Abstract. In ovarian cancer patients, chemotherapy resistance is the principal factor restricting long-term treatment. Paclitaxel (Pac) has been previously reported to be a ligand to Toll-like receptor 4 (TLR4). It was determined that TLR4 signaling is divided into the following two pathways: Myeloid differentiation factor 88 (MyD88)-dependent and MyD88-independent. The present study investigated the effect of TLR4 ligation by Pac in MyD88-positive (MyD88+) and MyD88-negative (MyD88-) human ovarian cancer cell lines. An RNA interference expression vector was specifically constructed to target TLR4 mRNA, which was stably transfected into the human ovarian cancer cell lines (SKOV3, OVCAR3, A2780 and 3AO). Cytokines, including interleukin (IL)-6 and IL-8, were detected. Cell proliferation and apoptosis were assessed in the cells transfected with scramble control and TLR4 shRNA to explore the possible functions of TLR4 in ovarian cancer cell growth. It was found that lipopolysaccharide and Pac significantly increase the secretion of IL-6 and IL-8 in the SKOV3 cell line. Similarly, Pac resulted in a significant upregulation of IL-6 and IL-8 in OVCAR3 cells, but not in A2780 and 3AO cells. These results suggested that in MyD88+ ovarian cancer cell lines, TLR4 depletion shows increased sensitivity to Pac treatment in inhibiting cell proliferation compared with in cells without TLR4 knockdown. On the contrary, such changes were not found in MyD88+ cells (A2780 and 3AO). TLR4 negatively regulates Pac chemotherapy, particularly in terms of cell proliferation, and TLR4 may be a novel treatment target in Pac-resistant ovarian cancer.

Introduction

Toll-like receptors (TLRs) are a family of pattern recognition receptors. To date, 11 human TLRs and 13 mouse TLRs have been identified (1). Mammalian TLRs recognize microbial products and initiate innate immune responses (2). Although a limited number of studies have analyzed the correlation between TLR expression and human malignancy, several studies concerning the expression of TLRs and cancer have been conducted (3).

The Toll-related proteins were first identified in mammals (4) and the mammalian TLR4 was rapidly demonstrated to be responsible for the recognition of lipopolysaccharide (LPS) (5). It has been previously reported that TLR4 negatively regulates the Salmonella-induced antitumor activity (6). Notably, TLR4 has been reported to be important in promoting the immune escape of human lung cancer cells by inducing immunosuppressive cytokines and apoptosis resistance (7).

In ovarian cancer patients, chemotherapy resistance is the principal factor restricting long-term treatment (8). Previously, paclitaxel (Pac) has been reported to be a ligand to TLR4 (9). The current study investigated the effects of TLR4 in ovarian cancer, particularly in Pac chemotherapy. Myeloid differentiation factor 88 (MyD88) was originally isolated as a gene that is induced rapidly during the interleukin (IL)-6-stimulated differentiation of M1 myeloleukemic cells into macrophages (10). IL-6 is considered to be involved in host immune responses to types of ovarian cancer (11,12). IL-6 has also been demonstrated to provide paracrine growth stimulation when monocytes are attracted to types of ovarian cancer that produce macrophage colony-stimulating factor (13). IL-6 signaling in ovarian cancer cells regulates tumor cell proliferation, invasion and angiogenesis (14,15) and IL-8 has also been reported to promote ovarian tumor growth in vivo (16). Previously, it has been reported that TLR4 signaling is divided into the following two pathways: MyD88-dependent and MyD88-independent (17,18).

Abbreviations: TLR4, Toll-like receptor 4; Pac, paclitaxel; MyD88, myeloid differentiation factor 88

Key words: ovarian cancer, TLR4, MyD88, paclitaxel, chemotherapy
The present study investigated the role of TLR4 in ovarian cancer cells and the effect of TLR4 ligand by Pac in MyD88+ and MyD88−human ovarian carcinoma in vitro.

**Materials and methods**

**Reagents.** Pac was purchased from Sigma-Aldrich (St. Louis, MO, USA) and the rabbit polyclonal antibodies against TLR4 and MyD88 were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA, USA).

**Cell culture and samples.** The in vitro experiments were performed with human ovarian cancer cell lines, SKOV3, OVCAR3, A2780 and 3AO. All the cell lines were obtained from the Basic Research Center, Shandong Cancer Hospital and Institute (Jinan, China). Cells were cultured in RPMI-1640 medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL) and incubated under standardized conditions (37°C; 5% CO₂ atmosphere).

Samples of normal ovarian tissue adjacent to tumor (n=12) and borderline (n=8) and malignant (n=24) tumors were collected with the approval of the Ethics Committee of the Shandong Cancer Hospital and Institute.

**RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR).** Total RNA was extracted using the TRIzol reagent kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Reverse transcription was performed using SYBR ExScript RT-PCR kit (Takara Bio, Inc., Shiga, Japan). The following set of primers were used for amplification: i) human TLR4 sense, 5’-AAATGGATCAAGGACCAGAGG-3’; and antisense, 5’-CAGCCAGACAGAAGCATCG-3’; and ii) human MyD88 sense, 5’-CGCCGGATGGTGGTGGTTGT-3’ and antisense, 5’-TGATGTCCGACAGCTGATGAACC-3’. The primers were used to amplify an 197-bp fragment of TLR4 cDNA and an 186-bp fragment of MyD88 cDNA. The following primers were used for β-actin: forward, 5’-TTGTATCGTGGAAGGACTCA-3’ and reverse, 5’-TGTGATCATGATGATGATGAACC-3’. The primers were used to amplify a 186-bp fragment of MyD88 cDNA. Thirty cycles of PCR were performed at 95°C for 30 sec, 63°C for 30 sec and 72°C for 45 sec. The primers for human MyD88 were used to amplify a 186-bp fragment of MyD88 cDNA. Thirty cycles of PCR were performed at 95°C for 30 sec, 61°C for 30 sec and 72°C for 45 sec.

**SDS-PAGE and western blot analysis.** Protein was denatured in sample buffer [2.5% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.15 mol/l Tris (pH 6.8) and 0.01% bromophenol blue] and subjected to 10-12% SDS-PAGE as previously described (6). The following antibodies were used: Rabbit anti-TLR4 (1:1,000), -MyD88 (1:1,000) and -actin (1:10,000) (Santa Cruz Biotechnology, Inc.). Signals were detected using ECL western blotting detection reagents (Pierce Biotechnology, Inc., Rockford, IL, USA) according to the manufacturer’s instructions.

**Immunohistochemistry.** Paraffin sections of tumor tissues were deparaffinized and microwaved while immersed in 0.01 M citrate buffer (pH 6.0) for 20 min. Sections were washed with PBS and incubated overnight at 4°C with polyclonal rabbit anti-human TLR4 antibody (1:50) or with polyclonal rabbit anti-human MyD88 (1:50; Santa Cruz Biotechnology, Inc.). Following washing, tissues were incubated with horseradish peroxidase-labeled anti-rabbit antibody for 1 h, followed by 3,3’-diaminobenzidine (Dako, Carpinteria, CA, USA). The results for TLR4 and MyD88 expression in tissues were scored by two independent investigators based on the following levels of staining intensity: None (-), weak (+), moderate (++), or strong (+++).

**Incubation of tumor cells with TLR4 ligands.** In all experiments testing the effects of LPS or Pac on tumor cells, LPS was used at a concentration of 10 µg/ml and Pac at 2 µM.

**Cytokine and chemokine production.** Cytokine and chemokine production by the human ovarian cancer cells was determined using a Luminex-100 System (Luminex, Austin, TX, USA). Supernatants of tumor cells seeded in 12-well plates at 5x10⁵ cells/well in 1 ml of LPS or Pac medium were collected following 36 h of incubation. The levels of IL-6 and IL-8 were measured using panels of capture antibody-coated beads and the labeled detection antibodies, which were pretested and qualified by the manufacturer to ensure the absence of cross-reactivity. The assay sensitivity varied between 5 and 15 pg/ml.

**Caspase-Glo 3/7 assay.** In total, 10 µg of protein in a 50 µl total volume was mixed with 50 µl equilibrated Caspase-Glo 3/7 reagents (Promega Corporation, Madison, WI, USA). Following incubation at room temperature for 1 h, luminescence was measured using TD 20/20 luminometer (Turner Designs, Inc., Sunnyvale, CA, USA). Blank values were subtracted and fold increase in activity was calculated based on the activity measured from untreated cells. Each sample was measured in triplicate.

**Construction of RNA interference targeting the TLR4 gene in the vector.** Small interfering RNA (siRNA) oligonucleotides specifically targeting TLR4 oligonucleotides were confirmed to be valid by the authors and were designed with the following sequences: Sense, 5’-GGTAAAGGAGGACCTAGA-3’ and antisense, 5’-TACTAGCTCATATTACCTACC-3’. The pGenesil-1 negative control vector (Wuhan Genesil Biotechnology Co., Ltd., Wuhan, China) was used as the negative control plasmid in all experiments as previously described (20).

**Transcription and production of stable clones.** The day prior to transfection, cells were trypsinized and plated at a density of 5x10⁵ cells/well in six-well tissue culture plates (Corning Inc., Corning, NY, USA). Cells were rinsed twice with serum-free RPMI-1640 when the density reached >90% confluence. Next, pGenesil-shTLR4 (RNA interference expression vectors) or pGenesil-shControl (control vector) and Lipofectamine 2000 mixtures, prepared in OptiMEM
(Invitrogen Life Technologies), were added. Following 5 h of incubation, the plasmid-Lipofectamine 2000 mixture was removed and RPMI-1640 plus 10% FBS and 800 µg/ml geneticin (Invitrogen Life Technologies) for SKOV3 and OVCAR3 cells, 500 µg/ml G418 for 3AO cells and 600 µg/ml geneticin for A2780 cells were added. All the non-transfected cells died within 7 days and a number of surviving transfected cells were harvested 21 days later.

MTT assay. Stable clone cells were seeded into 96-well culture plates (5,000/well) and treated with 2 µmol/l Pac (2 µM) for 24 h. As the control for normal cell proliferation, 0.1% ethanol was used. At the end of each treatment, cells were incubated as previously described (21).

Statistical analysis. Data are presented as the mean ± standard deviation for continuous variables, and the frequency and percentage for categorical variables. Results were statistically evaluated by analysis of variance. SPSS 17.0 software for Windows was used for statistical treatment (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of TLR4 and MyD88 in ovarian cancer tissues. Expression of TLR4 and MyD88 were assessed in situ on paraffin sections of normal ovarian tissue adjacent to tumor (n=12) and borderline (n=8) and malignant (n=24) tumors. TLR4 was found to exhibit moderate (++) or strong (+++) expression in malignant (20/24) and borderline (5/8) tumors and normal ovarian epithelium (6/12). However, the expression of MyD88 in malignant tumors was considerably greater (18/24) than that in normal ovarian tissue (1/8) or in borderline tumors (1/12) (Table I).

Expression of TLR4 and MyD88 signaling adapter protein in epithelial ovarian cancer (EOC) cell lines. Furthermore, the expression of TLR4 and MyD88 in EOC cells was evaluated by RT-PCR. As shown in Fig. 1, the mRNA of TLR4 were expressed in EOC cell lines. However, the expression of MyD88 was found to differ; SKOV3 and OVCAR3 cells were MyD88-positive, while A2780 and 3AO cells were MyD88-negative.

LPS- and Pac-induced cytokine production in MyD88+ cells. Supernatants of the four cell lines exposed to LPS or Pac for 36 h were analyzed for levels of inflammatory cytokines and growth factors. SKOV3 and OVCAR3 cells constitutively secreted a wide range of cytokines and chemokines, including IL-6 and IL-8. By contrast, A2780 and 3AO cells produced low levels of these cytokines. LPS and Pac significantly increased the secretion of IL-6 and IL-8 in SKOV3 cells. Similarly, Pac resulted in a significant upregulation of IL-6 and IL-8 in OVCAR3 cells, but not in A2780 and 3AO cells (Figs. 2 and 3).

Effect of shRNA on TLR4 gene expression. To investigate the potential role of TLR4 in EOC cell lines, pGenesil-shTLR4 directed at nucleotides 2,202-2,220 of TLR4 was used to selectively reduce TLR4 gene expression in EOC cell lines, SKOV3, OVCAR3, A2780 and 3AO. As shown in Fig. 4, the shTLR4 significantly reduced the expression of the TLR4 mRNA and

| Table I. Results for TLR4 and MyD88 expression in tissues. |
|---|---|---|
| Definite histology | TLR4 | MyD88 |
| Malignant tumor cases | | |
| 1 | ++ | - |
| 2 | +++ | ++ |
| 3 | +++ | ++ |
| 4 | ++ | - |
| 5 | +++ | +++ |
| 6 | ++ | +++ |
| 7 | +++ | ++ |
| 8 | + | +++ |
| 9 | +++ | ++ |
| 10 | ++ | - |
| 11 | ++ | ++ |
| 12 | +++ | ++ |
| 13 | +++ | ++ |
| 14 | +++ | ++ |
| 15 | ++ | + |
| 16 | ++ | + |
| 17 | ++ | + |
| 18 | + | + |
| 19 | ++ | + |
| 20 | +++ | + |
| 21 | + | + |
| 22 | ++ | + |
| 23 | + | + |
| 24 | +++ | + |
| Borderline cases | | |
| 1 | ++ | - |
| 2 | ++ | + |
| 3 | + | - |
| 4 | +++ | ++ |
| 5 | ++ | + |
| 6 | + | - |
| 7 | ++ | + |
| 8 | + | + |
| Normal ovarian epithelium cases | | |
| 1 | ++ | + |
| 2 | +++ | ++ |
| 3 | ++ | + |
| 4 | ++ | + |
| 5 | + | + |
| 6 | + | - |
| 7 | ++ | + |
| 8 | + | + |
| 9 | + | - |
| 10 | + | + |
| 11 | ++ | + |
| 12 | ++ | + |
| P-value | 0.022a | 0.001b |

Summary of the pathological characteristics of tissues. TLR4 and MyD88 expression were scored by the following levels of staining intensity: none (-); weak (+); moderate (++); or strong (+++). aP=0.022, vs. the proportion of TLR4-positive expression in normal ovarian epithelium and bP=0.001, vs. MyD88-positive expression in borderline and normal ovarian epithelium. TLR4, Toll-like receptor 4; MyD88, myeloid differentiation factor 88.
Figure 1. Expression of TLR4 and MyD88 in EOC cells at the mRNA level. TLR4 was expressed in all EOC cell lines; however, positive expression of MyD88 was identified in SKOV3 and OVCAR3 cells, while negative MyD88 expression was observed in A2780 and 3AO cells. TLR4, Toll-like receptor 4; MyD88, myeloid differentiation factor 88; EOC, epithelial ovarian cancer.

Figure 2. Levels of cytokines in tumor cell SNs. SNs were collected from tumor cells cultured at a density of 5x10^6 cells/well following 36 h of treatment and tested for levels of IL-6. Three independent experiments were performed. *P<0.05, vs. NT/SKOV3 and **P<0.05, vs. NT/OVCAR3. SNs, supernatants; IL, interleukin; LPS, lipopolysaccharide; Pac, paclitaxel. NT, cells not treated with LPS or Pac.

Figure 3. Levels of cytokines in tumor cell SNs. SNs were collected from tumor cells cultured at a density of 5x10^6 cells/well following 36 h of treatment and tested for levels of IL-8. Three independent experiments were performed. *P<0.05, vs. NT/SKOV3 and **P<0.05, vs. NT/OVCAR3. SNs, supernatants; IL, interleukin; LPS, lipopolysaccharide; Pac, paclitaxel. NT, cells not treated with LPS or Pac.

Figure 4. Effect of TLR4 knockdown in EOC cells using RT-PCR and western blot analysis. (A) Evaluation of mRNA expression of TLR4 in SKOV3, OVCAR3, A2780 and 3AO cell lines. (B) Western blot analysis for TLR4 protein expression in the four cell lines. shTLR4 cells were not treated with TLR4 interference vector transfection. TLR4, Toll-like receptor 4; EOC, epithelial ovarian cancer; RT-PCR, reverse transcription-polymerase chain reaction; NT, cells not treated with lipopolysaccharide or paclitaxel.

Figure 5. Effect of TLR4 knockdown on cell proliferation in MyD88" EOC cells. The effect of Pac on cell proliferation in SKOV3, OVCAR3, A2780 and 3AO cell lines was investigated. In Fig. 8, the growth inhibiting rate (GIR) of SKOV3/shTLR4 cells was ~60% following 24 h treatment of 2 µM Pac, which is significantly higher than those of the parental SKOV3 (29%) and SKOV3/shControl (31%) cells (P<0.001). In the OVCAR3 cell line, the same treatments were used as with the parental OVCAR3, OVCAR3/shControl and OVCAR3/shTLR4. The GIR was ~61% in OVCAR3/shTLR4, which was higher than that of the parental OVCAR3 (33%) and OVCAR3/shControl (35%) (P<0.001). However, no difference was observed in the
In the present study, TLR4 was found to exhibit moderate expression in malignant tumors in relation to its antitumor effects. Although it has been reported that LPS-stimulated tumor cell supernatants inhibit T cell proliferation and natural killer cell activity, blockade of the TLR4 pathway reverses the functional changes observed in ovarian cancer cells. MyD88, myeloid differentiation factor 88; EOC, epithelial ovarian cancer; Pac, paclitaxel; wtTLR4, wild-type Toll-like receptor 4; shControl, short hairpin RNA control; shTLR4, short hairpin RNA–targeted TLR4; LPS, lipopolysaccharide; Pac, paclitaxel. NT, cells not treated with LPS or Pac.

Discussion

The successful treatment of ovarian cancer remains a major challenge. Overall, >85% patients presenting with advanced disease are likely to relapse. Recurrence defines incurable disease in the majority of cases. The main obstacle to effective treatment is the failure of initial therapy to eradicate a sufficient number of tumor cells to prevent disease recurrence. Pac is a product of the Pacific yew and its antimitotic actions are due to its ability to bind and stabilize microtubules, which prevent accurate cell division during mitosis. In ovarian cancer, Pac induces the secretion of inflammatory cytokines in murine macrophages in a TLR4-dependent manner in addition to its antitumor effects. Although, the effect of Pac on human macrophages is controversial. Few previous studies have analyzed the contribution of innate immunity pathways to the mechanism of action of Pac. MyD88 has been reported to be a negative regulator of TLR signaling. It has been identified that MyD88 is an essential downstream component of the TLR4 signaling cascade mediating Pac resistance. In addition, it has been reported that LPS-stimulated tumor cell supernatants inhibit T cell proliferation and natural killer cell activity. Blockade of the TLR4 pathway reverses the functions of these cells in vitro and in vivo, delays tumor growth and, thus, prolongs the survival of tumor-bearing mice.

Previously, MyD88 has been reported to be a negative regulator of TLR signaling. It has been identified that MyD88 is an essential downstream component of the TLR4 signaling cascade mediating Pac resistance. In addition, it has been reported that LPS-stimulated tumor cell supernatants inhibit T cell proliferation and natural killer cell activity. Blockade of the TLR4 pathway reverses the functions of these cells in vitro and in vivo, delays tumor growth and, thus, prolongs the survival of tumor-bearing mice.
erably greater (18/24) than that in normal ovarian tissue (1/8) or borderline tumors (1/12). The results of the present study suggest that MyD88 acts as a downstream factor and combines with TLR4 to increase the proliferation of ovarian cancer cells.

If TLR4 signaling highlights a survival benefit to tumor cells and alters their sensitivity to Pac, then its silencing via siRNA must aid in identifying the molecular mechanisms responsible for LPS- and Pac-mediated effects. We hypothesized that in TLR4-MyD88 signaling, TLR4 is activated by Pac. MyD88+ human ovarian carcinoma cells (SKOV3 and OVCAR3) and MyD88+ ovarian carcinoma cell lines (A2780 and 3AO) were selected to investigate the TLR4 effects on apoptosis with Pac chemotherapy. The molecular mechanisms of chemotherapy resistance are considered to be associated with apoptosis inhibition (33,34). Acquired resistance to chemotherapy is a significant impediment to effective cancer therapy (35). Notably, silencing of TLR4 expression in MyD88+ EOC cells results in sensitization of the cells to Pac-induced apoptosis and this sensitization is accompanied by the inhibition of cytokine IL-6 and IL-8 production in response to Pac and LPS. In the current study, MyD88+ cells (SKOV3 and OVCAR3) constitutively secreted a wide range of cytokines including, IL-6 and IL-8. By contrast, MyD88− cells (A2780 and 3AO) produced low levels of these cytokines. LPS and Pac significantly increased the secretion of IL-6 and IL-8 in SKOV3 and OVCAR3 cells, but not in A2780 and 3AO cells (Figs. 2 and 3).

The present study used RNA interference to knock down the expression of TLR4 in SKOV3, OVCAR3, A2780 and 3AO cell lines. Cytokine production in response to LPS and Pac stimulation was significantly inhibited in SKOV3/shTLR4 and OVCAR3/shTLR4 cells. No changes in cytokine production were observed in A2780/shTLR4 and 3AO/shTLR4 cells (Figs. 5 and 6).

Caspa-Glo 3/7 assay was used to investigate the apoptosis of ovarian cancer cells. When TLR4 was knocked down, a positive increase in caspase-3/7 activity was identified following Pac treatment between MyD88+ and MyD88− cell lines. The mechanism responsible for Pac resistance in ovarian cancer is not completely understood. The present study confirmed that there is a negative correlation between MyD88 expression and Pac-induced apoptosis in TLR4 signaling, consistent with the results of a previous study (36).

In addition, RNA interference was used to knockdown the expression of TLR4 in SKOV3, OVCAR3, A2780 and 3AO cell lines. In the caspase-Glo 3/7 assay, a significant increase of caspase-3/7 activity was identified in SKOV3/shTLR4 and in OVCAR3/shTLR4 cells (Fig. 7).

The results of the current study indicated that TLR4-MyD88 signaling negatively regulates Pac treatment. Knockdown of TLR4 may increase Pac chemosensitivity in MyD88+ cells. In addition to the caspase-Gol 3/7 assay, cell proliferation was evaluated and a significant increase of G1R was identified in SKOV3/shTLR4 and OVCAR3/shTLR4 cell lines. However, in A2780/shTLR4 and 3AO/shTLR4 cells, no significant changes were observed compared with their control cells (Fig. 8). The present study revealed that the proliferation and survival of the cancer cells is regulated by a specific defense mechanism in TLR4/MyD88 signaling.

In conclusion, the observations of the current study imply that Pac activates TLR4-MyD88 signaling, which increases ovarian cancer cell proliferation. Although the results suggest that the knockdown of TLR4 inhibits cell growth and that IL-6 and IL-8 levels are associated with MyD88 (+) EOC cells, the precise underlying molecular mechanisms responsible for these observations remain to be established. As TLR4 is functionally associated with tumor progression, TLR4 is likely to be a promising target for tumor therapy in the future.

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