Abstract. Tumor-associated macrophages of the M2 phenotype 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 blotting. 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 phosphorylation 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 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 phosphorylation 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.

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, lymphocytes and macrophages (9-12). Tumor-associated macrophages (TAMs) can be classified into two phenotypes: M1, or classically activated macrophages, and M2, or alternatively activated macrophages. M2 macrophages are involved in tissue remodelling, 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 peritoneal 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 Bioreresources (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 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-cm² flasks (BD BioCoat, San Jose, CA, USA) and cultured in 10 ml of medium at 37°C in a humidified atmosphere of 5% CO₂ 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 1x10⁴/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, 5x10⁵ 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%
MKN45 cells were prepared by plating 5x10⁶ tumor cells in conditioned media of Histology and immunofluorescence. system (aTTO). (GE Healthcare, Ltd., Tokyo, Japan) and the Light-Capture were detected using an ECL western blotting detection kit with secondary antibodies, the antibody-antigen complexes diluted 1:10,000 (Sigma-aldrich, Inc.). Following incubation Rockford, IL, USa) according to the manufacturer's protocol. and Cytoplasmic Extraction Reagents (Thermo Scientific, alexa Fluor 488 (Life Technologies, Co.), diluted 1:400 for 1:200 (TransGenic, Inc.). The slides were washed in PBS and at 4̊C with mouse monoclonal anti-CD204 antibody, diluted in PBS) to block non-specific binding and incubated overnight acetone (1:1) for 15 min, incubated with normal goat serum (5% 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 electrophoresis. First stand cDNA was prepared from 2-μg aliquots of DNase-treated RNA using cDNA synthesis kits. Primers for VEGF-A (forward, 5'-CCTGCTGCTACTCCAGC-3' and reverse, 5'-ATGATTGCTGCTTCCTCTC-3'); VEGF-C (forward, 5'-CAGGACGCTACCTCACAGAGA-3' and reverse, 5'-GCTGCTGCTACACTGTTGTA-3'); IL-10 (forward, 5'-GTC ATGTTTCTTCCCTGTG-3' and reverse, 5'-ACTCTGAG TTTGATGATGCC-3'); and arg-1 (forward, 5'-TTCTT AAAGGAGCACCCAGC-3' and reverse, 5'-TCAACGAGACC AGCCTTTCTC-3'); CD163 (forward, 5'-AGGATGTGGAGT GATTGC3'- and reverse, 5'-CCAGCCGTCATCACATAT TG-3'); CD204 (forward, 5'-GGGAAACATTCTCAGACCT TG-3' and reverse, 5'-AATCTCTGGAGACCTACATTT-3'); PD-L1 (forward, 5'-GAACACTACCTCTGGACACATCT-3' and reverse, 5'-CATCACCTATCATTCTCCTTT-3'); and PD-L2 (forward, 5'-GTCTTGGGAGCCAGGTGAC-3' and reverse, 5'-TGAAAGTGCAAATGCGACA-3'); and TATA box binding protein (TBP; forward, 5'-TGACAGGGACCAAGA GTCAA-3' and reverse, 5'-CACATCACAGCTCCACCACA-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 reactions 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 5x10⁵ 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-molecule-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 independent 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
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 cells, but was increased when THP-1 macrophages were treated with 5 nM PTX and co-cultured with TMK-1 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
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
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-
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...
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/m² 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 antitumor effects, both as a cytotoxic agent and as an immunomodulator.

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References


