Low-dose paclitaxel suppresses the induction of M2 macrophages in gastric cancer

TAKAHISA YAMAGUCHI[1], SACHIO FUSHIDA[1], YASUHIKO YAMAMOTO[2], TOMOYA TSUKADA[1],
JUN KINOSHITA[1], KATSUNOBU OYAMA[1], TOMOHARU MIYASHITA[1], HIDEHIRO TAJIMA[1],
ITASU NINOMIYA[1], SEIICHI MUNESUE[2], AI HARASHIMA[2], SHINICHI HARADA[3],
HIROSHI YAMAMOTO[2] and TETSUO OHTA[1]

[1]Department of Gastroenterologic Surgery, Division of Cancer Medicine,
[2]Department of Biochemistry and Molecular Vascular Biology and [3]Center for Biomedical Research and
Education, Kanazawa University Graduate School of Medical Sciences, Kanazawa 920-8641, Japan

Abstract. Tumor-associated macrophages of the M2 pheno-type promote tumor proliferation and are associated with a poor prognosis in patients with various malignancies, including gastric cancer with peritoneal dissemination. The present study assessed whether paclitaxel (PTX) suppresses M2 macrophages, by acting as a Toll-like receptor 4 (TLR4) agonist. Macrophages derived from the THP-1 monocytic cell line and peripheral blood mononuclear cell (PBMC)-derived macrophages were cultured with gastric cancer cells in medium containing PTX, at a concentration that did not affect cell proliferation. The effects of PTX on macrophage expression of CD204, a marker of M2 macrophages and NOS2, a marker of M1 macrophages, was evaluated by western blot-ting. The ability of PTX to stimulate intranuclear translocation of NF-κB was determined by evaluating the expression of the p65 subunit of NF-κB. In THP-1 macrophages, low-dose PTX (1 and 5 nM) inhibited the expression of CD204, enhanced the expression of NOS2, and significantly suppressed the phos-phorylation of STAT3, which is essential for the M2 phenotype. Low-dose PTX also inhibited CD204 expression in primary macrophages derived from PBMCs. PTX treatment of THP-1 macrophages for 1 h induced marked intranuclear translocation of NF-κB p65. Low-dose PTX inhibited the M2 phenotype and induced the M1 phenotype via TLR4 signaling, suggesting that low-dose PTX can alter the macrophage phenotype, whereas clinical doses can kill cancer cells. These results suggest that the anticancer effects of PTX are due both to its cytotoxic and immunomodulatory activities.

Correspondence to: Dr Sachio Fushida, Department of Gastroenterologic Surgery, Division of Cancer Medicine, Kanazawa University Graduate School of Medical Sciences, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan
E-mail: fushida@staff.kanazawa-u.ac.jp

Introduction

Worldwide, gastric cancer is among the most common types of cancer, and one of the most frequent causes of cancer-related deaths (1). Peritoneal dissemination, the most frequently observed metastatic pattern of gastric cancer, is a critical indicator of poor patient prognosis, and is the main contributor to the failure of radical gastrectomy in patients with advanced gastric cancer (2,3). Despite developments in systemic and intraperitoneal chemotherapy, only a minority of patients with advanced disease respond to treatment (4-8), indicating the need for novel strategies, in addition to conventional surgery and chemotherapy, to treat this disease.

The connections between tumor cells and their surrounding microenvironment play important roles in tumor initiation and progression. The tumor microenvironment contains numerous types of cells, including endothelial cells, fibroblasts, lympho-cytes and macrophages (9-12). Tumor-associated macrophages (TAMs) can be classified into two phenotypes: M1, or classi-cally activated macrophages, and M2, or alternatively activated macrophages. M2 macrophages are involved in tissue remod-eling, the promotion of angiogenesis, and the suppression of adaptive immunity, as well as being involved in promoting tumor growth (13,14). The density of M2 macrophages has been associated with poor prognosis in patients with glioma, lymphoma and intrahepatic cholangiocarcinoma (15-17). TAM infiltration into gastric cancer tissue was found to positively correlate with the depth of invasion, nodal status and clinical stage (18). During peritoneal dissemination, we previously reported that a large number of M2 macrophages existed in the peritoneal cavity of gastric cancer patients with perito-neal dissemination and contribute to tumor progression (19). Therefore, M2 macrophages are considered promising targets in patients who have gastric tumors with peritoneal dissemination.

Paclitaxel (PTX) is an antineoplastic agent derived from the bark of the Pacific yew tree, *Taxus brevifolia*, and stabilizes polymerized microtubules and enhances microtubule assembly. Low and high doses of PTX arrest the cell cycle in the G0/G1 and G2/M phases, respectively, with

both leading to cell death (20,21). Clinically, PTX has been shown to be effective in treating patients with ovarian (22), non-small cell lung (23) and breast (24) cancers, and may be effective in patients with advanced or recurrent gastric cancer and peritoneal dissemination (25).

Although PTX is regarded as an antitumor agent, it may also act on other cell types. For example, PTX was found to significantly suppress transforming growth factor-β (TGF-β)/Smad signaling in human peritoneal cells by inhibiting Smad2 phosphorylation and reducing stromal fibrosis (26). At low concentrations, PTX has antiproliferative and antimigratory effects on vascular smooth muscle and endothelial cells (27,28), with PTX-eluting stents widely used in patients with cardiovascular stenosis (29). Furthermore, low-dose of PTX was shown to block tumor-induced polarization of conventional dendritic cells (DCs) into immunosuppressive regulatory DCs (30). At non-cytotoxic concentrations, PTX was found to block the immunosuppressive potential of myeloid-derived suppressor cells (MDSCs) in melanoma models (31). However, it has not yet been determined whether PTX modulates TAMs of the M2 phenotype, which contribute to tumor progression. PTX is an agonist of Toll-like receptor 4 (TLR4), a macrophage molecule involved in the innate immune system (32). TLR4 activation is a key to the induction of M1 macrophages and the possible suppression of M2 macrophages (33). We hypothesized that PTX can suppress the induction of M2 macrophages and upregulate M1 macrophages. The present study, therefore, evaluated the effects of low-dose PTX on induction of the M2 phenotype in macrophages derived from the THP-1 monocytic cell line and in primary macrophages derived from peripheral blood mononuclear cells (PBMCs).

Materials and methods

Cell lines and cell culture. The human monocytic THP-1 cell line was obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan) and cultured in RPMI-1640 medium (Life Technologies, Co., Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Nichirei Biosciences, Inc., Tokyo, Japan), 100 IU/ml penicillin, and 100 mg/ml streptomycin (Life Technologies, Co.). THP-1 cells were treated with 320 nM phorbol-12-myristate-13-acetate (PMA; EMD Millipore, Billerica, MA, USA) for 24 h to induce macrophages and washed three times with phosphate-buffered saline (PBS) to remove PMA. Monocytes were isolated from a healthy human donor, who provided written informed consent, in accordance with the protocols approved by the Ethics Committees at Kanazawa University Hospital (study no. 1463). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Peripheral blood was collected from the donor into conical tubes containing 1-2 ml heparin and mixed with an equal volume of saline. PBMCs were isolated using LymphoPrep tubes (Axis-Shield, Dundee, Scotland), according to the manufacturer's protocol, and cultured in RPMI-1640 supplemented with 10% heat-inactivated FBS, penicillin and streptomycin. After 24 h, the non-adherent cells were removed by gentle aspiration, and the adherent cells were regarded as monocytes. To induce M2 macrophages, these monocytes were cultured with monocyte colony stimulating factor (M-CSF; 100 ng/ml; Wako, Tokyo, Japan) for up to 5 days, followed by treatment with interleukin (IL)-4 (20 ng/ml; PeproTech, Rocky Hill, NJ, USA), IL-10 (20 ng/ml; MACS, Cambridge, MA, USA) and IL-13 (20 ng/ml; R&D Systems, Inc., Minneapolis, MN, USA) for 24 h (34). The human gastric cancer cell lines, TMK-1 (poorly differentiated adenocarcinoma) and MKN45 (poorly differentiated adenocarcinoma), were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured as described for THP-1 cells. After being grown to confluence, the cells were harvested by treatment with 0.25% trypsin/EDTA (Life Technologies, Co.). The cells were seeded in gelatin-coated 75-cm^2 flasks (BD BioCoat, San Jose, CA, USA) and cultured in 10 ml of medium at 37˚C in a humidified atmosphere of 5% CO_2 in air.

PTX. PTX was purchased from Bristol-Myers Squibb Co. (Tokyo, Japan), dissolved in distilled water at appropriate concentrations and stored at -20˚C until used.

Cell viability assay. The viability of THP-1 macrophages and PBMC-derived M2 macrophages treated with PTX was determined by a standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at 1x10^4/well in 96-well plates and incubated overnight at 37˚C. The supernatants were discarded and replaced with fresh serum-free medium. PTX dissolved in PBS was added to the cell culture medium at concentrations of 0-3,000 nM. After incubation for 24 and 48 h, the supernatants were discarded, MTT solution was added to each well (final concentration, 500 μg/ml) and the cells were incubated at 37˚C for 3 h. The supernatants were removed, 150 μl of dimethyl sulfoxide (Wako) was added to each well, and the absorbance of each at 535 nM was determined using a microplate reader (Bio-Rad 550; Bio-Rad, Tokyo, Japan). The percentage of inhibition was calculated by comparing the density of the drug-treated to the untreated control cells. All experiments were repeated a minimum of three times.

Interactions of THP-1 macrophages, gastric cancer cells and PTX. An *in vitro* co-culture system was used to assess the indirect effects of cancer cells on THP-1 macrophages. Briefly, 5x10^5 THP-1 macrophages were seeded into the lower chamber of each well of a 6-well Transwell dish and incubated in the absence (control) or presence of the same number of MKN45 or TMK-1 cells using 1-μm pore Boyden chambers (BD Falcon, San Jose, CA, USA). To investigate whether PTX modulates THP-1 macrophages, the cells were exposed to 1 and 5 nM PTX at the start of the co-culture.

Treatment of PBMC-derived M2 macrophages with PTX. M2 macrophages derived from PBMC preparations were incubated in the absence or presence of 1, 5 or 10 nM PTX for 48 h, followed by western blot analysis.

Western blotting. Cells were lysed in RIPA buffer [50 mmol/l Tris-HCl (pH 8.0), 150 mmol/l sodium chloride, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 1.0% (w/v) NP-40 substitute (Wako)] containing 1%

protease inhibitor cocktail and 1% phosphatase inhibitor (both from Sigma-Aldrich, Inc., St. Louis, MO, USA). The protein concentration of each lysate was measured using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Whole-cell lysates were boiled in SDS sample buffer and subjected to SDS polyacrylamide gel electrophoresis (Bio-Rad, Philadelphia, PA, USA). Proteins were transferred to polyvinylidene fluoride membranes (Bio-Rad) and blocked with commercial gradient buffer (EzBlock; ATTO, Corp., Tokyo, Japan) at room temperature for 30 min. The membranes were incubated with the following primary antibodies: mouse monoclonal anti-TLR4 IgG, diluted 1:200 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); mouse mono-clonal anti-CD204 IgG, diluted 1:200 (TransGenic, Inc., Kobe, Japan); mouse monoclonal anti-NOS2 IgG, diluted 1:200 (Santa Cruz Biotechnology, Inc.); rabbit monoclonal anti-pSTAT1 IgG, diluted 1:1,000; rabbit monoclonal anti-pSTAT3 IgG, diluted 1:1,000 (both from Cell Signaling Technology, Danvers, MA, USA); mouse monoclonal anti-NF-κB p65 IgG, diluted 1:200 (Santa Cruz Biotechnology, Inc.); rabbit mono-clonal anti-histone H3 IgG, diluted 1:1,000 (Cell Signaling Technology); and mouse monoclonal anti-β-actin IgG, diluted 1:10,000 (Sigma-Aldrich, Inc.). Following incubation with secondary antibodies, the antibody-antigen complexes were detected using an ECL western blotting detection kit (GE Healthcare, Ltd., Tokyo, Japan) and the Light-Capture system (ATTO).

Histology and immunofluorescence. Conditioned media of MKN45 cells were prepared by plating 5×10^6 tumor cells in 10 ml complete RPMI-1640 supplemented with FBS in 75-cm^2 flasks for 48 h, and harvesting and centrifuging the superna-tants. THP-1 macrophages (3×10^5 cells/slide) were seeded onto Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY, USA) for 24 h and exposed to 50% conditioned medium of MKN45 cells for 2 days in the absence or presence of 1 or 5 nM PTX. The cells were fixed in a mixture of methanol and acetone (1:1) for 15 min, incubated with normal goat serum (5% in PBS) to block non-specific binding and incubated overnight at 4°C with mouse monoclonal anti-CD204 antibody, diluted 1:200 (TransGenic, Inc.). The slides were washed in PBS and incubated with goat anti-mouse IgG antibody conjugated to Alexa Fluor 488 (Life Technologies, Co.), diluted 1:400 for 1 h at room temperature. The slides were counterstained with 4',6-diamidino-2-phenylindole (Life Technologies, Co.), diluted 1:2000, to visualize the nuclei and examined under a fluores-cence microscope (Olympus BX51; Olympus, Tokyo, Japan).

NF-κB assay. NF-κB activation was determined by measuring the increased expression of its p65 subunit by western blot-ting. Briefly, THP-1 macrophages (5×10^5 cells/well) were seeded into 6-well plates and cultured in RPMI-1640 medium containing 1% FBS for 24 h. The cells were stimulated with LPS (1,000 ng/ml; Wako), PTX (1 or 5 nM), 50% conditioned medium of MKN45 cells, or 50% conditioned medium of MKN45 cells plus 5 nM PTX for 1 h. The cells were harvested with trypsin-EDTA and centrifuged at 500 x g for 5 min. Nuclear extract was prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's protocol.

Real-time quantitative polymerase chain reaction (PCR). Total RNA was extracted from THP-1 or M2 macrophages using RNeasy Mini kits and treated with RNase-free DNase (both from Qiagen, Germantown, MD, USA). The integrity of isolated RNA was verified by analytical agarose gel electro-phoresis. First stand cDNA was prepared from 2-μg aliquots of DNase-treated RNA using cDNA synthesis kits. Primers for VEGF-A (forward, 5'-CCTTGCTGCTCTACCTCCAC-3' and reverse, 5'-ATGATTCTGCCCTCCTCCTT-3'); VEGF-C (forward, 5'-CACGAGCTACCTCAGCAAGA-3' and reverse, 5'-GCTGCCTGACACTGTGGTA-3'); IL-10 (forward, 5'-GTC ATCGATTTCTTCCCTGTG-3' and reverse, 5'-ACTCATGGC TTTGTAGATGCCT-3'); arginase (Arg)-1 (forward, 5'-TTCTC AAAGGGACAGCCACG-3' and reverse, 5'-TCAAGCAGACC AGCCTTTCT-3'); CD163 (forward, 5'-AGGATGCTGGAGT GATTTGC-3' and reverse, 5'-CCAGCCGTCATCACATAT TG-3'); CD204 (forward, 5'-GGGAACATTCTCAGACCT TG-3' and reverse, 5'-AATCCTCGTGGACCACTTTC-3'); PD-L1 (forward, 5'-GAACTACCTCTGGCACATCCT-3' and reverse, 5'-CACATCCATCATTCTCCCTTT-3'); PD-L2 (forward, 5'-GTCTTGGGAGCCAGGGTGAC-3' and reverse, 5'-TGAAAAGTGCAAATGGCAAGC-3'); and TATA box binding protein (TBP; forward, 5'-TGCACAGGAGCCAAGA GTGAA-3' and reverse, 5'-CACATCACAGCTCCCCACCA-3') were designed using Primer Express software. The expression of each of the above genes was normalized relative to that of 18S rRNA in the same sample. Each PCR mixture for VEGF-A, VEGF-C, IL-10, Arg-1 and TBP contained 2X SYBR-Green Master Mix (PE Biosystems, Foster City, CA, USA), cDNA template, and optimized primer concentrations, diluted to a final volume of 25 μl with nuclease-free water. All PCR reac-tions were performed using an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA).

Effect of TAK-242, a small-molecule-specific inhibitor of TLR4 signaling, on CD204 expression in M2 macrophages. M2 macrophages derived from PBMC were suspended in 10% FBS/RPMI-1640. Cells were plated at density of 5×10^5 cells/wells in 6-well culture plates. After removing the cell culture supernatants, M2 macrophages were stimulated with PTX (5 nM) in the presence or absence of TAK-242 (20 nM; ChemScene, Monmouth Junction, NJ, USA), a small-mole-cule-specific inhibitor of TLR4 signaling for 24 h, followed by RT-PCR as mentioned above.

Statistical analysis. Differences were analyzed by one-way analysis of variance or two-sided Student's t-tests using the computer software package SPSS 10.0 (SPSS, Inc., Chicago, IL, USA). All *in vitro* data represent the results of three inde-pendent experiments. A P-value <0.05 indicated statistical significance.

Results

Expression of TLR4 in THP-1 macrophages and PBMC-derived M2 macrophages. PTX has been shown to be a ligand of TLR4, a member of the Toll-like receptor family, a class of pattern recognition molecules in innate and acquired immune responses (33). Assays of TLR4 expression in THP-1 macrophages and PBMC-derived M2 macrophages by western
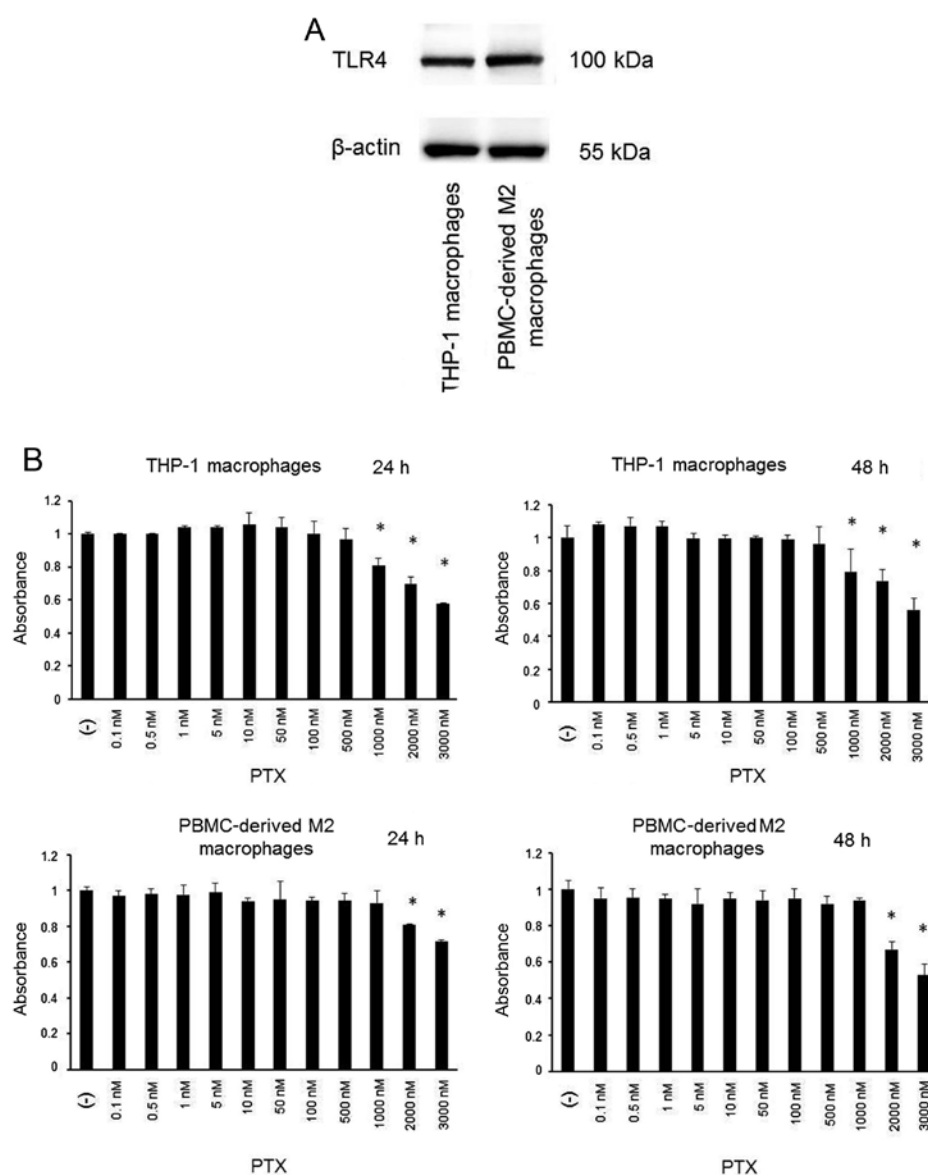
Figure 1. (A) TLR4 expression in human macrophages and (B) cytotoxic effects of paclitaxel (PTX) on THP-1 macrophages and PBMC-derived M2 macrophages. (A) Western blotting shows that TLR4 was expressed by THP-1 macrophages and PBMC-derived M2 macrophages. (B) Cell viability was assessed using MTT assays. Cells were treated with the indicated doses of PTX (0-3,000 nM) in serum-free medium for 24 and 48 h. *P<0.01 vs. the untreated control.

blotting showed that both cell types were positive for TLR4 expression (Fig. 1A).

Cytotoxic examination by MTT assay. MTT assays of THP-1 macrophages and PBMC-derived M2 macrophages were performed to determine the minimum concentrations of PTX that were cytotoxic towards these cells. The viability of THP-1 macrophages and PBMC-derived M2 macrophages at 48 h was significantly inhibited by 1,000 and 2,000 nM PTX, respectively (P<0.01 each; Fig. 1B). Since the concentrations of PTX in plasma and ascites are ~10 nM up to 72 h after intravenous administration of 80 mg/m² to patients with gastric cancer (35), subsequent experiments used PTX concentrations of 1, 5 and 10 nM.

Low-dose PTX inhibits the expression of CD204 in THP-1 macrophages. The interactions among macrophages, gastric cancer cells, and PTX were analyzed using an *in vitro* co-culture system. Western blot analysis showed that the expression of the M2 marker CD204 was increased when THP-1 macrophages were co-cultured with MKN45 or TMK-1 cells, and that this expression was suppressed by treatment with 1 or 5 nM PTX for 48 h (Fig. 2A). Densitometric analyses showed the same results (Fig. 2B). In contrast, expression of the M1 marker NOS2 was not altered when THP-1 macrophages were co-cultured with MKN45 or TMK-1 cells, but was increased when THP-1 macrophages were treated with 5 nM PTX and co-cultured with MKN45 cells, and when THP-1 macrophages were treated with 1 and 5 nM PTX and co-cultured with TMK-1 cells (Fig. 2A and C).

Immunofluorescence examination of CD204 expression in THP-1 macrophages. Although THP-1 macrophages cultured in the absence of conditioned medium of MKN45 cells did
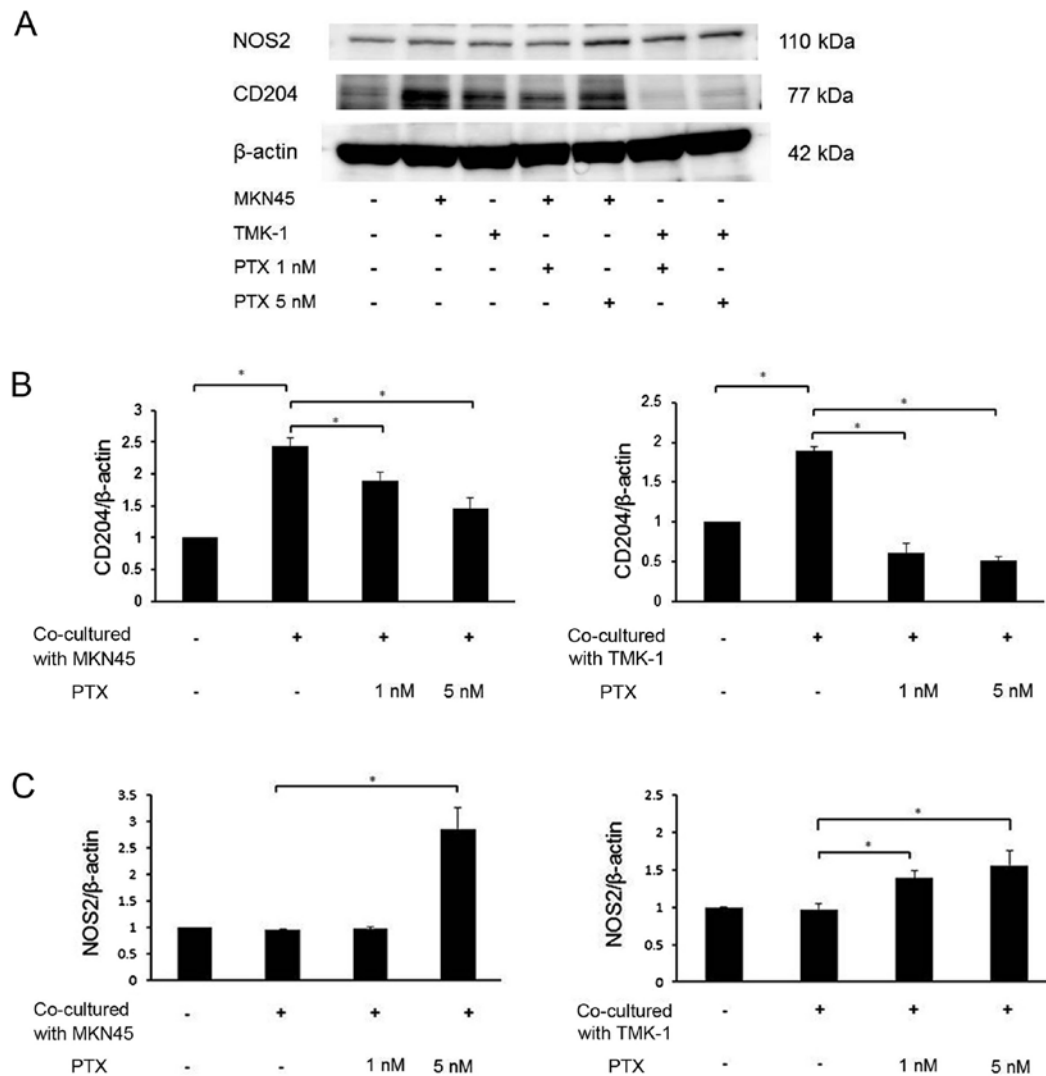
Figure 2. Western blot analysis of CD204 (77 kDa) and NOS2 (110 kDa) in THP-1 macrophages. (A) CD204 expression was increase when MKN45 or TMK-1 cells were co-cultured with the macrophages for 48 h, and CD204 expression was suppressed by treatment with 1 or 5 nM paclitaxel (PTX) for 48 h. Furthermore, NOS2 expression was not altered when MKN45 or TMK-1 cells were co-cultured with macrophages. Its expression was increased when macrophages treated with 5 nM PTX were co-cultured with MKN45 cells and when macrophages treated with 1 and 5 nM PTX were co-cultured with TMK-1 cells. (B) Densitometric analyses of CD204 expression in three independent experiments; data are expressed as means ± SD (*P<0.01). (C) Densitometric analyses of NOS2 expression in three independent experiments. Data are expressed as means ± SD (*P<0.01).

not express CD204 (Fig. 3A), cells cultured in the presence of conditioned medium showed induction of CD204 expression (Fig. 3B). Moreover, treatment with 1 and 5 nM PTX suppressed the expression of CD204 (Fig. 3C and D).

Phosphorylation of STAT1 and STAT3 in THP-1 macrophages. The signaling transducer and activator of transcription (STAT) pathways play pivotal roles in the transcriptional profile of macrophages. STAT1 has been shown essential for M1 tumoricidal activity and to trigger the expression of pro-inflammatory cytokines (36). In contrast, cells with M2 phenotype harboring activated STAT3 are not tumoricidal; rather, they facilitate tumor development (37). We, therefore, investigated whether STAT1 and STAT3 in the THP-1 macrophages are phosphorylated after co-culture with the gastric cancer cell lines for 12 h, and found that STAT3 was significantly phosphorylated (Fig. 3E). However, treatment with 1 and 5 nM PTX significantly

suppressed the phosphorylation of STAT3 while enhancing the phosphorylation of STAT1.

Low-dose PTX inhibits the expression of CD204 in PBMC-derived M2 macrophages. PBMC-derived M2 macrophages exhibited a spindle pattern morphology in the absence of PTX (Fig. 4A), but had a round shape in the presence of 1, 5 and 10 nM PTX (Fig. 4B-D), suggesting that treatment with PTX may have altered their morphology into M1-like macrophages. Western blot analysis showed that CD204 expression in the PBMC-derived M2 macrophages was suppressed by treatment with 1, 5 and 10 nM PTX (Fig. 4E). Moreover, STAT3 phosphorylation was significantly suppressed by treatment with PTX.

PTX-induced intranuclear translocation of NF-κB p65. As NF-κB signaling is one of the major pathways involved in LPS
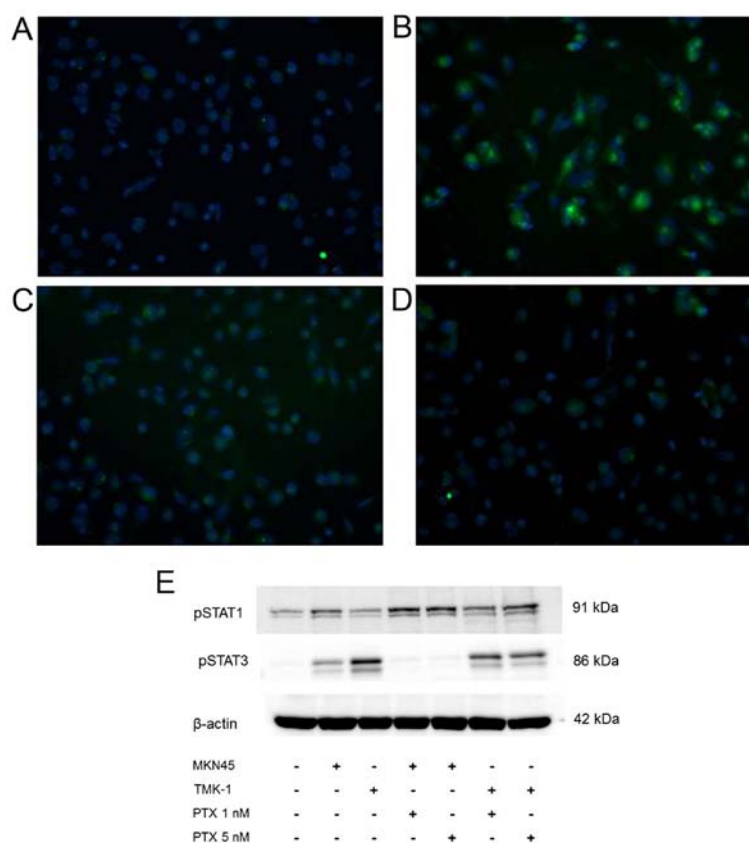
Figure 3. Immunofluorescence examination of CD204 expression and western blotting of phosphorylated STAT1 (91 kDa) and STAT3 (86 kDa) in THP-1 macrophages. (A) Control THP-1 macrophages showed no expression of CD204. (B) Incubation with conditioned media of MKN45 cells increased the expression of CD204, but that effect was suppressed in macrophages incubated with (C) 1 nM and (D) 5 nM paclitaxel (PTX). Original magnification, x200. Representative photomicrographs of THP-1 macrophages labeled with antibodies to CD204 (green). (E) Phosphorylation of STAT3 was detected after exposure to gastric cancer cell lines. Pre-treatment with 1 or 5 nM PTX inhibited phosphorylation of STAT3 and enhanced the phosphorylation of STAT1.

and PTX-TLR4-mediated inflammation in macrophages (38), the interactions of LPS and PTX with NF-κB signaling were examined in the THP-1 macrophages. Treatment of THP-1 macrophages with LPS or PTX for 1 h induced marked intranuclear translocation of NF-κB p65 (Fig. 5A). Whereas conditioned medium of MKN45 cells did not induce intranuclear translocation of NF-κB p65, conditioned medium plus 5 nM PTX resulted in significant intranuclear translocation of this protein. This result showed that TLR4-NF-κB p65 signaling is stimulated by co-culture with gastric cancer cells.

Effect of TLR4 antagonist TAK-242 in PBMC-derived M2 macrophages with or without PTX. Expression of CD204 mRNA in the M2 macrophages treated with PTX was significantly reduced, when compared to M2 macrophages without any treatments as determined by RT-PCR. However, treatment with TAK-242, a TLR4 antagonist, significantly cancelled the lower CD204 mRNA level (P=0.01; Fig. 5B). These data indicate that low-dose PTX can change M2 polarization to M1 polarization in human macrophages via TLR4.

Effect of PTX on VEGF-A, VEGF-C, IL-10, Arg-1, CD163, PD-L1 and PD-L2 production by THP-1 macrophages. M2 TAMs upregulate the expression of VEGF-A, VEGF-C, IL-10, Arg-1 and CD163 (39), making them target molecules

in cancer treatment. To investigate the mechanism of action of PTX in macrophages, the levels of expression of these cytokine mRNAs were assayed in macrophages co-cultured with gastric cancer cells in the presence or absence of 1 nM PTX. The levels of VEGF-A and VEGF-C mRNAs in macrophages were upregulated by co-culture with cancer cells, and downregulated by incubation with 1 nM PTX (P=0.02; Fig. 5C). IL-10, Arg-1 and CD163 mRNAs showed the same trends. These results suggest that PTX can alter macrophage phenotype and functions. We further analyzed PD-L1 and PD-L2 expression. As a result, we did not find any differences in PD-L1 expression among the groups. In contrast, the expression of PD-L2 mRNA was higher in the macrophages co-cultured with cancer cells than that in the macrophages cultured alone, while PTX treatment could not significantly inhibit the upregulated expression.

Discussion

The development of peritoneal dissemination is a multistep process. Peritoneal milky spot macrophages are reeducated by gastric cancer cells, generating M2 macrophages (40). These M2 macrophages induce apoptosis and fibrosis of human peritoneal mesothelial cells (HPMCs), resulting in the adhesion of cancer cells to submesothelial basement membrane leading to peritoneal dissemination (40,41). Inflammation in the perito-
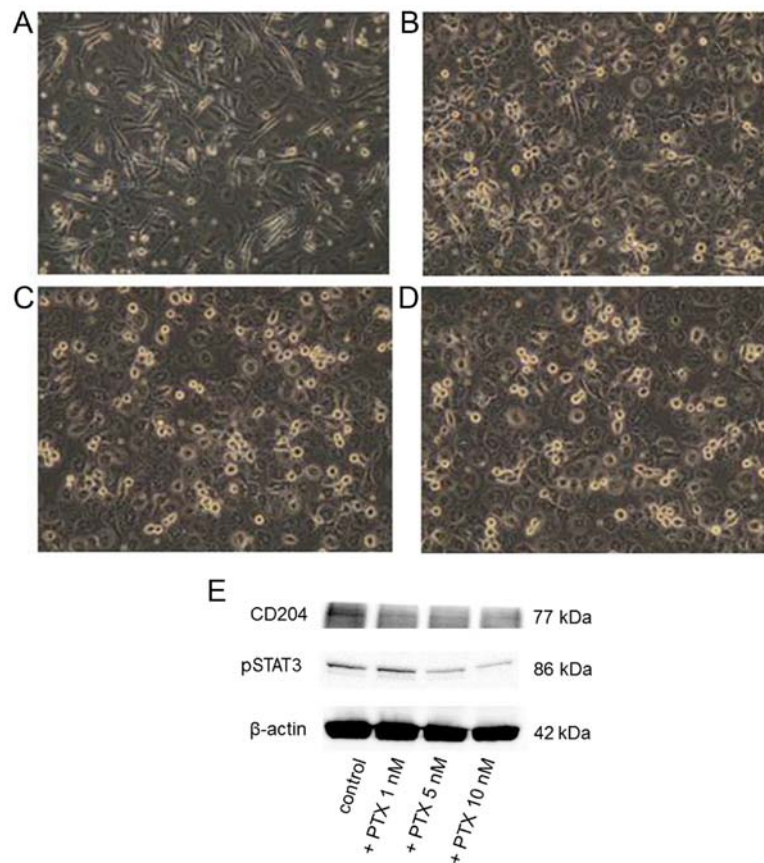
Figure 4. Effect of paclitaxel (PTX) in PBMC-derived M2 macrophages. (A) PBMC-derived M2 macrophages exhibited a spindle-shaped pattern. (B-D) PBMC-derived M2 macrophages incubated with 1, 5 and 10 nM PTX were more round. (E) Western blot analysis showing that CD204 expression and STAT3 phosphorylation in PBMC-derived M2 macrophages were suppressed by treatment with 1, 5 and 10 nM PTX.

neal cavity results in the mobilization of inflammatory cells, including macrophages and neutrophils, through small pores on the surface of milky spots. In ascites fluids, Th2 cytokines, including IL-10, IL-4 and IL-13, produced by tumor cells induce the M2 phenotype in macrophages, enhancing tumor progression and fibrosis (19,42). These processes result in the rapid progression of peritoneal dissemination, contributing to the poor prognosis observed in patients with disseminated gastric cancer.

Therapeutic approaches targeting M2 TAMs can be grouped into four categories: i) inhibition of macrophage recruitment; ii) suppression of TAM survival; iii) enhancement of the M1-like tumoricidal activity of TAMs; and iv) inhibiting the M2-like tumor-promoting activity of TAMs (43). The findings presented in the present study revealed that low-dose PTX increased the expression of the M1 marker NOS2 and suppressed the expression of the M2 marker CD204 in macrophages. Moreover, low-dose PTX was not cytotoxic to THP-1 or M2 macrophages derived from PBMC preparations, indicating that low-dose PTX does not suppress TAM survival while enhancing their M1-like tumoricidal activity and inhibiting their M2-like tumor-promoting activity.

The M1 macrophage marker NOS2 is a heme-containing enzyme that catalyzes the synthesis of NO and citrulline from arginine (44). NOS is expressed by various cells of the immune system, with its activation considered a hallmark of classically activated (M1) macrophages. In contrast, the M2 macrophage marker CD204 is a scavenger receptor with a wide range of ligand-binding specificities. CD204 recognizes various negatively charged macromolecules, including modified low-density lipoproteins and apoptotic cells (45). Expression of CD204 has been associated with an anti-inflammatory M2 macrophage phenotype and is believed useful for distinguishing M2 from M1 macrophages (15). Moreover, higher numbers of CD204-expressing TAMs have been associated with poor clinical outcomes in patients with esophageal, pancreatic and lung cancers (46-49). We found that co-culture of macrophages with gastric cancer cells enhanced CD204 expression, suggesting that conversion to the M2 phenotype was caused by cytokines released by gastric cancer cells. Conversely, we found that low-dose PTX treatment increased the expression of NOS2 and suppressed the expression of CD204 in macrophages, indicating that low-dose PTX may alter macrophage polarization.

PTX activates TLR4 by binding to MD2, an adaptor protein that makes the TLR4 cascade responsive to LPS. PTX also activates several signaling pathways, including the pathway involving NF-κB p65. The TLR4/NF-κB signaling pathway is critical in immune and inflammatory responses (50). NF-κB p65 can induce not only M1 cytokines (e.g., tumor necrosis factor-α, IL-1β and IL-12p70) but Socs3, which in turn inhibits the action of STAT3 (51), a key molecular in the transcriptional profiles of M2 macrophages. Our findings therefore suggest that TLR4/NF-κB p65 signaling
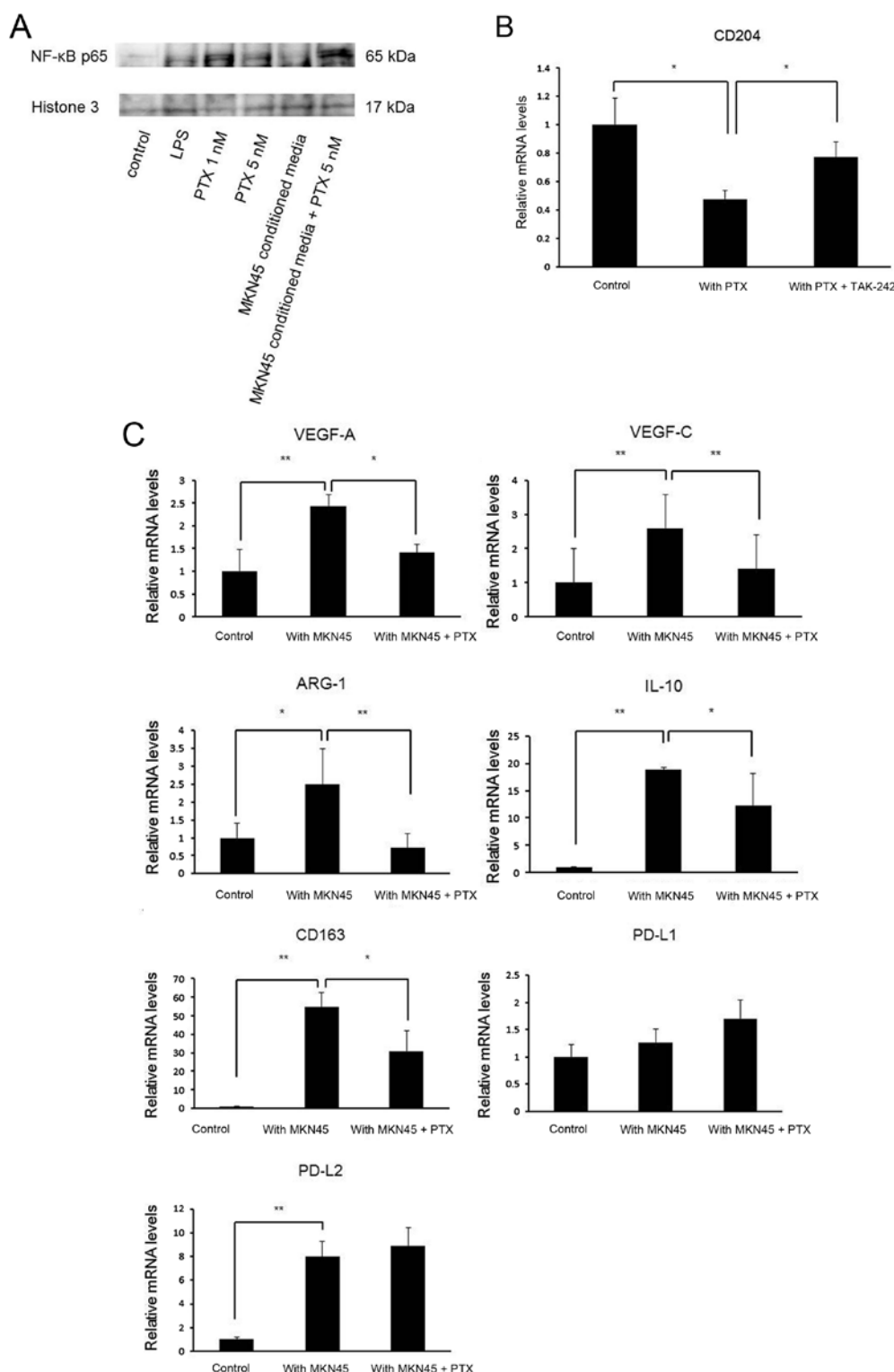
Figure 5. Paclitaxel (PTX) accelerates M1 macrophage polarization via the TLR4/NF-κB p65 signaling pathway. (A) Western blot analysis of the effect of PTX on the nuclear translocation of NF-κB p65. Treatment with LPS or PTX for 1 h markedly enhanced the intranuclear translocation of NF-κB p65. While conditioned media of MKN45 cells had no effect on the intranuclear translocation of NF-κB p65, conditioned media of MKN45 cells plus 5 nM PTX resulted in significant intranuclear translocation. (B) Effect of a TLR4 antagonist, TAK-242, on PBMC-derived M2 macrophage with PTX. Expression levels of CD204 mRNA were confirmed by RT-PCR (*P=0.01). (C) Relative quantification of VEGF-A, VEGF-C, IL-10, ARG-1, CD163, PD-L1 and PD-L2 mRNA levels by real-time quantitative PCR. VEGF-A, VEGF-C, IL-10, ARG-1 and CD163 mRNA levels were higher in macrophages co-cultured with cancer cells than in macrophages alone, but these levels of expression were suppressed by pretreatment with 1 nM PTX. Data are presented as means ± SD (*P<0.05, **P<0.01).

inhibits the M2 phenotype and promotes the M1 phenotype in macrophages. We found that PTX markedly enhanced the intranuclear translocation of NF-κB p65 in cells incubated with conditioned media from gastric cancer cell lines. In addition, we found that PTX could alter M2 polarization to M1 polarization in human PBMC derived macrophages via TLR4.

These findings suggest that low-dose PTX accelerates M1 macrophage polarization via the TLR4/NF-κB p65 signaling pathway.

Systemic administration of PTX has been shown effective in controlling gastric cancers with peritoneal dissemination (25,52). PTX has shown efficacy against diffuse-type adenocarcinoma, which can easily disseminate, with a high rate of transition into the peritoneal cavity. In addition, PTX has a bulky molecular structure, high molecular weight, and an affinity for proteins present at high concentrations in the peritoneal cavity, particularly in malignant ascites. The PTX concentration in ascites 72 h after intravenous administration of 80 mg/m^2 was found to be <10 nM (35). We previously reported that low-dose PTX (5-10 nM) is not only cytotoxic to gastric cancer cells, but inhibits fibrosis by inhibiting the TGF-β1 signaling pathway (26). The present study also found that low-dose PTX could also promote M1 polarization via the TLR4/NF-κB p65 signaling pathway. Thus, the dose and timing of PTX administration are essential for maximizing the potential additive and synergistic clinical activities of PTX.

In conclusion, PTX inhibits the M2 phenotype and induces the M1 phenotype in macrophages via TLR4 signaling. These results suggest that PTX has dual anticancer effects, both as a cytotoxic agent and as an immunomodulator.

Acknowledgements

References

1. Parkin DM: Global cancer statistics in the year 2000. Lancet Oncol 2: 533-543, 2001.
2. Yamazaki H, Oshima A, Murakami R, Endoh S and Ubukata T: A long-term follow-up study of patients with gastric cancer detected by mass screening. Cancer 63: 613-617, 1989.
3. Chen CY, Wu CW, Lo SS, Hsieh MC, Lui WY and Shen KH: Peritoneal carcinomatosis and lymph node metastasis are prognostic indicators in patients with Borrmann type IV gastric carcinoma. Hepatogastroenterology 49: 874-877, 2002.
4. Sadeghi B, Arvieux C, Glehen O, Beaujard AC, Rivoire M, Baulieux J, Fontaumard E, Brachet A, Caillot JL, Faure JL, et al: Peritoneal carcinomatosis from non-gynecologic malignancies: Results of the EVOCAPE 1 multicentric prospective study. Cancer 88: 358-363, 2000.
5. Fushida S, Kinoshita J, Yagi Y, Funaki H, Kinami S, Ninomiya I, Fujimura T, Nishimura G, Kayahara M and Ohta T: Dual anticancer effects of weekly intraperitoneal docetaxel in treatment of advanced gastric cancer patients with peritoneal carcinomatosis: A feasibility and pharmacokinetic study. Oncol Rep 19: 1305-1310, 2008.
6. Shirao K, Boku N, Yamada Y, Yamaguchi K, Doi T, Goto M, Nasu J, Denda T, Hamamoto Y, Takashima A, et al; Gastrointestinal Oncology Study Group of the Japan Clinical Oncology Group: Randomized Phase III study of 5-fluorouracil continuous infusion vs. sequential methotrexate and 5-fluorouracil therapy in far advanced gastric cancer with peritoneal metastasis (JCOG0106). Jpn J Clin Oncol 43: 972-980, 2013.
7. Fushida S, Kinoshita J, Kaji M, Hirono Y, Goda F, Yagi Y, Oyama K, Sudo Y, Watanabe Y and Fujimura T; Society for Study of Peritoneal Carcinomatosis in Gastric Cancer: Phase I/II study of intraperitoneal docetaxel plus S-1 for the gastric cancer patients with peritoneal carcinomatosis. Cancer Chemother Pharmacol 71: 1265-1272, 2013.
8. Fushida S, Oyama K, Kinoshita J, Tsukada T, Okamoto K, Tajima H, Ninomiya I, Kitagawa H, Fujimura T and Ohta T: Intraperitoneal chemotherapy as a multimodal treatment for gastric cancer patients with peritoneal metastasis. J Cancer Ther 4: 6-15, 2013.
9. Kalluri R and Zeisberg M: Fibroblasts in cancer. Nat Rev Cancer 6: 392-401, 2006.
10. Tlsty TD: Stromal cells can contribute oncogenic signals. Semin Cancer Biol 11: 97-104, 2001.
11. Zeisberg EM, Potenta S, Xie L, Zeisberg M and Kalluri R: Discovery of endothelial to mesenchymal transition as a source for carcinoma-associated fibroblasts. Cancer Res 67: 10123-10128, 2007.
12. Pietras K and Ostman A: Hallmarks of cancer: Interactions with the tumor stroma. Exp Cell Res 316: 1324-1331, 2010.
13. Coussens LM and Werb Z: Inflammation and cancer. Nature 420: 860-867, 2002.
14. Lewis CE and Pollard JW: Distinct role of macrophages in different tumor microenvironments. Cancer Res 66: 605-612, 2006.
15. Komohara Y, Ohnishi K, Kuratsu J and Takeya M: Possible involvement of the M2 anti-inflammatory macrophage phenotype in growth of human gliomas. J Pathol 216: 15-24, 2008.
16. Niino D, Komohara Y, Murayama T, Aoki R, Kimura Y, Hashikawa K, Kiyasu J, Takeuchi M, Suefuji N, Sugita Y, et al: Ratio of M2 macrophage expression is closely associated with poor prognosis for Angioimmunoblastic T-cell lymphoma (AITL). Pathol Int 60: 278-283, 2010.
17. Hasita H, Komohara Y, Okabe H, Masuda T, Ohnishi K, Lei XF, Beppu T, Baba H and Takeya M: Significance of alternatively activated macrophages in patients with intrahepatic cholangiocarcinoma. Cancer Sci 101: 1913-1919, 2010.
18. Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Okumura H, Matsumoto M, Miyazono F, Hokita S and Aikou T: Tumorassociated macrophage (TAM) infiltration in gastric cancer. Anticancer Res 23: 4079-4083, 2003.
19. Yamaguchi T, Fushida S, Yamamoto Y, Tsukada T, Kinoshita J, Oyama K, Miyashita T, Tajima H, Ninomiya I, Munesue S, et al: Tumor-associated macrophages of the M2 phenotype contribute to progression in gastric cancer with peritoneal dissemination. Gastric Cancer 19: 1052-1065, 2016.
20. Donaldson KL, Goolsby GL, Kiener PA and Wahl AF: Activation of p34^{cdc2} coincident with taxol-induced apoptosis. Cell Growth Differ 5: 1041-1050, 1994.
21. Gelmon K: The taxoids: Paclitaxel and docetaxel. Lancet 344: 1267-1272, 1994.
22. Noda K, Ikeda M, Kudo R, Nishiya I, Yajima A, Tanaka K, Kodama S, Takahashi T, Tokunaga A, Satoh I, et al: Phase II study of paclitaxel (BMS-181339) in patients with ovarian cancer by 3-hour intravenous infusion. Gan To Kagaku Ryoho 23: 317-325, 1996 (In Japanese).
23. Furuse K, Naka N, Takada M, Kinuwaki E, Kudo S, Takada Y, Yamakido M, Yamamoto H and Fukuoka M; West Japan Lung Cancer Group: Phase II study of 3-hour infusion of paclitaxel in patients with previously untreated stage III and IV non-small cell lung cancer. Oncology 54: 298-303, 1997.
24. Ito Y, Horikoshi N, Watanabe T, Sasaki Y, Tominaga T, Okawa T, Tabei T, Kuraishi Y, Tamura K, Abe R, et al: Phase II study of paclitaxel (BMS-181339) intravenously infused over 3 hours for advanced or metastatic breast cancer in Japan. BMS-181339 Breast Cancer Study Group. Invest New Drugs 16: 183-190, 1998.
25. Ishigami H, Kitayama J, Kaisaki S, Hidemura A, Kato M, Otani K, Kamei T, Soma D, Miyato H, Yamashita H, et al: Phase II study of weekly intravenous and intraperitoneal paclitaxel combined with S-1 for advanced gastric cancer with peritoneal metastasis. Ann Oncol 21: 67-70, 2010.
26. Tsukada T, Fushida S, Harada S, Terai S, Yagi Y, Kinoshita J, Oyama K, Tajima H, Ninomiya I, Fujimura T, et al: Low-dose paclitaxel modulates tumour fibrosis in gastric cancer. Int J Oncol 42: 1167-1174, 2013.
27. Jordan MA, Toso RJ, Thrower D and Wilson L: Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. Proc Natl Acad Sci USA 90: 9552-9556, 1993.
28. Axel DI, Kunert W, Göggelmann C, Oberhoff M, Herdeg C, Küttner A, Wild DH, Brehm BR, Riessen R, Köveker G, et al: Paclitaxel inhibits arterial smooth muscle cell proliferation and migration in vitro and in vivo using local drug delivery. Circulation 96: 636-645, 1997.

29. Colombo A, Drzewiecki J, Banning A, Grube E, Hauptmann K, Silber S, Dudek D, Fort S, Schiele F, Zmudka K, *et al*; TAXUS II Study Group: Randomized study to assess the effectiveness of slow- and moderate-release polymer-based paclitaxel-eluting stents for coronary artery lesions. Circulation 108: 788-794, 2003.

30. Ma Y, Shurin GV, Gutkin DW and Shurin MR: Tumor associated regulatory dendritic cells. Semin Cancer Biol 22: 298-306, 2012.

31. Sevko A, Michels T, Vrohlings M, Umansky L, Beckhove P, Kato M, Shurin GV, Shurin MR and Umansky V: Antitumor effect of paclitaxel is mediated by inhibition of myeloid-derived suppressor cells and chronic inflammation in the spontaneous melanoma model. J Immunol 190: 2464-2471, 2013.

32. Kawasaki K, Akashi S, Shimazu R, Yoshida T, Miyake K and Nishijima M: Mouse toll-like receptor 4.MD-2 complex mediates lipopolysaccharide-mimetic signal transduction by Taxol. J Biol Chem 275: 2251-2254, 2000.

33. Sica A and Mantovani A: Macrophage plasticity and polarization: In vivo veritas. J Clin Invest 122: 787-795, 2012.

34. Jaguin M, Houlbert N, Fardel O and Lecureur V: Polarization profiles of human M-CSF-generated macrophages and comparison of M1-markers in classically activated macrophages from GM-CSF and M-CSF origin. Cell Immunol 281: 51-61, 2013.

35. Kobayashi M, Sakamoto J, Namikawa T, Okamoto K, Okabayashi T, Ichikawa K and Araki K: Pharmacokinetic study of paclitaxel in malignant ascites from advanced gastric cancer patients. World J Gastroenterol 12: 1412-1415, 2006.

36. Sica A and Bronte V: Altered macrophage differentiation and immune dysfunction in tumor development. J Clin Invest 117: 1155-1166, 2007.

37. Weigert A, Sekar D and Brüne B: Tumor-associated macrophages as targets for tumor immunotherapy. Immunotherapy 1: 83-95, 2009.

38. Guha M and Mackman N: LPS induction of gene expression in human monocytes. Cell Signal 13: 85-94, 2001.

39. Alfano M, Graziano F, Genovese L and Poli G: Macrophage polarization at the crossroad between HIV-1 infection and cancer development. Arterioscler Thromb Vasc Biol 33: 1145-1152, 2013.

40. Liu XY, Miao ZF, Zhao TT, Wang ZN, Xu YY, Gao J, Wu JH, You Y, Xu H and Xu HM: Milky spot macrophages remodeled by gastric cancer cells promote peritoneal mesothelial cell injury. Biochem Biophys Res Commun 439: 378-383, 2013.

41. Tsukada T, Fushida S, Harada S, Yagi Y, Kinoshita J, Oyama K, Tajima H, Fujita H, Ninomiya I, Fujimura T, *et al*: The role of human peritoneal mesothelial cells in the fibrosis and progression of gastric cancer. Int J Oncol 41: 476-482, 2012.

42. Takaishi K, Komohara Y, Tashiro H, Ohtake H, Nakagawa T, Katabuchi H and Takeya M: Involvement of M2-polarized macrophages in the ascites from advanced epithelial ovarian carcinoma in tumor progression via Stat3 activation. Cancer Sci 101: 2128-2136, 2010.

43. Tang X, Mo C, Wang Y, Wei D and Xiao H: Anti-tumour strategies aiming to target tumour-associated macrophages. Immunology 138: 93-104, 2013.

44. Mantovani A, Sozzani S, Locati M, Allavena P and Sica A: Macrophage polarization: Tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Trends Immunol 23: 549-555, 2002.

45. Platt N and Gordon S: Is the class A macrophage scavenger receptor (SR-A) multifunctional? - The mouse's tale. J Clin Invest 108: 649-654, 2001.

46. Shigeoka M, Urakawa N, Nakamura T, Nishio M, Watajima T, Kuroda D, Komori T, Kakeji Y, Semba S and Yokozaki H: Tumor associated macrophage expressing CD204 is associated with tumor aggressiveness of esophageal squamous cell carcinoma. Cancer Sci 104: 1112-1119, 2013.

47. Kurahara H, Shinchi H, Mataki Y, Maemura K, Noma H, Kubo F, Sakoda M, Ueno S, Natsugoe S and Takao S: Significance of M2-polarized tumor-associated macrophage in pancreatic cancer. J Surg Res 167: e211-e219, 2011.

48. Yoshikawa K, Mitsunaga S, Kinoshita T, Konishi M, Takahashi S, Gotohda N, Kato Y, Aizawa M and Ochiai A: Impact of tumor-associated macrophages on invasive ductal carcinoma of the pancreas head. Cancer Sci 103: 2012-2020, 2012.

49. Hirayama S, Ishii G, Nagai K, Ono S, Kojima M, Yamauchi C, Aokage K, Hishida T, Yoshida J, Suzuki K, *et al*: Prognostic impact of CD204-positive macrophages in lung squamous cell carcinoma: Possible contribution of Cd204-positive macrophages to the tumor-promoting microenvironment. J Thorac Oncol 7: 1790-1797, 2012.

50. Zhao Q, Qian Y, Li R, Tan B, Han H, Liu M, Qian M and Du B: Norcantharidin facilitates LPS-mediated immune responses by up-regulation of AKT/NF-κB signaling in macrophages. PLoS One 7: e44956, 2012.

51. Liu Y, Stewart KN, Bishop E, Marek CJ, Kluth DC, Rees AJ and Wilson HM: Unique expression of suppressor of cytokine signaling 3 is essential for classical macrophage activation in rodents in vitro and in vivo. J Immunol 180: 6270-6278, 2008.

52. Tamura S, Miki H, Okada K, Takeno A, Uji K, Yoshida A, Suzuki R, Nakahira S, Egawa C, Nakata K, *et al*: Pilot study of a combination of S-1 and paclitaxel for patients with peritoneal metastasis from gastric cancer. Gastric Cancer 13: 101-108, 2010.