

The cytotoxic effect of TGF- β 1 on mesothelial cells via apoptosis in early peritoneal carcinomatosis

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Abstract. Peritoneal dissemination is one of the main causes of death in gastric cancer patients. We have previously reported that gastric cancer cells can induce peritoneal apoptosis, lead to damage of peritoneum integrity, and therefore promote peritoneal metastasis. However, the soluble factors secreted by cancer cells to trigger the damaging cascade remain unclear. TGF- β 1, a cytokine known for its capacity to induce proliferative and transformative changes of cells is found in significantly higher quantities correlated with peritoneal metastasis and TNM stages of gastric cancer. High levels of TGF- β 1 in the subperitoneal milieu may affect the morphology and function of mesothelial cells, so that the resulting environment becomes favorable for peritoneal metastases. We observed apoptosis induced by TGF- β 1 in mesothelial cells in peritoneal carcinomatosis. Knockdown of the Smad2 gene by siRNA silencing can partially inhibit these effects. TGF- β 1 could upregulate the expressions of Bax and suppress Bcl-2 in mesothelial cells. We conclude that TGF- β 1 could induce apoptosis of mesothelial cells, which involves the Smad2 signaling pathway in peritoneal carcinomatosis. Bcl-2 and Bax may contribute to this phenomenon.

Introduction

Peritoneal carcinomatosis frequently occurs at the later stages of gastric carcinoma, especially after surgery, which reflects

the peritoneal metastatic cascade of gastric cancer and significantly contributes to gastric cancer-related mortality (1,2). To date, the mechanisms by which gastric carcinoma undergoes peritoneal carcinomatosis has not yet been specified. Paget's 'seed and soil' theory of tumor metastasis may provide a clue useful for further investigation (3). This theory stated that the sites where metastasis occurs are defined not only by the tumor cells (seed) but also by the local microenvironment of the metastatic site (soil). In other words, the specific site of cancer cell metastasis is not simply due to the anatomic location of the primary tumor or proximity to the secondary sites but rather, it involves interactions between tumor cell and the local microenvironment at the secondary site (4).

It has been previously reported that prior to gastric cancer cell adhesion to the peritoneum, mesothelial cells become hemispherical, exfoliation occurs, and the naked areas of the submesothelial connective tissue are exposed to the peritoneal cavity, and the disrupted mesothelial cells will then promote tumor cells invasion. We have previously demonstrated that cancer cells can secrete soluble factors into the abdominal cavity to induce damage and apoptosis of peritoneal mesothelial cells, and this injured peritoneum provides a favorable environment for peritoneal metastasis (5). However, the soluble factors secreted by cancer cells to trigger the damaging cascade remains unclear.

One of the most potent apoptotic stimuli for mesothelial cells is the transforming growth factor- β 1 (TGF- β 1), which can participate in a broad array of biological activities such as normal development, wound healing and pathological processes (6-8). It can also regulate multiple cellular functions, including both inhibition and stimulation of proliferation, apoptosis and differentiation. Our previous study demonstrated that the TGF- β 1 levels in peritoneal lavage fluid are significantly correlated with peritoneal metastasis and TNM stages of gastric cancer (9,10). In addition, TGF- β 1 stimulates both the invasion and adhesion of gastric cancer cells to the peritoneum, thus resulting in an increase of the potential for peritoneal dissemination (11). Taken together, we hypothesize that disseminated gastric cancer cells infiltrate into the abdominal cavity, where they secrete abundant inflammatory factors, such as TGF- β 1, to induce apoptosis of peritoneal mesothelial cells. Thus, mesothelial cells become hemispherical and exfoliation occurs. Areas of the sub-mesothelial

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Abbreviations: HPMCs, human peritoneal mesothelial cells; TGF- β 1, transforming growth factor- β 1; AO/EB, acridine orange/ethidium bromide; siRNA, small interfering-RNA; FCS, fetal calf serum

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connective tissue are then exposed to the peritoneal cavity and this injured peritoneum provides a favorable environment for peritoneal metastasis.

Materials and methods

Reagents. Human TGF- β 1 was obtained from Sigma (USA). Acridine orange/ethidium bromide (AO/EB) stains were obtained from Fluka (USA). Propidium iodide was obtained from Biosharp (USA). Bcl-2, Bax, Smad2, phosphorylated Smad2 primary antibodies, as well as the secondary antibodies goat anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other laboratory reagents were obtained from Sigma (USA).

Cell line and cell culture. Human peritoneal mesothelial cells (HPMCs) were isolated from surgical specimens of human omentum as previously described (12). Briefly, small pieces of omentum were surgically resected under sterile conditions and were trypsinized at 37°C for 30 min. The suspension was then passed through a 200- μ m-pore nylon mesh to remove undigested fragments and centrifuged at 2,000 rpm for 5 min. The collected cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS). In the following experiments, cells were used during the second or third passage after primary culture. HPMCs were identified by immunostaining with mouse monoclonal antibodies against cytokeratin and vimentin. The donors had no evidence of peritoneal inflammation and/or malignancy. All patients provided written informed consent prior to participation in the study. Our study was approved by the Institutional Ethics Committee.

Transmission electron microscopy. After incubation in test solutions of TGF- β 1, the mesothelial cells were trypsinized and then fixed in ice-cold 2.5% electron microscopy grade glutaraldehyde in PBS (pH 7.3). The specimens were rinsed with PBS, post-fixed in 1% osmium tetroxide with 0.1% potassium ferricyanide, dehydrated through a graded series of ethanol (30-90%) and embedded in Epon. Semithin (300 nm) sections were cut using a Reichart Ultracut, stained with 0.5% toluidine blue, and examined under light microscopy. Ultrathin sections (65 nm) were stained with 2% uranyl acetate and Reynold's lead citrate, and examined on a transmission electron microscope at x5,000 magnification.

Flow cytometry. After incubations in test solutions, mesothelial cells were harvested by trypsinization. Cells were resuspended in PBS at a concentration of 1×10^6 /ml and fixed in 2 ml methanol for 30 min at 4°C. After mesothelial cells were fixed, the mixture was incubated in 0.5 ml of propidium iodide solution (0.05 mg/ml in 3.8 mol/l Na citrate) and 0.5 ml of RNase A (0.5 mg/ml) at room temperature for 30 min. Finally, the cells were resuspended in 1 ml PBS and analyzed by using flow cytometry according to the manufacturer's instructions. The cells in the subdiploid peak were considered apoptotic.

In situ detection of apoptosis. HPMCs were cultured to subconfluence in a 24-chamber slide with 10% FCS containing DMEM. The medium then was changed to test solutions.

After incubation for 24 or 48 h, apoptosis was quantified by fluorescent staining methods: the detection of acridine orange/ethidium bromide (AO/EB) condensed nuclei by fluorescent microscopy. AO/EB staining identifies alive, early apoptotic, late apoptotic and necrotic cells. Subconfluent cells (70-80% confluent) in 24-well uncoated plates were exposed to apoptotic stimuli for 48 h. Mesothelial cells in 24-wells were gently washed with PBS and immediately treated with AO (100 μ g/ml) for 5 min and EB (100 μ g/ml) for 5 min. Each well was then immediately examined under an epifluorescence microscope. For evaluation by fluorescence microscopy, an excitation wavelength of 455 nm was used. Apoptosis was defined by morphological criteria. Cells containing normal nuclear chromatin exhibited green nuclear staining. Cells containing fragmented nuclear chromatin exhibited orange to red nuclear staining.

Western blotting. HPMCs were cultured to subconfluence in a 50-cm² dish with 10% FCS-containing DMEM. The media were then changed to test solutions. The cells were lysed by ice-cold lysis solution (65 mmol/l Tris base, pH 8.0, containing 154 mmol/l NaCl, 1 mmol/l EDTA, 1% octylphenoxy polyethoxyethanol, 1 mmol/l phenylmethanesulfonyl fluoride, leupeptin (1 mg/ml), pepstatin (1 mg/ml), aprotinin (1 mg/ml) and 0.25% sodium deoxycholate). Samples were rotated for 15 min at 4°C and then centrifuged at 12,000 \times g for 5 min at 4°C. The supernatant was recovered, and the protein concentration was measured by using the bicinchoninic acid assay, with bovine serum albumin as the standard. Samples were incubated for 5 min at 95°C in loading buffer (12 mmol/l Tris-HCl, pH 6.8, with 25% glycerol, 2% sodium dodecyl sulfate, 14.4 mmol/l 2-mercaptoethanol and 0.1% bromophenol blue), and 50 mg of protein were loaded on SDS-polyacrylamide gels of different percentages (and exclusion limits) corresponding to the molecular weight of the target proteins. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membrane by electroblotting. The membrane was blocked in 1% BSA/0.05% Tween/PBS solution overnight at 4°C. Blots were developed by incubation in a chemiluminescence substrate and were exposed to X-ray film.

Small interfering-RNA (siRNA) treatment. The HPMCs were grown to a 70% confluence on culture dishes. Transient transfection was performed with specific stealth small interference RNA against Smad2, or control siRNA overnight, according to the manufacturer's instructions. The total of three siRNA sequences for Smad2 and control-siRNA were designed and synthesized from Invitrogen using the RNAi designer software program. The concentration of 300 nM was determined to be the most effective siRNA concentration for Smad2 silencing. The transfection medium was changed with culture medium containing 5% FBS for 24 h. TGF- β 1 at a final concentration of 5 ng/ml was added to the cell cultures in serum free medium with or without TGF- β 1 (control). The cells were harvested at 4, 24 and 72 h for further experiments.

Statistical analysis. The data are expressed as mean \pm SE. The Student's t-test was performed for comparison of the control and TGF- β 1 treatment groups. Differences were considered statistically significant when the P-value was ≤ 0.05 .

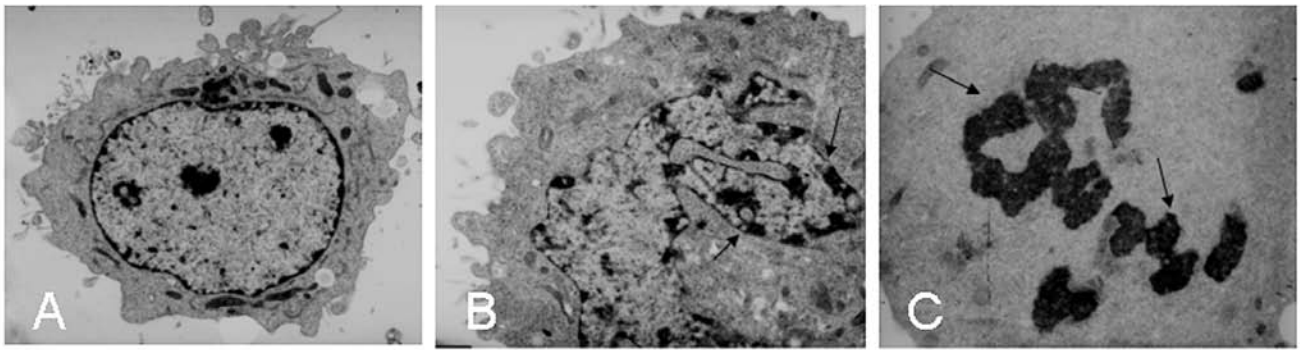


Figure 1. Effect of TGF- β 1 on the apoptosis of HPMCs examined by electron microscopy (original magnification, x5,000). (A) The control cell displayed a normal nucleus and endoplasmic reticulum. HPMCs incubated with TGF- β 1 for 24 h showed wrinkling of the nuclear membrane, and chromatin condensation along the nuclear membrane (arrow in B), and apoptotic bodies (arrows in C).

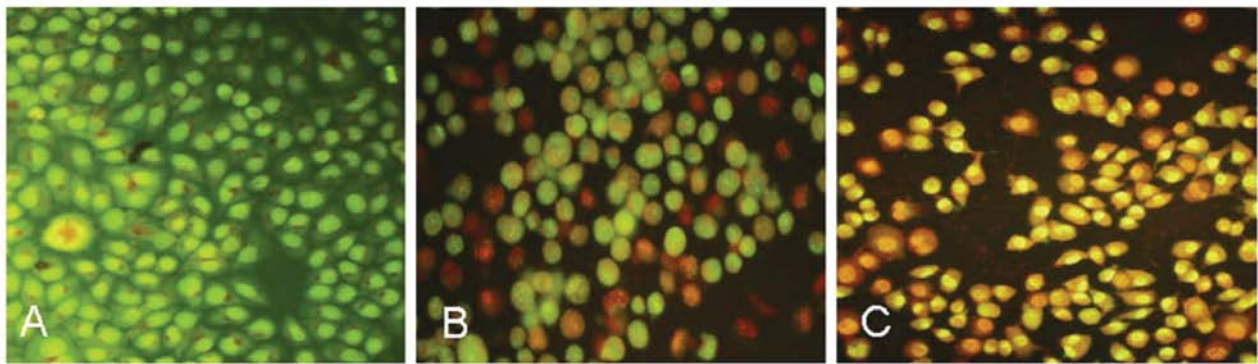


Figure 2. Cell apoptosis observed by acridine orange/ethidium bromide (AO/EB) staining. Cells containing normal nuclear chromatin exhibit green nuclear staining. Cells containing fragmented nuclear chromatin exhibit orange to red nuclear staining. (A) Mesothelial cells cultured in serum free DMEM as control; (B) mesothelial cells were treated with TGF- β 1 (5 ng/ml) for 24 h; (C) mesothelial cells were treated with TGF- β 1 (5 ng/ml) for 48 h.

Results

Transmission electron microscopy. After 24 h of TGF- β 1 treatment, the apoptotic features (such as condensation of nuclear chromatin, wrinkling of the nuclear membrane, dilation of the endoplasmic reticulum, and relatively normal structure of the mitochondria) were verified by using electron microscopy (Fig. 1). Under transmission electron microscope, the membrane of the nucleus was complete, and the chromatin concentrated into masses, on the boundary of the membranes or forming arches (Fig. 1B). We could also observe the phenomena of budding and the formation of apoptotic bodies (Fig. 1C).

In situ detection of apoptosis. After HPMCs were treated with TGF- β 1 for 24 h, marked morphological changes of cell apoptosis such as condensation of chromatin, nuclear fragmentations and apoptotic bodies were clearly observed using acridine orange/ethidium bromide (AO/EB) staining (Fig. 2B). Apoptotic cells significantly increased in HPMCs treated with TGF- β 1 for 48 h, while the number of red cells increased (Fig. 2C).

Effects of TGF- β 1 or siRNAi-Smad2 on Smad2 phosphorylation of HPMCs. Similarly to in our previous study (13) we observed that TGF- β 1 induced phosphorylation of Smad2 within 10 min of stimulation, and the level of Smad2

phosphorylation reached a maximum between 30-60 min after treatment and remained elevated for the duration of the experiment without affecting total Smad2 expression. siRNAi-Smad2#1 showed highly significant knockdown for Smad2 and phosphorylated Smad2 when compared to the other two siRNA's or control siRNA.

Silencing Smad 2 signaling blocks TGF- β 1 induced apoptosis of mesothelial cells. In order to confirm whether Smad2 is involved in TGF- β 1 induced apoptosis of mesothelial cells, siRNA were used to knock down the Smad2 gene in HPMCs. We evaluated the apoptosis of mesothelial cells by flow cytometry, after silencing Smad2 by using siRNA-Smad2 or control-siRNA in HPMCs treated with TGF- β 1. We obtained apoptosis peaks ahead the diploid peak when HPMCs were incubated with TGF- β 1. Treatment of HPMCs with siRNA-Smad2 prior to TGF- β 1 stimulation significantly inhibited the apoptosis of mesothelial cells when compared to the control (Fig. 3). This was true for various time points. Combination of siRNA-Smad2 could effective suppress mesothelial cells apoptosis induced by TGF- β 1 (Fig. 4).

Bcl-2 and Bax expression in mesothelial cells. We then sought to further delineate the mechanisms that underlie the combined effects of TGF- β 1 on the HPMCs apoptosis. We examined the

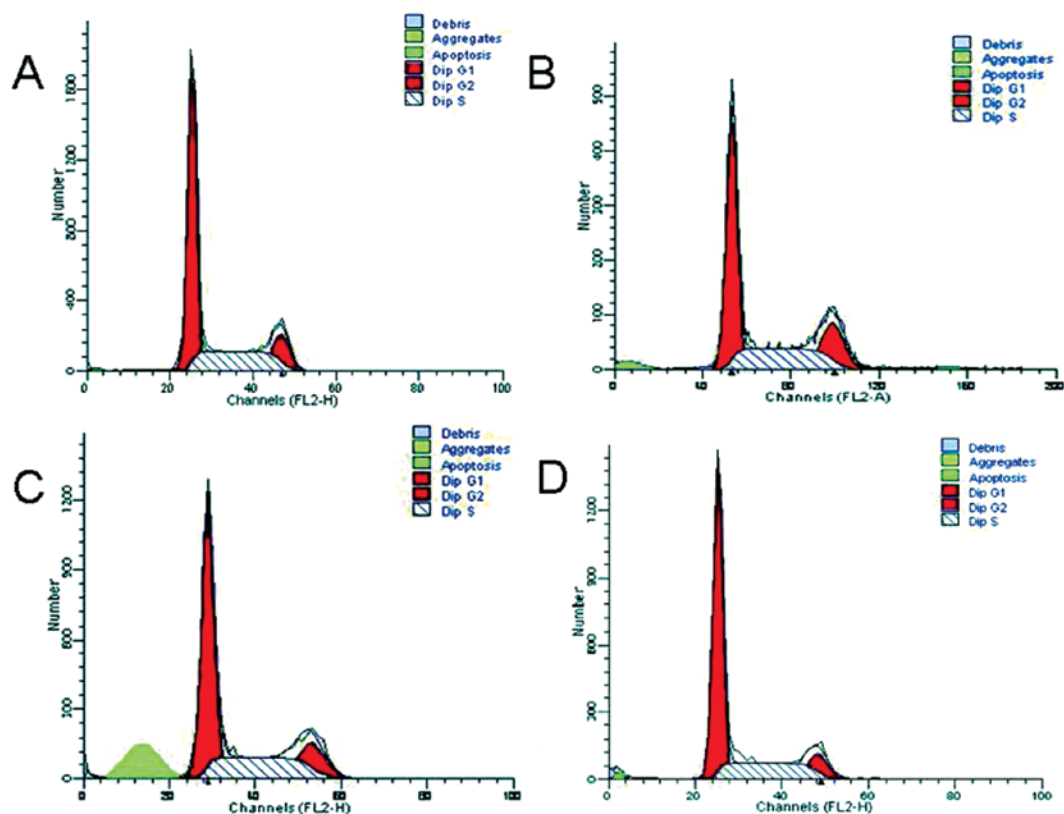


Figure 3. The effect of TGF- β 1 and/or Smad2 siRNA on cell-cycle distribution. Mesothelial cells (2×10^6) were treated with TGF- β 1, and/or Smad2 siRNA. Samples were analyzed by flow cytometry as described in Materials and methods. Apoptotic peaks are shown in green. (A) Mesothelial cells cultured in serum-free DMEM as control displayed 0.52% apoptosis. Mesothelial cells treated with TGF- β 1 (5 ng/ml) for (B) 12 h or (C) 24 h displayed 3.38% and 13.11% apoptosis, respectively. (D) Mesothelial cells treated with TGF- β 1 + Smad2 siRNA showed 1.21% apoptosis.

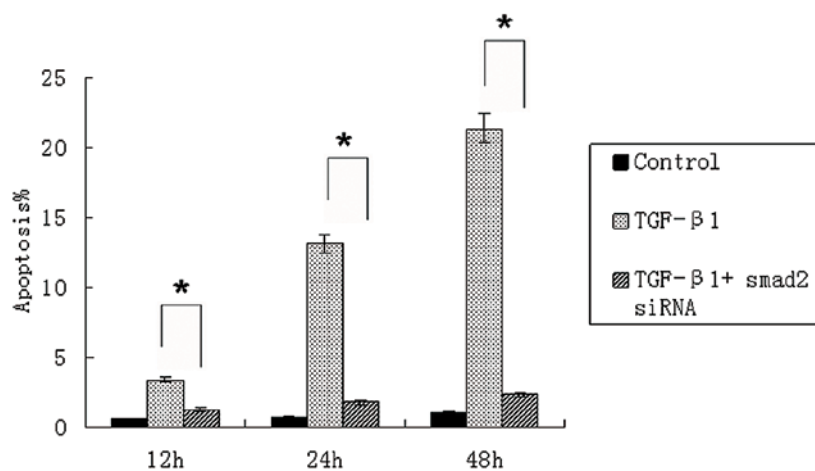


Figure 4. Percentages of human peritoneal mesothelial cells in the sub-G1 group (apoptosis) after treatment with control (mesothelial cells cultured in serum-free DMEM), TGF- β 1, TGF- β 1 + Smad2 siRNA for various time periods. *P < 0.01.

influence of TGF- β 1 or siRNA-Smad2 on the expression of the major anti-apoptotic protein Bcl-2 and apoptotic protein Bax in mesothelial cells. As shown in Fig. 5, TGF- β 1 reduced Bcl-2 and increased Bax expression in mesothelial cells significantly, while siRNA-Smad2 partially inhibit these effects. The data demonstrated that combinations of siRNA-Smad2 could augment the anti-apoptotic effects.

Discussion

Mesothelial cells have been reported to prevent cancer invasion and undergo morphological changes in response to factors released by cancer cells (14). Paget has explained this phenomenon by the 'seed and soil' theory: metastases occur when some tumor cells only live and grow in a congenial

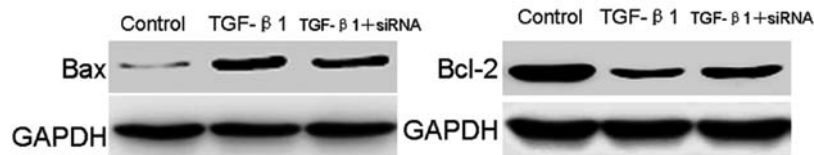


Figure 5. Western blot analysis of Bcl-2 and Bax proteins. Mesothelial cells were treated with serum-free DMEM as control; mesothelial cells were treated with TGF- β 1 (5 ng/ml) in the presence or absence of Smad2 siRNA for 24 h. The cells were harvested and processed for western blotting. GAPDH was used as the loading control.

environment (3). The peritoneum appears to be a congenial environment for scirrhous gastric cancer cells. It is conceivable that mesothelial cells prevent cancer cell infiltration into the submesothelial connective tissue. We hypothesized that cancer cells free-floating in the abdominal cavity and released primarily when gastric cancer tumors are exposed to the serosa, induce the release of early inflammatory factors, such as TGF- β 1, and induce apoptosis in peritoneal mesothelial cells (15,16). Mesothelial cells become hemispherical, exfoliation occurs, and the naked areas of the submesothelial connective tissue are exposed to the peritoneal cavity, and this injured peritoneum is a congenial environment for peritoneal metastasis (17-19).

Our present study demonstrated that plasmic atrophy, nuclear shrinkage, and formed extracellular and/or intracellular apoptotic bodies were observed under a transmission electron microscope. Furthermore, apoptosis was quantified by two methods: the detection of acridine orange/ethidium bromide-stained condensed nuclei by fluorescent microscopy and the detection of surface expression of phosphatidylserine by flow cytometry. The 24-h apoptotic indexes of mesothelial cells which were tested by flow cytometry with PI staining were $13.11 \pm 1.75\%$ in TGF- β 1 group. Compared with the control group, statistically significant differences were found. In the control group, spontaneous apoptosis occurred in mesothelial cells, but the apoptotic index was low. Therefore, we thought that TGF- β 1 could induce apoptosis of mesothelial cells, cause exfoliation, and eventually result in metastasis. These may be the mechanisms by which cancer cells could adhere to submesothelial connective tissue even though mesothelial cells are confluent.

Smads are a group of intracellular proteins that are critical for transmitting the TGF- β 1 signals from the cell surface to the nucleus to promote transcription of target genes (13,20,21). In the present study, we demonstrated *in vitro* that the Smad2 signaling pathway may be involved in apoptosis of mesothelial cells, and silence of Smad2 could block the effects of TGF- β 1. These findings suggested that apoptosis of mesothelial cells was dependent on Smad2 signaling, and provide a favorable environment for the dissemination of gastric cancer.

The signaling pathway of apoptosis on HPMCs has not been fully studied. In general, the signaling pathway of apoptosis is complex, but can be divided largely into a Fas/Fas ligand and a mitochondrial pathway (22). The tumor suppressor p53 is a transcriptional factor which is involved in apoptosis partly by inducing Bax expression (23,24). Bcl-2, an inhibitor of the mitochondrial apoptosis pathway, exerts its action by blocking proapoptotic counterparts, which in turn

prevents the release of cytochrome c and the activation of caspases (25,26). Our results showed that treating mesothelial cells with TGF- β 1 significantly inhibited the Bcl-2 expression in mesothelial cells. Bax is a death promoter, which is neutralized by heterodimerization with Bcl-2. Bax translocates into the outer mitochondrial membrane followed by a leakage of cytochrome c from the mitochondria into the cytosol (27). In the present study, we demonstrated that TGF- β 1 significantly reduced Bcl-2 and increased Bax expression in mesothelial cells, while siRNA-Smad2 can partially inhibit these effects. Consequently, there is a great possibility that TGF- β 1 mediated apoptosis of mesothelial cell is the result of regulation of Bcl-2 and Bax activation, and combinations of siRNA-Smad2 could augment the anti-apoptotic effects. Hence, identification of the target compounds is imminent.

To the best of our knowledge, this is the first study that demonstrates that HPMC apoptosis is induced via the TGF- β 1 and Smad2 signaling. It suggests the possibility of further developing anti-apoptosis drugs as a treatment option and as adjuvant chemotherapeutic agents in the therapy of gastric cancer peritoneal metastasis.

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