Advances in Brief

Transforming Growth Factor β_1 Induces Apoptotic Cell Death in Cultured Human Gastric Carcinoma Cells[1]

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Abstract

Transforming growth factor β_1 (TGF-β_1) is a potent growth inhibitor for many cell types, including tumor cells. We recently have reported the establishment and characterization of two human gastric scirrhous carcinoma cell lines, HSC-39 and HSC-43. Here we examined the effect of TGF-β_1 on the growth of these lines as compared to five other human gastric adenocarcinoma cell lines. Proliferation of HSC-39 and HSC-43 cells was strongly inhibited by TGF-β_1, whereas the other gastric adenocarcinoma cell lines were unresponsive to TGF-β_1. Both HSC-39 and HSC-43 cells gradually lost viability following exposure to TGF-β_1. This response was dose dependent up to 4 ng/ml. When TGF-β_1 was removed, the cells failed to exhibit regrowth, indicating an irreversible growth-inhibitory effect of this agent, leading to cell death. DNA fragments were observed consisting of multimers of approximately 180 base pairs 24 h after TGF-β_1 treatment. The chromatin condensation of each cell line was confirmed by Hoechst 33258 fluorochrome staining. Ultrastructurally, condensed and fragmented nuclei were observed in TGF-β_1-treated cells. These features are generally associated with apoptotic processes. Both cell death and DNA fragmentation were partially inhibited by cycloheximide, suggesting the requirement for new protein synthesis. Our results suggest that TGF-β_1 induces cell death in human gastric scirrhous carcinoma cells *in vitro* which is mediated by activation of a signal transduction pathway for apoptosis.

Introduction

TGF-β_1[3] is a polypeptide homodimer with a molecular weight of 25,000 that is present in platelets and in other normal and cancerous cells and that is a member of a larger family of growth and differentiation regulatory peptides (see current review in Refs. 1 and 2). While stimulating the proliferation of mesenchymal cells, TGF-β_1 inhibits the growth of most epithelial cells (1, 2), including certain tumor cells such as colon carcinoma (3), breast carcinoma (4), and gastric carcinoma (5). The inhibitory effect of TGF-β_1 has generally been considered fully reversible in some experimental systems (6, 7). However, recent evidence indicates that TGF-β_1 participates in the enhancement and induction of programmed cell death of a variety of normal epithelial cells *in vitro* (8–10). Moreover, these studies have indicated that apoptotic cell death can be induced by exogenous TGF-β_1. However, it is still unknown whether a similar response can be induced in tumor cells by TGF-β_1. Additionally, the mechanisms underlying TGF-β_1-mediated signal transduction and growth-inhibitory action are only poorly understood.

We have recently reported the establishment and characterization of two human stomach scirrhous cancer cell lines[4] (11). Therefore, in this study, we have examined the effect of TGF-β_1 on these cultured gastric cancer cells and demonstrate that TGF-β_1 induces cell death by an apoptotic process.

Materials and Methods

Materials. Purified human TGF-β_1 was purchased from R & D Systems, Inc. (Minneapolis, MN). The lyophilized preparations were reconstituted in 4 mM HCl containing 1 mg/ml of BSA. Recombinant human TGF-β_1 was obtained from Otsuka Assay Laboratories (Tokushima, Japan). Dilutions were made in 5 mM HCl containing 1 mg/ml of BSA (globulin free). Cycloheximide was obtained from Sigma Chemical Co. (St. Louis, MO). Hoechst 33258 was purchased from Flow Laboratories, Inc. (McLean, VA). TGF-β-neutralizing antibody (chicken anti-TGF-β) was purchased from R & D Systems, Inc.

Cell Line and Culture. The human signet ring cell gastric cancer cell line, HSC-39, was originally established and maintained in α-MEM supplemented with 10% FCS as previously described (11). In order to further characterize these subpopulations, the cell lines were adapted to continuous culture in serum-free CDM, which consisted of DMEM: Ham's F12 (1:1) supplemented with 0.05% BSA (Sigma). HSC-39 cells at 20 to 50% confluency were transferred from 10% FCS containing α-MEM to serum-free CDM. After approximately 10 passages, the growth rates of HSC-39 cells had stabilized with a doubling time of 36 to 38 h. The HSC-43 cell line was established from a human gastric scirrhous carcinoma in serum-free CDM by us.[4] These lines were routinely cultured in serum-free CDM supplemented and antibiotics. The 5 other human gastric cancer cell lines (MKN-1, adenosquamous cell carcinoma; MKN-7, MKN-28, and MKN-74, well-differentiated adenocarcinoma; and MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr. T. Suzuki (Fukushima Medical University, Japan). These lines were maintained in DMEM (GIBCO Laboratories, Grand Island, NY) containing 10% heat-inactivated FCS (Hazleton Research Products, Lenexa, KS).

Effect of TGF-β_1 on Cell Growth. In order to determine the effect of TGF-β_1 on cell proliferation, cells were seeded at 1, 2, or 5 × 10⁴ cells/ml per well with FCS-containing or serum-free CDM in 24-well plates. TGF-β_1 was added after 24 h. Medium containing growth factor was replaced every 72 h. Trypan blue dye exclusion was used to assay for cell viability. Cells resuspended in culture medium were stained by addition of an equal volume of 0.4% trypan blue in 0.7% saline and counted on a hemocytometer. The extent of the cell growth was also determined by a crystal violet dye elution assay as described previously (12). Viability was expressed as a percentage relative to the untreated cells. Each experiment was repeated at least 3 times.

DNA Fragmentation Assay. Cells were seeded at 1 × 10⁶ cells per 100-mm plate with 10% FCS-containing or serum-free CDM, and they were treated with 1 ng/ml of TGF-β_1. DNA was isolated from the cultured cells as described previously (11). Electrophoresis was carried out on 1.6% agarose gel in Tris-borate buffer (pH 8.0) containing 1 mM EDTA. Gels were stained with ethidium bromide.
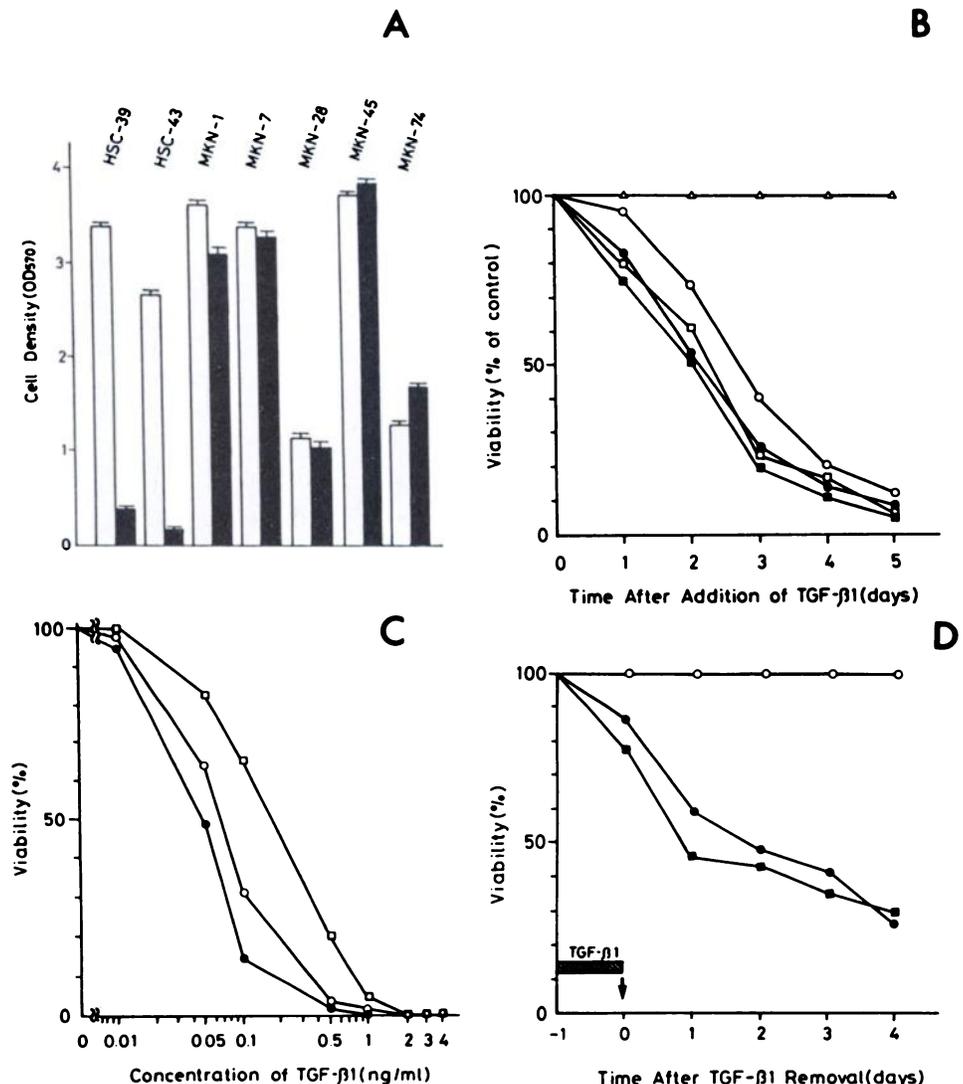
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Results and Discussion

We have previously established and characterized two human scirrhous-type gastric carcinoma cell lines, HSC-39 (11) and HSC-43.[4] In order to determine whether the responsiveness to specific peptide growth regulators was altered in scirrhous-type gastric carcinoma cells relative to that in non-scirrhous-type gastric carcinoma cells, we compared the effect of TGF-β_1 on the growth of gastric carcinoma cell lines which are representative of the two groups. Fig. 1A compares the growth inhibition produced by TGF-β_1 in the scirrhous-type carcinoma cell lines HSC-39 and HSC-43 to the TGF-β_1-induced, growth-inhibitory response in the non-scirrhous-type carcinoma cell lines MKN-1, MKN-7, MKN-28, MKN-45, and MKN-74. Cell proliferation of HSC-39 and HSC-43 cells was strongly inhibited by 1 ng/ml of TGF-β_1 in medium containing either 1% or 5% FCS. This inhibitory effect could be blocked by the addition of a polyclonal TGF-β-neutralizing antibody (data not shown). In other gastric adenocarcinoma cell lines, MKN-1, MKN-7, MKN-28, MKN-45, and MKN-74, cell growth was not significantly affected by the addition of TGF-β_1. These results indicated that two scirrhous carcinoma cells were sensitive to exogenous TGF-β_1, whereas gastric adenocarcinoma cells were insensitive to TGF-β_1. Accordingly, it was of interest to determine the kinetics of the inhibition of cell proliferation by TGF-β_1 in the two scirrhous-type carcinoma cell lines.

To study the kinetics in the decrease in cell growth produced by TGF-β_1, cultures were exposed to 1 ng/ml of TGF-β_1 in medium containing 1% FCS for 1 to 5 days (Fig. 1B). The loss of cell viability was assayed by the loss of ability of the cells to exclude trypan blue. Exogenous TGF-β_1 leads to the death of the HSC-39 and HSC-43 cells. Both cell lines were also grown in serum-free CDM and were found to be slightly more sensitive to the cytocidal effect of TGF-β_1 as compared to cells grown in serum-containing medium (Fig. 1B). The magnitude of the cytocidal effect of natural or recombinant TGF-β on HSC-39 and HSC-43 cells was dose dependent with a maximum effect observed at 1 ng/ml (Fig. 1C). To determine whether the cytocidal effect was reversible, cells were incubated with TGF-β_1 (3 ng/ml) for 24 h, after which TGF-β_1 was removed from the medium. Both HSC-39 and HSC-43 cells failed to show regrowth, confirming the irreversible nature of the effect of TGF-β_1 (Fig. 1D). These results are of interest because they suggest that TGF-β_1 induces a programmed cell death in human gastric carcinoma cells *in vitro*.

Generally, the mechanisms of cell death can occur by either apoptosis (programmed cell death) or by necrosis (13). Apoptosis has been described extensively in embryogenesis, morphogenesis, hematopoiesis, and clonal selection in the thymus (14–16), where it performs an essential role in various physiological processes. Apoptosis also occurs in a variety of other systems

A

B

Fig. 1. Effect of TGF-β_1 on proliferation of cultured human gastric cancer cells. A, growth-inhibitory effect of TGF-β_1 in various cell lines. Cells (2 × 10⁴ cells/ml) were cultured without (□) or with (■) 1 ng/ml of TGF-β_1 in 1% FCS-containing medium for 7 days. Cell density was determined by the crystal violet elution assay as described previously (12). B, time course of the effect of continuous exposure to TGF-β_1 on HSC-39 and HSC-43 cells. The cells (2 × 10⁴ cells/ml) were incubated with 1 ng/ml of TGF-β_1 in 1% FCS-containing medium or CDM. Viable cells were counted by the trypan blue exclusion method and were expressed as the percentage relative to the untreated cells. △, control; ○, HSC-39 (1% FCS); ●, HSC-39 (CDM); □, HSC-43 (1% FCS); ■, HSC-43 (CDM). C, concentration response of TGF-β_1 on the viability of HSC-39 and HSC-43 cells. Cells (2 × 10⁴ cells/ml) were treated with varying concentrations of TGF-β_1 for 7 days. The cell density of viable cells was determined and expressed as the percentage relative to the untreated control cultures. □, HSC-39 (natural TGF-β_1); ○, HSC-43 (natural TGF-β_1); ●, HSC-43 (recombinant TGF-β_1). D, irreversible effect of TGF-β_1 on HSC-39 and HSC-43 cells. Both types of cells were seeded at 2 × 10⁴ cells/ml in CDM and were incubated overnight. Then 3 ng/ml of TGF-β_1 was added. After 24 h, cells were washed and incubated in CDM. Viability was expressed as described in Fig. 1B. ○, control; ●, HSC-39; □, HSC-43.

C

D

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Fig. 3. Inhibition of TGF-β_1-mediated cell death by cycloheximide. HSC-43 cells were seeded at 2×10^4 cells/ml in CDM and treated with 0.25 ng/ml of TGF-β_1 6 h later. Then cultures were incubated in the presence of varying concentrations of CHX for 7 days. Viability was expressed as a percentage of the ratio in untreated control cultures by cell density. *Columns*, mean; *bars*, SD.

and in response to different stimuli (17, 18). In all of these systems, apoptotic cells have a distinct morphology and show a characteristic "ladder" pattern of DNA fragmentation resulting from cleavage of nuclear DNA in internucleosomal regions (19).

To discriminate between apoptosis or necrosis that might be induced by the TGF-β_1, we have examined the morphological change of TGF-β_1-treated HSC-39 and HSC-43 cells by Hoechst 33258 fluorochrome staining. As shown in Fig. 2*A*, chromatin condensation is visible after a 24-h incubation with 0.5 ng/ml of TGF-β_1. Such a morphological change was not observed in untreated cells. Furthermore, transmission electron microscopy of TGF-β_1-treated HSC-39 or HSC-43 cells showed extensive condensation of chromatin, fragmentation of nuclei, and the presence of much necrotic debris (Fig. 2*B*). In contrast, untreated control cells were viable and showed a normal morphology (data not shown).

DNA fragmentation was assessed in the HSC-39 and HSC-43 cells after incubation with 1 ng/ml of TGF-β_1 (Fig. 2*C*). This is one of the few specific biochemical features that is characteristic of apoptosis (19). When HSC-39 cells are cultured with TGF-β_1 in CDM or in 1% FCS-supplemented CDM for 24 h, DNA is converted to a "ladder" pattern of fragments of integer multiples of roughly of 180 base pairs (*Lanes 2* and *3*). A similar pattern of DNA fragmentation was observed in the HSC-43 cells following the same condition at 24 or 48 h (*Lanes 7* and *8*). No chromosomal DNA cleavage was observed in untreated control cells (*Lanes 1* and *6*). These observations are consistent with cell death occurring by the process of apoptosis, or programmed cell death.

Apoptosis is an active process requiring new protein synthesis (20). The effect of CHX on the TGF-β-induced death was therefore investigated. HSC-43 cells were incubated with various concentrations of CHX following exposure to 0.25 ng/ml of TGF-β_1. The effect of CHX was not observed at high doses of TGF-β_1 (1 or 2 ng/ml). However, at lower TGF-β_1 concentrations, CHX partially prevented TGF-β_1-induced cell death in a
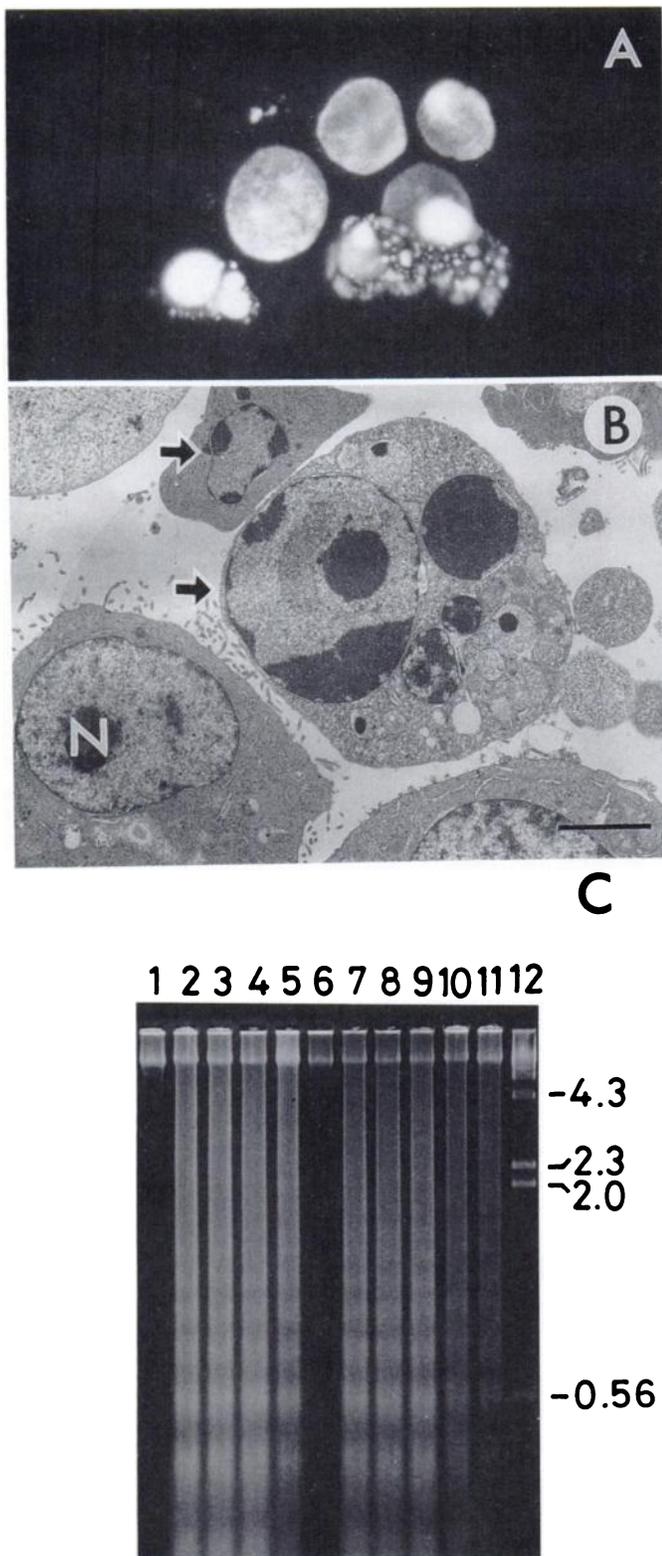

Fig. 2. Apoptotic features of TGF-β_1-induced cell death. In *A*, a fluorescence micrograph shows four fragmented apoptotic cell nuclei and three intact nuclei. The HSC-43 cells were incubated with TGF-β_1 at 0.5 ng/ml after 24 h in CDM plus 1% FCS on tissue culture chamber/slides (Lab-Tek). Cultured cells were fixed with Carnoy's fixative (glacial acetic acid: absolute methanol, 1:3) and stained for 30 min for DNA with Hoechst 33258 fluorochrome (0.1 μg/ml in distilled water). Slides were then mounted with Aquamount and coverslips. Photographs were obtained with a Nikon EFD2 fluorescence microscope ($\times$ 400). In *B*, a transmission electron micrograph shows condensed and fragmented nuclei. The HSC-39 cells were incubated for 24 h with 1 ng/ml of TGF-β_1. The electron micrograph was taken using a JEM-1200 as described previously (11). *N*, normal nucleus; →, apoptotic cell nuclei ($\times$ 4200); *bar*, 3 μm. *C*, DNA fragmentation in TGF-β_1-treated cells. Exponentially growing cells were treated with 1 ng/ml of TGF-β_1 for 24 or 48 h (*Lanes 2, 3*, and *7* to *9*). Each DNA sample (5 μg) was
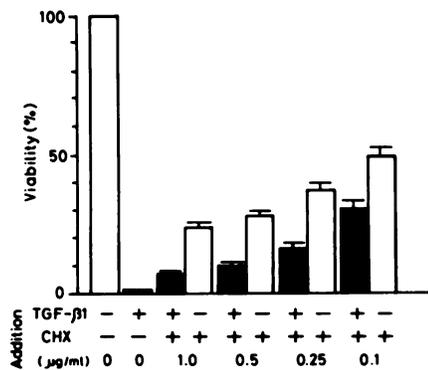
electrophoresed through a 1.6% agarose gel. DNA bands were visualized by staining with ethidium bromide. *Lanes: 1*, DNA from untreated HSC-39 cells, 48 h, CDM; *2*, TGF-β_1-treated HSC-39 cells, 24 h, CDM; *3*, TGF-β_1-treated HSC-39 cells, 48 h, 1% FCS; *4*, 0.25 ng/ml of TGF-β_1-treated HSC-39, 72 h, CDM; *5*, 0.25 ng/ml of TGF-β_1- and 0.1 μg/ml of CHX-treated HSC-39, 72 h; *6*, DNA from HSC-43 cells, 48 h, CDM; *7*, TGF-β_1-treated HSC-43 cells, 24 h, CDM; *8*, TGF-β_1-treated HSC-43 cells, 48 h, 1% FCS; *9*, TGF-β_1-treated HSC-43 cells, 48 h, CDM; *10*, 0.25 ng/ml of TGF-β_1-treated HSC-43 cells, 72 h, CDM; *11*, 0.25 ng/ml of TGF-β_1- and 0.1 μg/ml of CHX-treated HSC-43, 72 h; and *Lane 12*, restriction enzyme *Hin*dIII digest of λ-phage DNA.

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dose-dependent fashion (Fig. 3). These results suggest that CHX can provide protection from TGF-β_1-induced cell death within the limits of its own toxicity. This was also correlated with a reduction in DNA fragmentation produced by TGF-β_1 (Fig. 2C, Lane 11). Similar results were observed on the HSC-39 cells (Fig. 2C, Lane 5). Therefore, under these conditions, CHX also appears to protect from TGF-β_1-induced DNA cleavage. This DNA fragmentation has been shown to result from activation of a Ca^{2+}- and Mg^{2+}-dependent endonuclease present within the cell nucleus which selectively hydrolyzes DNA at regions located between nucleosomal units, thus resulting in a ladder of DNA fragments in various systems (19, 21, 22). The present data also suggest that exogenous TGF-β_1 can possibly induce the activation of endonuclease at a very early step in the process of apoptosis. This process is analogous to the glucocorticoid-induced apoptosis of lymphocytes and indicates a potentially common signal transduction pathway. This endonuclease activation has been demonstrated to be triggered by a sustained elevation in the intracellular free Ca^{2+} concentration initiated early in the process of apoptosis (23, 24). There is recent evidence that the calcium ionophore prevents apoptosis in interleukin 3-dependent pre-B-cells (25). It is possible that TGF-β_1 is involved in the regulation of intracellular calcium homeostasis.

As this paper was being prepared for submission, Lin and Chou (26) also reported apoptosis in a human hepatoma cell line induced by TGF-β_1. However, our experimental system differs from theirs by several criteria: (a) when serum-starved hepatoma cells were exposed with TGF-β_1; (b) although DNA fragmentation was observed, chromatin condensation was not found; and (c) treatment of CHX had no effect upon the TGF-β-mediated hepatoma cell death. Lin and Chou therefore conclude that TGF-β_1 causes early minor apoptotic effects in hepatoma cells. Our data are in agreement with recent studies which have demonstrated that elevation of TGF-β_1 gene expression is closely associated with some apoptotic cell death (27, 28). Finally, the present study provides the first evidence to suggest that TGF-β_1 causes apoptotic cell death in human gastric carcinoma cells. However, the explanation and physiological significance for this cell death phenomenon of cancer cells by TGF-β_1 are not clear. Further studies are required for delineating the mechanisms of this form of cell death.

Acknowledgments

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