastric mucosal injury. Although the precise mechanisms underlying NSAID-induced gastropathy remain unknown, several possibilities

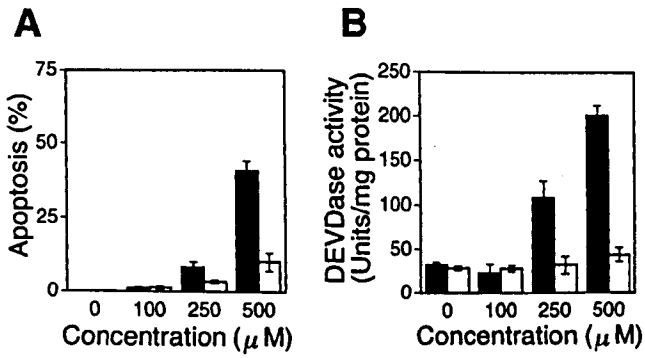

Figure 5. Apoptosis and DEVDase activities in RGM1 cells exposed to NS-398. (A) Dose-response of the ratio of apoptotic RGM1 cells in cultures exposed to IND (solid columns) or NS-398 (open columns). Cells were incubated with 0, 100, 250, or 500 μ*M* IND or NS-398 for 48 hr at 37°C under a 5% CO_2, 95% air atmosphere. (B) Dose-response of DEVDase activities to IND or NS-398. Apoptotic cells and DEVDase activities were determined as described in the legend to Figure 1. Data points represent the mean ± SEM of three separate experiments. Note: some columns and error bars are too small to visualize.

have been considered. First, NSAIDs inhibit the COXs and thus reduce the production of protective PGs. They also increase leukocyte infiltration into the gastric mucosa (4), and this is followed by ROS production (5). In addition, they disturb the microcirculation in the gastric mucosa (16).

Recently IND has been reported to induce apoptosis in rat gastric mucosal cells in the absence of serum (7). Serum contains many growth factors (17) and antitoxic substances such as antioxidants (18). However, it has been well documented that serum deprivation *per se* can induce apoptosis in a variety of cells (19–24). Therefore, we investigated whether IND can trigger apoptosis in RGM1 cells in the presence of serum. We found that IND was indeed able to induce apoptosis in RGM1 cells in the presence of serum, and that this occurred in a dose- and time-dependent fashion. In our experiments, the most significant potential criticism is that 500 μM IND used to induce apoptosis seems slightly high. However, 1,000 μM IND has been used to induce apoptosis in RGM1 cells even under the condition of serum deprivation (7). As described above, serum deprivation required a lower concentration of IND to cause apoptosis and also increased necrotic cells in our experiments. It is well known that IND is a highly albumin-bound protein (25). Therefore, binding to plasma proteins may influence the concentration of free IND available to be taken up by the cells when serum is present.

Mounting evidence now shows that certain cysteine proteases, named caspases, play a key role in apoptosis (26). In particular, caspase-3 seems to function as a primary effector in the execution phase of apoptosis (26). Since we also determined previously that the apoptotic signal mediated by hydrogen peroxide in HL-60 cells was transduced to caspase-3 independently of caspase-1 (6), to investigate the roles of individual caspases in IND-induced apoptosis, we determined whether their activities changed independently in IND-treated RGM1 cells. Caspase-3-like protease activity increased in a dose- and time-dependent manner in these cells, and these increases paralleled a rise in the number of apoptotic cells. By contrast, caspase-1-like protease activity remained unchanged (Figs. 1 and 2). These data indicate that caspase-3-like protease activation is involved in IND-induced apoptosis in RGM1 cells, whereas caspase-1-like protease does not participate in the apoptosis resulting from IND treatment. To further confirm the contribution of caspase-3-like protease activation to IND-induced apoptosis, we examined the effects of Z-VAD-FMK, a pan-caspase inhibitor, on caspase activities and apoptosis. Twenty-five μM Z-VAD-FMK prevented both the increase in caspase-3-like protease activity and apoptosis in IND-treated RGM1 cells, supporting the idea that caspase-3-like protease, but not caspase-1-like protease, is required for the execution of IND-mediated apoptosis. This finding is in accordance with several lines of evidence indicating that caspase-1-like protease is not involved in apoptosis triggered by diverse stimuli in various cell types (6, 24, 27). However, a previous study using Ac-YVAD-CHO, a caspase-1-like protease inhibitor, implicated caspase-1-like protease in producing some degree of DNA fragmentation in NSAID-treated RGM1 cells (7), although caspase-1-like protease activity was not measured.

Recently, mitochondria have been identified as playing a central role in apoptosis (8, 28). In many cases, during the apoptotic process, opening of the permeability transition pore (PTP) and subsequent loss of the mitochondrial transmembrane potential ($\Delta\psi m$) have been found to occur (28, 29). Cytochrome *c* release from mitochondria into the cytosol, which is associated with the induction of apoptosis, might result from the opening of the PTP (29, 30). However, it has been reported that cytochrome *c* release can occur before or in the absence of loss of $\Delta\psi m$ in some cases (31, 32), suggesting that two regulatory pathways, PTP-dependent and PTP-independent pathways, may exist in cytochrome *c* efflux from mitochondria. To determine the contribution of mitochondria to IND-induced apoptosis, we examined the leakage of cytochrome *c* from mitochondria into the cytosol in IND-treated RGM1 cells. After, or at the same time as, mitochondrial cytochrome *c* efflux, caspase-3-like protease became activated. Subsequently, apoptotic DNA fragmentation (an increase in the sub-G1 population) was induced (Fig. 2). This time course indicated that the release of cytochrome *c* from mitochondria into the cytosol is an early event in the apoptotic process. This is the first report of mitochondrial cytochrome *c* release during IND-induced apoptosis in nontransformed gastric cells and is consistent with the previous observation that cytochrome *c* release may be required for caspase activation during the induction of apoptosis (33–36). Although the precise mechanism driving the translocation of cytochrome *c* from mitochondria to the cytosol is not defined, it has been reported that BH3 interacting domain death agonist (Bid) becomes cleaved and activated by caspase-8, and the COOH-terminal part of Bid is likely causing cytochrome *c* efflux in some cases, especially in receptor-mediated apoptosis (37, 38). However, in apoptosis induced by chemicals such as etoposide and staurosporine, a mechanism other than cleavage of Bid seems to be responsible for the release of cytochrome *c* (38). The present study also showed that Z-VAD-FMK failed to block the cytochrome *c* efflux induced by IND treatment. This suggests that the IND-triggered apoptotic signal is not transmitted to mitochondria *via* upstream initiator caspases.

Though it is not clear how IND functions as an apoptosis inducer upstream of mitochondria, a recent report has shown that elevation of arachidonic acid (AA) levels resulting from inhibition of COX is related to the induction of apoptosis by NSAIDs in colorectal cancer cell lines (39). Therefore, we examined the effect of AA on the proportion of sub-G1 cells in a cytofluorometric study, together with caspase activities, in RGM1 cells. Treatment of the cells with 100 or 250 μM AA had no effect on these two hallmarks of apoptosis (data not shown). Accordingly, it is unlikely (at least in nontransformed cells such as RGM1 cells)

that an increase in AA is a main mechanism for NSAID-induced apoptosis.

Classical NSAIDs such as IND are known to inhibit COX-1 (constitutively expressed) and COX-2 (inducible isoform) simultaneously, and to cause gastropathy by inhibiting COX-1 but not COX-2 (40). Recently, selective COX-2 inhibitors have been highlighted as cancer preventive agents because the COX-2 isoform is upregulated in cancer (40–42). As shown in Figure 5, NS-398 little caused apoptosis and caspase-3-like protease activation in RGM1 cells. Thus, selective COX-2 inhibition does not appear to induce apoptosis effectively in nontransformed cells. Indeed, based on the observations that AA does not induce apoptosis in RGM1 cells, as described above, and that 16, 16-dimethyl PGE_2 administered concomitantly with an NSAID does not reverse NSAID-induced DNA fragmentation in RGM1 cells (7), the initiation of IND-induced apoptosis in nontransformed cells may be due to mechanisms independent of both COX-1 and COX-2. Further studies will be required to elucidate the role of IND in the early steps of apoptotic signaling.

Given that IND alone induced apoptosis in RGM1 cells under more physiological conditions in our experiments, the apoptosis of gastric mucosal cells could be part of a series of events involved in the pathogenesis of NSAID-induced gastric mucosal injury. Moreover, the release of cytochrome *c* from mitochondria into the cytosol and subsequent caspase-3-like protease activation may play a pivotal role in the regulation and execution of NSAID-induced apoptosis in nontransformed gastric cells. Therefore, manipulation of these steps in the apoptotic process by pharmaceutical agents might improve the efficacy of treatment for NSAID-induced gastropathy.

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