Transforming growth factor ß1 produced in autocrine/paracrine manner affects the morphology and function of mesothelial cells and promotes peritoneal carcinomatosis

DI NA[1], ZHI-DONG LV[1], FU-NAN LIU[1], YAN XU[1], CHENG-GANG JIANG[1], ZHE SUN[1], ZHI-FENG MIAO[1], FENG LI[2] and HUI-MIAN XU[1]

[1]Department of Surgical Oncology, The First Affiliated Hospital, China Medical University; [2]Department of Cell Biology, China Medical University, Shenyang 110001, Liaoning Province, P.R. China

Received March 9, 2010; Accepted April 15, 2010

DOI: 10.3892/ijmm_00000469

Abstract. Human peritoneal mesothelial cells (HPMCs) in intact mesothelium have been demonstrated to protect against tumor peritoneal metastasis. We have previously reported that gastric cancer cells can induce peritoneal apoptosis, lead to damage of peritoneum integrity, and therefore promote peritoneal metastasis. In this study, we investigated the effects of TGF-ß1 on tumor-mesothelial interaction. Briefly, the levels of various soluble factors, in particular TGF-ß1, were measured. HMrSV5 cells, a human peritoneal mesothelial cell line, were co-incubated with TGF-ß1, gastric cancer cells, or gastric cancer cells and TGF-ß1 receptor inhibitor SB431542. The expressions of smad 2/3 and phosphorylated smad 2/3, indicator of TGF-ß/Smads pathway activation, were evaluated. Then the morphological changes of HPMCs were observed. The cell damage was quantitatively determined by fluorescent microscopy and flow cytometry. Tumor-mesothelial cell adhesion was also examined. Results showed a significant elevation of TGF-ß1 expression, which is companied by dramatically increased phosphorylated-smad 2/3 levels, after mesothelial cell co-culture with the gastric cancer cell line. In addition, mesothelial cells exposed to gastric cancer cells or TGF-ß1 became exfoliated and exhibited signs of injury, while blocking TGF-ß1 can partially inhibit these effects. These results indicate that soluble factors, such as TGF-ß1, produced in autocrine/paracrine manner in the peritoneal cavity, affect the morphology and function of mesothelial cells so that the resulting environment becomes favorable for peritoneal metastases.

Correspondence to: Dr Hui-Mian Xu, Department of Surgical Oncology, The First Affiliated Hospital, China Medical University, Shenyang 110001, Liaoning Province, P.R. China
E-mail: xuhuimian@126.com; xuhuimian@yahoo.cn

Abbreviations: HPMCs, human peritoneal mesothelial cells; AO/EB, acridine orange/ethidium bromide; SF-CM, serum-free conditional media

Key words: peritoneal carcinomatosis, stomach neoplasms, mesothelial cell, transforming growth factor ß1

Introduction

Peritoneal carcinomatosis remains a major obstacle that severely limits the further improvement of gastric cancer patients' prognosis after surgery (1). Peritoneal carcinomatosis is a peritoneal metastatic cascade that is composed of a series of events. It usually occurs at the late stage of tumor development and significantly contributes to gastric cancer-related mortality. The mechanisms of peritoneal metastasis of diffusely infiltrating carcinoma are not yet clearly understood.

Over 100 years ago, Paget *et al* proposed a 'seed and soil' theory: metastasis only occurs when tumor cells (seeds) survive and grow in a favorable organ/tissue micro-environment (soil) (2). It is conceivable that peritoneal carcinomatosis occurs as the peritoneal stroma environment promotes tumor cells proliferation by providing various growth factors and chemokines, thus promotes tumor metastasis. On the contrary, the healthy, intact mesothelial cells prevent cancer cells from infiltrating into the sub-mesothelial connective tissue by forming a layer of peritoneum barrier. Yashiro *et al* (1) have previously demonstrated that the layer of confluent, intact mesothelial cells hindered cancer cell invasion to the abdominal cavity. However, once the integrity of such barrier is disrupted, metastasis may occur because peritoneum provides a favorable environment for gastric cancer cells to grow. For example, Kiyasu *et al* (3) reported that mesothelial cells became hemispherical and exfoliated from the peritoneum prior to the peritoneal infiltration of cancer cells.

One of the most potent apoptotic and fibrotic stimuli for mesothelial cells is transforming growth factor ß1 (TGF-ß1), which belongs to the TGF-ß super-family. The TGF-ß super-family also includes other TGF factors, bone morphogenic proteins and activin families, all of which share similar structures, signaling pathways, and an overlap in biologic functions. TGF-ß is a 25 kD homodimeric polypeptide that can participate in a broad array of biologic activities such as normal development, wound healing and pathologic processes (4). It can regulate multiple cellular functions, including both inhibition and stimulation of proliferation, apoptosis and differentiation. TGF-ß is also an inducer of extracellular matrix (ECM) protein synthesis and has been implicated as the key mediator of fibrogenesis in various tissues (5,6).

Our previous study demonstrated that the TGF-ß1 level in peritoneal lavage fluid is significantly correlated with peritoneal metastasis and TNM stages of gastric cancer (7). Collectively, we hypothesize that disseminated gastric cancer cells infiltrate into abdominal cavity, where they secrete abundant inflammatory factors, such as TGF-ß1, to induce apoptosis of peritoneal mesothelial cells. Thus, mesothelial cells become hemispherical and exfoliation occurs. Areas of the sub-mesothelial connective tissue are then exposed to peritoneal cavity and this injured peritoneum provides a favorable environment for peritoneal metastasis (8-14). In this study, we investigated the effects of TGF-ß1 on the morphology and function of human peritoneal mesothelial cells to provide more information on the reciprocal interaction between tumor and mesothelial cells during peritoneal gastric cancer metastasis.

Materials and methods

Reagents. ELISA kits for TGF-ß1, IL-8, uPA, VEGF, KGF, MMP-7, MMP-9, HGF, FGF, and EGF were obtained from R&D Systems, USA. Calcein-AM was brought from Calbiochem, UK. Acridine orange/ethidium bromide (AO/EB) was obtained from Fluka, USA. Smad2, Smad3, phosphorylated-Smad2, and phosphorylated-Smad3, actin antibodies, as well as second antibodies (goat anti-mouse IgG) were purchased from Santa Cruz Biotechnology Inc., USA. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco BRL (Grand Island, USA). Propidium iodide (PI) was from Biosharp, USA. Human TGF-ß1 was obtained from Sigma, USA. Phase contrast microscope (Japan Nikon), fluorescence microscope (Japan Olympus, Japan), and flow cytometer (FACScalibur, Becton Dickson, USA) were employed.

Cell line and cell culture. The human peritoneal mesothelial cell line HMrSV5 was obtained from the Department of Cell Biology, China Medical University, China. HMrSV5 was originally isolated from human omentum. Briefly, omentum collected from consenting non-uraemic patients undergoing elective abdominal surgery was incubated in 0.05% (w/v) trypsin and 0.01% (w/v) EDTA for 20 min at 37˚C. The harvested mesothelial cells were centrifuged at 150 x g for 5 min and then transferred into 75 cm^2 tissue culture flasks and cultured in humidified 5% CO_2 incubator. DMEM was supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l L-glutamine and 20 mmol/l hydroxyethyl piperazine ethanesulfonic acid (HEPES, Gibco BRL). Medium was changed every two or three days.

The human gastric carcinoma cell lines, MKN-45, MKN-1, SGC-7901, BGC-823 and MGC-803 were obtained from the Department of Cell Biology, China Medical University. These cells were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l L-glutamine in humidified 5% CO_2 incubator at 37˚C.

Preparation of serum-free conditional media (SF-CM). Conditional media from gastric cancer cells or HPMC cells was prepared as previously reported (1). Briefly, 5.0x10^5 cells were seeded in a 100-mm tissue culture dish with regular medium for 3 days. Then the cells were washed twice with PBS and incubated with 3 ml of serum-free DMEM. Two days later, the SF-CM was collected and centrifuged at 1000 x g for 5 min, passed through filters (pore size, 0.45 μm) and stored at -20˚C until use.

Enzyme-linked immunoassay (ELISA). The levels of TGF-ß1, IL-8, uPA, VEGF, KGF, MMP-7, MMP-9, HGF, FGF and EGF in both the SF-CM and cell lysates of MKN-45, MKN-1, SGC-7901, BGC-823, MGC-803 and HMrSV5 cell lines were measured using human Quantikine ELISA kits (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

To evaluate the effect of co-culture of both gastric tumor cells and mesothelial cells on TGF-ß1 secretion, 8x10^4 mesothelial cells/well were first cultured in flat-bottomed 96-well plates to sub-confluence. Then 4x10^4 SGC-7901 cells were washed 3 times, added to the mesothelial cells and then co-cultured for additional 72 h. The supernatant was collected for ELISA test.

Western blotting. Human peritoneal mesothelial cells were cultured to sub-confluence in a 60-mm culture dish with 10% FCS-containing DMEM. The medium were then changed to either 1) SF-CM from gastric cancer cells SGC-7901 or 2) serum-free DMEM serving as control. Protein was extracted in a standard lysis buffer with proteinase inhibitors (sodium orthovanadate, phenylmethylsulfonyl fluoride, leupeptin, and aprotinin obtained from BioShop, Burlington, ON, Canada). Protein lysate (20 μg) was electrophoresed with 12% SDS-PAGE gel, transferred to a nylon membrane, and probed with an antibody for Smad2, phosphorylated-Smad2, Smad3, and phosphorylated-Smad3. Following the incubation with secondary antibody, blots were developed by ECL Western blot substrate kit (Abcam, USA).

Morphological evaluation of mesothelial cells under various conditions by a phase contrast microscope. Human peritoneal mesothelial cells were cultured to sub-confluence in a 60-mm dish with 10% FCS containing DMEM. The medium was then changed to either 1) serum-free DMEM; 2) medium containing TGF-ß1 (100 ng/ml); 3) SF-CM from gastric cancer cells or 4) SF-CM from gastric cancer cells supplemented with TGF-ß1 receptor inhibitor SB431542. Twenty-four hours later, all groups were examined under a phase contrast microscope for alterations in size, shape, and integrity of cell membrane, cytoplasm and nucleus.

Detection of apoptosis. Human peritoneal mesothelial cells were cultured to sub-confluence in a 24-chamber plate with 10% FCS containing DMEM. The medium was then changed to either 1) serum-free DMEM; 2) medium containing TGF-ß1 (100 ng/ml); 3) SF-CM from gastric cancer cells or 4) SF-CM

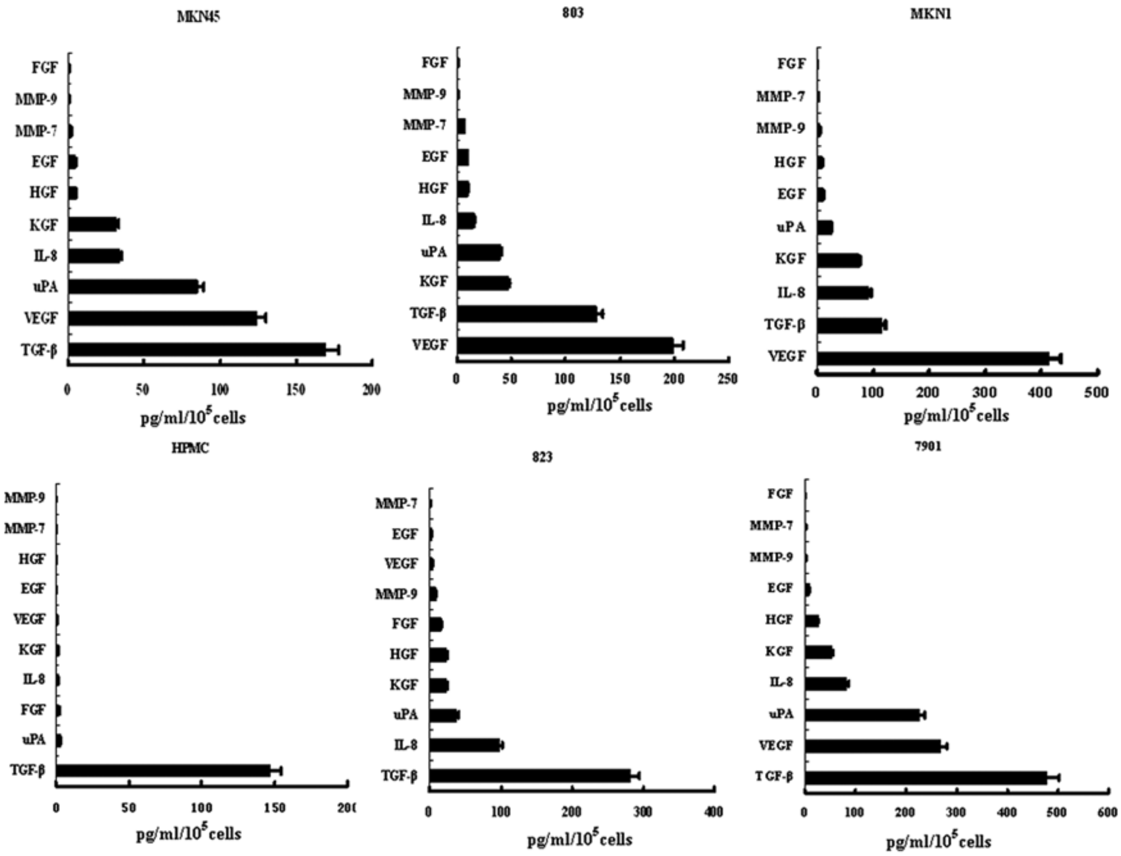
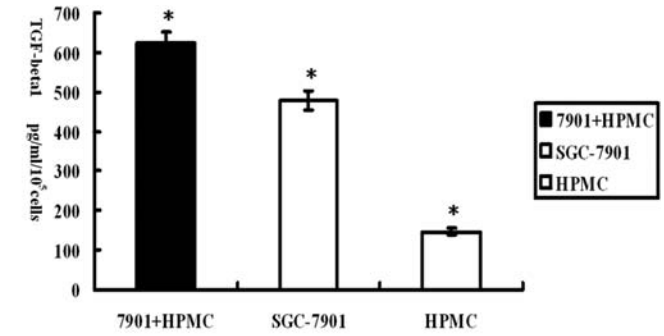


Figure 1. The level of various soluble factors, including TGF-ß1, IL-8, uPA, VEGF, KGF, MMP-7, MMP-9, HGF, FGF, and EGF from the SF-CM of MKN-45, MKN-1, SGC-7901, BGC-823, MGC-803 and HPMC cells. SF-CM was prepared from gastric cancer cells or HPMC for ELISA. Cell and supernatant volumes were measured when SF-CM were collected. The levels of various soluble factors were statically analyzed. The data shown are the means ± SD (pg/ml/10^5 cells) of four determinations.

from gastric cancer cells supplemented with TGF-ß1 receptor inhibitor SB431542. Forty-eight hours later, apoptosis was quantified by the following two methods: 1) Fluorescent methods by AO/EB staining to quantify alive, early apoptotic, late apoptotic, and necrotic cells. Briefly, both adherent and non-adherent cells were harvested and washed twice with PBS, and then immediately treated with acridine orange (100 μg/ml) for 5 min and ethidium bromide (100 μg/ml) for 5 min. Cells were then examined under fluorescence microscope. Cells containing normal nuclear chromatin will exhibit green nuclear staining. Cells containing fragmented nuclear chromatin (apoptotic cells) will exhibit orange to red nuclear staining. 2) Flow cytometry methods. Briefly, both adherent and non-adherent cells were harvested, re-suspended in PBS at a concentration of $1x10^6$/ml and then fixed in 2-ml methanol for 30 min at 4˚C. After fixation, cells were re-suspended with PBS, treated with RNAse (50 μg/ml), and re-suspended in PBS containing propidium iodide (PI) (0.05 mg/ml in 3.8 mol/l natrium citrate) at room temperature for 30 min. Then, the HPMCs were spun down and re-suspended in 1-ml PBS and analyzed using flow cytometry according to the manufacturer's instructions. The cells in the sub-diploid peak were considered as apoptotic cells.

Adhesion assay. Mesothelial cells/well ($8x10^4$) were incubated in flat-bottomed 96-well plates that were previously coated with 0.5% gelatin (Sigma Chemical Co., UK) and cultured to sub-confluence. Gastric carcinoma cells were detached with non-enzymatic cell dissociation solution (Sigma Chemical Co.), washed twice and incubated with DMEM containing 5 μM Calcein-AM (Calbiochem) at 37˚C for 30 min. Calcein-AM is a cell-permeable, non-fluorescent and hydrophobic compound, which is rapidly hydrolysed by cytoplasmic esterases, releasing the membrane-impermeable hydrophilic and highly fluorescent calcein (15,16). Then, $4x10^4$ labeled tumor cells were washed 3 times with serum complete medium and added to the mesothelial cells for co-culture at 37˚C for 3 h. Wells were then washed 3 times with 200 μl of serum complete medium to remove the non-adherent tumor cells. The remaining adherent tumor cells were measured for fluorescence intensity using a cyto-fluorometer (Titertek Fluoroskan II, Flow Laboratories, McLean, VA, USA). Another plate was seeded with labeled tumor cells for 3 h as positive control and its fluorescence intensity was considered as 100%.

The adhesion percentage was calculated as: 100x fluorescence intensity of the experimental group/positive control. The experimental groups are: 1) mesothelial cells incubated with gastric cancer cells; 2) mesothelial cells pre-treated with TGF-ß1 (100, 50, 10 ng) for 2 h, and then incubated with gastric cancer cells; 3) mesothelial cells pre-treated with TGF-ß1 receptor inhibitor SB431542 for 2 h, and then incubated with gastric cancer cells; 4) gastric cancer cells incubated without mesothelial cells were prepared as a



Group	SGC-7901+HPMC	SGC-7901	HPMC
TGF-β1 (pg/ml/10^5cells)	622.04±31.10	478.71±24.59	147.15±8.46

Figure 2. The level of TGF-ß1 expression before and after tumor-mesothelial co-culture. Mesothelial cells were incubated in flat-bottomed 96-well plates and cultured to sub-confluence. SGC-7901 cells were added to the mesothelial cells and then co-incubated for 72 h. TGF-ß1 level was then measured by ELISA. The image shows the level of TGF-ß1 expression in supernatants from HPMC (open bars) or SGC-7901 (open bars) or co-culture (closed bars). Bars represent mean ± SD of three independent experiments (four replicates per experiment), P<0.05 as compared to each group.

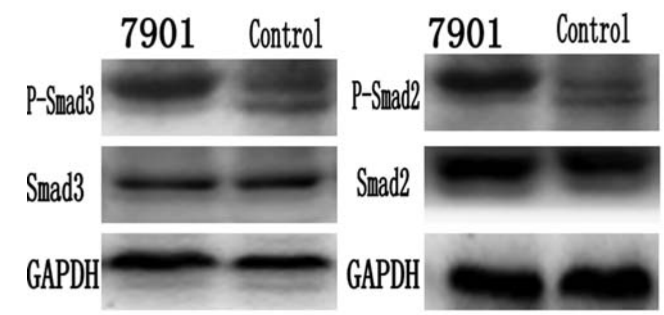


Figure 3. Western blot analyses of TGF-ß1/smad2, 3 proteins. Mesothelial cells were treated for 24 h with either gastric cancer cells SGC-7901 and serum-free DMEM.

positive control group. All the experiments were repeated 3 times.

Statistical analysis. All data are expressed as mean ± SD. Statistical analysis was performed using the Student's t-test. P<0.05 was considered as significant.

Results

Enzyme-linked immunoassay (ELISA). We examined the levels of various soluble factors, including TGF-ß1, IL-8, uPA, VEGF, KGF, MMP-7, MMP-9, HGF, FGF, and EGF from multiple cancer cell lines including MKN-45, MKN-1, SGC-7901, BGC-823, MGC-803 and a human peritoneal mesothelial cell line HMrSV5. Interestingly, all five gastric cancer cell lines showed abundant levels of TGF-ß1 (Fig. 1). In addition, we also observed a resonable level of TGF-ß1 from HPMCs cells. This result indicated that the TGF-ß1 pathway might be active in the normal HPMC biological functions (Fig. 1).

It is widely accepted that TGF-ß1 plays a critical role in tumor invasion and metastasis. We then further investigated the role of TGF-ß1 in the reciprocal interaction between gastric tumor cells and HPMCs. We co-cultured both gastric tumor cells and HPMCs cells for 72 h and found that TGF-ß1 expression was greatly increased in the co-culture system compared to individual culture condition (Fig. 2). The TGF-ß1 level in co-culture was 4 times higher when compared to HPMCs cell culture alone. This indicates that TGF-ß1 may be actively involved in the reciprocal communication between gastric tumor and mesothelial cells, and both

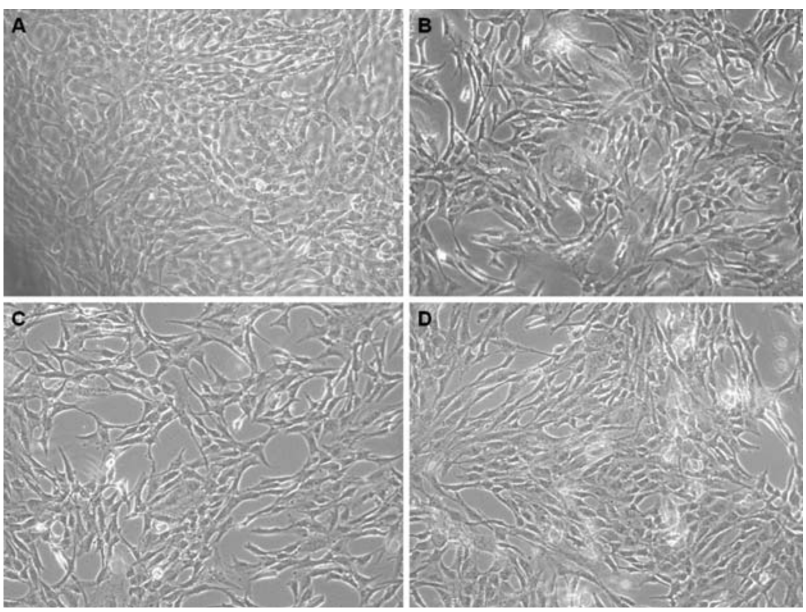
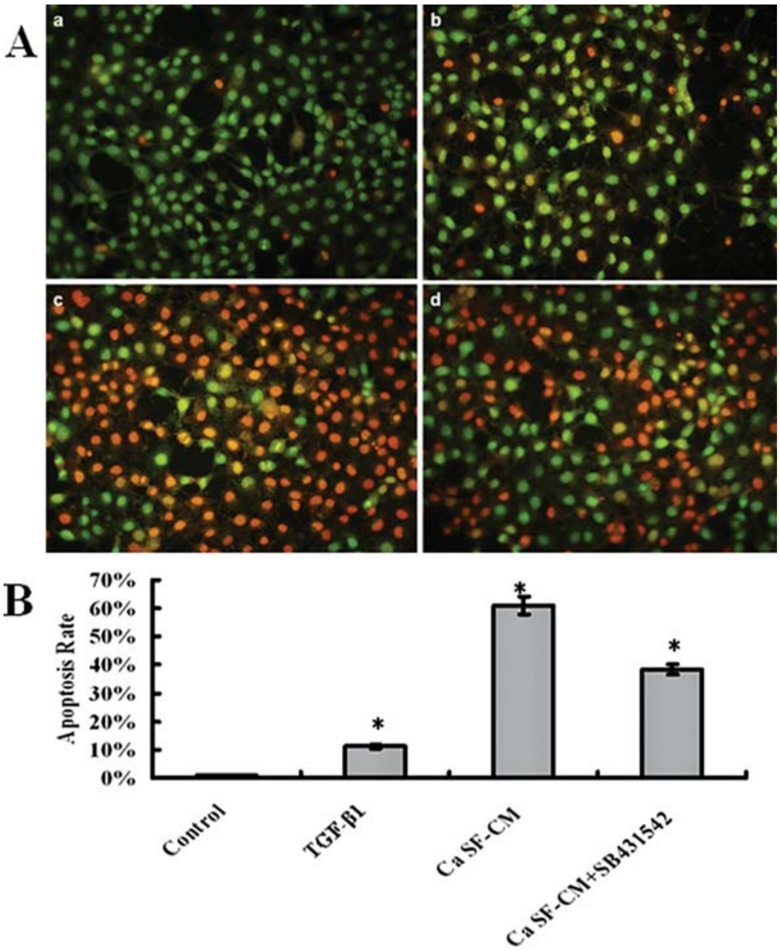


Figure 4. Morphological changes in human peritoneal mesothelial cells under phase contrast microscopy (x40). (A) Morphology of mesothelial cells cultured in serum-free DMEM; (B) Exfoliation and naked areas of mesothelial cells after treatment with 100 ng/ml TGF-ß1; (C) Morphological changes in mesothelial cells after treatment with gastric cancer cells; (D) Morphological changes were partly suppressed in mesothelial cells after treatment with gastric cancer cells + TGF-ß1 receptor inhibitor SB431542 (IC_{50} 94 nmol/l).



Group	Control	TGF-β 1 (100ng/ml)	Cancer SF-CM	Cancer SF-CM+SB431542
Apotosis Rate %	0.7±0.16	11.11±0.71	60.98±3.07	38.46±1.92

Figure 5. Gastric cancer released factors, especially TGF-ß1, induce apoptosis on mesothelial cells. (A) Apoptosis was determined by fluorescent microscope. Mesothelial cells were treated for 48 h with serum-free DMEM (a), 100 ng/ml TGF-ß1 (b), gastric cancer cells SGC-7901 (c), gastric cancer cells + TGF-ß1 receptor inhibitor SB431542 (d). The cells were stained by AO/EB. Cells containing normal nuclear chromatin exhibit green nuclear staining. Cells containing fragmented nuclear chromatin exhibit orange to red nuclear staining. (B) Apoptosis was quantified by flow cytometry. Percentages of HPMC in sub-G1 group (apoptosis) after treatment with serum-free DMEM, TGF-ß1, SF-CM from gastric cancer cells, gastric cancer cells + TGF-ß1 receptor inhibitor SB431542 for 48 h. P<0.01 as compared to control.

autocrine and paracrine TGF-ß1 may contribute to the disrupted mesothelial integrity, therefore facilitating peritoneal invasion/metastasis.

Western blotting. To further investigate the role of abundant TGF-ß1 in gastric cancer cell infiltration through mesothelial cells, we next examined the activation of TGF-ß/Smad pathway, which is known to be involved in promoting cell invasion. Interestingly, we observed a dramatic upregulation of both phosphorylated-smad2 and phosphorylated-smad3 levels after HPMCs co-culture with gastric cancer cells SGC-7901, while the total smad2 and smad3 levels remained similar (Fig. 3). These results clearly demonstrated that the TGF/Smad pathway is highly activated when mesothelial cells were co-cultured with gastric tumor cells, and which may contribute to the damaging effect on mesothelial cell function and integrity in preventing gastric tumor invasion/metastasis.

Morphological alterations of HPMCs after co-culture with gastric tumor cells. In addition, we observed dramatically different morphology of HPMCs after co-culture with gastric tumor cells. In particular, the non-treated HPMCs exhibited the typical polygonal and cobblestone-like morphology (Fig. 4A). In contrast, cells treated with gastric cancer cells or TGF-ß1 for 24 h underwent significant morphological alterations (Fig. 4B and C). Cells were spindle-like with reduced cytoplasm volume, and with a scattered distribution indicating reduced cell-cell adhesion. Moreover, some cells rounded up and detached from the culture dish, leading to exposed surface area (Fig. 4B and C). Interestingly, the above morphological alterations were effectively inhibited by the TGF-ß1 inhibitor treatment (Fig. 4D).

Detection of apoptosis. We then characterized the effect on apoptosis of HPMC cells after co-culture with gastric tumor cells. In control group, serum-free DMEM was not able to

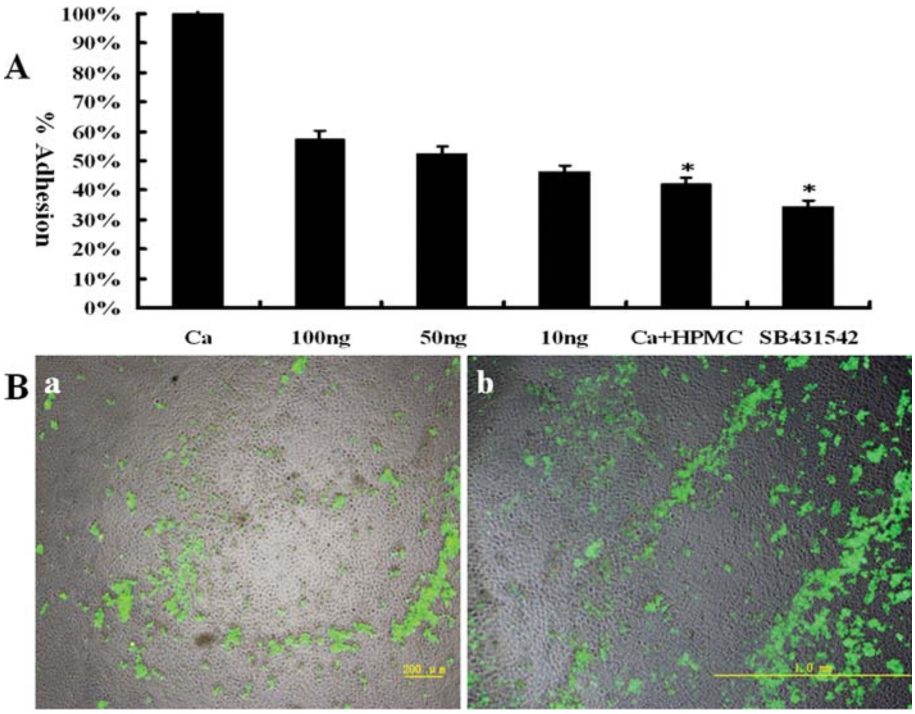


Figure 6. (A) Mesothelial cells were incubated with TGF-ß1 (100, 50, 10 ng/ml), or TGF-ß1 receptor inhibitor SB431542 for 2 h prior to the addition of cancer cells. Adhesion was evaluated after 3 h of incubation. Blocking TGF-ß1 by SB431542 reduced the ability of tumor cells adhesion to the mesothelial cells. $P<0.01$ as compared to each other. (B) Adhesion of gastric cancer cells to mesothelial cells with (a) or without (b) TGF-ß1 receptor inhibitor SB431542. Fluorescently labeled gastric cancer cells were overlaid on mesothelial cells and incubated for 3 h. Gastric cancer cells containing Calcein-AM exhibit green staining. The background cells are mesothelial cells.

induce apoptosis of mesothelial cells (Fig. 5A, a). After HPMC line HMrSV5 cells were treated with gastric cancer cell conditional medium or TGF-ß1 for 48 h, we observed significant morphological indications of cell apoptosis, such as condensation of chromatin, nuclear fragmentation and apoptotic bodies, by AO/EB staining (Fig. 5A, b and c). Interestingly, TGF-ß1 receptor inhibitor SB431542 could partly suppress the apoptotic morphological changes (Fig. 5A, d). The above apoptotic effect of co-culture and TGF-ß1 treatment was also confirmed by flow cytometric analysis. As is shown in Fig. 5B, the apoptotic rate of mesothelial cells incubated with gastric cancer conditional medium and TGF-ß1 was 60.98±3.07% and 11.11±0.71%, respectively. In contrast, apoptosis in control group was significantly lower at 0.70±0.16%. Most importantly, TGF-ß1 receptor inhibitor SB431542 treatment dramatically suppressed apoptosis induced by gastric tumor cell conditional medium. This clearly indicated that the apoptotic effect on mesothelial cells by gastric tumor cells are contributed by TGF-ß1 upregulation and the TGF/Smad pathway activation.

Adhesion assay. To determine the effect of TGF-ß1 on tumor-mesothelial cell adhesion, a monolayer of mesothelial cells were incubated with TGF-ß1 (100, 50, and 10 ng/ml), gastric tumor cell conditional medium, or both gastric tumor cell conditional medium and TGF-ß1 receptor inhibitor SB431542 for 2 h. Then fluorescence labeled tumor cells were added to the monolayer of mesothelial cells for another 3 h. After rinsing away the non-adherent tumor cells from the co-culture, the fluorescence intensity was measured to represent the remaining adherent tumor cells on the mesothelial layer. We found that TGF-ß1 can promote the adhesion of gastric tumor cells to mesothelial cells in a dose-dependent manner (Fig. 6A). We also found that less gastric tumor cells were able to adhere to mesothelial cell monolayer when compared to the control non-coated plate (42.19±2.10% compared to 100% in the control). The presence of TGF-ß1 receptor inhibitor SB431542 significantly inhibited the adhesion of tumor cells to mesothelial cells, indicating that the TGF-ß1 produced by autocrine/paracrine pathways during co-culture plays a critical role in promoting the tumor cell adhesion to mesothelial cells (Fig. 6A and B). There is a positive correlation between dose of TGF-ß1 and adhesion.

Discussion

According to the 'seed and soil' theory; metastases only occur when tumor cells encounter a favorable micro-environment where they can survive and proliferate rapidly. It has been previously reported that healthy mesothelial cells can prevent tumor cell invasion and metastasis by providing an intact barrier. Interestingly, tumor cells are also known to secrete various factors to induce damage or apoptosis in mesothelial cells and the disrupted mesothelial cells will then promote tumor cells invasion (17-19). We hypothesize that the damaged peritoneum may provide such a micro-environment for scirrhoid gastric cancer cell metastasis. We have previously demonstrated that cancer cells can secrete soluble factors into abdominal cavity to induce damage and apoptosis of peritoneal mesothelial cells (20,21). Then the mesothelial cells became hemispherical, and detached from the mesothelial layer, leading to the expose of sub-mesothelial connective tissue. This injured peritoneum may function as a favorable environment for peritoneal metastasis (22,23). However, what these soluble factors secreted by cancer cells are that trigger the damaging cascade remain unclear.

Over the past decade, significant progress has been made to better understand TGF-ß signaling in both physiological and pathological scenarios (24). In particular, TGF-ß1 has been demonstrated to be a key mediator of fibrosis in both experimental and human peritoneal dialysis-induced mesothelial injury (25,26). Whether the introduction of TGF-ß1 can promote gastric tumor-dissemination and infiltration into peritoneum, and if so whether TGF-ß1 inhibitor can prevent such an event remain poorly understand.

Our study demonstrated a significant level of TGF-ß1 expression in all gastric cancer cell lines we examined. We also observed a decent level of TGF-ß1 in HPMC, which indicates that TGF-ß1 pathway and its related pathways may be involved in normal HPMC biological functions. We observed a dramatic increase of TGF-ß1 from HPMC cells when HPMC cells were co-cultured with gastric tumor cells than the individual HPMC cell culture alone. This indicates that TGF-ß1 pathway may play a role in the reciprocal communication of gastric tumor cells and mesothelial cells, and it potentially contributes to gastric tumor cell invasion through mesothelial cell layer. More importantly, the higher TGF-ß1 expression after co-culture also leads to increased p-smad2 and p-smad3 levels, which clearly indicate the TGF/Smad pathway activation.

It is known that after TGF-ß1 ligand binding with TGF-ß receptors on the cell membrane, the receptor kinase is activated and then leads to receptor Smads (both smad2 and smad3) phosphorylation. The p-smad2/3 will then be translocated into nucleus where they form heteromeric complex with smad4, and functions as transcription factors to regulate various downstream genes expression (27). The TGF/Smad pathway can regulate multiple cellular functions including inhibition and stimulation of cell growth, cell death or apoptosis, and cellular differentiation. In this study, we found that the p-smad2 and p-smad3 levels in HPMCs are significantly elevated, while the total level of smad 2, 3 remain similar, after co-culture with gastric cancer cells SGC-7901. The results indicate that elevated TGF-ß1 expression can lead to TGF/Smads pathway activation in HPMCs.

Considering the significant function of TGF/Smads in regulating cell proliferation and apoptosis, we hypothesized that the TGF-ß1, produced by both autocrine and paracrine via gastric tumor cells and mesothelial cells respectively, can affect mesothelial cell viability. We observed under phase contrast microscope that mesothelial cells became hemispherical and exfoliation occurred when gastric cancer cell or TGF-ß1 were added into mesothelial cell culture. Furthermore, both fluorescent microscopy and flow cytometric analysis confirmed that apoptosis of HPMCs was dramatically increased in response to TGF-ß1 treatment and gastric cancer cell co-culture. This indicated that factors, in particular TGF-ß1, secreted in abdominal cavity by invading cancer cells, induce damage and apoptosis of mesothelial cells, lead to exfoliation, and result in eventual metastasis after the barrier of metastasis is severely disrupted. TGF-ß1 receptor inhibitor SB431542 can partially suppress the above effects, supporting the involvement of TGF pathway

To investigate whether the introduction of TGF-ß1 could enhance tumor-mesothelial adhesion and if TGF-ß1 receptor inhibitor SB431542 could reduce this adhesion, we incubated the mesothelial cells with TGF-ß1, or with cancer cell conditional medium with and without TGF-ß1 receptor inhibitor for 2 h. Then, we added tumor cells for another 3 h to evaluate the adhesion ability to mesothelial cells under various conditions. Interestingly, TGF-ß1 increases tumor-mesothelial adhesion in a dose-dependent manner. Consistently, the inhibition of TGF/Smad pathway by SB431542 led to a significant decrease of tumor-mesothelial adhesion. The findings are consistent with previous studies that TGF-ß1 may enhance tumor-mesothelial cell adhesion (28,29).

It should be noted that the effects of gastric cancer were stronger than TGF-ß1 alone; this indicates that some other soluble factors secreted by gastric cancer cells may also contribute to the effect. However, blocking TGF-ß1 pathway activation can partially inhibit these effects. This indicated that while various soluble factors contributed to tumor-mesothelial interaction, TGF-ß1 might be a key regulator. Further studies are required to elucidate the detailed mechanisms of the TGF-ß/Smad signaling activation induced by gastric cancer cells.

In conclusion, soluble factors, such as TGF-ß1, produced by autocrine/paracrine in peritoneal cavity can regulate the morphology and behavior of mesothelial cells so that the altered mesothelial cells may provide a favorable environment for the peritoneal dissemination of cancer cells (30-33).

Acknowledgements

This study was supported by National Science Foundation of China. NSFC (30672050, 30873043 and 30901419). We thank Dr Yan Song for technical assistance and Dr Chun-Yu Wang, Dr Jian Zhang, Dr Tong Liu for precious advice.

References

1. Yashiro M, Chung YS, Nishimura S, Inoue T and Sowa M: Fibrosis in the peritoneum induced by scirrhous gastric cancer cells may act as 'soil' for peritoneal dissemination. Cancer 77: 1668-1675, 1996.
2. Paget S: The distribution of secondary growths in cancer of the breast. Lancet 1: 571-573, 1889.
3. Kiyasu Y, Kaneshima S and Koga S: Morphogenesis of peritoneal metastasis in human gastric cancer. Cancer Res 41: 1236-1239, 1981.
4. Attisano L and Wrana JL: Signal transduction by members of the transforming growth factor-beta superfamily. Cytokine Growth Factor Rev 7: 327-339, 1996.
5. Border WA and Noble NA: Transforming growth factor-beta in tissue fibrosis. N Engl J Med 331: 1286-1292, 1994.
6. Ihn H: Pathogenesis of fibrosis: role of TGF-beta and CTGF. Curr Opin Rheumatol 14: 681-685, 2002.
7. Su XH, You ZY, Xu HM and Zhao YL: Relationship between the pathology and TGF-ß1 presented in gastric cancer and peritoneal lavage fluid. J China Med Univ 34: 164-166, 2005.
8. Kosaka K, Yashiro M, Sakate Y, *et al*: A synergistic antitumor effect of interleukin-2 addition with CD80 immunogene therapy for peritoneal metastasis of gastric carcinoma. Dig Dis Sci 52: 1946-1953, 2007.

9. Li ZR, Wang Z, Zhu BH, *et al*: Association of tyrosine PRL-3 phosphatase protein expression with peritoneal metastasis of gastric carcinoma and prognosis. Surg Today 37: 646-651, 2007.
10. Fujiwara Y, Doki Y, Taniguchi H, *et al*: Genetic detection of free cancer cells in the peritoneal cavity of the patient with gastric cancer: present status and future perspectives. Gastric Cancer 10: 197-204, 2007.
11. Foussat A, Balabanian K, Amara A, *et al*: Production of stromal cell-derived factor 1 by mesothelial cells and effects of this chemokine on peritoneal B lymphocytes. Eur J Immunol 31: 350-359, 2001.
12. Nasreen N, Mohammed KA, Hardwick J, *et al*: Polar production of interleukin-8 by mesothelial cells promotes the transmesothelial migration of neutrophils: role of intercellular adhesion molecule-1. J Infect Dis 183: 1638-1645, 2001.
13. Warn R, Harvey P, Warn A, *et al*: HGF/SF induces mesothelial cell migration and proliferation by autocrine and paracrine pathways. Exp Cell Res 267: 258-266, 2001.
14. Heath RM, Jayne DG, O'Leary R, *et al*: Tumor-induced apoptosis: a novel mechanism of peritoneal invasion. Br J Surg 87 (Suppl 1): 14, 2000.
15. Alkhamesi NA, Ziprin P, Pfistermuller K, Peck DH and Darzi AW: ICAM-1 mediated peritoneal carcinomatosis, a target for therapeutic intervention. Clin Exp Metastasis 22: 449-459, 2005.
16. Bellingan GJ, Xu P, Cooksley H, *et al*: Adhesion molecule-dependent mechanisms regulate the rate of macrophage clearance during the resolution of peritoneal inflammation. J Exp Med 196: 1515-1521, 2002.
17. Yashiro M, Chung YS, Inoue T, Nishimura S, Matsuoka T, Fujihara T and Sowa M: Hepatocyte growth factor (HGF) produced by peritoneal fibroblasts may affect mesothelial cell morphology and promote peritoneal dissemination. Int J Cancer 67: 289-293, 1996.
18. Mutsaers SE, Whitaker D and Papadimitriou JM: Stimulation of mesothelial cell proliferation by exudate macrophages enhances serosal wound healing in a murine model. Am J Pathol 60: 681-692, 2002.
19. Michailova KN: Mesothelial lamellar bodies in norm and experimental conditions. Transmission and scanning electron microscopic observations on the peritoneum, pleura and pericardium. Anat Embryol (Berl) 208: 301-309, 2004.
20. Na D, Liu FN, Miao ZF, Du ZM and Xu HM: Astragalus extract inhibits destruction of gastric cancer cells to mesothelial cells by anti-apoptosis. World J Gastroenterol 15: 570-577, 2009.
21. Na D, Liu F, Miao Z, Du Z and Xu H: Destruction of gastric cancer cells to mesothelial cells by apoptosis in the early peritoneal metastasis. J Huazhong Univ Sci Technolog Med Sci 29: 163-168, 2009.
22. Heath RM, Jayne DG, O'Leary R, Morrison EE and Guillou PJ: Tumour-induced apoptosis in human mesothelial cells: a mechanism of peritoneal invasion by Fas Ligand/Fas interaction. Br J Cancer 90: 1437-1442, 2004.
23. Bird SD: Mesothelial primary cilia of peritoneal and other serosal surfaces. Cell Biol Int 28: 151-159, 2004.
24. Yanez-Mo M, Lara-Pezzi E, Selgas R, *et al*: Peritoneal dialysis and epithelial-to-mesenchymal transition of mesothelial cells. N Engl J Med 348: 403-413, 2003.
25. Yang AH, Chen JY and Lin JK: Myofibroblastic conversion of mesothelial cells. Kidney Int 63: 1530-1539, 2003.
26. Wilm B, Ipenberg A, Hastie ND, Burch JB and Bader DM: The serosal mesothelium is a major source of smooth muscle cells of the gut vasculature. Development 132: 5317-5328, 2005.
27. Massague J and Chen YG: Controlling TGF-beta signaling. Genes Dev 14: 627-644, 2000.
28. van Grevenstein WM, Hofland LJ, Jeekel J and van Eijck CH: The expression of adhesion molecules and the influence of inflammatory cytokines on the adhesion of human pancreatic carcinoma cells to mesothelial monolayers. Pancreas 32: 396-402, 2006.
29. Takatsuki H, Komatsu S, Sano R, Takada Y and Tsuji T: Adhesion of gastric carcinoma cells to peritoneum mediated by alpha3beta1 integrin (VLA-3). Cancer Res 64: 6065-6070, 2004.
30. Margetts PJ, Kolb M, Galt T, Hoff CM, Shockley TR and Gauldie J: Gene transfer of transforming growth factor-beta1 to the rat peritoneum: effects on membrane function. J Am Soc Nephrol 12: 2029-2039, 2001.
31. Alkhamesi NA, Ziprin P, Pfistermuller K, Peck DH and Darzi AW: ICAM-1 mediated peritoneal carcinomatosis, a target for therapeutic intervention. Clin Exp Metastasis 22: 449-459, 2005.
32. Yao V, Platell C and Hall JC: Lavage enhances the production of proinflammatory mediators by peritoneal mesothelial cells in an experimental model. Dis Colon Rectum 48: 560-566, 2005.
33. Ren J, Xiao YJ, Singh LS, *et al*: Lysophosphatidic acid is constitutively produced by human peritoneal mesothelial cells and enhances adhesion, migration, and invasion of ovarian cancer cells. Cancer Res 66: 3006-3014, 2006.