agarose gel electrophoresis in the presence of ethidium bromide. As shown in Figure 1B, incubation period-dependent DNA fragmentation was observed in the presence of 10% FCS, but not 0.5% FCS. The pattern of DNA fragmentation was typical of that seen in apoptotic DNA fragmentation based on the results from electrophoresis with a DNA ladder marker (data not shown). The time course of the apoptotic DNA fragmentation (Fig. 1B) in the presence of 10% FCS correlated to that of the decrease in cell viability (Fig. 1A).

We further examined the characteristics of the cell death reported in Figure 1A by double-staining experiments with PI and Ho 342. Because necrotic cells lose their membrane integrity, PI staining causes red nuclear staining in necrotic cells, whereas living cells and apoptotic cells are not stained with PI (24). Chromatin condensation, which is one of characteristic features of apoptosis, can be visualized by Ho 342 staining (24). Cells cultured for 24 hr in the presence of 10% FCS showed condensed chromatin but not red nuclear staining (Fig. 1C), suggesting that the decrease in cell viability evident in Figure 1A is mediated mainly by the induction of apoptosis. Based on these results, we concluded that cultured gastric pit cells undergo spontaneous apoptosis in the presence of 10% FCS but not 0.5% FCS. As such, we considered that we could examine the molecular mechanism of the spontaneous apoptosis by comparing cells cultured in the presence of 10% FCS and 0.5% FCS.

Activation of Caspases upon Spontaneous Apoptosis. Most apoptotic events are mediated by the activation of caspases (27). Among them, caspase-3 is located downstream in the biochemical pathway and directly activates proteins responsible for DNA fragmentation and chromatin condensation (28, 29). Therefore, activation of caspase-3 is a principle indicator for apoptosis. Caspase-8 and caspase-9 activate caspase-3 (30), although some reports have suggested that caspase-3 can be activated without activation of either caspase-9 or caspase-8 (31). We recently showed that each of a series of gastric stressors tested (ethanol, hydrogen peroxide, and hydrochloric acid) induced apoptosis in cultured guinea pig gastric pit cells via a common pathway in which caspase-3, caspase-8, and caspase-9 were all activated equally (32). Therefore, we examined aspects of the activation of these caspases upon spontaneous apoptosis and compared our findings with those seen with gastric stressor-induced apoptosis. Caspase activities were determined by use of fluorogenic peptide substrates (Ac-DEVD-MCA [caspase-3], Ac-IETD-MCA [caspase-8], and Ac-LEHD-MCA [caspase-9]) as described in "Materials and Methods." Because these peptides can be cleaved by other caspases (for example, caspase-7 can recognize and cleave Ac-DEVD-MCA [33]), we should describe caspase-3-, caspase-8-, or caspase-9-like activity, instead of caspase-3, caspase-8, or caspase-9 activity, respectively.

As shown in Figure 2A, according to culture in the presence of 10% FCS, caspase-3-like activity was stimulated strongly (about 10-fold), confirming that apoptosis was induced under these conditions. The time course of the

stimulation of caspase-3-like activity in the presence of 10% FCS correlated to that of the decrease in the cell viability and the apoptotic DNA fragmentation (Fig. 1, A and B), suggesting that this activation is responsible for the spontaneous apoptosis. By employing immunoblotting experiments using specific antibodies against caspase-3, we observed caspase-3 cleavage in the presence of 10% FCS but not 0.5% FCS (Fig. 2C). Caspase-8- and caspase-9-like activities were slightly activated (about 3-fold) during culture in the presence of 10% FCS (Fig. 2A).

We recently reported that treatment of the gastric pit cells with 4% ethanol for 4 hr caused apoptosis (32). Here, we confirmed that when gastric pit cells induce apoptosis upon being exposed to a gastric stressor (4% ethanol), the activities of all of these caspases were stimulated rapidly and equally under the condition of 0.5% FCS (Fig. 2B). We obtained results that were similar for other gastric stressors tested (hydrogen peroxide and hydrochloric acid; data not shown). Therefore, results in Figure 2 suggest that spontaneous apoptosis is mediated by a different pathway from that of gastric stressor-induced apoptosis.
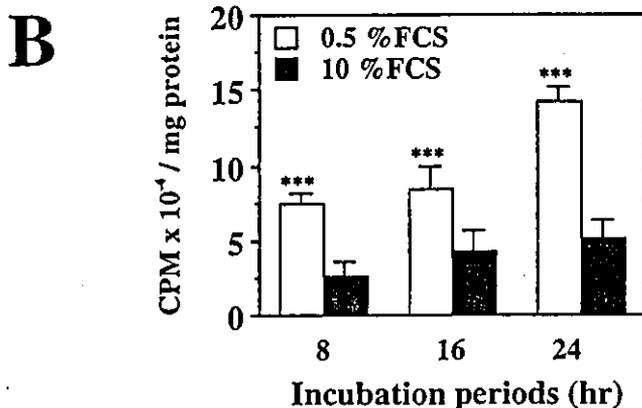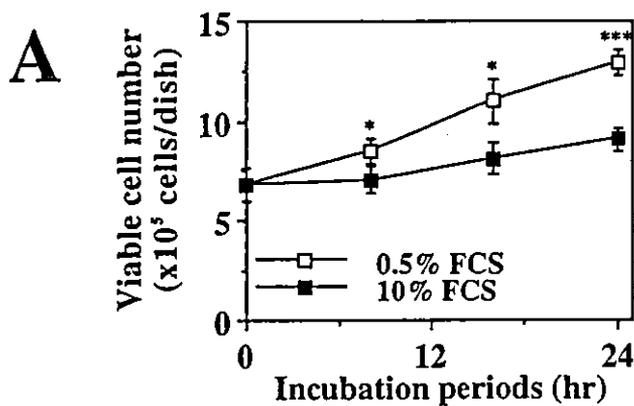
Effect of Serum Concentrations on Cell Growth. As shown in Figure 3A, the rate of increase in the cell number was much more rapid in the presence of 0.5% FCS than that in the presence of 10% FCS. The cell number after 24 hr of culture in the presence of 10% FCS was about 60% of that in the presence of 0.5% FCS. Considering differences in cell viability between the 10% and 0.5% FCS culture conditions (Fig. 1A), we considered that the difference in cell number (Fig. 3A) was due not only to the apoptotic cell death, but also to the inhibition of cell growth.

To test whether cell growth was inhibited in the presence of 10% FCS, we examine the rate of DNA synthesis in the presence of 10% or 0.5% FCS by measuring methyl-[^{3}H]thymidine incorporation into acid-insoluble fractions. As shown in Figure 3B, the amount of incorporated thymidine was lower in the presence of 10% FCS than 0.5% FCS at every 8-hr incubation period. These data show that the cell growth was partially inhibited by 10% FCS, suggesting that some factors in FCS inhibit the growth of gastric pit cells in primary culture.

Determination of the Amounts of TGF-β1 in Serum and Culture Medium. TGF-β1 is known to induce apoptosis and to inhibit cell growth of mammalian cells *in vivo* and *in vitro* (11, 34). TGF-β1 is a highly conserved protein among various mammalian species (35). It was shown that TGF-β1 inhibits cell growth in cultured guinea pig gastric pit cells (15) and also that it induced apoptosis in a human gastric carcinoma cell line (36). Thus, based on results in Figures 1 through 3, we considered that TGF-β1 in FCS might be responsible for both the spontaneous apoptosis and inhibition of cell growth observed in the presence of 10% FCS. Therefore, we initially determined the concentration of TGF-β1 in culture medium and its alteration according to the cell culture (10% or 0.5% FCS) using the ELISA method.
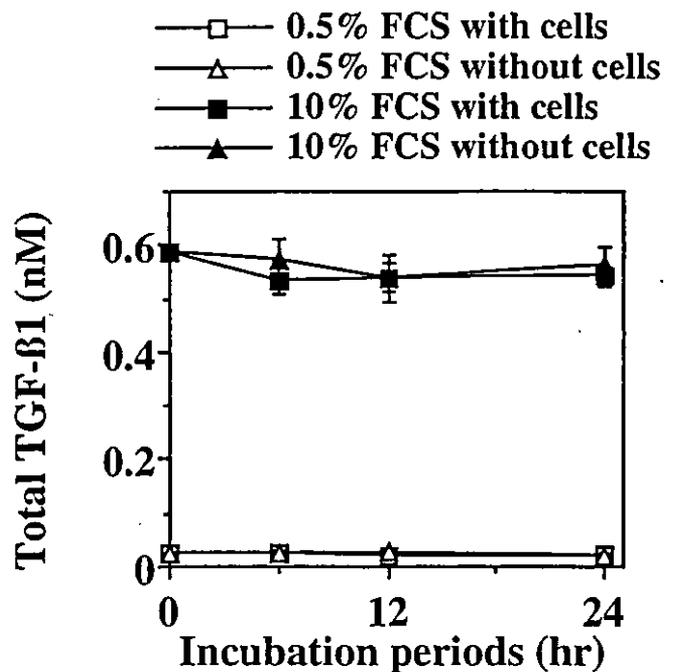
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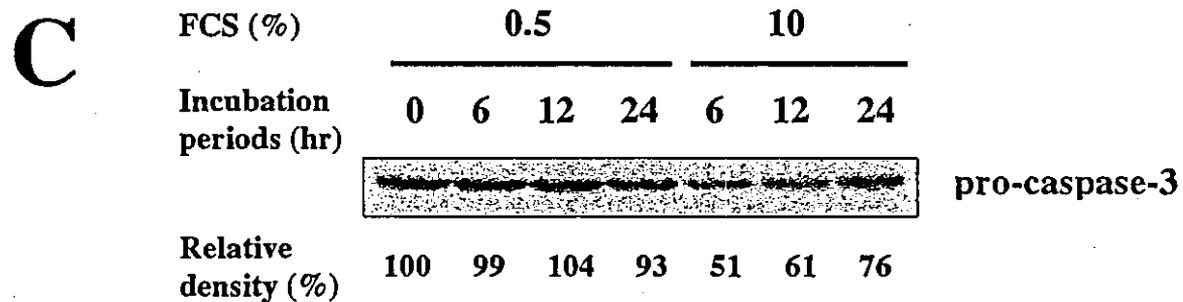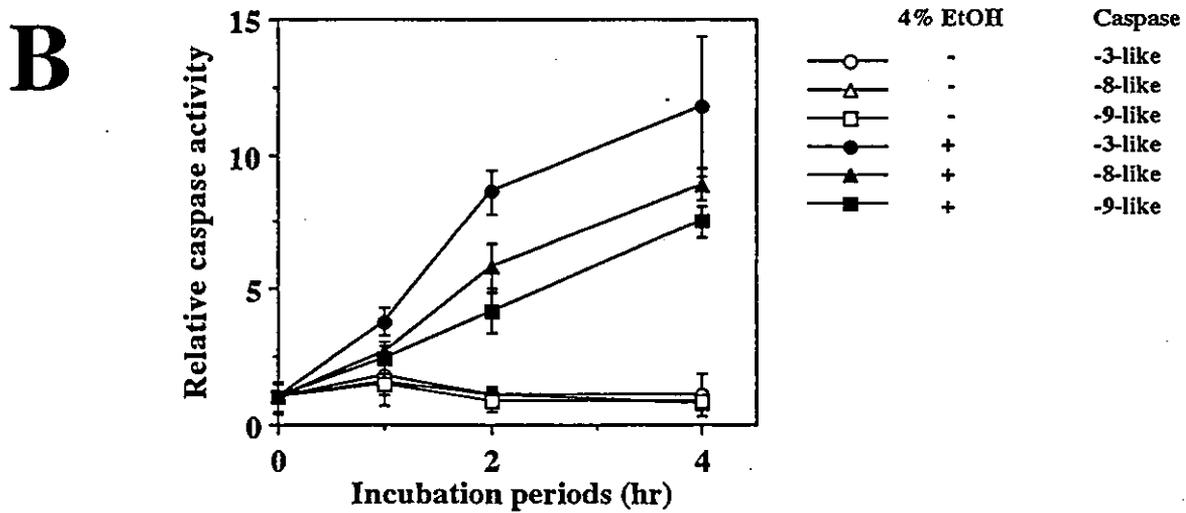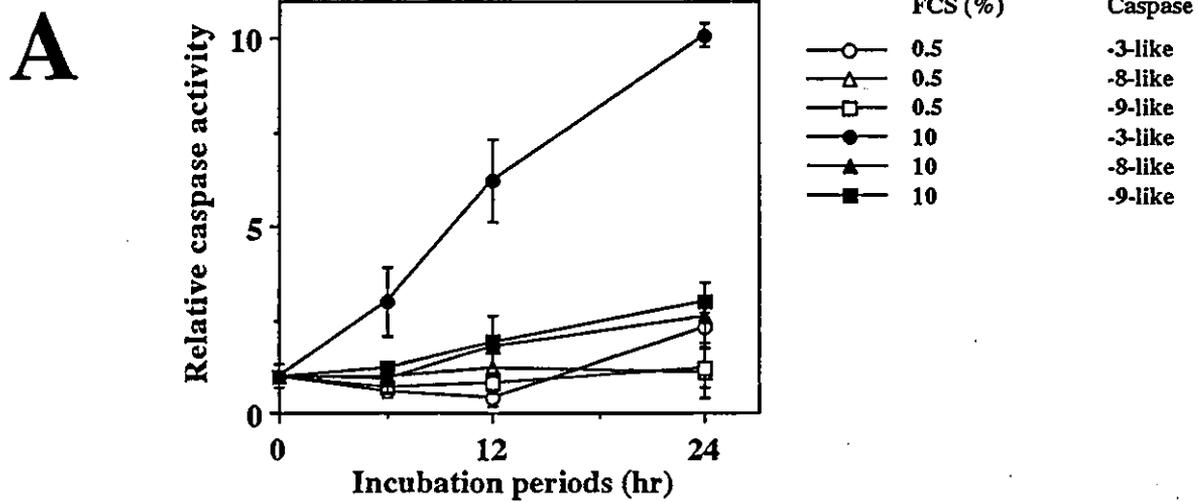
Figure 2. Activation of caspases upon spontaneous apoptosis and gastric stressor-induced apoptosis. Gastric pit cells were cultured with 10% or 0.5% FCS for indicated periods (A). Cells were cultured with or without 4% ethanol under the conditions of 0.5% FCS for indicated periods (B). Caspase-3-, caspase-8-, and caspase-9-like activities were measured with the aid of 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 pM AMC/min/mg protein. Caspase-3 cleavage was monitored by immunoblotting with specific antibody against caspase-3 (C). Values are relative to those at time zero and are shown as mean $\pm$ SD ($n = 3$). Data are representative of four similar experiments.

40

A

B

Figure 3. Effects of serum concentrations on cell growth. Gastric pit cells were cultured with 10% or 0.5% FCS for indicated periods and cell numbers were determined by counting with a hemacytometer (A). Gastric pit cells were cultured with 10% or 0.5% FCS for indicated periods. Eight hours before the end of culture, methyl-[^{3}H]thymidine was added and pulse-labeled for 8 hr. Incorporated methyl-[^{3}H]thymidine was determined as described in "Materials and Methods" (B). Values are mean ± SD (n = 3). *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$. Data are representative of three similar experiments.

Figure 4. Alteration in amounts of TGF-β1 upon *in vitro* culture. Culture medium containing 10% or 0.5% FCS was incubated with or without gastric pit cells for indicated periods. Culture medium was collected and amounts of TGF-β1 were determined by ELISA as described in "Materials and Methods." Values are mean ± SD (n = 3). Data are representative of four similar experiments.

As shown in Figure 4, the initial concentrations of TGF-β1 are about 0.6 and 0.03 nM in the culture medium containing 10% and 0.5% FCS, respectively. Because the latent form of TGF-β1 was activated by acid before the ELISA, these concentrations represent the total of both the latent and active forms. In the absence of acid activation, the concentration of TGF-β1 in the culture medium containing 10% FCS was estimated to be 0.02 nM, showing that the concentration of the active form of TGF-β1 is much lower than the latent form. The concentrations of total TGF-β1 and the active form only did not change during the cell culture for up to 24 hr (Fig. 4 and data not shown).

We determined the concentrations of TGF-β1 in FCS to be 4.89 ± 0.18 nM (n = 5). Therefore, the TGF-β1 in the culture medium should be derived mainly from the FCS, meaning that only very small quantities of TGF-β1 would

be released from cells compared with that in FCS. We also determined the concentrations of TGF-β1 in the serum of guinea pig to be 2.81 ± 0.21 nM (n = 5).

Induction of the Spontaneous Apoptosis and Inhibition of Cell Growth by TGF-β1. In order to test whether TGF-β1 is responsible for both the spontaneous apoptosis and inhibition of cell growth observed in the presence of 10% FCS, we first examined the effects of recombinant human TGF-β1 on apoptosis and cell growth under the conditions of 0.5% FCS. As shown in Figure 5A, recombinant human TGF-β1 decreased cell viability in a dose-dependent manner. Because apoptotic DNA fragmentation was also observed in a dose-dependent manner of recombinant human TGF-β1 (Fig. 5B), the decrease in cell viability observed in Figure 5A must be due to the induction of apoptosis. We also examined the effect of recombinant human TGF-β1 on cell growth under the conditions of 0.5% FCS in the culture medium. As shown in Figure 5C, recombinant human TGF-β1 inhibited cell growth in a dose-dependent manner. All these data support the notion that TGF-β1 in the culture medium is responsible for both the spontaneous apoptosis and inhibition of cell growth observed in the presence of 10% FCS.

We performed similar experiments using the latent form of recombinant human TGF-β1. As shown in Figure 5, D through F, the latent form of TGF-β1 (up to 2 nM) did not cause decrease in the cell number, apoptotic DNA fragmentation, and inhibition of cell growth compared with control. These data suggest that activation of the latent form of
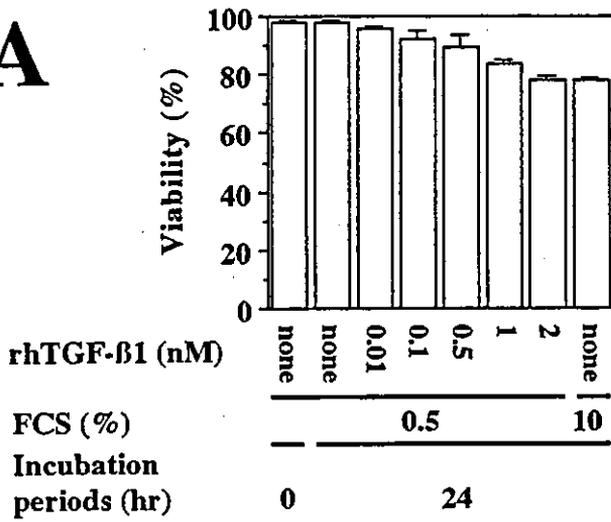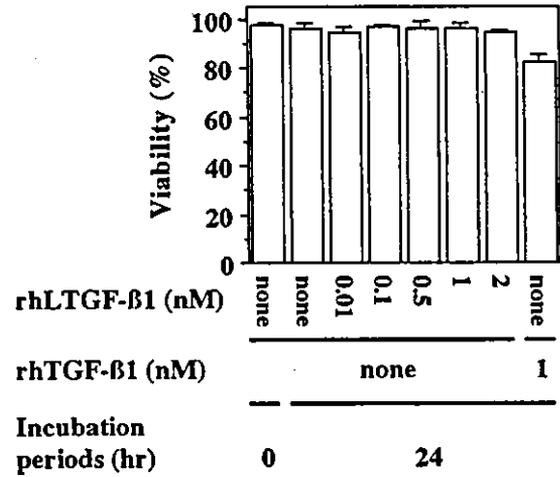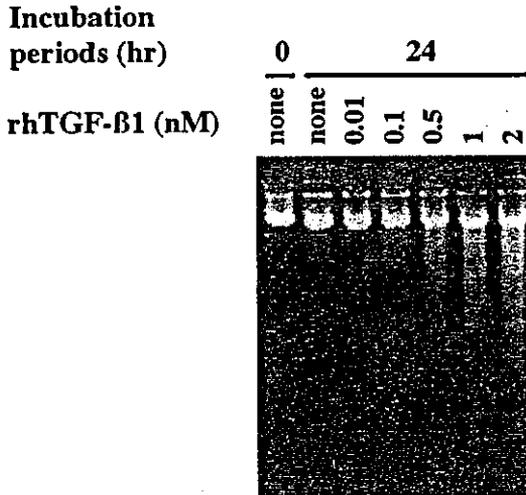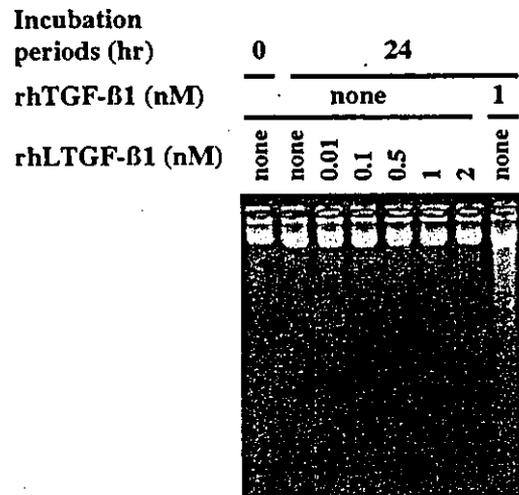
SPONTANEOUS APOPTOSIS AND TGF-β1   407

A

Viability (%)

100, 80, 60, 40, 20, 0

rhTGF-ß1 (nM): none, none, 0.01, 0.1, 0.5, 1, 2, none

FCS (%): — 0.5 10

Incubation periods (hr): 0 24

D

Viability (%)

100, 80, 60, 40, 20, 0

rhLTGF-ß1 (nM): none, none, 0.01, 0.1, 0.5, 1, 2, none

rhTGF-ß1 (nM): none 1

Incubation periods (hr): 0 24

B

Incubation periods (hr): 0 24

rhTGF-ß1 (nM): none, none, 0.01, 0.1, 0.5, 1, 2

E

Incubation periods (hr): 0 24

rhTGF-ß1 (nM): none 1

rhLTGF-ß1 (nM): none, none, 0.01, 0.1, 0.5, 1, 2, none

C

Relative cell growth

1.0, 0.5, 0

rhTGF-ß1 (nM): none, 0.01, 0.1, 0.5, 1, 2, none

FCS (%): 0.5 10

F

Relative cell growth
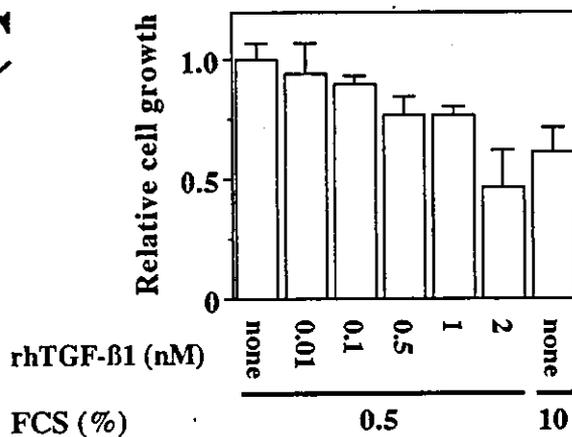
1.0, 0.5, 0

rhLTGF-ß1 (nM): none, 0.01, 0.1, 0.5, 1, 2, none

rhTGF-ß1 (nM): none 1

Figure 5. Effect of TGF-β1 on spontaneous apoptosis and cell growth. Gastric pit cells were cultured with indicated concentrations of recombinant human TGF-β1 (rhTGF-β1; A–C) or the latent form of recombinant human TGF-β1 (rhLTGF-β1; D–F) under the conditions of 0.5% FCS for 24 hr. Control experiments were performed as indicated. Cell viability was determined by the trypan blue exclusion test (A and D). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (B and E). Relative cell growth was measured by the MTT assay (C and F). Values are mean ± SD (n = 3). Data are representative of three similar experiments.

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TGF-β1 was not so vigorous, which is consistent with results that the concentration of active form of TGF-β1 was constant upon cell culture (see above). We consider that the spontaneous apoptosis and growth inhibition in the presence of 10% FCS is mainly mediated by TGF-β1 present in the serum as the active form.

**Requirement of TGF-β1 for Both the Spontaneous Apoptosis and Inhibition of Cell Growth.** We further examined the requirement of TGF-β1 for both the spontaneous apoptosis and inhibition of cell growth observed in the presence of 10% FCS using an immunodepletion technique. TGF-β1 in FCS was immunodepleted by the use of anti-TGF-β1 antibody and protein A-Sepharose. ELISA showed that only 20% of the latent form of TGF-β1 was depleted by this method (data not shown), but more than 70% of the active form of TGF-β1 was depleted. This is due to the fact that this antibody recognizes the active form more efficiently than it does the latent form (37). Due to the detection limitation of the ELISA method, we could not accurately determine the efficiency of immunodepletion of the active form of TGF-β1.

We monitored cell viability according to culture conditions in the presence of the immunodepleted FCS and control FCS. Figure 6A shows that the effect of 10% FCS on cell viability was diminished by the immunodepletion of TGF-β1. We did the same experiments as in Figure 6A using several different lots of FCS, and we obtained similar results in all experiments (data not shown). We also observed that the spontaneous apoptotic DNA fragmentation observed in the presence of 10% FCS was also diminished by the immunodepletion (Fig. 6B). These data suggest that TGF-β1 in FCS is required for inducing spontaneous apoptosis.

We also examined the effect of the immunodepletion on growth of cells cultured in the presence of 10% FCS. As shown in Figure 6C, the cell growth inhibited by 10% FCS recovered to levels seen with culture in the presence of 0.5% FCS when immunodepletion was used. All these data support the notion that TGF-β1 is responsible for both the spontaneous apoptosis and inhibition of cell growth observed in the presence of 10% FCS.

Discussion

In this paper, we suggest that TGF-β1 plays an important role in the spontaneous apoptosis of gastric pit cells in
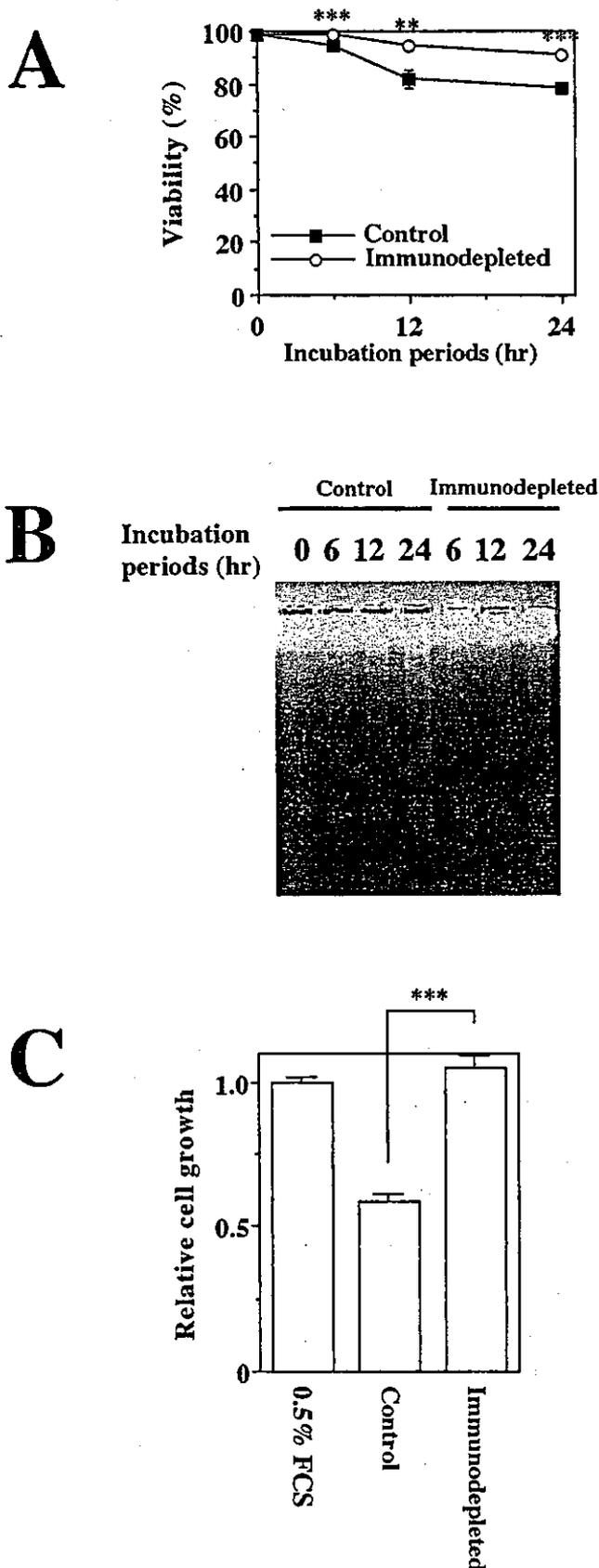
Figure 6. Effect of immunodepletion of TGF-β1 on spontaneous apoptosis and cell growth. TGF-β1 in FCS was immunodepleted using anti-TGF-β1 antibody and protein A-Sepharose as described in "Materials and Methods." Gastric pit cells were cultured with immunodepleted or control FCS (10%) for indicated periods. Cell viability was determined by the trypan blue exclusion test (A). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (B). Relative cell growth was measured by the MTT assay. Control experiment was performed as indicated (C). Values are mean ± SD ($n = 3$). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Data are representative of three similar experiments.

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primary culture. We found that the spontaneous apoptosis of cultured guinea pig gastric pit cells is observed in the presence of 10% FCS, but not 0.5% FCS, and that FCS (and also serum from guinea pig) contains relatively high concentrations of TGF-β1. Furthermore, recombinant human TGF-β1 induced spontaneous apoptosis in cells cultured under the conditions of 0.5% FCS, whereas immunodepletion of TGF-β1 in FCS inhibited the spontaneous apoptosis in the presence of 10% FCS. However, in relation to the concentration of the active form of TGF-β1, these results have some contradictions. We estimated the concentration of active form of TGF-β1 to be 0.02 nM in culture medium containing 10% FCS; however, more than 0.5 nM recombinant human TGF-β1 was required for the induction of spontaneous apoptosis. We cannot explain this discrepancy clearly. It may be due to a relatively low activity or instability of recombinant human TGF-β1 compared with the TGF-β1 in FCS.

TGF-β1 is known to induce apoptosis in various types of cells, including prostate epithelial cells, podocytes, and hematopoietic cells (9, 10, 38, 39). Therefore, the apoptosis by TGF-β1 is not specific for gastric pit cells. However, at present, it is not clear whether or not TGF-β1 induces apoptosis in other various kinds of cells than gastric pit cells in culture. As for the mechanism of apoptosis by TGF-β1, it was recently reported that mitogen-activated protein kinase (MAPK) and bcl-2 are involved in the TGF-β1-dependent apoptosis (38, 39). We assume that these proteins are involved in the apoptosis by TGF-β1 in gastric pit cells in primary culture.

The primary culture of guinea pig gastric pit cells has been established and well characterized (21). Although these cells have been reported to proliferate slowly, mature, and undergo spontaneous apoptosis in the presence of 10% FCS (20), it was recently reported that these cells do not undergo such processes in the absence of FCS (15). Here, we have shown that in the presence of 0.5% FCS, these cells proliferate rapidly, but do not undergo spontaneous apoptosis. Because gastric pit cells have a rapid rate of turnover *in vivo*, we consider that the condition of 10% FCS is more physiological than that of 0.5% FCS. However, results in this paper suggest that the level of TGF-β1 present can regulate the number of gastric pit cells *in vivo*. Recently, it was reported that the concentration of TGF-β1 around the gastric mucosa is increased by *H. pylori* infection (40). This increase may decrease the number of gastric pit cells present by stimulating spontaneous apoptosis and inhibiting cell proliferation and cause the development of atrophic gastritis.

The mechanisms of anti-ulcer drugs, which are currently in clinical use, are either elimination of aggressive factors or increase in defensive factors. Examples of the former are proton pump inhibitors and H$_2$-blockers, whereas those of the latter include non-toxic inducers of heat shock proteins and prostaglandin-related drugs (21, 41–43). We recently found that various gastric stressors (NSAIDs, alco-

hol, oxidative stressors, and acids) decrease the number of viable gastric pit cells by inducing both apoptosis and necrosis (44, 45). Thus, increasing the number of gastric pit cells could provide a new target for anti-ulcer drugs. In fact, growth factors for gastric mucosa were shown to be effective in combating ulcers in rats by increasing the number of gastric pit cells present (46). We propose here that antagonists of TβRII or inhibitors of the activation of TGF-β1 could provide the basis of new types of anti-ulcer drugs, which increase the number of gastric pit cells by inhibiting their spontaneous apoptosis and stimulating their cell proliferation.

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Geranylgeranylacetone Protects Guinea Pig Gastric Mucosal Cells from Gastric Stressor-Induced Apoptosis

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TATSUYA HOSHINO, BS, TOMOFUSA TSUCHIYA, PhD, and TOHRU MIZUSHIMA, PhD

Various stressors induce apoptosis in gastric mucosal cells, which may cause gastric mucosal lesions *in vivo*. We recently reproduced gastric stressor-induced apoptosis *in vitro*, using primary cultures of guinea pig gastric mucosal cells. Geranylgeranylacetone is an antiulcer drug with heat-shock protein-inducing properties. The purpose of this study is to examine the effect of geranylgeranylacetone on gastric stressor-induced apoptosis *in vitro*. Ethanol, hydrogen peroxide, and hydrochloric acid all induced, in a dose-dependent manner, apoptotic DNA fragmentation. Pretreatment of cells with geranylgeranylacetone inhibited the apoptotic DNA fragmentation caused by each of these gastric stressors. Pretreatment of cells with a low concentration of ethanol, a procedure that is also known to induce heat-shock proteins, made cells resistant to the apoptotic DNA fragmentation. These results suggest that heat-shock proteins could be at least partly involved in the inhibitory effect of geranylgeranylacetone against apoptosis of gastric mucosal cells caused by these gastric stressors.

KEY WORDS: geranylgeranylacetone; gastric mucosal cells; apoptosis; heat-shock proteins; gastric stressors.

The gastric mucosa can be damaged by various types of stressors (such as alcohol, acid, oxidative stressors, drugs, and bacteria). These gastric stressors were thought to cause gastric mucosal injury by stimulating the onset of necrosis (1, 2). However, recent studies suggest that apoptosis is also partly involved in the gastric mucosal injury caused by gastric stressors *in vivo* (3). For example, at the onset of gastric ulceration, stimulated apoptosis of gastric mucosal cells was reported (4). The increased rate of apoptosis

associated with *Helicobacter pylori* infection was suggested to induce the atrophic gastritis caused by *H. pylori* infection (5). In order to understand the molecular mechanism governing gastric stressor-induced apoptosis and to establish a clinical protocol for overcoming mucosal damage caused by gastric stressor-induced apoptosis, a system for reproducing such apoptosis *in vitro* is necessary.

In several recent studies we were able to reproduce apoptosis in guinea pig gastric mucosal cells in primary culture by exposure of cells to various gastric stressors (ethanol, hydrogen peroxide, and hydrochloric acid) (Tsutsumi et al, submitted). It was found that all of these gastric stressors induce apoptosis through a common pathway, in which caspase-8 plays an important role (Tsutsumi et al, submitted). Thus, inhibitors of this common pathway can suppress apoptosis by gastric stressors in general, which may be subsequently therapeutically

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A

Incubation periods (hr) 0   1   4

EtOH concentrations (%) 0 0 1 2 3 0 1 2 3

lanes 1 2 3 4 5 6 7 8 9

B

Incubation periods (hr) 0   1   4

H2O2 concentrations (mM) 0 0 0.1 0.3 0.5 0 0.1 0.3 0.5

lanes 1 2 3 4 5 6 7 8 9

C

Incubation periods (hr) 0   4   8

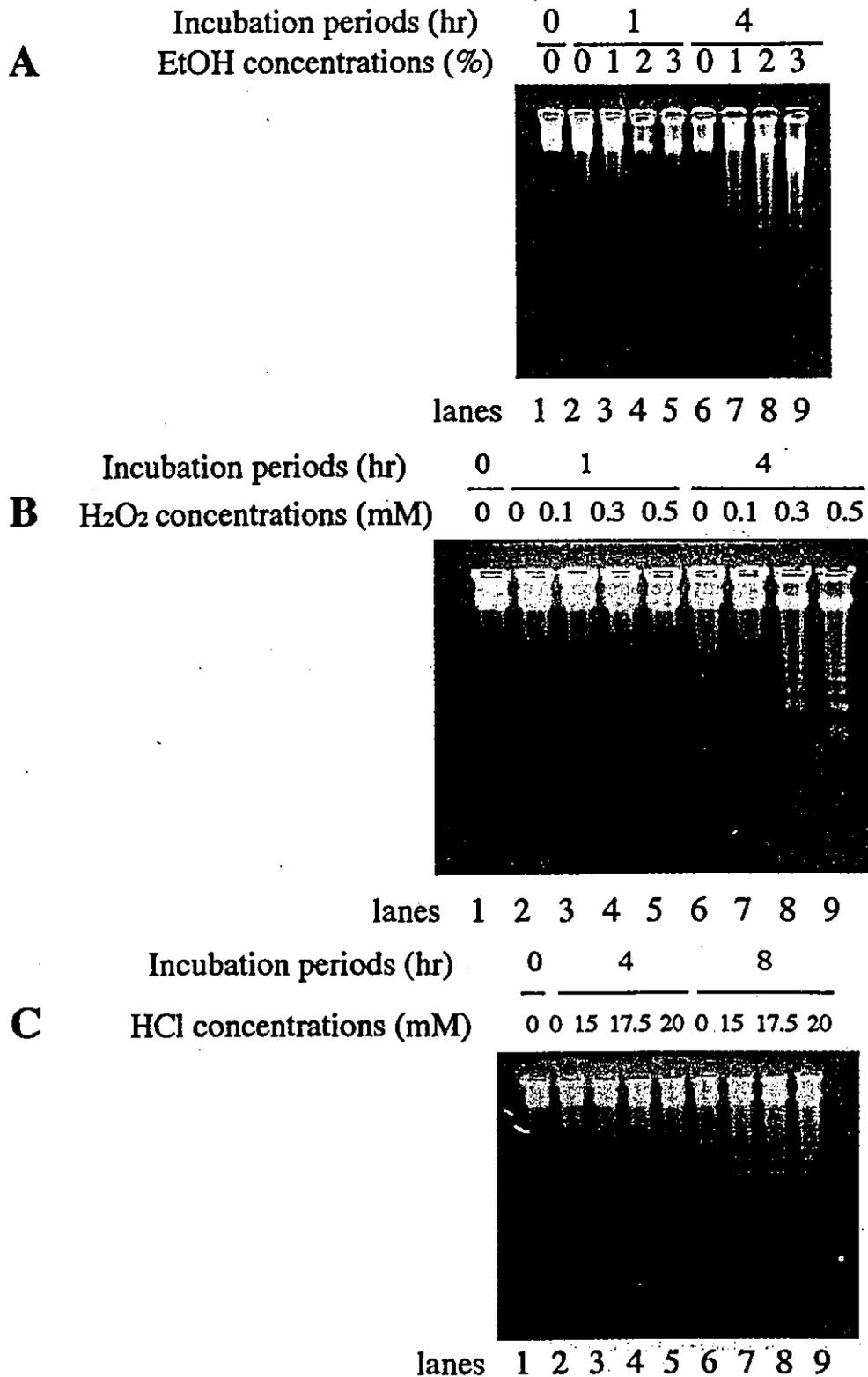HCl concentrations (mM) 0 0 15 17.5 20 0 15 17.5 20
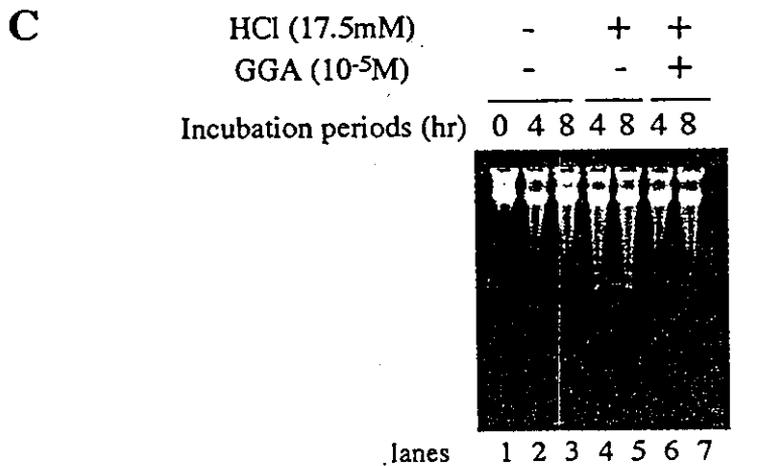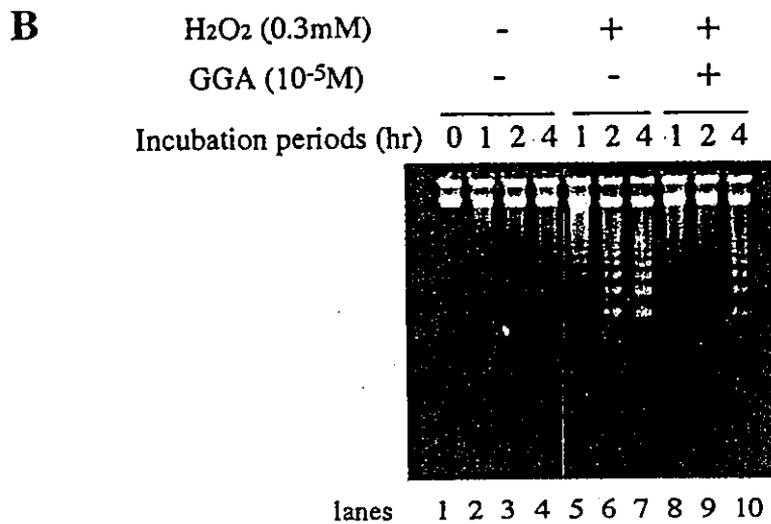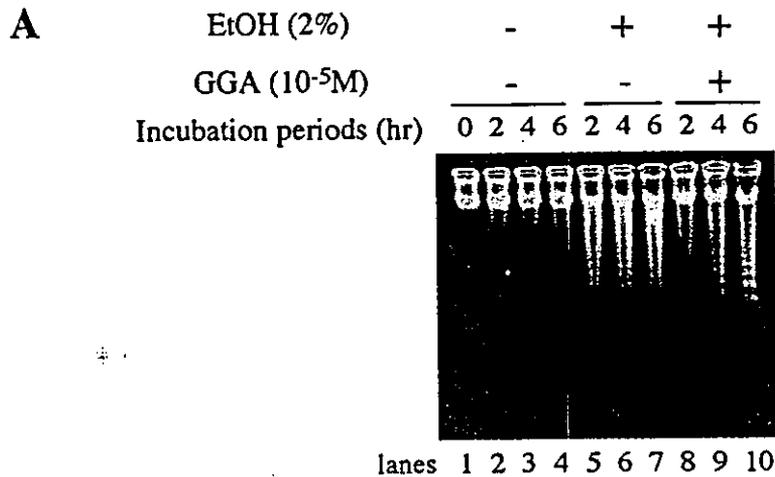
lanes 1 2 3 4 5 6 7 8 9

Fig 1. Apoptotic DNA fragmentation induced by various gastric stressors. Cultured gastric mucosal cells were incubated with indicated concentrations of ethanol (EtOH) (A), hydrogen peroxide (H2O2) (B), or hydrochloric acid (HCl) (C) for indicated periods. Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis.

A

EtOH (2%)	-	+	+
GGA (10⁻⁵M)	-	-	+

EtOH (2%)　　　　　-　　　+　　　+

GGA (10^{-5}M)　　-　　　-　　　+

Incubation periods (hr)　0 2 4 6　2 4 6　2 4 6

lanes　1 2 3 4 5 6 7 8 9 10

B

H₂O₂ (0.3mM)　　　-　　　+　　　+

GGA (10^{-5}M)　　-　　　-　　　+

Incubation periods (hr)　0 1 2 4　1 2 4　1 2 4

lanes　1 2 3 4 5 6 7 8 9 10

C

HCl (17.5mM)　　　-　　　+　　+

GGA (10^{-5}M)　　-　　　-　　+

Incubation periods (hr)　0 4 8　4 8　4 8

lanes　1 2 3 4 5 6 7

Fig 2. Effect of GGA on apoptotic DNA fragmentation induced by various gastric stressors. Cultured gastric mucosal cells were pre-incubated with or without 10^{-5} M GGA for 2 h. Cells were further incubated with or without ethanol (EtOH) (A), hydrogen peroxide (H_2O_2) (B), or hydrochloric acid (HCl) (C) for indicated periods in the presence or absence of 10^{-5} M GGA. Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis.

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beneficial for protecting gastric mucosal cells *in vivo* from gastric stressors.

Geranylgeranylacetone (GGA), an antiulcer drug, has been shown to directly induce transcriptional activation of heat-shock proteins (HSPs) in cultured gastric mucosal cells and in intact gastric mucosa (6). HSPs make cells resistant to various stressors and are thought to be important for gastric mucosal defense (7). Several reports have shown that some HSPs have antiapoptotic activity (8-10). Therefore, it would appear that preinduction of HSPs by GGA in gastric mucosal cells should protect the cells from gastric stressor-induced apoptosis. We show here that pretreatment of cells with GGA made cells resistant to apoptosis upon their exposure to various gastric stressors (ethanol, hydrogen peroxide, and hydrochloric acid). We consider that this antiapoptotic activity of GGA can explain, at least in part, the cytoprotective effect of this drug on gastric mucosa *in vivo*.

MATERIALS AND METHODS

Chemicals and Media. RPMI 1640 was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal calf s2erum (FCS) and trypsin solution were purchased from Gibco (Grand Island, New York, USA). Pronase E and type I collagenase were purchased from Kaken Pharmaceutical Co. (Kyoto, Japan) and Nitta Gelatin Co. (Osaka, Japan), respectively. Sodium-*N*-lauroylsarcosinate was from Wako Co. (Tokyo, Japan). Proteinase K and RNase A were from Sigma Co. (Tokyo, Japan). GGA and α-tocopherol were kindly provided by Eisai Co. (Tokyo, Japan).

The experiments and procedures described here were approved by the Animal Care Committee of Okayama University.

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 units/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 nonadherent cells by washing with RPMI 1640, cells that were attached to the plate at about 80% confluence were used. Guinea pig gastric mucosal cell preparations under these conditions have been previously characterized, with the majority (about 90%) of cells being identified as pit cells (11).

Treatment of Cells with Gastric Stressors and GGA. Cells were exposed to gastric stressors (ethanol, hydrogen peroxide, and hydrochloric acid) by changing the entire bathing medium. For some experiments, cells were preincubated with 10^{-5} M GGA or 1% ethanol for 2 h. After removing these chemicals by changing the bathing medium, cells were further incubated with one of gastric stressors as described above.

DNA Fragmentation Assay. Apoptotic DNA fragmentation was monitored as previously described (12, 13). Cells were collected with a rubber policeman and suspended in 30 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. RNase A was then added to give a final concentration of 1 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.

RESULTS AND DISCUSSION

Effects of GGA on Apoptosis by Various Gastric Stressors. We previously obtained preliminary data showing that GGA inhibited the ethanol-induced apoptosis of guinea pig gastric mucosal cells in primary culture in the presence of 10% FCS (14). We also recently found that the gastric mucosal cells undergo maturation-dependent spontaneous apoptosis in the presence of 10% serum, even when gastric stressors were not present (13). Thus, in order to exclude the contribution to the experimental findings of the effect of the serum-induced spontaneous apoptosis, we examined here the effects of GGA on gastric stressor-induced apoptosis in the presence of a low serum concentration (0.3%) whereupon spontaneous apoptosis is inhibited (13). We recently reported that all gastric stressors tested (ethanol, hydrogen peroxide, and hydrochloric acid) induced apoptosis in guinea pig gastric mucosal cells in primary culture for experimental conditions where 0.3% serum was used (Tsutsumi et al, submitted).

We first attempted to reconfirm that the gastric stressors stated above induce apoptosis. Guinea pig gastric mucosal cells in primary culture were incubated with various concentrations of ethanol, hydrogen peroxide, or hydrochloric acid for various periods and chromosomal DNA was extracted and analyzed by agarose gel electrophoresis in the presence of ethidium bromide. As shown in Figure 1A, in the presence of ethanol at concentrations higher than 2%, DNA fragmentation was induced after 4 h of incubation. The pattern of DNA fragmentation was typical of that of apoptotic DNA fragmentation, based on the results from electrophoresis with a DNA ladder marker (data not shown). Hydrogen peroxide and hydrochloric acid also induced the apoptotic DNA fragmentation in both a dose- and incubation period-dependent manner (Figure 1B, C).

We subsequently examined the effect of GGA on apoptosis caused by gastric stressors. When cells were pretreated with 10^{-5} M GGA for 2 h, the apoptotic

DNA fragmentation seen in the above experiment following treatment with 2% ethanol was clearly inhibited (Figure 2A). GGA did not induce apoptotic DNA fragmentation in the absence of stressors (data not shown). Similar results with respect to apoptotic DNA fragmentation were observed when the gastric stressor used was 0.3 mM hydrogen peroxide or 17.5 mM hydrochloric acid (Figures 2B, C). Given that the GGA was dissolved in ethanol containing 0.25% α-tocopherol, we confirmed in control experiments that these compounds, which were used at final concentrations of 0.00025 and 0.1%, respectively, did not affect the apoptosis induced by the gastric stressors (data not shown). These results suggest that GGA can suppress apoptosis by gastric stressors in general.

Requirement of Prior Treatment of Cells with GGA for Its Inhibitory Effect on Apoptosis. As for experiments in Figure 2, gastric mucosal cells were first incubated with GGA (preincubation step) and then incubated with various gastric stressors in the presence of the same concentrations of GGA (incubation step). As such, we examined whether both preincubation and incubation with GGA were required for the inhibitory effect of GGA on the gastric stressor-induced apoptosis. When GGA was omitted in the preincubation step, the ethanol-induced apoptosis was not inhibited by GGA (Figure 3A, lane 4). On the other hand, even when GGA was omitted in the incubation step (but included in the preincubation step), the ethanol-induced apoptosis was inhibited by GGA (Figure 3A, lane 5) to much the same extent as that when GGA was included in both the preincubation and incubation steps (Figure 3A, lane 6). Thus, preincubation of gastric cells with GGA is required for the inhibitory effects of GGA on ethanol-induced apoptosis. Interestingly, when cells were not preincubated with GGA, the apoptotic DNA fragmentation by 2% ethanol was slightly more apparent in the presence of GGA at the incubation step than in its absence (compare lane 3 to lane 4 in Figure 3A). These data suggest that, in addition to having an inhibitory effect, GGA also has a stimulatory effect on the ethanol-induced apoptosis depending on the experimental conditions used. At present, we are unsure of the mechanism of this stimulatory effect of GGA on apoptosis. We also observed similar results for apoptosis induced by either 0.3 mM hydrogen peroxide or 17.5 mM hydrochloric acid (Figure 3B, C).

Effect of Low Concentrations of Ethanol on Gastric Stressor-Induced Apoptosis. As described above, GGA must be included at the preincubation step in order for its inhibitory effect on the gastric stressor-induced apoptosis to be seen, suggesting that GGA induced some proteins, thereby making the cells resistant to each of the gastric stressors tested. HSPs are a candidate for such proteins, because HSPs make cells resistant to various stressors and are thought to be important for gastric mucosal defense (7). To test this possibility, we examined whether or not the induction of HSPs by means other than by GGA protects gastric mucosal cells from gastric stressor-induced apoptosis. We used as our experimental protocol the treatment of cells with low concentrations of ethanol, given that we previously reported the induction of HSPs by low concentrations of ethanol under the same culture conditions as those used to initiate gastric stressor-induced apoptosis (15). We found that, compared to nontreated cells, the pretreatment of gastric mucosal cells with 1% ethanol inhibited the apoptosis that would otherwise have been observed following treatment with any of these gastric stressors (Figure 4). These results support the notion that preinduction of HSPs by GGA inhibited the apoptosis by gastric stressors.
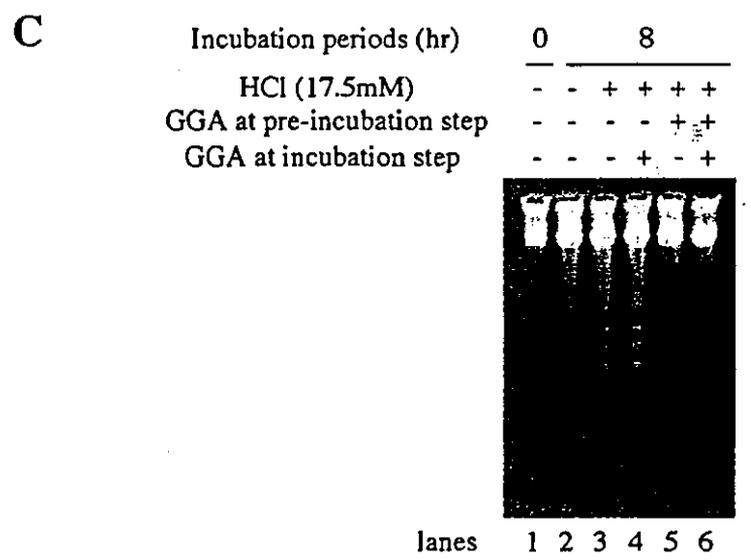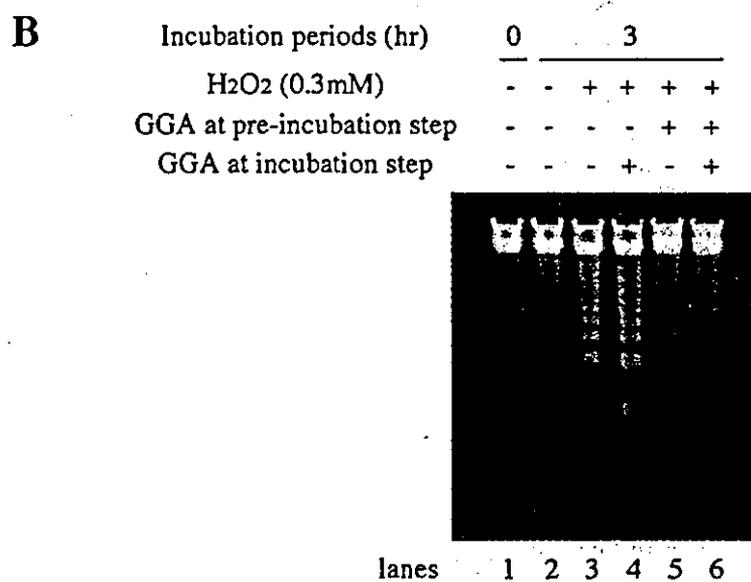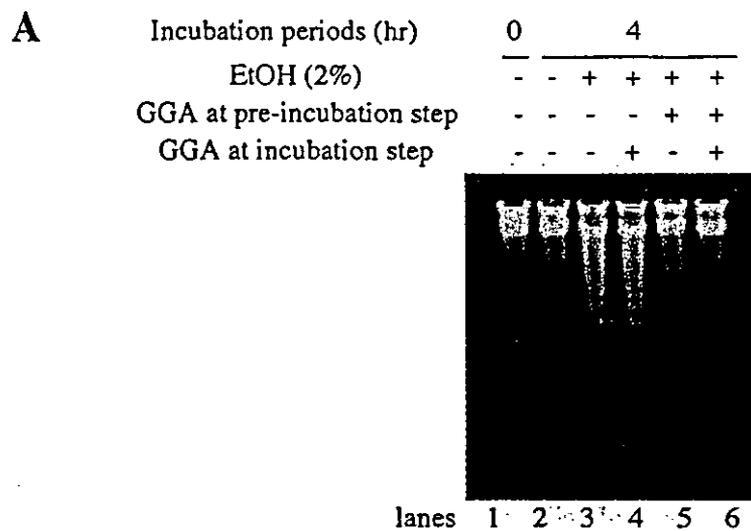
Various gastric stressors seem to damage the gastric mucosa by inducing both necrosis and apoptosis in vivo (1, 2, 16, 17). Thus, for protecting gastric mucosa from various gastric stressors, both apoptosis and necrosis by gastric stressors should be suppressed. We recently reproduced this necrosis and apoptosis in vitro, using primary cultures of guinea pig gastric mucosal cells (18). This in vitro system may be useful to identify target molecules for drugs in the prevention of mucosal damage caused by gastric stressors. The induction of HSPs is one potential target for such drugs, because HSPs make cells nonspecifically resistant to various stressors (7).
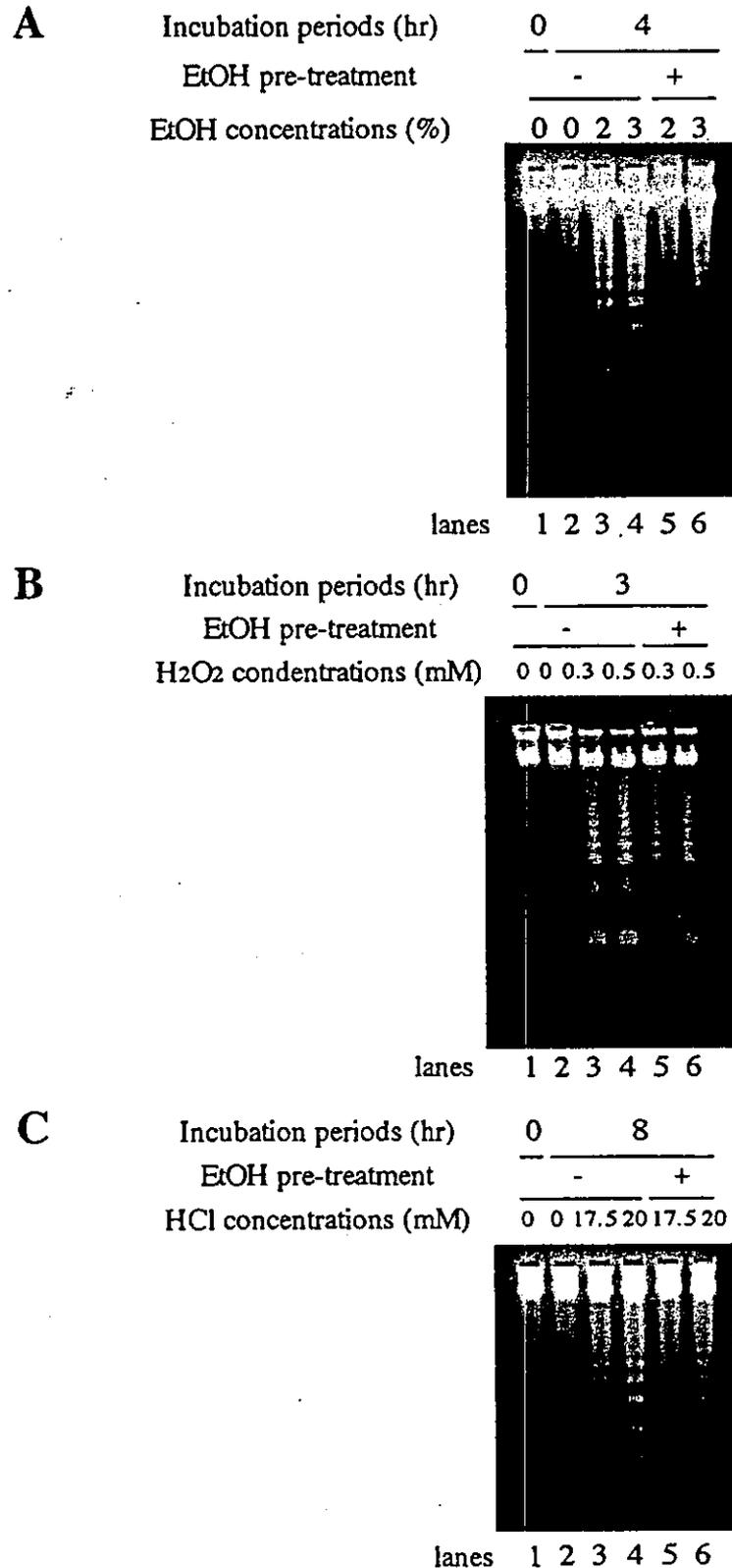
In this paper, we have shown that GGA, which has HSP-inducing properties, protected cultured gastric mucosal cells from gastric stressor-induced apooptosis. Since another HSP inducer (low concentrations of ethanol) also inhibited gastric stressor-induced apoptosis, HSPs induced by GGA could be responsible for its anti-apoptotic activity.

We recently showed that HSPs induced by GGA protected gastric mucosal cells from necrotic cell

Fig 3. (Opposite) Rbequirement of prior incubation with GGA for its inhibitory effect on gastric stressor-induced apoptotic DNA fragmentation. Cultured gastric mucosal cells were preincubated with or without 10^{-5} M GGA for 2 h. Cells were further incubated with or without indicated concentrations of ethanol (EtOH) (A), hydrogen peroxide (H_2O_2) (B), or hydrochloric acid (HCl) (C) for indicated periods in the presence or absence of 10^{-5} M GGA. Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis.

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A

Incubation periods (hr)	0		4			
EtOH (2%)	-	-	+	+	+	+
GGA at pre-incubation step	-	-	-	-	+	+
GGA at incubation step	-	-	-	+	-	+

lanes 1 2 3 4 5 6

B

Incubation periods (hr)	0		3			
H2O2 (0.3mM)	-	-	+	+	+	+
GGA at pre-incubation step	-	-	-	-	+	+
GGA at incubation step	-	-	-	+	-	+

lanes 1 2 3 4 5 6

C

Incubation periods (hr)	0		8			
HCl (17.5mM)	-	-	+	+	+	+
GGA at pre-incubation step	-	-	-	-	+	+
GGA at incubation step	-	-	-	+	-	+

lanes 1 2 3 4 5 6

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A

Incubation periods (hr)	0	4				
EtOH pre-treatment	-			+		
EtOH concentrations (%)	0	0	2	3	2	3

lanes 1 2 3 4 5 6

B

Incubation periods (hr)	0	3				
EtOH pre-treatment	-			+		
H2O2 condentrations (mM)	0	0	0.3	0.5	0.3	0.5

lanes 1 2 3 4 5 6

C

Incubation periods (hr)	0	8				
EtOH pre-treatment	-			+		
HCl concentrations (mM)	0	0	17.5	20	17.5	20

lanes 1 2 3 4 5 6

Fig 4. Effect of pretreatment with a low concentration of ethanol on apoptotic DNA fragmentation induced by various gastric stressors. Cultured gastric mucosal cells were preincubated with (lanes 5 and 6) or without (lanes 1–4) 1% ethanol for 2 h. Cells were further incubated with (lanes 3–6) or without (lanes 1 and 2) indicated concentrations of ethanol (EtOH) (A), hydrogen peroxide (H_2O_2) (B), or hydrochloric acid (HCl) (C) for indicated periods. Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis.

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death due to ethanol, hydrogen peroxide, hydrochloric acid, or nonsteroidal anti-inflammatory drugs (15). Thus, it seems that preinduction of HSPs in gastric mucosal cells protects cells from both necrotic and apoptotic cell death caused by gastric stressors. Recently, however, it was suggested that some HSPs (such as HSP60) stimulate apoptosis (20). Thus, the identification of particular HSPs, which would be responsible for the antiapoptotic activity of GGA, and the screening of drugs that are capable of strongly inducing only these HSPs may be very important if drugs are to be identified for protecting gastric mucosal cells from various gastric stressors.

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Spontaneous Apoptotic DNA Fragmentation in Cultured Guinea Pig Gastric Mucosal Cells

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The purpose of this study was to elucidate the mechanism of spontaneous and rapid cell death of cultured gastric pit cells. Gastric pit cells have a rapid cell turnover rate *in vivo*. We here show that guinea pig gastric pit cells in culture undergo spontaneous and rapid apoptotic DNA fragmentation, which may represent the rapid cell turnover cycle of gastric pit cells *in vivo*. This spontaneous apoptotic DNA fragmentation required the presence of fetal calf serum in the culture media. Furthermore, the spontaneous apoptotic DNA fragmentation was prevented by protein synthesis and caspase inhibitors.

KEY WORDS: apoptosis; pit cell lineage; caspase; gastric mucosal cells.

Gastric pit cells (surface mucous cells) present along pit walls and the gastric surface have a rapid cell turnover rate *in vivo*. The short turnover cycle is the result of rapid proliferation of progenitor cells at the isthmus, maturation that occurs during upward migration, and cell death at the gastric surface (1). In the normal stomach, there is a balance between the proliferation of the immature cells and the death of senescent cells at the top of the surface. Alteration in the proliferation and death of gastric pit cells may cause various gastric diseases. For example, alterations in the proliferation and death of the gastric pit cells by *Helicobacter pylori* was suggested to be in-

volved in the development of atrophic gastritis and gastric carcinogenesis associated with *H. pylori* infection (2–5).

A number of lines of evidence suggest that multicellular organisms tend to eliminate unneeded cells by induction of apoptosis (6, 7). For example, the elimination of mature small intestine mucosal cells is done by induction of apoptosis (8). Mature gastric pit cells were also suggested to be eliminated by phagocytosis or direct extrusion of apoptotic cells *in vivo* (1, 9). Holl et al have shown that apoptosis may account for most of the cell loss in the normal stomach (10). In addition, it has been suggested that the acceleration of apoptosis of surface mucous cells plays an important role in the development of atrophic gastritis associated with *H. pylori* infection (3–5). Thus, an understanding of the features characteristic for mucous cell apoptosis is needed to understand the mechanism controlling pit cell dynamics, as well as the pathophysiology of gastric mucosal damage caused by *H. pylori* infection.

We have provided several lines of evidence that primary cultures of guinea pig gastric mucosal cells partly mimic the rapid turnover of gastric pit cells *in*
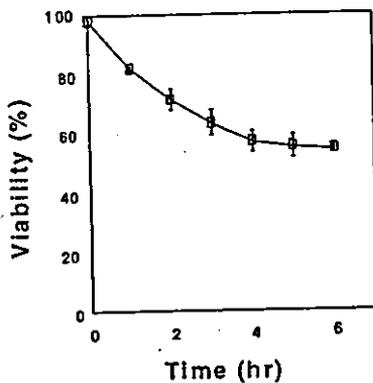
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Fig 1. Rapid cell death of cultured gastric mucosal cells. Gastric mucosa cells prepared from guinea pigs were cultured in RPMI 1640 medium containing 10% FCS. After removal of unattached cells, cells were further incubated in the same medium for the indicated periods, and cell viability was determined by the trypan blue exclusion test. Values are mean ± SD, $N = 3$.

conditions. The majority (about 90%) of the cells cultured with 10% FCS were pit cells (13, 14), while prepit cells composed about 80% of the total attached cells in serum-free conditions (12).

Examination of Apoptotic DNA Fragmentation. Apoptotic DNA fragmentation was monitored as described elsewhere (15). Cells were collected with a rubber policeman and suspended with 20 μl of lysis buffer, consisting of 50 mM Tris HCl (pH 7.8), 10 mM EDTA, and 0.5% sodium-

A

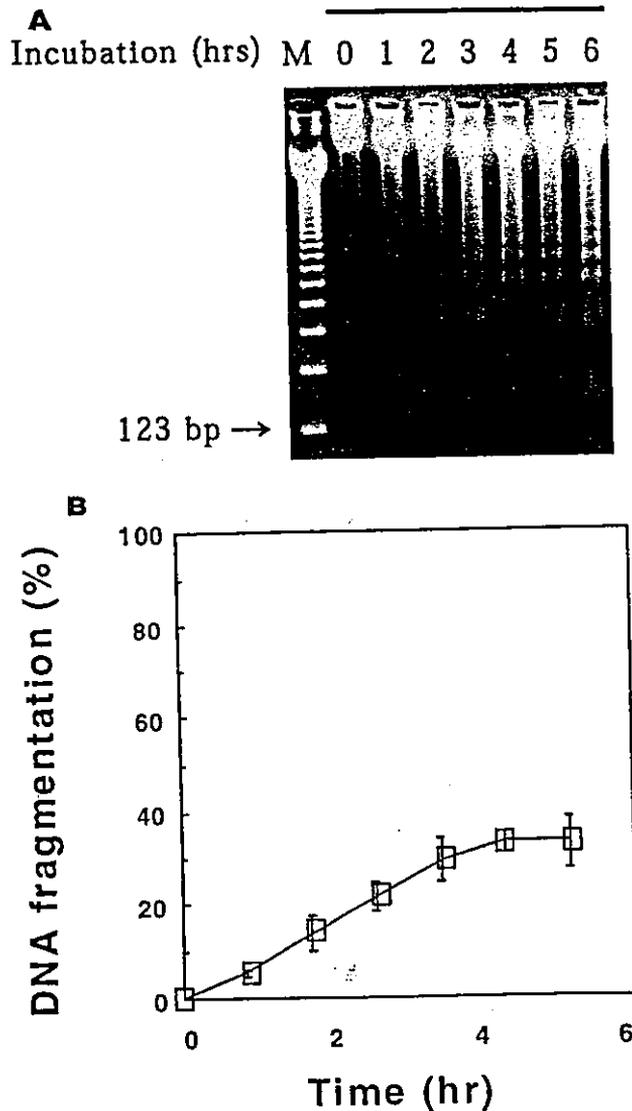Incubation (hrs) M 0 1 2 3 4 5 6

123 bp →

B

Fig 2. Spontaneous apoptotic DNA fragmentation in cultured gastric mucosal cells. Gastric mucosal cells prepared from guinea pigs were cultured, and the attached cells were further incubated in serum-containing RPMI 1640 medium for the indicated periods (A). Apoptotic DNA fragmentation was analyzed as described in Materials and Methods. Lane M indicates the DNA ladder marker (123 bp and its polyploids) (Takara) (B). The DNA size distribution was expressed as the percent of the total DNA that was in fragments shorter than 5 kb. Values are mean ± SD, $N = 3$.

vivo (11–13). In this study, we found that cultured guinea pig gastric mucosal cells showed spontaneous and rapid apoptotic DNA fragmentation, which is considered to mimic the rapid death of gastric pit cells *in vivo*. This DNA fragmentation required *de novo* protein synthesis and caspase activities.

MATERIALS AND METHODS

Chemicals and Media. RPMI 1640 was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Fetal calf serum (FCS) and trypsin solution were from Gibco (Grand Island, New York). Pronase E and type I collagenase were purchased from Kaken Pharmaceutical Co. (Kyoto, Japan) and Nitta Gelatin Co. (Osaka, Japan), respectively. Sodium-*N*-lauroylsarcosinate was from Wako Co. (Tokyo, Japan). Proteinase K, benzyloxycarbonyl-Val-Ala-Asp (Z-VAD-fmk), cycloheximide, and RNase A were from Sigma Co. (Tokyo, Japan).

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 (13, 14). Isolated gastric mucosal cells were cultured for 24 hr in RPMI 1640, containing 10% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin in plastic culture plates under the conditions of 10% CO_2/90% air. After removing non-adherent cells by washing with RPMI 1640, the cells that were attached to the plate at about 50% confluence were used to study spontaneous cell death. In some experiments, isolated cells, suspended in DMEM/Ham's F-12 (1:1) mixture, containing 15 mM HEPES, 0.2% BSA, 10 μg/ml transferrin, and 2.5 ng/ml sodium selenite, were placed in collagen type 1-coated culture dishes, as described previously (12). The cells that had reached 50% confluence (after an average of 48 hr of culture) were used to study the effect of FCS on apoptosis. We previously characterized guinea pig gastric mucosal cell preparations under these
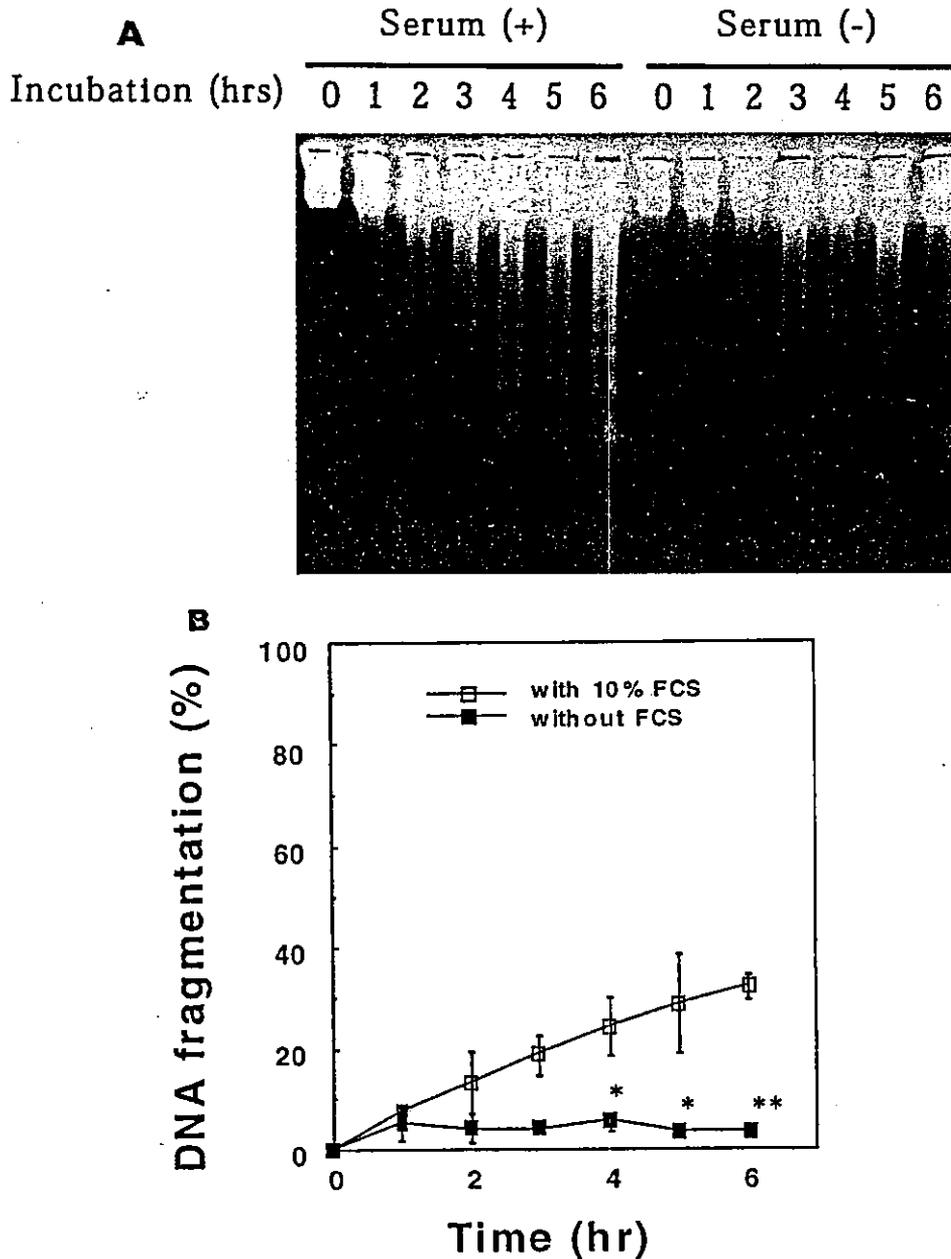
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Fig 3. Requirement of serum for spontaneous apoptotic DNA fragmentation. Gastric mucosal cells prepared from guinea pigs were placed on collagen type 1-coated dishes and cultured for 48 hr in DMEM/Ham's F-12 (1:1) mixture, containing 15 mM HEPES, 0.2% BSA, 10 μg/ml transferrin, and 2.5 ng/ml sodium selenite, with or without 10% FCS. After removal of nonadherent cells, attached cells were further incubated in the same conditions for the indicated periods (A). Analysis of apoptotic DNA fragmentation was performed as described in the legend of Figure 2 (B). The DNA size distribution was expressed as the percent of the total DNA that was in fragments shorter than 5 kb. Values are mean $\pm$ SD, $N = 3$. ** $P < 0.01$; * $P < 0.05$ by Student's t test compared with the control value at each time point.

N-lauroylsarcosinate. Proteinase K was added to a final concentration of 1 mg/ml, and the lysate was incubated at 50°C for 2 hr. Then, RNase A was added to a final concen- tration of 0.5 mg/ml and incubated at 50°C for 30 min. These samples were analyzed by 2% agarose gel electro- phoresis in the presence of 0.5 μg/ml ethidium bromide.

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RESULTS AND DISCUSSION

Spontaneous Apoptotic DNA Fragmentation in Cultured Guinea Pig Gastric Mucosal Cells. An *in vitro* culture system of guinea pig gastric mucosal cells has been established and has provided a number of important results about the functions of gastric mucosal cells. In FCS-containing media, guinea pig gastric mucosal cells, which are dispersed by protease digestion, rapidly attached to plastic culture dishes within several hours (11–13). After removing nonadherent cells at 24 hr, the attached cells at about 50% confluence continued to grow and mature into surface mucous cells in the presence of 10% FCS (11–13). The mature cells lost their mitotic activity, and the number of total cells started to gradually decrease from day 3 (11–13).

Isolated gastric mucosal cells were cultured in 10% FCS-containing RPMI 1640 for 24 hr, and then nonadherent cells were removed. The attached cells continued to grow in the same FCS-containing medium until they became confluent. However, when the viability of the cells was carefully monitored by the trypan blue exclusion test, we found that a significant population of the cells continued to die (Figure 1), and the number of floating cells increased in a time-dependent manner. To examine the mechanism of this rapid cell death, chromosomal DNA was extracted periodically, and the change in the size of chromosomal DNA was analyzed by agarose gel electrophoresis in the presence of ethidium bromide. Figure 2 shows the time-dependent appearance of DNA fragmentation in cultured gastric mucosal cells. The pattern of the DNA fragmentation (ie, the small molecular sizes of the fragments) was typical of that of apoptotic DNA fragmentation. The time-course of this apoptotic DNA fragmentation was well correlated to that of cell death (Figure 1), suggesting that the rapid cell death of cultured guinea pig gastric mucosal cells is mediated by apoptosis.

Serum-Dependent Spontaneous Apoptotic DNA Fragmentation. The mucosal cells used in this study were mixed mucosal cell populations; however, the majority of the cells (about 90%) were composed of a pit cell lineage at different stages of maturation (12, 13). We considered that the spontaneous apoptosis *in vitro* might reflect the maturation-dependent apoptosis that is operated *in vivo* (1, 9). To address this issue, we applied the serum-free culture of gastric mucosal cells (12), since the cells maintained in our serum-free culture system did not exhibit mitotic activity, and a great majority of the cells were in a prepit cell stage

A Incubation (hrs) 0   4

Cycloheximide (g/ml) 0 0 10⁻⁹10⁻⁸10⁻⁷10⁻⁶10⁻⁵

Cyclohex imide (g/ml)   0   0 10⁻⁹10⁻⁸10⁻⁷10⁻⁶10⁻⁵

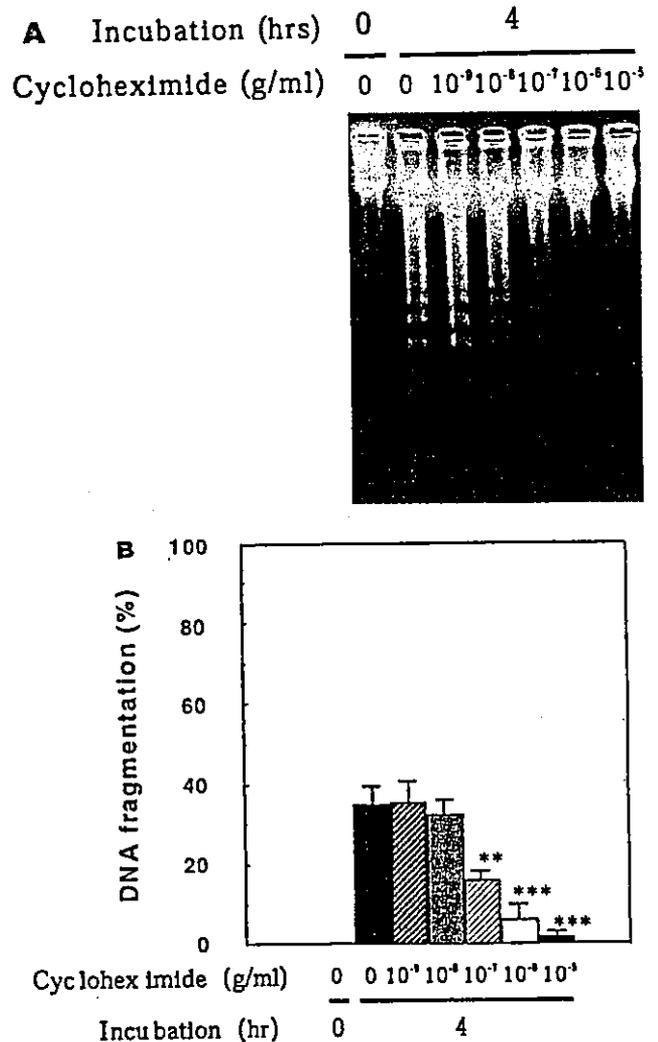Incubation (hr)   0   4

Fig 4. Effects of cycloheximide on spontaneous apoptotic DNA fragmentation. Gastric mucosal cells prepared from guinea pigs were cultured in RPMI 1640 medium containing 10% FCS in the presence of the indicated amount of cycloheximide and further incubated in the same conditions for 4 hr (A). Analysis of apoptotic DNA fragmentation was performed as described in the legend of Figure 2 (B). The DNA size distribution was expressed as the percent of the total DNA that was in fragments shorter than 5 kb. Values are mean ± SD, $N = 3$. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ by Student's t-test compared with the control value (no cycloheximide).

(12). As shown in Figure 3, addition of serum to the cells cultured on collagen type 1-coated dishes in the serum-free medium induces the spontaneous apoptotic DNA fragmentation. In the absence of serum in the medium, the cells were 100% alive, and no apparent DNA fragmentation was seen up to 6 hr (Figure 3). When the prepared gastric mucosal cells were first cultured in 10% FCS-containing RPMI 1640 and then in the serum-free medium, the extent of the
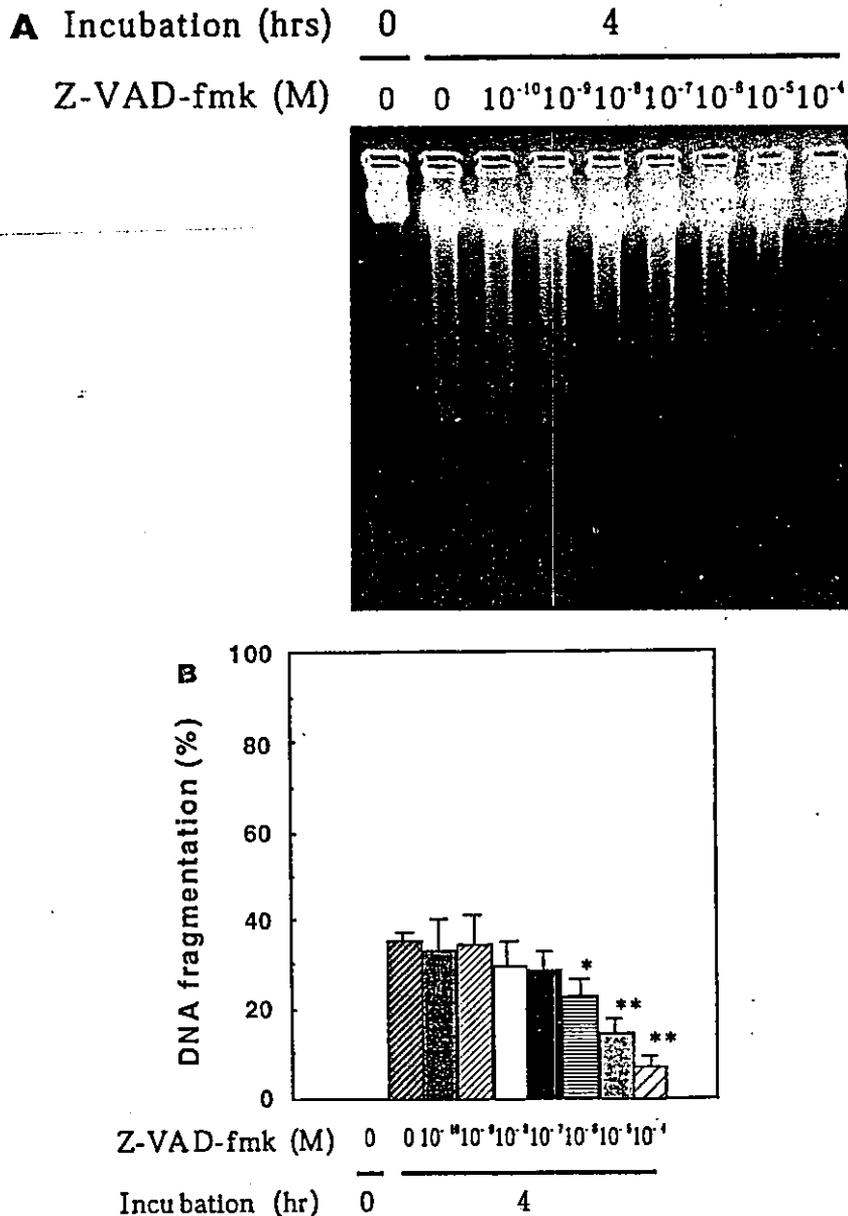
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Fig 5. Effects of Z-VAD-fmk on spontaneous apoptotic DNA fragmentation. Gastric mucosal cells prepared from guinea pigs were cultured in RPMI 1640 medium containing 10% FCS in the presence of indicated amount of Z-VAD-fmk and further incubated in the same conditions for 4 hr (A). Analysis of apoptotic DNA fragmentation was performed as described in the legend of Figure 2. (B) The DNA size distribution was expressed as the percent of the total DNA that was in fragments shorter than 5 kb. Values are mean ± SD, $N = 3$. **, $P < 0.01$; *, $P < 0.05$ by Student's t-test compared with the control value (no Z-VAD-fmk).

apoptotic DNA fragmentation was much the same as that seen in Figure 2 (data not shown). These results suggest that distinct factor(s) in serum may prime the cells for processing the maturation-dependent apoptosis pathway.

Inhibition of Spontaneous Apoptotic DNA Fragmentation by Protein Synthesis and Protease Inhibitors. There are two types of apoptosis pathway; one is dependent on de novo protein synthesis and the other is not (6). Treatment of cells with cycloheximide