Mouse forestomach carcinoma cells immunosuppress macrophages through transforming growth factor-β1

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Abstract. Peritoneal implantation metastasis of gastric cancer cells is associated with poor prognosis. Peritoneal macrophages are the most important immune cells in the abdominal cavity to control tumor metastasis. In the present study, the immunosuppressive effects of mouse forestomach cells on macrophages were examined. Conditioned medium from mouse forestomach cell cultures were used to treat isolated peritoneal macrophages. A colorimetry-based phagocytosis assay was performed to investigate the functional change of macrophages. The alteration of cytokine secretion by macrophages was measured by ELISA assay. Specific markers of macrophage polarization were analyzed by real-time RT-PCR. TGF-β1 signaling was evaluated by western blotting. Neutralization experiments were performed using an anti-TGF-β1 antibody. Conditioned medium reduced the phagocytotic capability of macrophages. Lower TNF-α and IL-1β levels and higher IL-10 and VEGF levels were observed. Real-time RT-PCR showed increased mRNA levels of M2 macrophage markers. Further study revealed that TGF-β1 was significantly elevated in the conditioned medium and TGF-β1 signaling was activated in the macrophages by the treatment of conditioned medium. Neutralization of TGF-β1 reversed the immunosuppressive effects on macrophages. Immunosuppressive macrophages can be induced by conditioned medium from mouse forestomach cell cultures. These effects appeared to occur through the production of TGF-β1 by the tumor cells. Targeted TGF-β1 intervention may help to control peritoneal metastasis of gastric cancers.

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Abbreviations: MFCs, mouse forestomach cells; CM, conditioned medium; TGF, transforming growth factor; TNF, tumor necrosis factor; IL, interleukin; VEGF, vascular endothelial growth factor; iNOS, inducible nitric oxide synthase; CXCL, chemokine (C-X-C motif) ligand; Fizz1, found in inflammatory zone 1; IFN, interferon

Introduction

Gastric cancer is the most common malignant tumor of the digestive tract. It has a poor prognosis and results in frequent death caused by postoperative relapse and metastasis (1,2). Peritoneal implantation metastasis of gastric cancers constitutes nearly 50% of postoperative relapses and is a leading cause of death in patients with gastric cancer (3,4). However, the detailed mechanisms of peritoneal metastasis of gastric cancer have not been fully understood. A better strategy to prevent and treat peritoneal metastasis is also required.

Peritoneal immune cells, including T lymphocytes, neutrophils, natural killer cells and macrophages, are cellular components of innate immunity which protect against tumor cells (5). Peritoneal macrophages are the most important immune cells in the abdominal cavity, and the function of macrophages is critical to prevent the peritoneal implantation metastasis of gastric cancer cells (6). Classically activated M1 macrophages are capable of phagocytosing microorganisms and tumor cells, antigen processing and presentation and producing proinflammatory cytokines (7). Therefore, they are involved in the peritoneal immunity against infection and tumor cell invasion and play a critical role in the cellular immunity against gastric cancer. On the contrary, alternatively activated M2 macrophages display a distinct function from M1 macrophages. M2 macrophages cannot prevent tumor progression, but rather facilitate tumor cell proliferation, angiogenesis and tissue remodeling (7,8). It has been reported that tumor cells can secrete inhibitory cytokines to evade immune surveillance (9). Peritumoral macrophages exhibit alternative activation possibly through the action of various cytokines secreted by tumor cells.

In this study, mouse forestomach cells (MFCs) (10) and isolated peritoneal macrophages were recruited to investigate the immunosuppressive effects of gastric carcinoma cells on macrophages. MFC conditioned medium (CM) was collected and used to treat peritoneal macrophages. The phagocytotic ability, cytokine secretion and M1/M2 macrophage markers were analyzed. Further examination disclosed that transforming growth factor (TGF)-β1 may be the key cytokine through which MFCs modify macrophage functions.

Materials and methods

Isolation of peritoneal macrophages and cell culture. Eight-week-old male C57BL6 mice were purchased from the Institute

of Laboratory Animal Science of the Chinese Academy of Medical Science. Mice were sacrificed by cervical dislocation. Five milliliters of precooled RPMI-1640 medium was injected into the abdominal cavity using a syringe. After injection, gentle massage was performed on the peritoneum to dislodge attached cells. The peritoneal fluid was collected into another syringe and the peritoneal cavity was washed twice. The fluid and wash solution were centrifuged and the macrophages were purified using the adherence method. All the protocols were reviewed and approved by the Animal Care and Use Committee of the Third Military Medical University.

Both macrophages and MFCs were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in a humidified, 5% CO_2 atmosphere. MFCs ($1x10^6$) were plated into a 6-well plate. After culture for 2 days, the supernatant of the MFCs was collected as MFC CM and stored at -20˚C until use. Macrophages were treated with CM alone or together with 1 μg/ml TGF-β1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 days and then underwent subsequent analysis.

Phagocytosis assay. The phagocytotic ability of macrophages was measured by the CytoSelect phagocytosis assay kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's instructions. Zymosan was used as a substrate in this assay. The absorbance of each sample was read at 405 nm.

Measurement of cytokine secretion. After treatment, the supernant of the macrophages was collected and the concentrations of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-10 and vascular endothelial growth factor (VEGF) were measured by ELISA kit (Invitrogen, Carlsbad, CA).

RNA extraction and real-time RT-PCR. Total RNA was isolated from the macrophages by TRIzol reagent (Invitrogen) and first strand cDNA was generated by AMV reverse transcriptase (Takara, Dalian, China) with Oligo dT-Adaptor Primer (Takara). Gene specific primers for inducible nitric oxide synthase (iNOS) (forward, 5'-TTCTGTGCTGTCCCA GTGAG-3'; reverse, 5'-TGAAGAAAACCCCTTGTGCT-3'), chemokine (C-X-C motif) ligand (CXCL) 11 (forward, 5'-CGCCCCTGTTTGAACATAAG-3'; reverse, 5'-CTGCTGA GATGAACAGGAAGG-3'), arginase-1 (forward, 5'-TTTTTC CAGCAGACCAGCTT-3'; reverse, 5'-AGAGATTATCGG AGCGCCTT-3') and found in inflammatory zone 1 (Fizz1) (forward, 5'-CTGGATTGGCAAGAAGTTCC-3'; reverse, 5'-CCCTTCTCATCTGCATCTCC-3') were used for expression analysis by real-time PCR on the ABI 7500 thermocycler (Applied Biosystem, Foster City, CA) using SYBR-Green mix (Applied Biosystem). β-actin (forward, 5'-ATGGAGGGGAAT ACAGCCC-3'; reverse, 5'-TTCTTTGCAGCTCCTTCGTT-3') was used for normalization.

Western blot analysis. After treatment, macrophages were washed with ice-cooled phosphate-buffered saline twice and lysed with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Inc., Rockford, IL). Then, 20 μg of total protein was separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA). After blocking with 5% skim milk, antibodies for Smad2 or

phosphorylated-Smad2 (p-Smad2) (Santa Cruz Biotechnology) were incubated with the membranes overnight at 4˚C. Horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) was used to detect the protein levels.

Statistical analysis. All values are expressed as the means ± SEM. Statistical analyses were performed using the Student's t-test. Differences having a p-value <0.05 were considered statistically significant.

Results

MFC CM induces immunosuppression of macrophages. Mouse peritoneal macrophages were isolated, and flow cytometric assay revealed that >90% of the total cells were CD68-positive cells, which indicated that the isolated cells were macrophages (data not shown). To determine the indirect effects of MFCs on macrophages, the MFC CM was used to the treat macrophages for 2 days. Phagocytosis assay was performed to evaluate the functional change in macrophages. As shown in Fig. 1A, the CM significantly reduced the phagocytotic capability of macrophages. Macrophages also secreted much less TNF-α and IL-1β after CM treatment (Fig. 1B). Meanwhile, secretion of IL-10, which is an immunosuppressive cytokine, was elevated nearly 3-fold compared to the control cells. Notably, VEGF, which is able to promote angiogenesis and accelerate tumor growth, was also greatly increased by CM treatment. These data suggest that MFC-derived soluble factors generate a microenvironment which suppresses the innate immunity of macrophages and induces angiogenesis, thus supporting tumor progression.

MFC CM induces macrophage polarization. The immunosuppressive state of macrophages is always accompanied by the increase in alternatively activated macrophages, also called M2 macrophages. Therefore, real-time RT-PCR was performed to examine the markers of M1 and M2 macrophages. As shown in Fig. 2, CM down-regulated the mRNA levels of M1 macrophage marker, iNOS, but not CXCL11. The M2 macrophage markers, arginase-1 and Fizz1, were increased by treatment of CM for 2 days, indicating CM stimulated the M2 macrophage polarization.

Increased level of TGF-β1 in CM activates TGF-β1 signaling in macrophages. Many different types of cytokines respond to macrophage immunosuppression. We further measured the concentration of these cytokines in the CM, including macrophage inhibitory cytokine-1, soluble colony-stimulating factor, TGF-β1, IL-4 and IL-10. Among them, only the TGF-β1 level was dramatically elevated in the CM (3.05±0.58 pg/ml in RPMI-1640 medium, 107.56±4.82 pg/ml in CM, n=4, p<0.01 and data not shown). Next, the downstream TGF-β1 signaling was examined by western blotting. As shown in Fig. 3, CM treatment increased the expression level of p-Smad2. Moreover, when macrophages were treated with CM and the TGF-β1 antibody together, the increased level of p-Smad2 was significantly diminished.

Neutralization of TGF-β1 restores macrophage functions. Since CM was collected 2 days after culturing with MFCs,
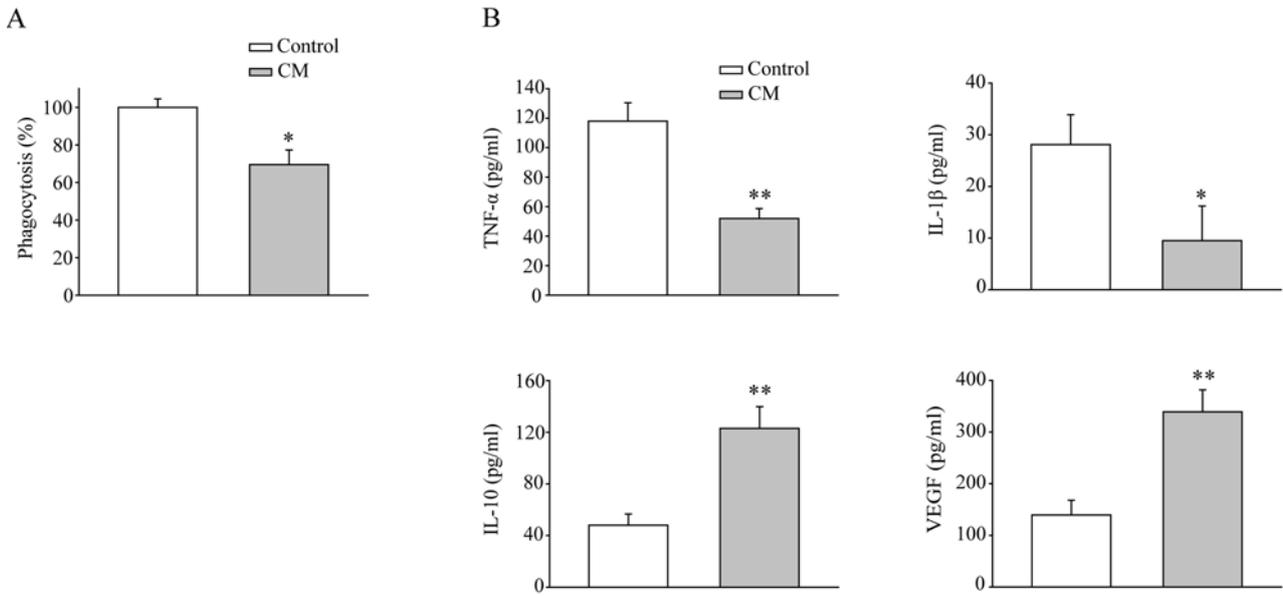
Figure 1. Conditioned medium (CM) induces immunosuppressive macrophages. (A) Isolated peritoneal macrophages were treated with CM for 2 days, and the phagocytosis assay was performed. (B) Concentrations of cytokines secreted by macrophages were measured by ELISA. $^{*}p<0.05$; $^{**}p<0.01$ vs. control, n=3-5.
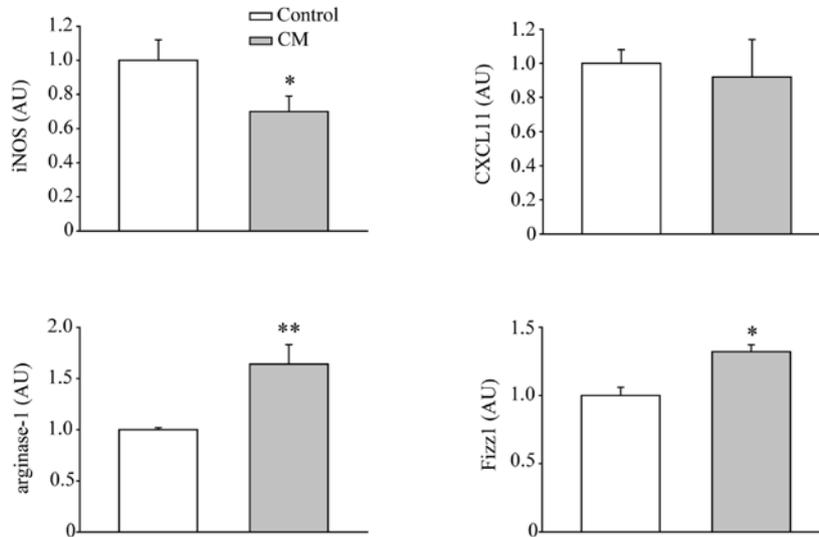
Figure 2. Conditioned medium (CM) induces M2 macrophage polarization. After treatment of CM for 2 days, RNA was extracted and gene expression of markers of M1 and M2 macrophages were analyzed by real-time RT-PCR with specific primers. $^{*}p<0.05$; $^{**}p<0.01$ vs. control, n=3. AU, arbitrary unit.

the nutrient contents and growth factors in the CM may have been depleted due to MFC consumption, consequently interfering with the macrophage functions. To further confirm the specific role of TGF-β1 in the inhibitory effects of CM on macrophages, the TGF-β1 antibody was added to the CM to neutralize TGF-β1. The phagocytosis assay revealed that the TGF-β1 antibody reversed the suppressive effects of CM on macrophages (Fig. 4A). The effects of CM on cytokine secretion was also blocked by the TGF-β1 antibody (Fig. 4B). These data suggest that TGF-β1 is the key cytokine secreted by MFCs to induce immunosuppressive macrophages.

Discussion

In the present study, we demonstrated that the immune functions of isolated peritoneal macrophages was restrained by CM, which contained inhibitory cytokines secreted by MFCs. The macrophages treated with CM showed weaker phagocytosis, less TNF-α and IL-1β production, increased secretion of IL-10 and VEGF and gain of M2 macrophage phenotypes. Among the different cytokines, the level of TGF-β1 in the CM was greatly increased and the TGF-β1 signaling was activated in the macrophages, evidenced by the phosphorylation of Smad2.
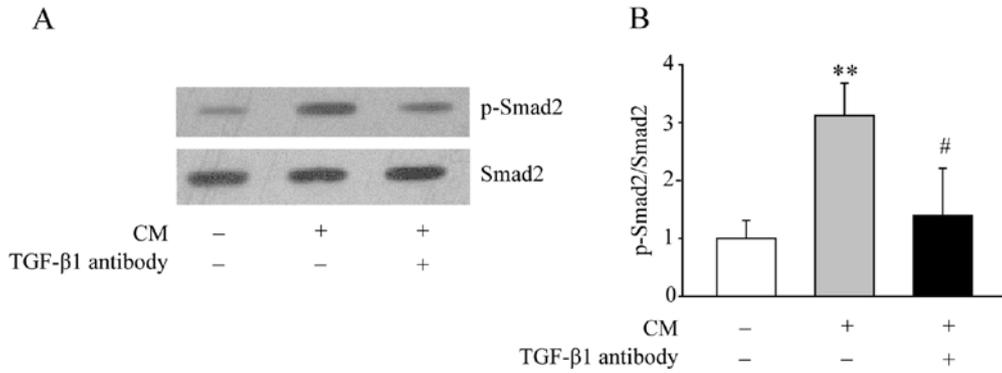
Figure 3. TGF-β1 signaling is activated in the macrophages by conditioned medium (CM) treatment. (A) Macrophages were treated with CM alone or together with the TGF-β1 antibody. The protein levels of Smad2 and phosphorylated Smad2 were examined by western blotting. (B) Densitometric analysis of the western blotting data. **p<0.01 vs. control; $^{#}$p<0.05 vs. CM-treated group, n=3.
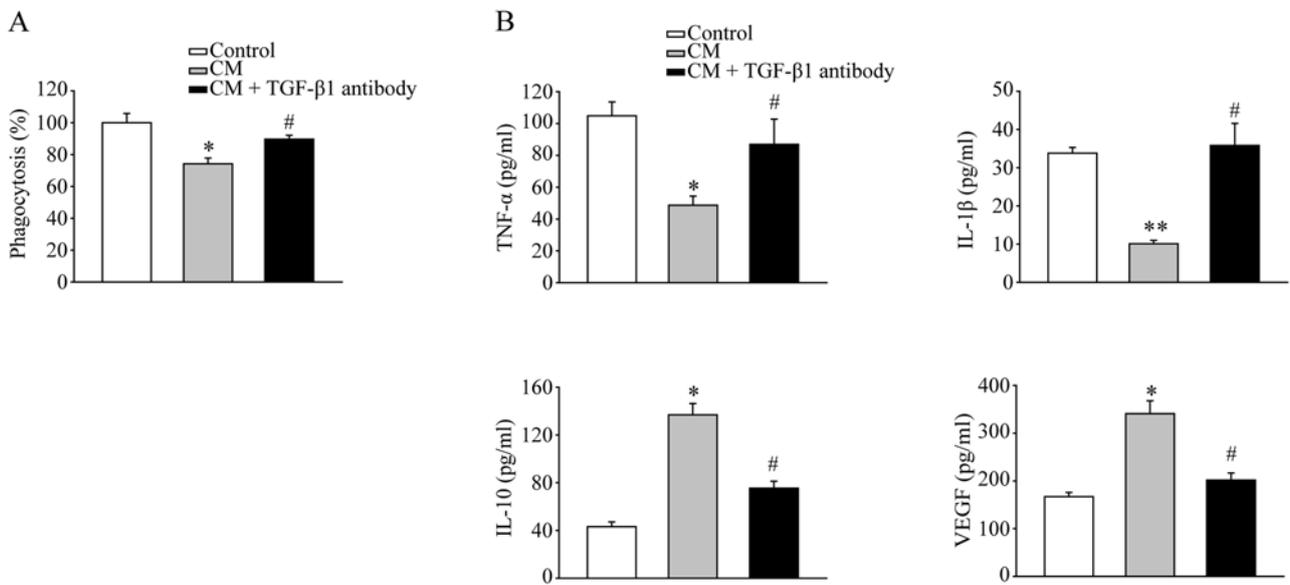
Figure 4. Inhibitory effects of conditioned medium (CM) was alleviated by the TGF-β1 antibody. Macrophages were treated with CM alone or together with the TGF-β1 antibody. All experimental procedures were the same as that described in Fig. 1 legend. *p<0.05; **p<0.01 vs. control; $^{#}$p<0.05 vs. CM-treated group, n=4-5.

Neutralization of TGF-β1 by its antibody helped macrophages retain their functions.

Macrophages are characterized by their remarkable versatility, heterogeneity and plasticity. They can response to different cytokines and certain microbial products that are present in the microenvironment (11). Activated macrophages induced by interferon (IFN)-γ, either alone or in combination with LPS, were found to produce a large amount of toxic agents, such as nitric oxide and reactive oxygen species, with strong antigen presentation capability and they further activated type I immune response (7,8,11). Macrophages which can be alternatively activated by IL-4 and IL-10, are known as M2 macrophages (12). M2 macrophages restrict inflammation and type I adaptive immunity, scavenge residues, promote angiogenesis and participate in tissue remodeling and repair (7). In the present study the increased expression levels of arginase-1 and Fizz1, as well as reduced iNOS expression, indicated the polarization

of M2. However, the M1 macrophage marker CXCL11, which is an IFN-γ inducible gene (13), did not show a difference between the two groups. Several other types of tumors are also capable of inducing M2 macrophages and promoting tumor progression, including glioma cancer (14), breast cancer (15) and hepatocellular carcinoma (16).

Our data suggest that TGF-β1 played a central role in the CM to regulate macrophage functions. Blocking TGF-β1 signaling by its antibody decreased p-Smad2 expression and antagonized the immunosuppressive effects of the CM. TGF-β1 is a member of a class of multifunctional polypeptide growth factors that play important regulatory roles in cell proliferation and differentiation, extracellular matrix production, angiogenesis, apoptosis and the immune system. TGF-β1 regulates cellular processes by binding to its cell-surface receptors and initiates intracellular signaling by phosphorylating several transcription factors known as Smads (17). TGF-β1 has dual

roles during tumorigenesis, both as a tumor suppressor and a tumor promoter. In the early stage, TGF-β1 controls cell growth and cell cycle progression. As tumor cells acquire certain genetic and epigenetic changes in the genome, they switch the TGF-β1 response from inhibition of proliferation to promotion of growth, motility and invasion (18). The interference of phagocytosis by MFC-derived TGF-β1, together with reduced levels of proinflammatory cytokines and elevated levels of anti-inflammatory cytokines, contribute to the escape of immune surveillance. Moreover, the CM induced higher VEGF secretion from the macrophages, suggesting that macrophages ameliorate tumor growth by enhanced angiogenesis. In gastric cancer patients, an elevated serum TGF-β1 was observed and correlated with venous invasion (19). In addition, TGF-β1 receptor inhibitors down-regulated the invasion, migration and epithelial-to-mesenchymal transition of scirrhous gastric cancer cells, suggesting the autocrine role of TGF-β1 during tumorigenesis (20). Approaches targeting TGF-β1 signaling may have beneficial effects on both tumor cells and macrophages.

In summary, the secreted factors by MFCs were able to induce immunosuppression of macrophages, thus avoiding immune restrictions and transforming macrophages into the tumor-promoting phenotype. These effects were mostly, if not totally, through TGF-β1 secretion. The present study provided preliminary *in vitro* evidence of the central role of TGF-β1 in the interaction between MFCs and peritoneal macrophages. The components of TGF-β1 signaling may be promising candidates for the prevention and management of the peritoneal metastasis of gastric cancers.

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