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

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

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Received December 3, 2001.
Accepted February 27, 2002.

1535-3702/02/2276-0402$15.00

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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.

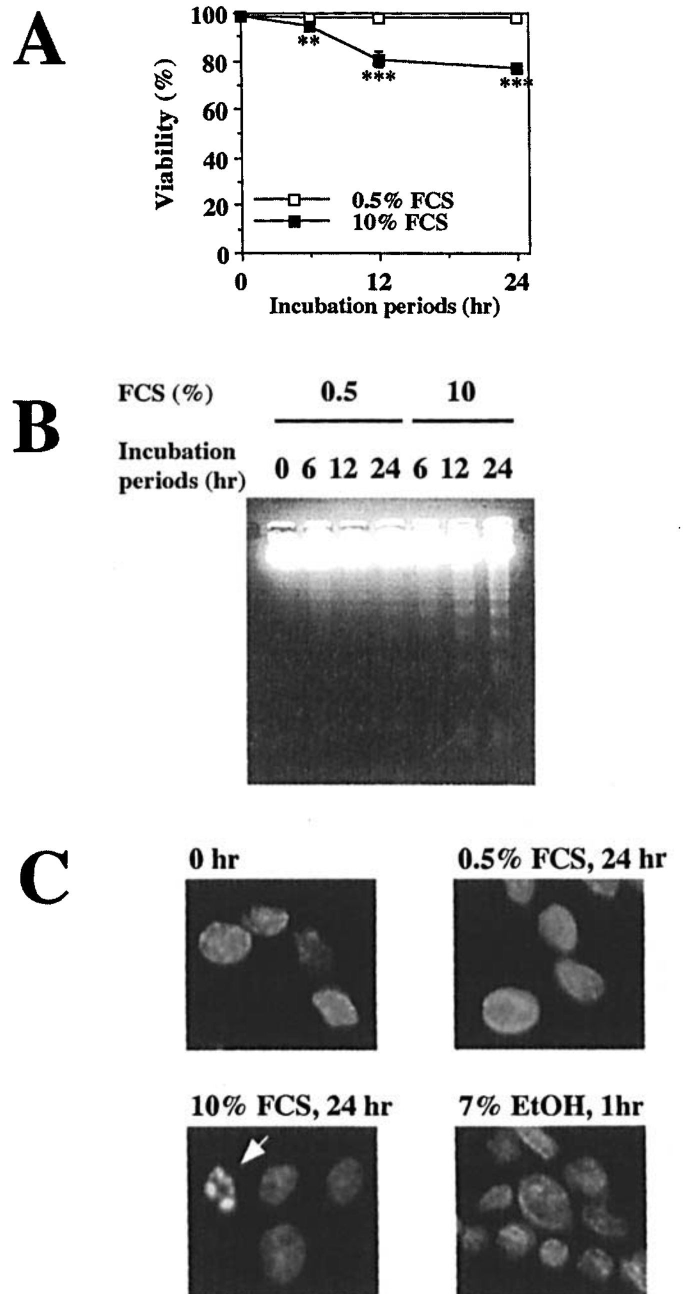


Figure 1. Effect of serum concentrations on spontaneous apoptosis. Gastric pit cells were cultured with 10% or 0.5% FCS for indicated periods. Cell viability was determined by the trypan blue exclusion test (A). Chromosomal DNA was extracted and analyzed by 2% agarose gel electrophoresis (B). Cells were 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

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.

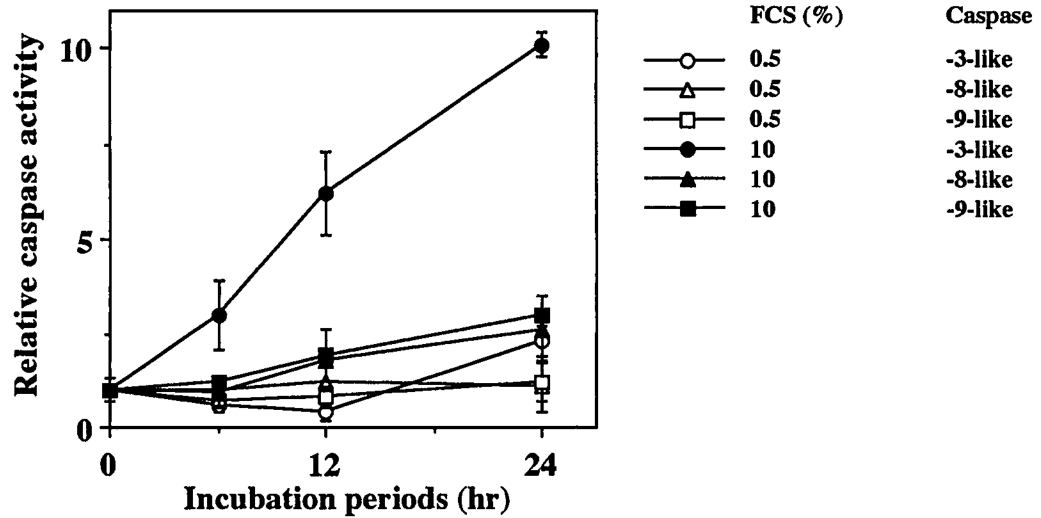
As shown in Figure 2A, according to culture in the presence of 10% FCS, caspase-3-like activity was stimulated strongly (about 10-fold), confirming that apoptosis was induced under these conditions. The time course of the stimulation of caspase-3-like activity in the presence of 10% FCS correlated to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

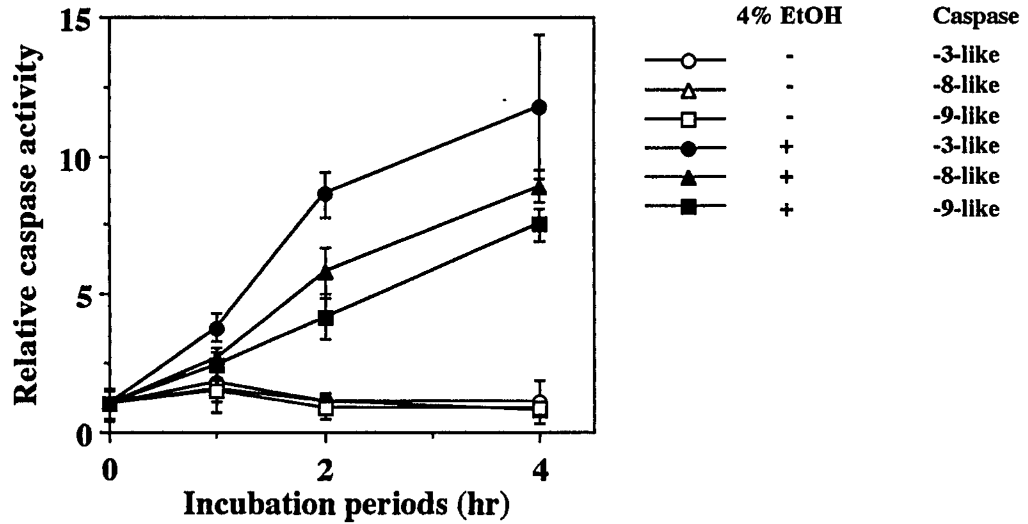
We recently reported that treatment of the gastric pit cells with 4% ethanol for 4 hr caused apoptosis (32). Here, we confirmed that when gastric pit cells induce apoptosis upon being exposed to a gastric stressor (4% ethanol), the activities of all of these caspases were stimulated rapidly and equally 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.





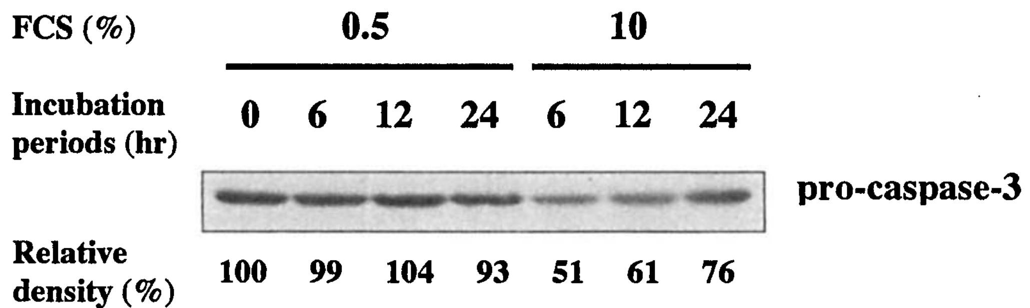
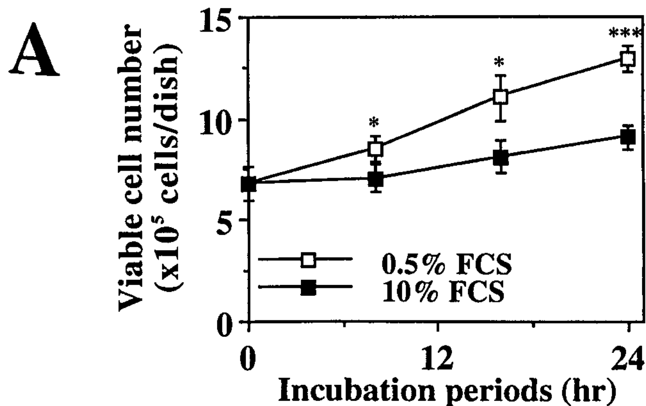


Figure 2. Activation of caspases upon spontaneous apoptosis and gastric stressor-induced apoptosis. Gastric pit cells were cultured with 10% or 0.5% FCS for indicated periods (A). Cells were cultured with or without 4% ethanol under the conditions of 0.5% FCS for indicated periods (B). Caspase-3-, caspase-8-, 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 p*M* 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 ± SD ($n = 3$). Data are representative of four similar experiments.



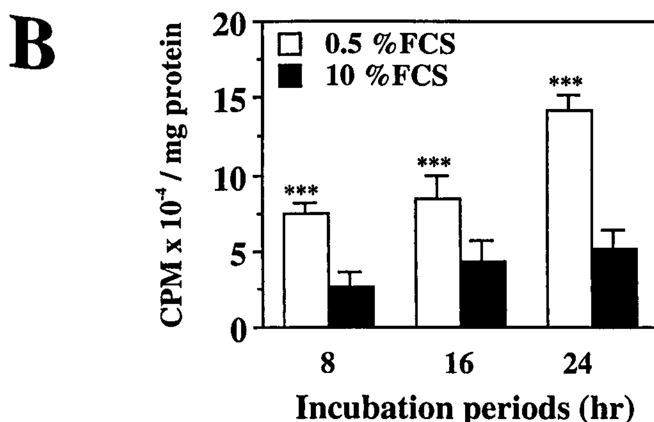


Figure 3. Effects of serum concentrations on cell growth. Gastric pit cells were cultured with 10% or 0.5% FCS for indicated periods and cell numbers were determined by counting with a hemacytometer (A). Gastric pit cells were cultured with 10% or 0.5% FCS for indicated periods. Eight hours before the 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.

As shown in Figure 4, the initial concentrations of TGF-β1 are about 0.6 and 0.03 n*M* 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 n*M*, 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 n*M* (*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

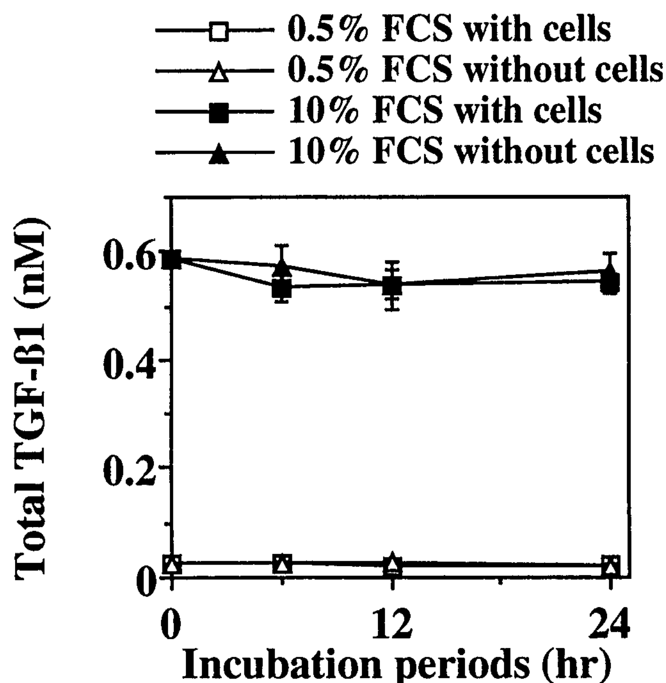


Figure 4. Alteration in amounts of TGF-β1 upon *in vitro* culture. Culture medium containing 10% or 0.5% FCS was incubated with or without gastric pit cells for indicated periods. Culture medium was collected and amounts of TGF-β1 were determined by ELISA as described in "Materials and Methods." Values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

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 n*M* (*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 n*M*) 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

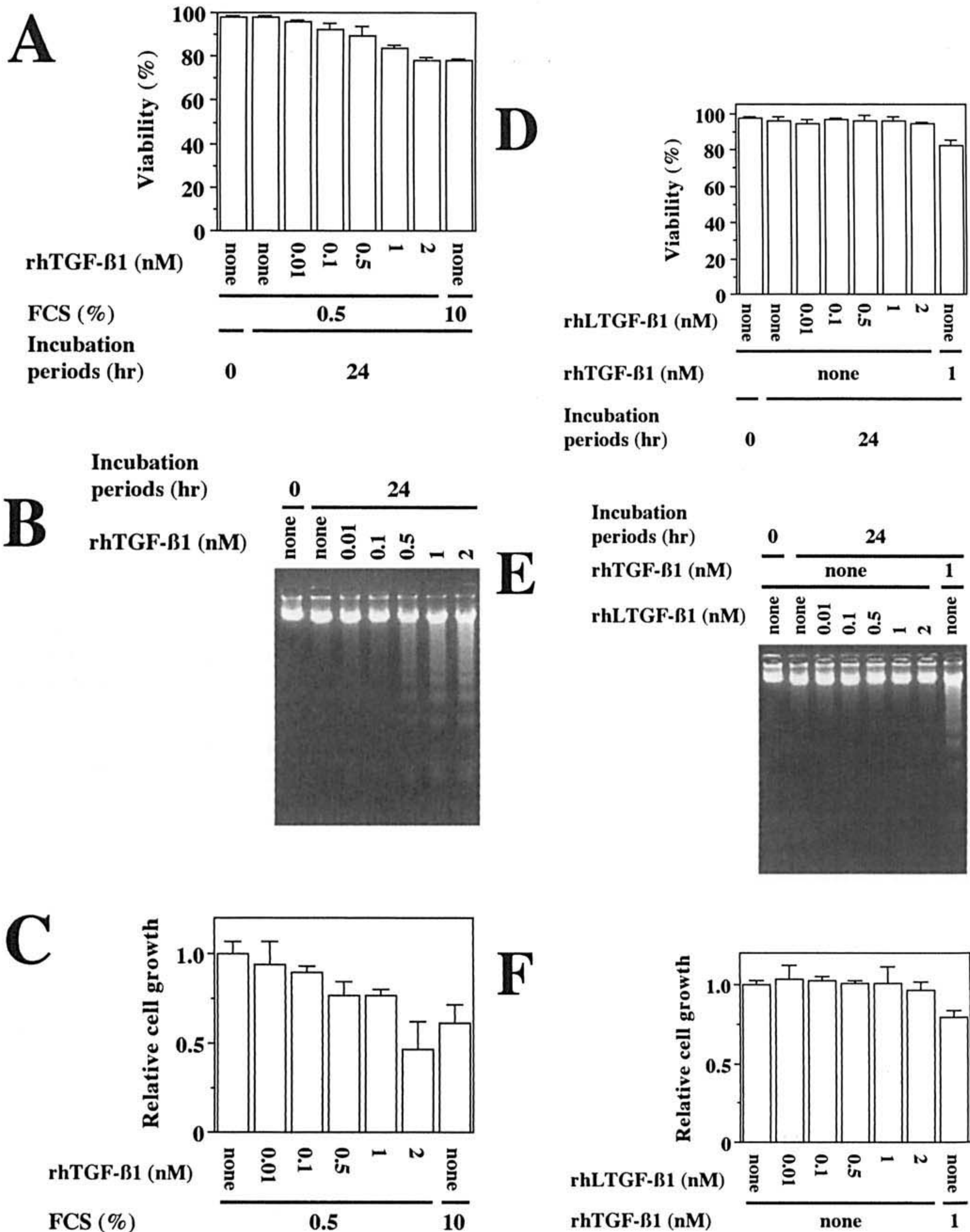


Figure 5. Effect of TGF-β1 on spontaneous apoptosis and cell growth. Gastric pit cells were cultured with indicated concentrations of recombinant human TGF-β1 (rhTGF-β1; A–C) or the latent form of recombinant human TGF-β1 (rhLTGF-β1; D–F) under the conditions of 0.5% FCS for 24 hr. Control experiments 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.

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-β1was 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

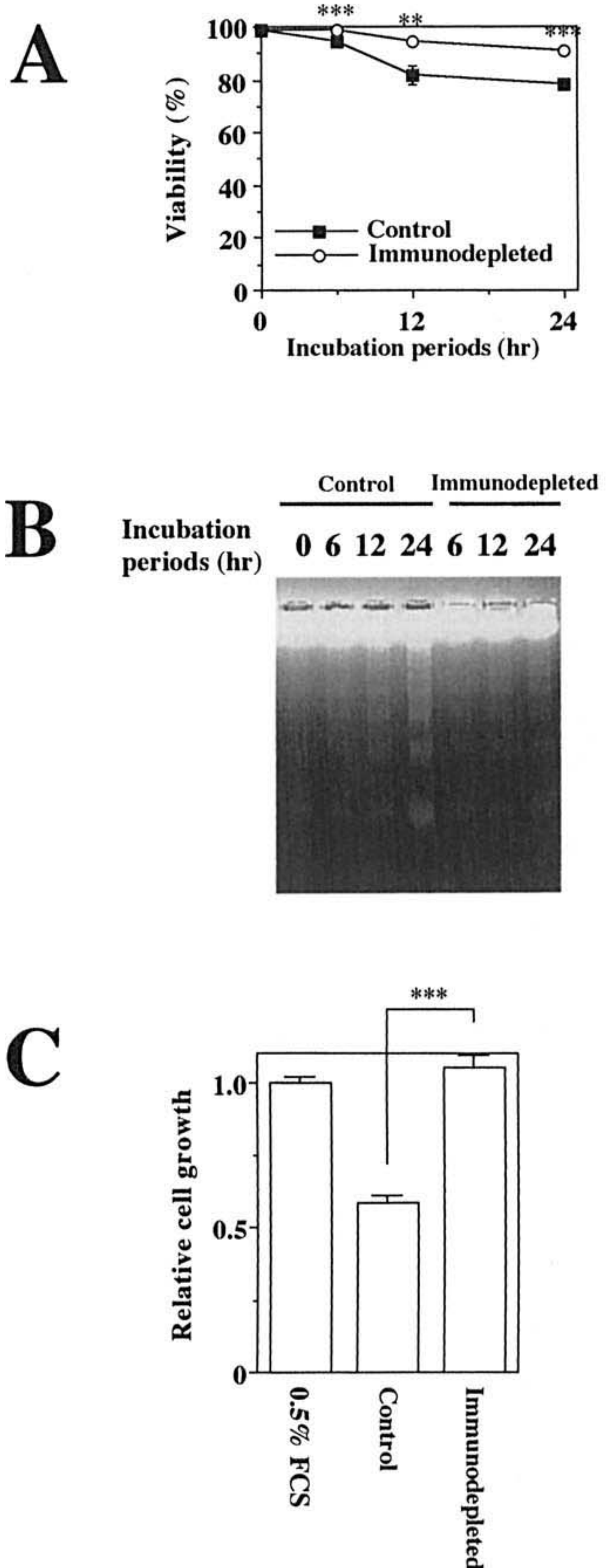


Figure 6. Effect of immunodepletion of TGF-β1 on spontaneous apoptosis and cell growth. TGF-β1 in FCS was immunodepleted using anti-TGF-β1 antibody and protein A-Sepharose as described in "Materials and Methods." Gastric pit cells were cultured with immunodepleted or control FCS (10%) for indicated periods. 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.

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 n*M* in culture medium containing 10% FCS; however, more than 0.5 n*M* 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 (24, 41–43). We recently found that various gastric stressors (NSAIDs, alcohol, 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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