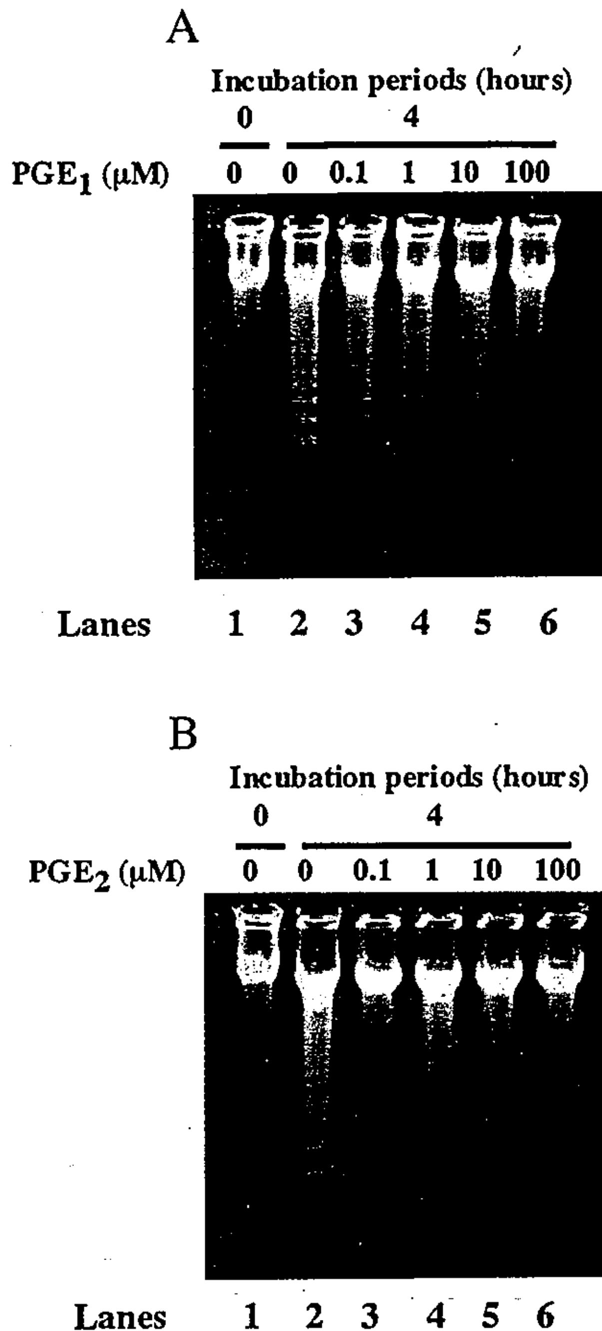

Fig 1. Effects of PGE_1 and PGE_2 on spontaneous apoptosis in cultured gastric mucosal cells. Gastric mucosa cells prepared from guinea pig fundic glands were preincubated in RPMI 1640 medium containing 10% FCS in the presence of indicated concentrations of PGE_1 (A) or PGE_2 (B). After removal of nonadhering cells, the remaining cells were incubated in the same medium for 4 hr. Apoptotic DNA fragmentation was analyzed as described in Materials and Methods. DNA obtained from cells harvested soon after the washing step is shown in lane 1.

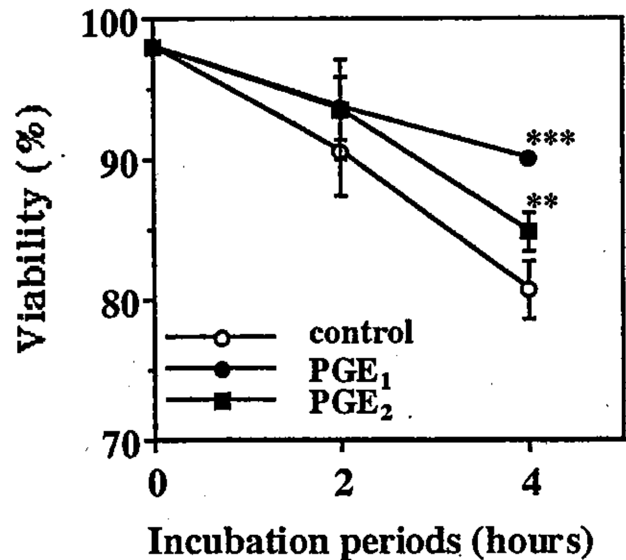

Fig 2. Effects of PGE_1 and PGE_2 on cell viability of cultured gastric mucosal cells. Gastric mucosa cells prepared from guinea pig fundic glands were preincubated in RPMI 1640 medium containing 10% FCS in the absence or presence of 100 μM PGE_1 or PGE_2. After removal of nonadhering cells, remaining cells were incubated in the same medium for indicated periods. Cell viability was determined by the trypan blue exclusion test. Values are mean ± SD, $N = 3$. $^{***}P < 0.001$; $^{**}P < 0.01$

We also examined the effects of PGE_1 and PGE_2 on cell viability. As shown in Figure 2, PGE_1 and PGE_2 increased the viability of gastric mucosal cells compared to controls. The presence of 100 μM PGE_1 resulted in a greater increase in the cell viability than that seen for the same concentration of PGE_2. These results strongly suggest that both PGE_1 and PGE_2 inhibit spontaneous apoptosis and, as a result, increase the viability of gastric mucosal cells in primary culture.

As described above for results shown in Figures 1 and 2, gastric mucosal cells were incubated with PGE_1 or PGE_2 at both the preincubation and incubation steps. We subsequently examined whether or not the presence of PGE_1 at both the preincubation and incubation steps were required for the inhibitory effect of PGE_1 on the spontaneous apoptosis to be seen. Even when PGE_1 was omitted in the preincubation step, the spontaneous apoptosis was inhibited by PGE_1 (Figure 3A) to much the same extent as that seen in the positive control experiments where PGE_1 was included in the medium for both the preincubation and incubation steps (Figure 1A). Thus, the preincubation of gastric cells with PGE_1 is not a prerequisite for the inhibitory effects of PGE_1 on spontaneous apoptosis in gastric mucosal cells. On the other hand, when PGE_1 was included in the preincubation step but omitted from the incubation step, the spontaneous apoptosis was not inhibited by

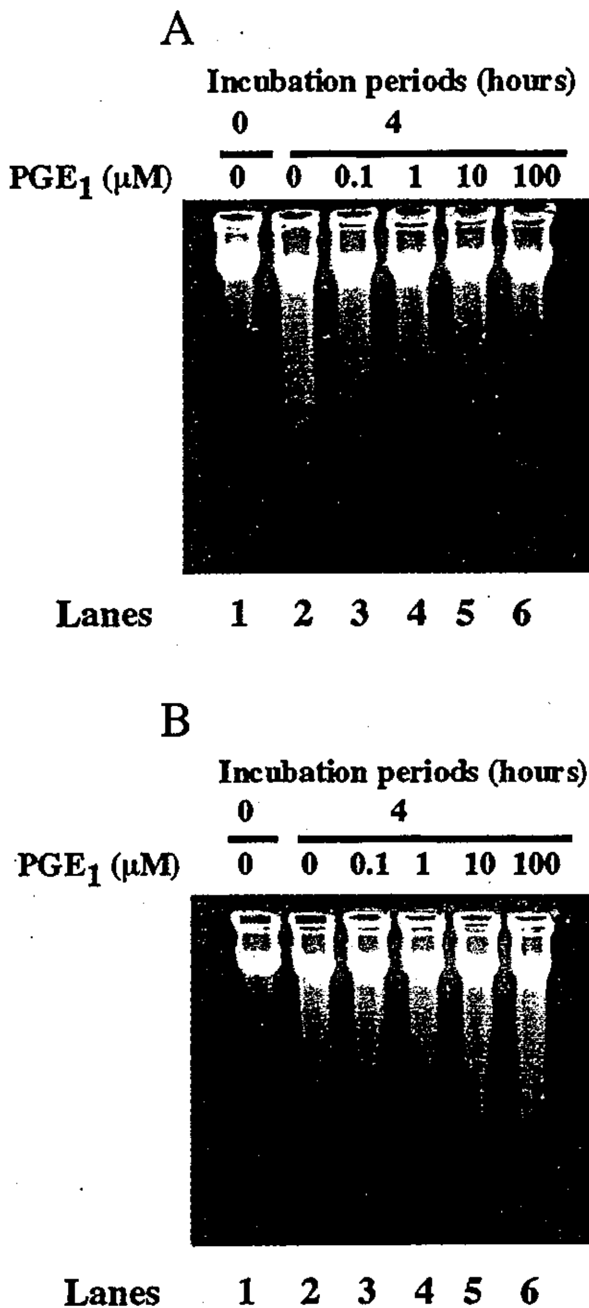

Fig 3. Requirement of presence of PGs in the preincubation and incubation steps for highlighting their inhibitory effects on spontaneous apoptosis. Gastric mucosa cells prepared from guinea pig fundic cells were preincubated in RPMI 1640 medium containing 10% FCS in the absence (A) or presence (B) of the indicated concentrations of PGE_1. After removal of nonadhering cells, attached cells were incubated in RPMI 1640 medium containing 10% FCS in the absence (B) or presence (A) of indicated concentrations of PGE_1 for 4 h. Apoptotic DNA fragmentation was analyzed as described in Materials and Methods. DNA obtained from cells harvested soon after the washing step is shown in lane 1.

PGE_1 (Figure 3B). Thus, the presence of PGE_1 in the incubation step only is essential for PGE_1 to demonstrate its inhibitory effects on the spontaneous apoptosis. Much the same results as those seen for were PGE_1 were obtained for experiments where PGE_2 was used (data not shown). These results suggest that the inhibitory effects of PGE_1 and PGE_2 on the spontaneous apoptosis are rapid and transient.

Effects of Various Antiulcer Drugs on Spontaneous Apoptosis. PGs are well known for their protective effects towards the gastric mucosa. Thus, a number of clinically used antiulcer drugs are related in their action to achieving increased intracellular levels of PGs. Plaunotol, rebamipide, and ecabet sodium, for example, have previously been shown to increase endogenous PG levels in gastric mucosal cells both *in vivo* and *in vitro* (16–18). Ornoprostil, on the other hand, is a synthetic PGE_1 analog (19). We examined here the effects of these antiulcer drugs on the spontaneous apoptosis of cultured gastric mucosal cells. As shown in Figure 4, all of these antiulcer drugs inhibited the spontaneous apoptosis in a dose-dependent manner. Although the concentrations of the drugs required for the inhibition of spontaneous apoptosis varied depending on drugs, the concentration employed for each drug corresponded to that required for an increase in PGs in cells or for other effects related to PGs (16–19). Therefore, their effects on PG concentrations in cells readily explain the inhibitory effects of these antiulcer drugs on spontaneous apoptosis in cultured gastric mucosal cells.

It is unknown whether the maturation-dependent death of gastric mucosal cells associated with rapid cell turnover cycle should be enhanced or attenuated in order to protect gastric mucosa against various stresses. One way to approach this issue is to examine the effects of gastric mucosa cytoprotective factors on the rate of such cell death. The major endogenous cytoprotective factors for gastric mucosa are PGs and heat shock proteins (HSPs). We previously reported that geranylgeranylacetone (GGA), a clinically used antiulcer drug and a HSP-inducer, inhibited the maturation-dependent spontaneous apoptosis in cultured gastric mucosal cells (20). In the present study, we have shown that another group of cytoprotective factors, PGs, also inhibited spontaneous apoptosis in cultured gastric mucosal cells. We also showed that each of the PG-inducing anti-ulcer drugs tested here inhibited the spontaneous apoptosis. These results suggest that maturation-dependent cell death at the gastric surface should be attenuated to some extent

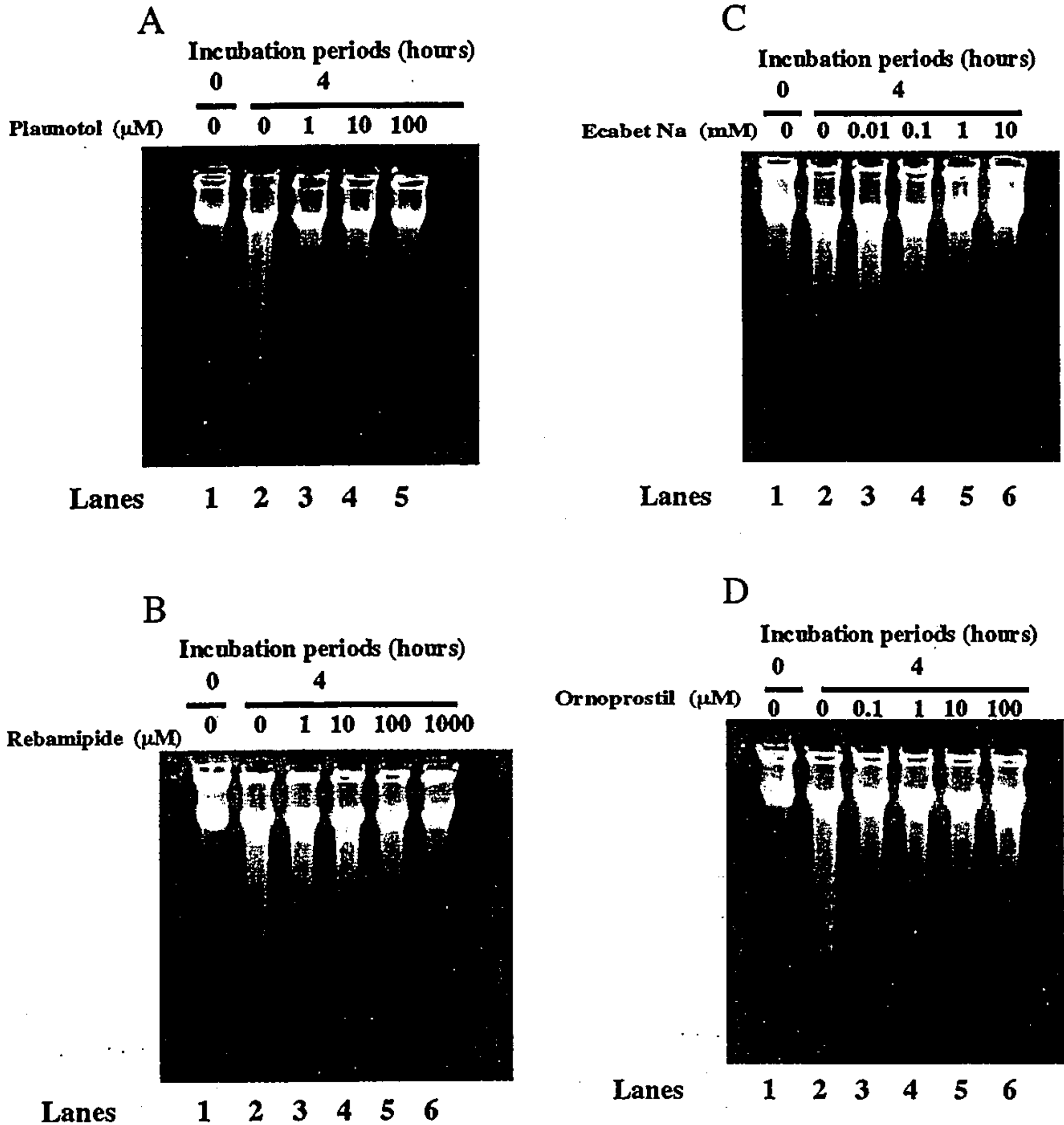

Fig 4. Effects of various antiulcer drugs on the spontaneous apoptosis observed in cultured gastric mucosal cells. Gastric mucosa cells prepared from guinea pig fundic glands were preincubated in RPMI 1640 medium containing 10% FCS in the presence of indicated concentrations of plaunotol (A), rebamipide (B), ecabet sodium (C), and ornoprostil (D). After removal of unattached cells, remaining cells were incubated in the same medium for 4 hr. Apoptotic DNA fragmentation was analyzed as described in Materials and Methods. DNA obtained from cells harvested soon after the washing step is shown in lane 1.

for protection of gastric mucosa against various stresses.

The mechanisms of action of antiulcer drugs currently in clinical use are either the result of an elimination of aggressive factors or an increase in defensive factors. As a novel mechanism for antiulcer drugs, growth factors for gastric mucosa have been shown to be effective by increasing the overall number

of gastric mucosal cells present at the gastric surface (21). It is thus possible that the inhibition of maturation-dependent spontaneous apoptosis in gastric mucosal cells also increases the number of gastric mucosal cells. We therefore consider that the inhibition of maturation-dependent spontaneous apoptosis should become a new target of antiulcer drugs.

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Gastric irritant-induced apoptosis in guinea pig gastric mucosal cells in primary culture

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Abstract

When the gastric mucosa is exposed to various irritants, apoptosis and subsequent gastric mucosal lesion can result in vivo. We here show that gastric irritants induced apoptosis in gastric mucosal cells in primary culture and examined its molecular mechanism. Ethanol, hydrogen peroxide, and hydrochloric acid all induced, in a dose-dependent manner, cell death, apoptotic DNA fragmentation, and chromatin condensation, suggesting that each of these gastric irritants induced apoptosis in vitro. Since each of these irritants decreased the mitochondrial membrane potential and stimulated the release of cytochrome *c* from mitochondria, gastric irritant-induced apoptosis seems to be mediated by mitochondrial dysfunction. Caspase-3, caspase-8, and caspase-9-like activities were all activated simultaneously by each of these irritants and the activation was concomitantly with cell death and apoptotic DNA fragmentation. Furthermore, pre-treatment of gastric mucosal cells with an inhibitor of caspase-8 suppressed the onset of cell death as well as the stimulation of caspase-3- and caspase-9-like activities caused by each of these gastric irritants. Based on these results, we consider that caspase-8, an initiator caspase, plays an important role in gastric irritant-induced apoptosis. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Apoptosis; Gastric mucosal cell; Caspase; Gastric irritant

1. Introduction

Gastric mucosa is frequently exposed to various types of irritant (such as alcohol, acid, oxidative irritant, drugs, and bacteria). These irritants cause gastric mucosal injury at least in part by stimulating apoptosis of gastric epithelial cells [1]. For example, stimulated apoptosis of gastric mucosal cells was documented especially at the onset of gastric ulceration [2]. The increased rate of apoptosis associated with *Helicobacter pylori* infection was suggested to be involved in the development of atrophic gastritis caused by *H. pylori* infection [3]. Thus, the molecular mechanism governing gastric irritant-induced apoptosis needs to be elucidated in order to understand the mechanism of gastropathy and to establish a clinical protocol for overcoming mucosal damage caused by gastric irritants.

Caspases are a family of specific cysteine proteases whose sequential activation plays a central role in apoptosis in general [4]. Among the more than a

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dozen of members of the caspases, caspase-3 is located downstream of the cascade and directly activates proteins responsible for DNA fragmentation and chromatin condensation (CAD (caspase activated DNase) and Acinus, respectively) [5,6]. Caspase-3 is activated primary by two pathways, in which either caspase-8 or caspase-9 plays an important role [4]. Mitochondrial dysfunction (such as a decrease in mitochondrial membrane potential) stimulates the release of cytochrome *c* from the mitochondria to make its complex with Apaf-1, which in turn activates caspase-9 [7]. On the other hand, caspase-8 can directly activate caspase-3 in a manner independent of mitochondrial function [8]. Thus, apoptotic pathways were thought to be separable into mitochondria-dependent and -independent processes, which involve caspase-9 and caspase-8, respectively. However, recently, a number of other pathways and cross talk between these two pathways have been reported [9]. For example, caspase-8 promoted release of cytochrome *c* from mitochondria by inducing cleavage of bid, one of bcl-2 family proteins under some apoptotic conditions [10]. It seems that apoptotic pathways vary depending both on cell type and apoptotic stimuli. Thus, in order to understand the mechanism of apoptosis associated with gastric mucosal lesions due to gastric irritants, the mechanism of apoptosis induced in this way should be examined using cultured gastric mucosal cells in vitro. However, to date, there are no reports in the literature that have systematically investigated the mechanism of apoptosis in response to gastric irritants in cultured gastric mucosal cells.

The use of cells in primary culture is thought to mimic cells in vivo better than that achieved using cell lines, in addition to which the primary culture of guinea pig gastric mucosal cells has been established and well characterized [11]. This system mimics various physiology of gastric mucosa in vivo, including mucin synthesis [11]. On the other hand, the similarities and differences between the mechanisms for various forms of irritant-induced apoptosis in gastric mucosal cells should be revealed to identify a target for drugs to inhibit apoptosis in gastric mucosa by various gastric irritants in general. Thus, we here reproduced apoptosis by various gastric irritants (ethanol, hydrogen peroxide, and hydrochloric acid) using primary cultures of guinea pig gastric mucosal cells and examined their molecular mechanisms. Results obtained from analyses of activities of caspases, mitochondrial membrane potential, and effects of caspase inhibitors on the apoptosis suggested that all of these gastric irritants induce apoptosis through a common pathway, in which caspase-8 plays an important role.

2. Materials and methods

2.1. Chemicals and media

RPMI 1640 was obtained from Nissui Pharmaceutical Co. Fetal calf serum (FCS), trypsin solution, and trypan blue were purchased from Gibco. Pronase E and type I collagenase were purchased from Kaken Pharmaceutical Co. and Nitta Gelatin Co., respectively. Sodium-*N*-lauroylsarcosinate was from Wako Co. Proteinase K, RNase A, Hoechst 33342 (Ho 342), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and atractyloside were from Sigma Co. 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidaozolylcarbocyanine iodide (DiPsipher) was from Trevigen. Peptides for the assay and inhibition of caspases were from Peptide Institute. Cytochrome *c* ELISA kit was from R&D Systems. Anti-caspase 3, 8, and 9 antibodies were from Santa Cruz Biotechnology.

2.2. Preparation and culture of gastric mucosal cells

Male guinea pigs (4 weeks of age) were purchased from Shimizu Co. (Kyoto, Japan). Gastric mucosal cells were isolated from guinea pig fundic glands, as described previously [11]. Isolated gastric mucosal cells (1×10^6 cells/dish) were cultured for 48 h in RPMI 1640, containing 0.3% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin in type-I collagen-coated plastic culture plates (Iwaki) under the conditions of 5% CO_2/95% air and 37°C. After removing non-adherent cells by washing with RPMI 1640, cells that were attached to the plate at about 50% confluence were used. We have previously characterized guinea pig gastric mucosal cell preparations under these conditions, with the majority (about 90%) of cells being identified as pit cells [11].

2.3. Treatment of cells with gastric irritants and inhibitors of caspases

Cells were exposed to gastric irritants (ethanol, hydrogen peroxide, and hydrochloric acid) by changing the entire bathing medium. For some experiments, cells were pre-incubated with 20 μM z-VAD-fmk (a broad-spectrum inhibitor of caspases), 10 μM Ac-DEVD-cho (caspase-3 inhibitor), 10 μM Ac-IETD-cho (caspase-8 inhibitor), and 10 μM Ac-LEHD-cho (caspase-9 inhibitor) for 30 min, following which cells were treated with various irritants in the absence of these inhibitors.

2.4. Cell viability assay

Cell viability was examined by the trypan blue exclusion test or MTT assay. Trypan blue exclusion test: After treatment with gastric irritants, cells were treated with 1% trypsin and collected by centrifugation. Cells were re-suspended in phosphate-buffered saline (PBS) containing 0.2% trypan blue dye and observed under a light microscope. MTT assay: After the treatment with gastric irritants, cells were incubated with MTT solution at the final concentration of 1 mg/ml in PBS for 2 h. Isopropanol and hydrochloric acid were added to the final concentrations of 50% and 20 mM, respectively. The optical density at 570 nm was determined by spectrophotometer using a reference wavelength of 630 nm [12].

2.5. DNA fragmentation assay

Apoptotic DNA fragmentation was monitored on agarose gel electrophoresis. Cells were collected with a rubber policeman and suspended in 20 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 the lysate 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.

2.6. Nuclear staining assay for apoptosis

After gastric irritants treatment, cells were washed with PBS and fixed with 10% v/v formaldehyde for 4 h. Cells were then washed with PBS, stained with 0.17 mM Ho 342, and observed under a fluorescence microscope as described previously [13].

2.7. Caspase activity assay

The activities of caspase-3, caspase-8, and caspase-9 were determined as described previously [14,15]. 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 substrates (Ac-DEVD-MCA (caspase-3), Ac-IETD-MCA (caspase-8), and Ac-LEHD-MCA (caspase-9)) 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°C. The release of amino-methyl-coumarin (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 per minute.

Caspase-3, caspase-8 and caspase-9 cleavage was monitored by immunoblotting with specific antibodies against these caspases.

2.8. Mitochondrial membrane potential assay

Mitochondrial membrane potential was assayed using a fluorometric mitochondrial permeability assay kit (Trevigen) [16]. Briefly, after gastric irritants treatment, cells were treated with DiPsipher (5 μg/ml) for 20 min at 37°C and observed under a fluorescence microscope with 590 nm for red emission and 530 nm for green emission. For control measurements, cells were treated with 5 mM atractyloside for 4 h before the DiPsipher-treatment.

2.9. Assay for the release of cytochrome c from mitochondria

After gastric irritants treatment, cells were washed twice in fractionation buffer (250 mM sucrose, 20

mM HEPES–KOH (pH 8.0), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA) and resuspended in the same buffer supplemented with protease inhibitors. After homogenization on ice using a tight-fitting Dounce homogenizer, nuclei were spun down at 2500 rpm for 10 min. The supernatant was further spun at 14 000 rpm for 25 min to separate into cytosolic fraction and membrane fractions. Amounts of cytochrome *c* in cytosolic fraction was determined by a sandwich cytochrome *c* ELISA kit (R&D Systems), according to the manufacturer's specifications.

2.10. Statistical analysis

All values are expressed as the mean ± standard error (S.E.M.). A 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 $P < 0.05$.

3. Results

3.1. Effects of gastric irritants on cell viability and size of chromosomal DNA

We selected ethanol, hydrogen peroxide, and hydrochloric acid as gastric irritants to be tested on the basis of the following reasons. Ethanol is the most typical external gastric irritant, whose effect on gastric mucosa has been examined thoroughly both in vivo and in vitro [17,18]. Furthermore, we previously reported that ethanol induced apoptosis in gastric mucosal cells in primary culture [19]. When defensive factors protecting gastric mucosa are decreased by irritant (for example, mental irritant), the gastric mucosa is damaged by gastric acid (hydrochloric acid), resulting in ulceration [2,18]. With the onset of gastritis, reactive oxygen species released from activated neutrophils and macrophages damage gastric mucosa in vivo [20].

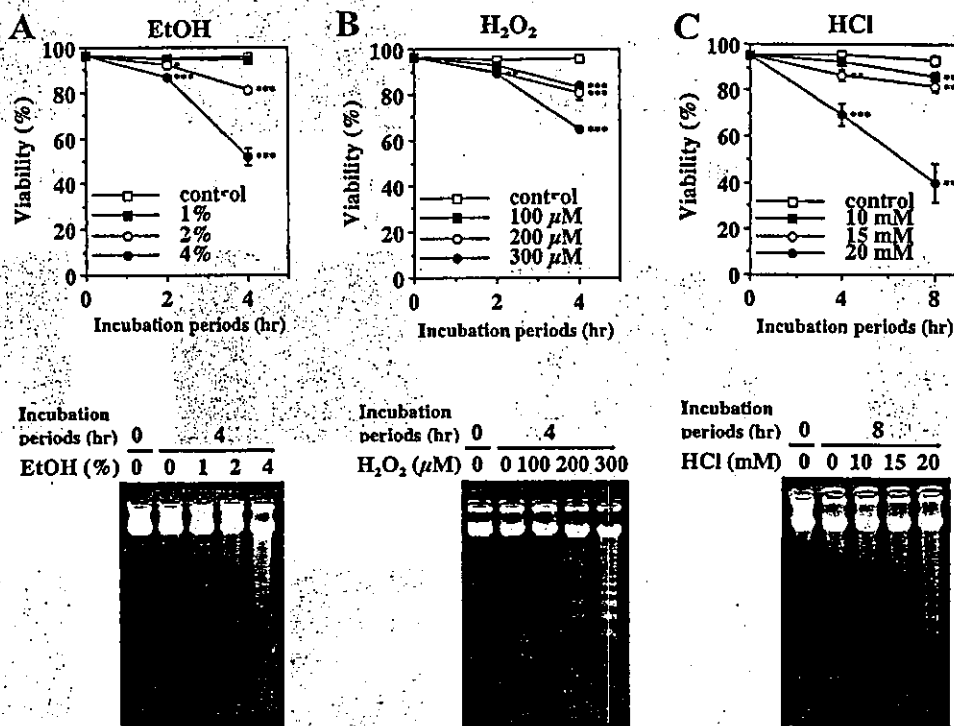


Fig. 1. Effects of gastric irritants on cell viability and chromosomal DNA. Cultured gastric mucosal cells were incubated with indicated concentrations of ethanol (EtOH) (A), hydrogen peroxide (H_2O_2) (B), or hydrochloric acid (HCl) (C) for indicated periods. Cell viability was determined by the trypan blue exclusion test. Values shown are mean ± S.D. ($n = 3$) ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ (upper panel). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (lower panel).

We examined here the effects of these gastric irritants on gastric mucosal cells cultured in the presence of low serum concentrations (0.3%), being different from our previous reports about ethanol-induced apoptosis (10%). This is because in the presence of high serum concentrations (e.g., 10%), gastric mucosal cells in primary culture undergo maturation-dependent spontaneous apoptosis, which mimic the rapid turnover of gastric mucosal cells in vivo [21,22].

We first examined the effects of these gastric irritants on cell viability. Ethanol and hydrogen peroxide rapidly decreased cell viability in a dose-dependent manner (Fig. 1A,B). Treatment with 4% ethanol or 300 μM hydrogen peroxide for 4 h decreased the cell viability to about 50% or 60%, respectively. Further incubation with these concentrations of ethanol and hydrogen peroxide up to 8 h did not cause any further decrease in the cell viability (data not shown). On the other hand, cell death by hydrochloric acid was relatively slow; a linear decrease in the cell viability by hydrochloric acid was observed over 8 h (Fig. 1C). Thus, in experiments described below, we used an incubation period of 4 h for ethanol and hydrogen peroxide treatment, while for the hydrochloric acid treatment, 8-h incubation periods were used as noted.

To understand the mechanism of cell death induced by these gastric irritants, we examined the state of chromosomal DNA under the conditions used. As shown in Fig. 1, all of gastric irritants tested here induced DNA fragmentation in a dose dependent manner. The pattern of DNA fragmentation (i.e., the small molecular sizes of the fragments) was typical of that of apoptotic DNA fragmentation,

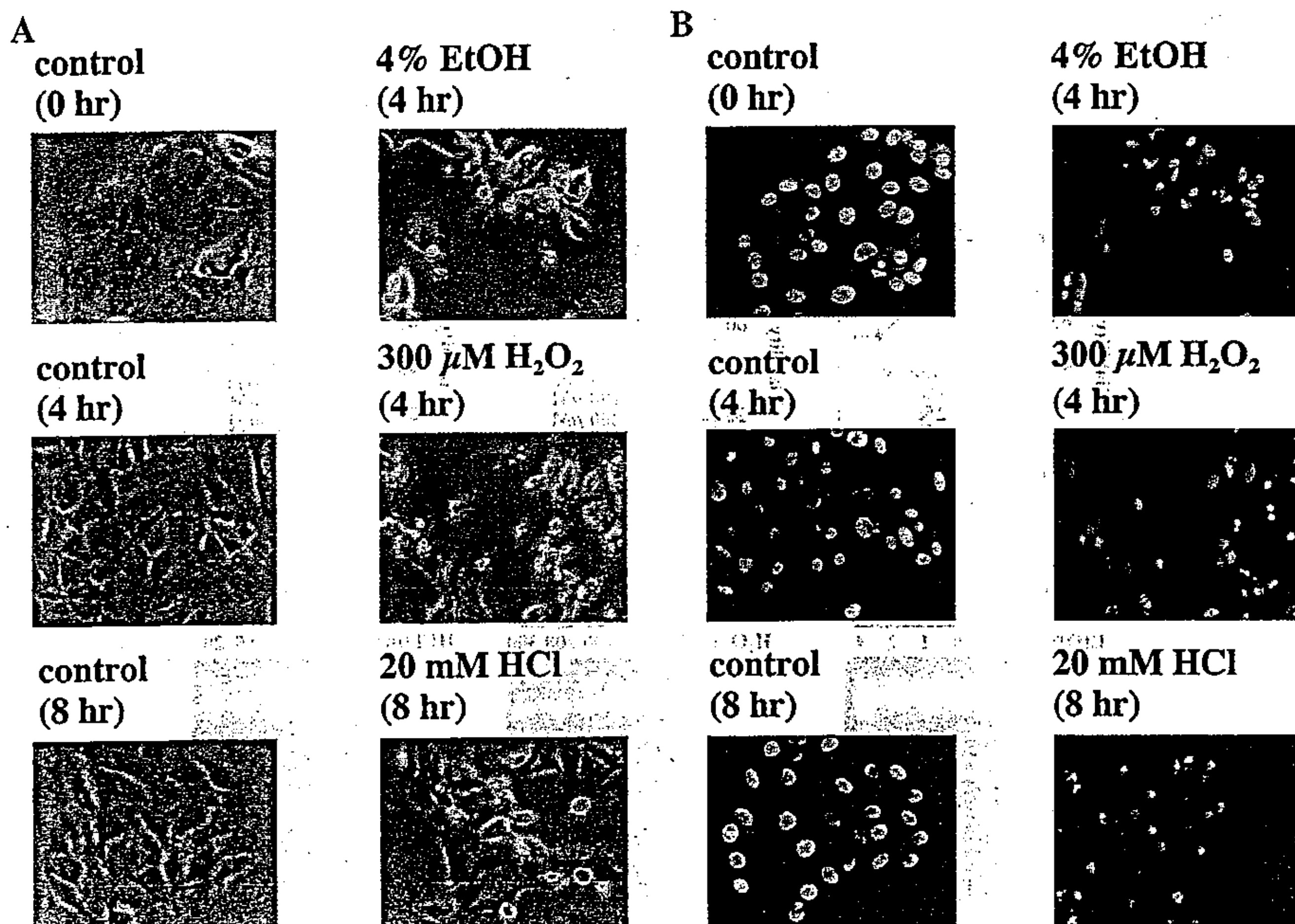


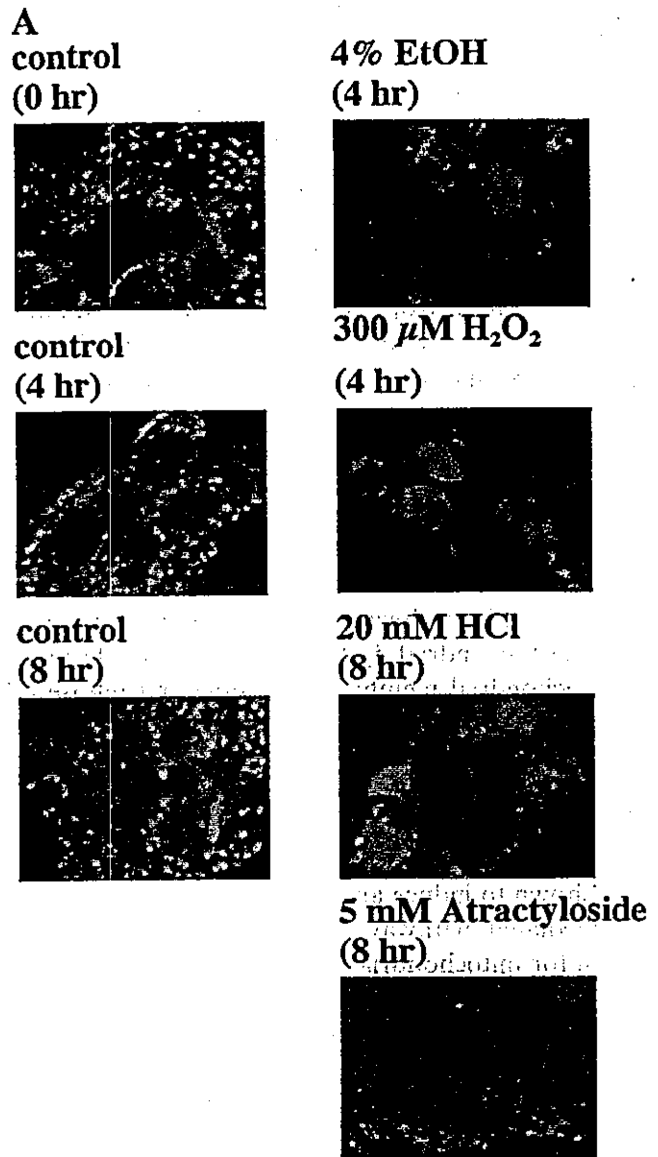
Fig. 2. Apoptotic morphological changes in cells induced by gastric irritants. Cultured gastric mucosal cells were incubated with indicated concentrations of gastric irritants for indicated periods. After staining with Ho 342, cells were observed under a phase-contrast (A) or fluorescence (B) microscope.

based on the results from electrophoresis with the DNA ladder marker (data not shown). The dose-dependency of the apoptotic DNA fragmentation by these gastric irritants correlated to that of decreased cell viability, suggesting that all of these gastric irritants decrease cell viability mainly by the induction of apoptosis.

3.2. *Effects of gastric irritants on cell morphology and chromatin structure*

Since DNA fragmentation is observed even in necrotic cells in some cases, we could not simply conclude, based only on the data shown in Fig. 1, that cell death by these gastric irritants was mediated by apoptosis. Thus, we examined characteristic features of apoptosis other than apoptotic DNA fragmentation, such as morphological change of cells and chromatin condensation. As shown in Fig. 2A, treatment of cells with 4% ethanol, 300 μM hydrogen peroxide, or 20 mM hydrochloric acid caused apoptotic cell rounding and cell shrinkage. We could also observe apoptotic bodies in cells under these conditions (Fig. 2A). The appearance of cell rounding, cell shrinkage, and apoptotic bodies were dependent on both the incubation period employed and concentrations of the compounds used (data not shown), which corresponded to the time-course and dose-response of the decrease in cell viability by these gastric irritants (Fig. 1).

The state of chromatin in cells treated with these gastric irritants was examined using Ho 342 staining. Fluorescence microscopy observations showed that all of these gastric irritants tested clearly induced chromatin condensation (Fig. 2B). The appearance of condensed chromatin was dependent on both the duration of the incubation period and concentrations of compound used (data not shown), which also corresponded to the time course and dose response of the decrease in cell viability (Fig. 1). Based on results



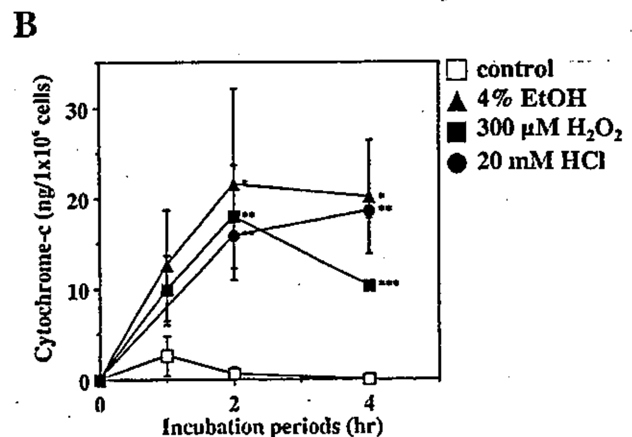


Fig. 3. Effects of gastric irritants on mitochondrial functions. Cultured gastric mucosal cells were incubated with indicated concentrations of gastric irritants for indicated periods (A,B). Mitochondrial membrane potential was analyzed as described in Section 2 (A). The release of cytochrome *c* from mitochondria was monitored as described in Section 2. Values shown are mean ± S.D. ($n = 3$). ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

of apoptotic DNA fragmentation, apoptotic morphological changes of cells, and chromatin condensation, we concluded that these gastric irritants (ethanol, hydrogen peroxide, and hydrochloric acid) all induced apoptosis in gastric mucosal cells in primary culture. We consider that such apoptosis in vitro may mimic apoptosis observed in gastric mucosa damaged by gastric irritants in vivo, which is associated with gastropathy. We, therefore, examined the molecular mechanism of the in vitro gastric irritant-induced apoptosis in following experiments.

3.3. Effects of gastric irritants on mitochondrial function

Apoptotic pathways can be distinguished by their involvement of mitochondria [23]. In a mitochondria-mediated pathway, the apoptotic stimulus causes mitochondrial dysfunction (such as a decrease in mitochondrial membrane potential and release of cytochrome *c* from mitochondria) and stimulates the release of cytochrome *c* from mitochondria, which in turn activates the downstream apoptotic pathway [23]. In cell types other than gastric mucosal cells, such as hepatocyte, ethanol and hydrogen peroxide were shown to induce apoptosis through a mitochondria-mediated pathway [24]. Thus, using an assay system for mitochondrial permeability [25], we examined whether or not these gastric irritants affect mitochondrial membrane potential. After treatment with gastric irritants, cells were treated with DiPsipher (5 μg/ml), a mitochondrial dye, for 20 min at 37°C and then observed under a fluorescence microscope. This mitochondrial dye normally exists in solution as a monomer emitting green fluorescence (530 nm) and forms a dimmer emitting red fluorescence (590 nm) in the reaction driven by changes in the mitochondrial membrane potential [25]. Atractyloside, an inhibitor of the ATP-ADP carrier, is known to decrease the mitochondrial membrane potential [25]. As shown in Fig. 3A, red fluorescence was apparent in control cells (in the absence of gastric irritants and atractyloside) especially in peripheral areas of each colony, while the red fluorescence was markedly decreased in atractyloside-treated cells. All of the gastric irritants tested (4% ethanol, 300 μM hydrogen peroxide, or 20 mM hydrochloric acid) decreased the red fluorescence (Fig. 3A), suggesting that the mitochondrial membrane potential was decreased by each of these gastric irritants. Since gastric mucosal cells in primary culture tend to aggregate on plastic plates to form colonies [11], only the mitochondria of cells which were located in peripheral area of each colony emitted red fluorescence; the dye may not reach the center of each colony (Fig. 3A). Upon treatment with the gastric irritants, cell–cell adhesion became weak. Thus, all gastric mucosal cells were stained with DiPsipher and emitted green fluorescence under the conditions of gastric irritants (Fig. 3A).

We also monitored the release of cytochrome *c* from mitochondria. As shown in Fig. 3B, All of the gastric irritants tested (4% ethanol, 300 μM hydrogen peroxide, or 20 mM hydrochloric acid) stimulated the release of cytochrome *c* from mitochondria. From these observations, we consider that gastric irritant-induced apoptosis is driven by a mitochondria-mediated pathway.

3.4. Activation of caspases by gastric irritants

Most apoptotic events are mediated by the sequential activation of caspases [12]. Among them, caspase-3 is located downstream of the pathway [12]. In a mitochondria-mediated apoptosis pathway, mitochondrial dysfunction (such as a decrease in mitochondrial membrane potential) stimulates the release of cytochrome *c* from mitochondria, which in turn activates caspase-9 in collaboration with Apaf-1, resulting in the activation of caspase-3 [26]. Since results obtained above suggest that gastric irritant-induced apoptosis is caused by a mitochondria-mediated pathway, we predicted that these gastric irritants activate caspase-9 and caspase-3. Since mitochondria-mediated apoptosis pathways can be further distinguished on the basis of whether or not caspase-8 is activated, we also examined whether these gastric irritants activate caspase-8 in addition to caspase-3 and caspase-9. After treatment with each gastric irritant, cells were extracted and their caspase activities were examined by use of fluorogenic peptide substrates (Ac-DEVD-MCA (caspase-3), Ac-IETD-MCA (caspase-8), and Ac-LEHD-MCA (caspase-9)). Since these peptides can be cleaved by other caspases (for example, caspase-7 can recognize and cleave Ac-DEVD-MCA [15,27], we should de-

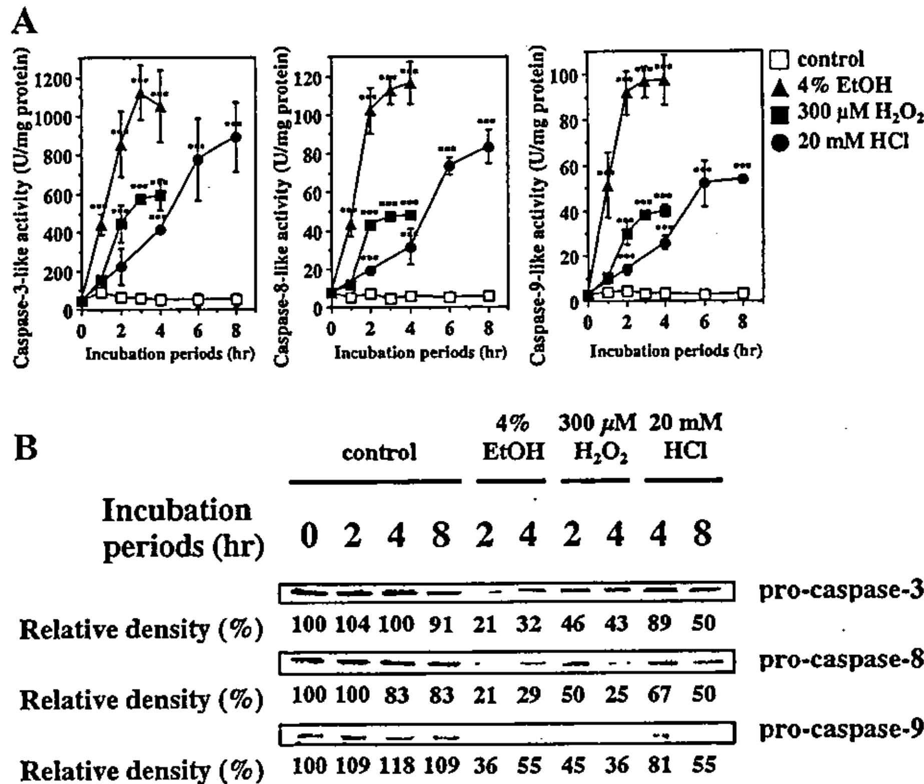


Fig. 4. Activation of caspases by gastric irritants. Cultured gastric mucosal cells were incubated with indicated concentrations of gastric irritants for indicated periods (A,B). Cell lysates were prepared and their activities of caspase-3, caspase-8, and caspase-9 were measured by a fluorometric assay using Ac-DEVD-MCA, Ac-IETD-MCA and Ac-LEHD-MCA, respectively. One unit of protease activity was defined as the amount of enzyme required to release 1 pmol AMC per minute. Values shown are mean ± S.D. ($n = 3$). ***$P < 0.001$ (A). Caspase-3, caspase-8 and caspase-9 cleavage was monitored by immunoblotting with specific antibodies against these caspases. The relative intensity of each band to control (time 0 without gastric irritants) is shown (B).

scribe caspase-3-, caspase-8-, or caspase-9-like activity, instead of caspase-3, caspase-8, or caspase-9 activity, respectively. As shown in Fig. 4A, all of the reagents increased caspase-3-like activity. Among the irritants tested, the extent of activation of caspase-3-like activity by 300 μM hydrogen peroxide was weaker than that by 4% ethanol or 20 mM hydrochloric acid (Fig. 4A). The time course of the activation of caspase-3-like activity by 20 mM hydrochloric acid was linear in the first 8 h after exposure, whereas that by 4% ethanol or 300 μM hydrogen peroxide reached a plateau level after 3 h (Fig. 4A). Furthermore, it should be noted that the time-dependent change with each reagent was quite similar among the individual caspase-like activity (Fig. 4A). By employing immunoblotting experiments, using specific antibodies against these caspases, we observed caspase-3, caspase-8 and caspase-9 cleavage in the presence of all these gastric irritants tested (Fig. 4B). Thus, these data showed that caspase-3, caspase-8 and caspase-9 were all activated simultaneously by each of all these gastric irritants. Since the time course of activation of each of these caspases corresponded to that of cell death and apoptotic DNA fragmentation (Fig. 1), apoptosis induced by these gastric irritants may be mediated commonly by the activation of caspases.

3.5. *Effects of caspase inhibitors on cell death induced by gastric irritants*

In order to further confirm that gastric irritant-induced apoptosis is mediated by the activation of caspases and to identify the critical caspases, which are responsible for the apoptosis, we examined the effects of pre-treatment of gastric mucosal cells with various caspase inhibitors on the apoptosis. Initially

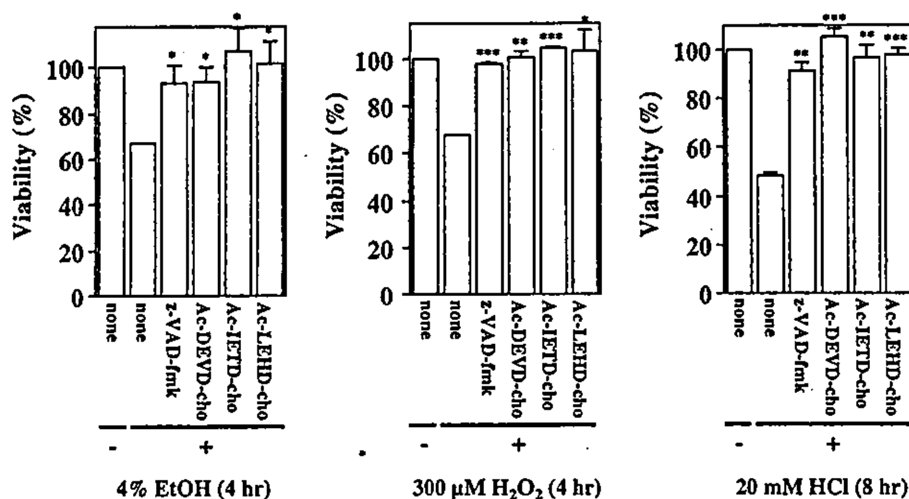


Fig. 5. Effect of caspase inhibitors on gastric irritant-induced cell death. Cultured gastric mucosal cells were pre-incubated with 20 μM z-VAD-fmk (a broad-spectrum caspase inhibitor), 10 μM Ac-DEVD-cho (caspase-3 inhibitor), 10 μM Ac-IETD-cho (caspase-8 inhibitor), or 10 μM Ac-LEHD-cho (caspase-9 inhibitor) for 30 min and then treated with indicated concentrations of gastric irritants for indicted periods. Cell viability was determined by MTT assay. Values shown are mean ± S.D. ($n = 3$). $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

we used a broad-spectrum caspase inhibitor, z-VAD-fmk, which inhibits the activities of most of caspases, including caspase-3, -8, and -9 [28]. As shown in Fig. 5, pre-treatment of gastric mucosal cells with 20 μM z-VAD-fmk for 30 min almost completely inhibited the cell death. We confirmed that 20 μM z-VAD-fmk inhibited activation of caspase-3, -8, and -9-like activities by these gastric irritants (data not shown). To identify essential caspases for gastric irritant-induced cell death, we then used a specific inhibitor for each caspase (Ac-DEVD-cho (caspase-3 inhibitor), Ac-IETD-cho (caspase-8 inhibitor), and Ac-LEHD-cho (caspase-9 inhibitor)). All of these inhibitors also clearly inhibited the cell death by each of the gastric irritants (Fig. 5). We also found that pre-treatment of cells with 20 μM z-VAD-fmk, 10 μM Ac-DEVD-cho, 10 μM Ac-IETD-cho, or 10 μM Ac-LEHD-cho for 30 min almost completely inhibited apoptotic DNA fragmentation by each of these gastric irritants (data not shown). These results suggest that activation of all of caspase-3, caspase-8 and caspase-9 is essential for apoptosis caused by these gastric irritants.

3.6. *Effect of an inhibitor of caspase-8 on caspase-3 and caspase-9 activation by gastric irritants*

As described above, caspase-3 is the effector caspase and is activated by both caspase-8 and caspase-9 [4]. Recently, several reports have shown that caspase-8 can activate caspase-9, through mitochondrial dysfunction [29,30]. It was also suggested that caspase-3 could activate caspase-8 [8]. Thus, if it can be assumed that these three caspases are activated sequentially one by one, then there are at least two possibilities for the pathway of gastric irritant-induced apoptosis. One is that gastric irritants initially activate caspase-8, which then activates caspase-9, resulting in caspase-3 activation. The other possibility is that gastric irritants cause directly mitochondrial dysfunction following which caspase-3, which is activated by caspase-9, induces the activation of caspase-8. However, the latter possibility is unlikely, since we showed above that an inhibitor of caspase-8 almost completely inhibited cell death induced by gastric irritants (Fig. 5). Given that caspase-3 is the effector caspase, caspase-8 is not necessary for apoptosis if caspase-3 can be activated without caspase-8 activation. Thus, the results obtained here suggest that the former possibility is the most likely. If this is the case, caspase-3 and caspase-9 activation should be dependent on the activation of caspase-8. We therefore examined whether or not an inhibitor of caspase-8 also inhibits caspase-3 and caspase-9 activation caused by these gastric irritants. Pre-treatment of cells with 10 μM Ac-IETD-cho partially in-

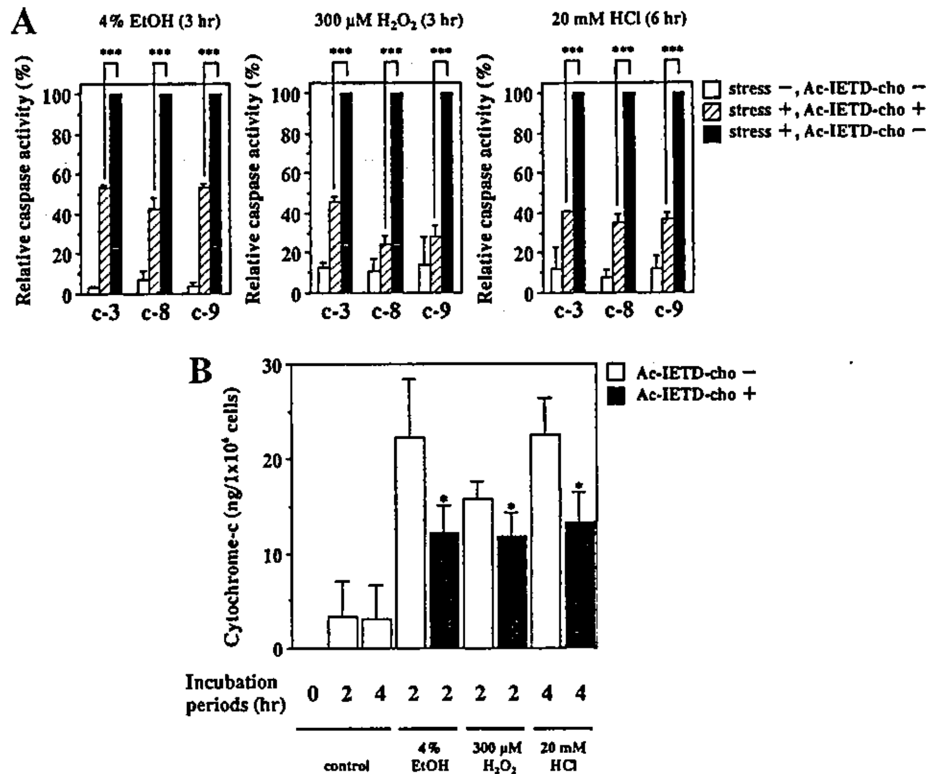


Fig. 6. Effect of a caspase-8 inhibitor on the activity of each caspase and the release of cytochrome *c* from mitochondria. Cultured gastric mucosal cells were pre-incubated with or without 10 μM Ac-IETD-cho (caspase-8 inhibitor) for 30 min and then treated with indicated concentrations of gastric irritants for indicated periods. Activities of caspase-3 (c-3), caspase-8 (c-8) and caspase-9 (c-9) or the release of cytochrome *c* from mitochondria were determined as described in the legend of Fig. 4 or Fig. 3, respectively. Values shown are mean ± S.D. ($n = 3$). ***$P < 0.001$; *$P < 0.05$.

hibited the activation of caspase-8-like activity by exposure to 4% ethanol (Fig. 6A). Under the present conditions, the activation of caspase-8-like activity by ethanol could not be completely inhibited by Ac-IETD-cho even at higher concentrations of Ac-IETD-cho (data not shown). We also found that Ac-IETD-cho inhibited the activation of caspase-3- and -9-like activities by ethanol to much the same extent as that of caspase-8-like activity (Fig. 6A). We also examined the effect of Ac-IETD-cho on the activation of caspases by hydrogen peroxide or hydrochloric acid and obtained the basically same results as that found with ethanol (Fig. 6A). These results suggest that the activation of caspase-3 and -9 by each of all these gastric irritants is partially dependent on caspase-8 activation.

We also examined the effect of Ac-IETD-cho on the release of cytochrome *c* from mitochondria. As shown in Fig. 6B, the release of cytochrome *c* from mitochondria by these gastric irritants was partially suppressed by Ac-IETD-cho, suggesting that caspase-8 is partially involved in the mitochondrial dysfunction. These data also imply that these gastric irritants directly affect mitochondrial function.

4. Discussion

In this paper, we clearly showed that all of the gastric irritants tested here (ethanol, hydrogen peroxide, and hydrochloric acid) induced apoptosis in gastric mucosal cells in primary culture. These conclusions are made on the basis of results obtained from experiments in which we examined cell viability, apoptotic DNA fragmentation, apoptotic morphological changes of cells, and chromatin condensation. The concentration of ethanol required for apoptosis in vitro was 4%. Studies on oral administration of ethanol in rats showed that ethanol concentration higher than 20% caused gastric injury [31].

Considering that the administered solution would be diluted about 2–3 times in the stomach [32], the concentration of ethanol in vitro (4%) is physiologically relevant in vivo. There are several reports showing that ethanol induces apoptosis in gastric mucosa in vivo [33,34]. Since primary culture of gastric mucosal cells is thought to closely mimic gastric mucosal cells in vivo, we consider that the apoptosis by ethanol in vitro reproduces ethanol-induced apoptosis in gastric mucosa in vivo. There are many papers describing that gastric acid secretion and production of reactive oxygen species from infiltrated neutrophils and macrophages damage gastric mucosa through induction of apoptosis [20,35]. In the present experiments, the pH value of the bathing medium required for the induction of apoptosis was determined to be 3.8 (data not shown). Since the pH value of gastric acid is 1–2 [36], the concentration of hydrochloric acid used here (20 mM) seems to be within physiological level. Breakage of acid-neutralising barrier, such as mucus coat, leads to the exposure of gastric mucosal cells to hydrochloric acid. Thus, it is also possible that apoptosis by hydrogen peroxide and hydrochloric acid in vitro mimics apoptosis in vivo at the onset of gastric ulceration and gastritis.

We also examined the molecular mechanism of the gastric irritant-induced apoptosis by measuring mitochondrial membrane potential, as well as the activities of caspases, and the effects of caspase inhibitors on apoptosis. All of our results supported the notion that these gastric irritants induce apoptosis through a common pathway. We have already suggested that another type of gastric irritant, non-steroidal anti-inflammatory drugs (NSAIDs), also induce apoptosis through the same pathway [37]. Since gastric mucosal cells are exposed to various types of irritants and induced apoptosis, resulting in gastric mucosal injury, a drug which can suppress all forms of gastric irritant-induced apoptosis may be therapeutically benefit for preventing gastric mucosal injury. Thus, it is particularly important to reveal the common pathway for all forms of gastric irritant-induced apoptosis and to find out a target molecule for such a drug. It is also important to reveal a pathway of physiological (non-irritant conditions) apoptosis in gastric mucosa and to find a molecule, which is involved in irritant-induced apoptosis but not in a physiologically induced apoptosis. Gastric mucosal cells have a rapid cell turnover rate in vivo; the short turnover cycle being the result of rapid cell death at the gastric surface through apoptosis [38]. We recently established in vitro system, which mimics this apoptosis [22]. Using this system, we are now investigating the molecular mechanism of physiological apoptosis in gastric mucosa cells.

Previously, apoptotic irritants were thought to directly affect the mitochondria and activate caspase-9, resulting in caspase-3 activation [23]. The present findings that all of gastric irritants tested decreased the mitochondrial membrane potential support this notion. However, they activated caspase-8-like activity, besides caspase-9 and caspase-3 (Fig. 4). Furthermore, the activation of caspase-8 was necessary for the induction of apoptosis by gastric irritants (Fig. 5). Thus, apoptosis observed here cannot be simply explained only by the classic irritant-induced apoptosis pathway. Recently, caspase-8 was shown to be able to cause mitochondrial dysfunction and stimulate the release of cytochrome *c* from mitochondria, which activates caspase-9. For example, in a death-receptor mediated apoptosis pathway, caspase-8 stimulated the release of cytochrome *c* by inducing cleavage of Bid, a bcl-2-related protein [39]. These findings suggest that these gastric irritants may at first activate caspase-8 and cause mitochondrial dysfunction to trigger the cytochrome *c*-dependent pathway, in which both caspase-9 and caspase-3 are activated. In fact, we showed that caspase-8 activation was necessary for the activation of both caspase-3 and caspase-9 (Fig. 6). At present, the mechanism by which these gastric irritants induce caspase-8 activation is unclear. It was reported that *H. pylori* infection stimulated the amount of tumor necrosis factor (TNF)-α and induced apoptosis in gastric cell line (Kato III) [40]. We examined the effect of these of gastric irritants on the amount of TNF-α in primary culture of gastric mucosal cells. Treatment of cells with 4% ethanol, 300 μM hydrogen peroxide, or 20 mM hydrochloric acid caused 0.3-, 1.6-, or 1.2-fold increase in the amount of TNF-α (data not shown), suggesting that TNF-α is not involved in the common pathway for apoptosis by these gastric irritants. Since the inhibitor of caspase-8 (Ac-IETD-cho) could not completely suppress the release of cytochrome *c* from mitochondria by these gastric irritants (Fig. 6B), these gastric irritants also seem to

directly affect mitochondrial function of caspase-8 in an independent manner.

The mechanisms of anti-ulcer drugs, which are currently in clinical use, are either elimination of aggressive factors or an increase in defensive factors. As a novel mechanism for anti-ulcer drugs, growth factors for gastric mucosa were shown to be effective in combating ulcers in rats by increasing the number of gastric mucosal cells [41]. It is possible that inhibition of irritant-induced apoptosis in gastric mucosal cells also increases the number of gastric mucosal cells. Thus, we consider that drugs which can inhibit gastric irritant-induced apoptosis may become a new type of anti-ulcer drugs.

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Transforming Growth Factor-β1 Is Responsible for Maturation-Dependent Spontaneous Apoptosis of Cultured Gastric Pit Cells

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In this study, we established a system of high concentration serum-dependent spontaneous apoptosis of guinea pig gastric pit cells in primary culture, which seems to mimic the spontaneous apoptosis of matured gastric pit cells at gastric surface *in vivo*. In addition to induction of the spontaneous apoptosis, cell growth was inhibited in the presence of 10% serum compared with 0.5% serum. Transforming growth factor-β1 (TGF-β1), which is known to cause both apoptosis and growth inhibition in mammalian cells, was present in serum of both fetal calf and guinea pig. The addition of recombinant TGF-β1 to the culture medium containing 0.5% fetal calf serum caused both induction of apoptosis and inhibition of cell growth. On the other hand, immunodepletion of TGF-β1 from fetal calf serum caused inability to induce both the spontaneous apoptosis and inhibition of cell growth. These data suggest that TGF-β1 is involved in the spontaneous apoptosis of guinea pig gastric pit cells in primary culture. [Exp Biol Med Vol. 227(6):402–411, 2002]

Key words: apoptosis; gastric mucosal cells; caspases; transforming growth factor-β1

Transforming growth factor β1 (TGF-β1) is a member of the TGF-β family, which is involved in regulation of cell growth, cell death, and cell development (1). TGF-β1 is secreted as a latent form and is activated by various factors (acid, plasmin, thrombospondin-1, and other undefined factors) (2–5). Activated TGF-β1 binds to a dimeric cell surface serine/threonine kinase receptor, the TGF-β type II receptor (TβRII), with the signal then transmitted to the cell nucleus via phosphorylation of TGF-β type I receptors and Smad proteins (6). Although TGF-β1 was originally identified as a growth inducer for normal rat fibroblast (7), it is now recognized as a multifunctional growth regulator (6). TGF-β1 inhibits cell growth by arresting the cell cycle at the G1 phase (8). TGF-β1 also induces apoptosis in various types of cells (9, 10). According to these activities, TGF-β1 is postulated to be involved in some diseases, particularly cancer (11), for which it has been shown that cancer cells have a tendency to release much more TGF-β1 than normal cells (12). Mutations in TβRII that render it unable to bind TGF-β1 have been reported in some clinically identified cancer cells (13). Therefore, TGF-β1 released from cancer cells seems to inhibit cell growth and to induce apoptosis selectively in normal cells near to tumors, thereby aiding in the progression of cancer (14). TGF-β1 seems to play important roles not only these pathological conditions, but also in the regulation of cell growth under some normal physiological conditions, such as cell proliferation of hematopoietic stem cells and gastric pit cells (9, 15). However, the precise action of TGF-β1 in these regulatory roles is yet to be fully elucidated.

Gastric pit cells (gastric mucosal cells) have a rapid rate of turnover, which makes them unique among mammalian cell types. This short turnover cycle is the result of rapid proliferation of progenitor cells at the isthmus, cell maturation that occurs during the upward migration of cells, and rapid apoptotic cell death at the gastric surface (16). In order to maintain homeostasis of the gastric mucosa, the proliferation of progenitor cells and the apoptotic cell death of mature cells needs to be well balanced, given that alterations to this balance can cause various types of gastropathy. For example, stimulation of the apoptosis of mature gastric pit cells by inflammatory cytokines (such as interferon-γ) seems to cause progression of gastritis into gastric ulcers (17). Stimulation of the apoptosis and the proliferation of gastric pit cells by *Helicobacter pylori* infection was suggested to be involved in the development of atrophic gastritis and gastric cancer, respectively (18, 19).

Therefore, in order to understand gastric physiology and pathology, the molecular mechanisms governing this

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short turnover cycle of gastric pit cells, in particular the rapid apoptotic cell death of mature cells at the gastric surface, need to be elucidated. For this purpose, a suitable *in vitro* model that can adequately reproduce rapid apoptotic cell death is necessary. We consider that the system of primary culture of guinea pig gastric pit cells is useful as such an *in vitro* system because these cells spontaneously mature and induce apoptosis in a serum-dependent manner, which is thought to mimic the rapid apoptotic cell death that takes place at the gastric surface *in vivo* (20). Therefore, we examined in the present study the molecular mechanism of spontaneous apoptosis by using primary cultures of guinea pig gastric pit cells. We found that spontaneous apoptosis occurs in the presence of 10% serum but not 0.5% serum, and is accompanied by inhibition of cell growth. We also suggest that based on experiments using recombinant TGF-β1 and an immunodepletion technique, TGF-β1 in serum is essential and sufficient for inducing the spontaneous apoptosis and growth inhibition.

Materials and Methods

Chemicals and Media. RPMI 1640 medium was obtained from Nissui Pharmaceutical (Tokyo, Japan). Fetal calf serum (FCS) and trypsin solution were purchased from Invitrogen (Carlsbad, CA) Pronase E and type I collagenase were purchased from Kaken Pharmaceutical (Kyoto, Japan) and Nitta Gelatin (Osaka, Japan), respectively. Proteinase K, RNase A, 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), Hoechst 33342 (Ho 342), propidium iodide (PI), and protein A-Sepharose were obtained from Sigma (Tokyo, Japan). Peptides for the assay of caspases were from Peptide Institute (Osaka, Japan). Recombinant human TGF-β1, a latent form of recombinant human TGF-β1, and a sandwich TGF-β1 ELISA kit were from R&D Systems (Abingdon, UK). Anti-TGF-β1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Male guinea pigs (4 weeks of age) were purchased from Shimizu (Kyoto, Japan). Methyl-[^{3}H]thymidine (3 TBq/m*M*) was from Amersham (Tokyo, Japan).

The Animal Care Committee of Okayama University approved all experiments and procedures described here.

Preparation and Culture of Gastric Pit Cells. Gastric mucosal cells were isolated from guinea pig fundic glands, as described previously (21). Isolated gastric mucosal cells (3×10^6 cells/dish) were cultured in type-I collagen-coated plastic culture plates in RPMI 1640 medium containing 0.5% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin under the conditions of 5% CO_2/95% air and 37°C for 1 day. After removing nonadherent cells by washing with RPMI 1640, cells that remained attached to plates were used. We characterized cell types of our gastric mucosal cell preparations by periodic acid Schiff (PAS) staining method. Results showed that 93% of cells were identified as pit cells.

Cell Viability and Cell Growth Assay. Cell viability was examined using the trypan blue exclusion test. Cells were treated with 1% trypsin and were collected by centrifugation. Cells were resuspended in phosphate-buffered saline (PBS) containing 0.2% trypan blue dye and were observed with the aid of a light microscope.

Cell growth was analyzed by MTT assay, which measures the metabolic activity of mitochondria. Cells were incubated with MTT (1 mg/ml) in PBS for 2 hr to change MTT to formazan. Isopropanol and hydrochloric acid were then added at final concentrations of 50% and 20 m*M*, respectively, to solubilize the formed colored formazan product (22). The optical density of the solution at 570 nm (absorbed by formazan dye) was determined using a spectrophotometer with a reference wavelength of 630 nm (22).

DNA Fragmentation Assay. Apoptotic DNA fragmentation was monitored as previously described (23). Briefly, cells were collected with a rubber policeman and were suspended in 100 μl of lysis buffer consisting of 50 m*M* Tris-HCl (pH 7.8), 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 hr. RNase A 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 for Apoptosis and Necrosis. Cells were washed with PBS and incubated with 0.17 m*M* Ho 342 and 100 μg/ml PI for 20 min, following which cells were analyzed using a fluorescence microscopy, as described previously (24).

Caspase Activity Assay. The activities of caspase-3, caspase-8, and caspase-9 were determined as described previously (25). 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 substrates (Ac-DEVD-MCA [caspase-3], Ac-IETD-MCA [caspase-8], and Ac-LEHD-MCA [caspase-9]) in reaction buffer (100 m*M* HEPES-KOH [pH 7.5], 10% sucrose, 0.1% CHAPS, and 1 mg/ml bovine serum albumin [BSA]) for 15 min at 37°C. The release of amino-methyl-cumarin (AMC) was determined using a fluorescence spectrophotometer. One unit of protease activity was defined as the amount of enzyme required to release 1 p*M* AMC/min/mg protein.

Caspase-3 cleavage was monitored by immunoblotting with specific antibody against caspase-3.

Thymidine Uptake Assay. Cells were incubated with culture medium containing 25 μ*M* methyl-[^{3}H]thymidine (3 TBq/m*M*) for 8 hr. The reaction was terminated by the addition of TCA to give a final concentration of 10%. Samples were passed through Whatman GF/C glass-fiber filters. The level of radioactivity on the filters was measured with a liquid scintillation counter.

Quantification of TGF-β1. Concentrations of TGF-β1 in culture media and sera were determined by a sandwich TGF-β1 ELISA kit (R&D Systems), according to the manufacturer's specifications. Briefly, culture medium was incubated in the presence of 1 *N* HCl for 10 min at room temperature (RT) to convert the latent form of TGF-β1 to its active form (26). As for serum, it was incubated in the presence of 2.5 *N* acetic acid and 10 *M* urea for the same purpose, according to manufacturer's specifications. After neutralization, samples were loaded on microtiter plates coated with TβRII and were incubated at RT for 3 hr. After washing with PBS, horseradish peroxidase-conjugated antibody against TGF-β1 was added and incubated for 1.5 hr at RT. Following further washing, hydrogen peroxide and chromogen were added and incubated for 20 min at RT. The activity of peroxidase trapped in each well was monitored on a microtiter plate reader (BMG Labtechnologies). In order to detect only the active form of TGF-β1, in some experiments, both steps of acid treatment and neutralization were omitted, as described previously (26).

Immunodepletion of TGF-β1 from Serum. A culture medium containing 10% FCS (1.5 ml) was incubated with anti-TGF-β1 antibody (2 μg) and 120 μl of protein A-Sepharose (50% slurry) for 30 min at 4°C. After centrifugation, supernatants were used as an immunodepleted sample. For control, the medium was treated similarly except for the presence of the antibody.

Statistical Analysis. All values are expressed as the mean ± SEM.. A Student's *t* test for paired results was performed for the evaluation of differences between groups. Differences were considered to be significant for values of $P < 0.05$.

Results

Establishment of a System to Examine Spontaneous Apoptosis. We previously reported that guinea pig gastric pit cells in primary culture undergo spontaneous apoptosis in the presence of 10% FCS (20). Because it had already been reported that these cells mature *in vitro* under the conditions (15), we considered that this spontaneous apoptosis mimics the apoptotic cell death of mature gastric pit cells at the gastric surface *in vivo*, which is associated with a rapid *in vivo* cell turnover rate (20). In the absence of serum, these cells showed neither spontaneous apoptosis nor cell proliferation (15). Thus, we needed to identify culture conditions where the spontaneous apoptosis is inhibited, but where cells can still grow in order that the molecular mechanism of the spontaneous apoptosis could be examined. We found in the present experiments that cell viability did not decrease for up to 24 hr when gastric pit cells were cultured in the presence of 0.5% FCS (Fig. 1A), and that cells could proliferate under these conditions (see below). In the presence of 10% FCS, cell viability gradually decreased (Fig. 1A), as described previously (20). We performed the same experiments as in Figure 1A, using several different lots of FCS, and obtained similar results in all experiments (data not shown). To confirm that the cell death in the presence of 10% FCS is mediated by apoptosis, we examined the state of chromosomal DNA under the conditions used.

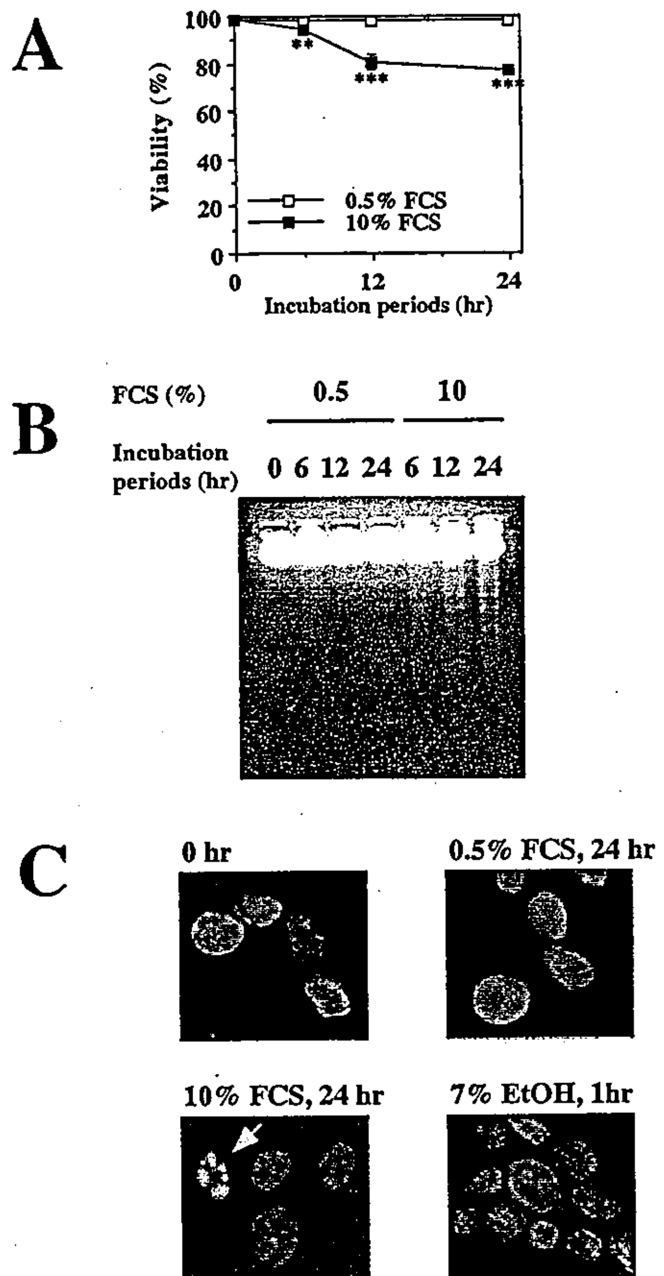

Figure 1. Effect of serum concentrations on spontaneous apoptosis. Gastric pit cells were cultured with 10% or 0.5% FCS for indicated periods. Cell viability was determined by the trypan blue exclusion test (A). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (B). Cells were stained with PI and Ho 342 and were observed under a fluorescence microscope. Cells were incubated with 7% ethanol (EtOH) for 1 hr (C) as a positive control for necrosis because we recently reported that treatment of the gastric pit cells with more than 7% ethanol for 1 hr induced necrosis (Ref. 44). An arrow indicates the apoptotic cell. Values are mean ± SD ($n = 3$) $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$. Data are representative of three similar experiments.

Chromosomal DNA was extracted and analyzed by