Abstract. Ovarian cancer is the number one cause of death from gynaecological malignancy. Platinum-based and taxol-based chemotherapy has been used as a standard therapy, but intrinsic and acquired resistance to chemotherapy is a major obstacle to treat the disease. In the present study, we found that in the chemoresistant ovarian cancer SKOV3/TR cells, interleukin-6 (IL-6), IL-6 receptor and signal transducers and activators of transcription 3 (STAT3) expression as well as STAT3 phosphorylation were upregulated compared to those in parental cells. Silencing of IL-6 using IL-6 siRNA was found to suppress IL-6 production, STAT3 and phosphoSTAT3 levels, which eventually reduced proliferation and clonogenicity of taxol-resistant SKOV3/TR cells. In addition, statitic, a STAT3 inhibitor, was found to result in decrease of cell viability and clonogenicity of these cells, indicating that the elevated IL-6 and STAT3, phosphoSTAT3 levels are associated with the development of taxol resistance. Next, we found anti-proliferative effect of apigenin on both SKOV3 and SKOV3/TR cells. RT-PCR and western blot results showed that apigenin significantly reduced the expression of Axl and Tyro3 receptor tyrosine kinases (RTKs) at mRNA and protein level, which account for its cytotoxic activity. We further found that apigenin decreased Akt phosphorylation and the level of B-cell lymphoma-extra large (Bcl-xl or BCL2-like 1 isoform 1), an inhibitor of apoptosis. On the contrary to these results, apigenin had no effect on IL-6 production, STAT3 and phosphoSTAT3 protein levels, suggesting that apigenin exerts its anti-proliferative activity via downregulation of Axl and Tyro3 expression, Akt phosphorylation and Bcl-xl expression, but not modulation of IL-6/STAT3 axis. Taken together, our data suggest that inhibition of IL-6/STAT3 signaling pathway and downregulation of Axl and Tyro3 RTKs expression might be a therapeutic strategy to overcome taxol resistance in ovarian cancer cells.

Introduction

Ovarian cancer is in the fifth place in respect of cancer-related death in woman (1). Worldwide the annual number of new cases and deaths of ovarian cancer is estimated to be around 0.22 and 0.14 million, respectively (2). Combination of surgery and chemotherapy has been used as a standard therapy for the treatment of ovarian cancer patients, but overall 5-year survival of the patients with stage III and IV still remains at only 20 to 40%. Such poor prognosis of advanced stage ovarian cancer is accounted for by the intrinsic and acquired chemoresistance, since 30% of patients with advanced stages have been reported not to respond to the first-line chemotherapy, paclitaxel and cisplatin/carboptatin, and ~80% of the initial responders eventually relapse and develop chemoresistance (3). However, the underlying molecular mechanisms of chemoresistance in ovarian cancer are not fully understood.

The signal transducers and activators of transcription (STAT) family proteins have been reported to be fairly upregulated and constitutively activated in many tumors (4) and known to be resuluted from the upregulation of upstream signaling molecules such as interleukin-6 (IL-6) (5). Of note, in half of ovarian cancers, constitutive activation of STAT3 has been observed and considered to play an important role for growth, cell cycle progression and invasion of these cancer cells (4). Therefore, targeting IL-6/STAT3 signaling...
Cells were grown in RPMI-1640 (Gibco-BRL, Grand Island, NY, USA) containing 10% FBS, 2 mM L-glutamine, 10 U/ml penicillin and 10 g/ml streptomycin at 37°C in 5% CO2 in a water-saturated atmosphere. The taxol-resistant SKOV3/TR cells were established by stepwise exposure of the parental SKOV3 cells to escalating concentrations of taxol, ranging from 1.5 to 24 nM for more than 6 months.

**RT-PCR.** Cells (3x10^6) were seeded in 60-mm culture dish and grown overnight at 37°C and then treated with the indicated concentrations of apigenin for the 24 h. Total RNA was extracted using TRI reagent and subjected to the cDNA synthesis and PCR. The specific primers were as follows: Axl, sense 5'-AACCTTCAACTCCTGCTTTCTC-3' and antisense 5'-CAGCTTTCTCTCTAGCTTAC-3'; Tyro3, sense 5'-GTGTTGCTGCTATCCGAC-3' and antisense 5'-CACGTCTCCAACACACTTG-3'; IL-6, sense 5'-ATGACTCTCTTCCACAAGCGC-3' and antisense 5'-GAGAGCCCCAACTTTGCT-3'; STAT3, sense 5'-CATTGCCATTGTCTCCACAAGCGC-3' and antisense 5'-GAAGAGCCCTCAATGCAGGAC-3'; GAPDH, sense 5'-GGAGCAAAGGGTCATCATC-3' and antisense 5'-GTGTTGCTGATCGGTGGT-3'.

**Western blot analysis.** Cells were treated with the indicated concentration of apigenin or statin for 24 h. Total cell lysates were prepared from those cells using lysis buffer (1% Triton X-100, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM PMSF, 1 mM Na3VO4, and protease inhibitor cocktail). Protein concentrations were determined using Bio-Rad protein assays. Proteins from cell lysates (20-40 µg) were separated on 12% SDS-PAGE, and electrotransferred to nitrocellulose membranes. Membranes were blocked for 30 min at room temperature in Tris-buffered saline-0.05% Tween-20 (TTBS) containing 5% non-fat dry milk, and then incubated with TTBS containing a primary antibody for 4 h at room temperature. After 3 x 10-min washes in TTBS, membranes were incubated with peroxidase-conjugated secondary antibody for 1 h. Following 3 additional 10-min washes with TTBS, protein bands of interest were visualized using an enhanced chemiluminescence detection system (Amersham).

**siRNA transfection.** RNA interference silencing was performed to inhibit IL-6 production. SKVO3/TR cells (1x10^6) were seeded in 100-mm culture dish and grown overnight and then transfected with 50 nM siRNA against IL-6 (sc-39627; Santa Cruz Biotechnology, Dallas, TX, USA), or control siRNA (sc-37007; Santa Cruz Biotechnology). At 48 h post-transfection, cells were harvested and the number of viable cells were counted and IL-6 level in conditioned media were determined by ELISA. STAT3 and phosphorylated STAT3 protein levels were determined by western blot analysis using whole cell lysates.

**Clonogenic assay.** Cells were seeded in 35-mm culture dishes (2x10^5 cells/dish) and allowed to grow for 7-10 days in the presence of and/or absence of apigenin or statin to form colonies. Colonies of >50 cells were visualized by crystal violet (in 60% methanol; Junsei Chemical Co., Ltd., Tokyo Japan) staining...
and images were taken by RAS 3000 image analysis system (Fuji Film, Tokyo, Japan).

**Cell viability assay.** The viability of cells was measured using Cell Counting Kit-8 assay kit (Dojindo Laboratories, Kumamoto, Japan). Cells (1-2x10³ cells/well) were seeded in 96-well plates and grown overnight at 37˚C and then treated with the indicated concentrations of stattic for the 24 h. At the end of the treatment, 10 µl of CCK-8 solution was added and further incubated for 4 h. The absorbance at 450 nm was measured using a microplate reader (Model 680 microplate reader; Bio-Rad Laboratories). Values are the mean ± SD for triplicate wells and normalized to that of control group to determine the % of viability.

**ELISA.** The level of IL-6 in culture media was measured using ELISA kit from R&D Systems according to the manufacturer's protocol. Cells were transfected with siRNAs, siCtrl and siIL-6 or treated with apigenin for 24 h. Conditioned media were harvested and assayed for IL-6. The data are representative of three independent experiments.

**Statistical analysis.** Data are expressed as the mean ± SD of triplicate samples or at least three independent experiments. For statistical significance, Student's t-test was used with a threshold of P-values which is <0.05.

**Results**

**IL-6, STAT3 and phosphorylated STAT3 levels are elevated in taxol-resistant ovarian cancer cells.** To understand the molecular mechanisms underlying taxol resistance in ovarian cancer cells, we established a taxol-resistant subline, SKOV3/TR cells, by long-term and stepwise exposure of taxol to parental SKOV3 cells. Since elevated production of interleukin-6 (IL-6) and IL-6-mediated activation of signal transducers and the activators of transcription 3 (STAT3) have been reported to lead to chemoresistance to several chemotherapeutic drugs in various cancers (29), we examined IL-6, IL-6 receptor, STAT3, and phosphorylated STAT3 levels in both SKOV3 and SKOV3/TR cells. RT-PCR result showed that IL-6 and IL-6 receptor mRNA levels in SKOV3/TR cells were increased compared to those of SKOV3 cells, respectively (Fig. 1A). In addition, enzyme-linked immunosorbent assay (ELISA) result also showed that the level of IL-6 in culture media of SKOV3/TR cells was higher than that in culture media of parental cells, which is consistent with the transcriptional upregulation of IL-6 in SKOV3/TR cells (Fig. 1B).

Next, expression and phosphorylation status of STAT3 were examined. As shown in Fig. IC, STAT3 mRNA level in SKOV3/TR cells was found to be increased compared to that in parental cells. Western blot results also showed that in SKOV3/TR cells, both STAT3 protein and phosphoSTAT3 level were significantly elevated (Fig. 1D), indicating the induction of STAT3 expression and its activation might be responsible for the development of resistance to chemotherapy.

**Silencing of IL-6 and inhibition of STAT3 reduce proliferation of taxol-resistant cells.** The biological relevance of the increase of IL-6 production, STAT3 protein level, and its phosphorylation status in SKOV3/TR cells was examined by silencing of IL-6 via siRNA and inhibition of STAT3 using stattic, a small molecule inhibitor of STAT3. SKOV3/TR cells were transfected with IL-6-specific siRNA, siIL-6 or control siRNA, siCtrl and assessed IL-6 level in culture media by ELISA. As shown in Fig. 2A, silencing of IL-6 via siIL-6
in SKOV3/TR cells resulted in significant decrease of IL-6 production. Western blot results further showed that phosphorylation and expression of STAT3 was also reduced in SKOV3/TR cells transfected with siIL-6 (Fig. 2B), confirming that STAT3 is a downstream effector of IL-6-mediated signaling pathway.

Next, we examined the effect of siIL-6 on cell proliferation. As shown in Fig. 2C, viability of SKOV3/TR cells transfected with siIL-6 was fairly reduced compared to that transfected with siCtrl.

The effect of stattic, a small molecule inhibitor of STAT3, on cell viability was also examined. We first found that both expression and phosphorylation of STAT3 were decreased by stattic treatment in SKOV3/TR cells (Fig. 3A). Then, both SKOV3 and SKOV3/TR cells were treated with 1, 3 and 10 µM stattic for 24 h and CCK assay results showed a dose-dependent decrease of the cell viability (Fig. 3B). Consistent with the CCK assay results, we also found that clonogenicity of stattic-treated cells was considerably reduced (Fig. 3C), confirming its anti-proliferative activity. Taken together, these results demonstrated that upregulation of IL-6 and STAT3 expression as well as the increased phosphorylation of STAT3 play a critical role in proliferation of SKOV3/TR cells and are associated with taxol resistance of SKOV3/TR cells.

Apigenin suppresses proliferation of both parental and taxol-resistant cells. Since we previously reported that apigenin
targets Axl receptor tyrosine kinase (RTK), one of TAM family members, which accounts for its anti-proliferative effects on non-small cell lung carcinoma (NSCLC) cell lines, we asked whether apigenin was cytotoxic in parental and taxol-resistant ovarian cancer cells, which might result from downregulation of TAM expression. As shown in Fig. 4A, apigenin treatment decreased the viability of both SKOV3 and SKOV3/TR cells in a dose-dependent manner. Of note, treatment with 40 µM apigenin for 24 h showed only 41.5% (SKOV3), and 28% (SKOV3/TR) survival of these cells, respectively (Fig. 4A), indicating a more profound anti-proliferative effect of apigenin on SKOV3/TR cells than parental SKOV3 cells. Colony-forming assay further demonstrated cytotoxic activity of apigenin on SKOV3 and SKOV3/TR cells. As shown in Fig. 4B, treatment of these cells with 40 µM apigenin was found to reduce not only the number of colonies but also the size of each colony.

Anti-proliferative effect of apigenin is mediated by the dysregulation of TAM RTKs and downstream effectors, but not IL-6/STAT3 axis. Since TMA family of RTKs, Axl, Tyro3 and Mer is known to be involved in cell survival, growth and proliferation, we then examined the effect of apigenin on TAM RTKs expression. Especially, in SKOV3/TR cells, Axl expression was found to be slightly reduced, while Tyro3 expression was increased, compared to those in parental SKOV3 cells, respectively. Both SKOV3 and SKOV3/TR cells were treated with 40 µM apigenin for 24 h and then expression of Axl and Tyro3 was examined at mRNA and protein level. RT-PCR results showed that apigenin treatment led to significant reduction of Axl and Tyro3 mRNA level in both parental and taxol-resistant cells (Fig. 5A). Downregulation of Axl and Tyro3 expression in apigenin-treated cells was further confirmed by western blot analysis. As shown in Fig. 5B, the protein levels of Axl and Tyro3 were decreased by apigenin treatment, which is consistent with RT-PCR results.

We next examined several downstream effectors which might be affected after apigenin-mediated inhibition of Axl and Tyro3 expression and subsequent reduction of cell proliferation. Western blot results showed that apigenin treatment decreased the level of phosphorylated Akt which transduces a strong signal for cell cycle progression and is fairly increased in SKOV3/TR cells (Fig. 5C). In addition, apigenin was also found to reduce the level of B-cell lymphoma-extra large (Bcl-xl, or BCL2-like 1 isoform 1) which is regulated by Akt and inhibits apoptosis (Fig. 5D). These data demonstrate that apigenin causes not only reduction of Axl and Tyro3 expression but also the decrease of Akt phosphorylation and Bcl-xl expression.
We further examined if apigenin affects IL-6/STAT3 axis, which is associated with cell viability. ELISA results showed that total amount of IL-6 in culture media was slightly decreased by apigenin treatment (Fig. 6A), whereas IL-6 production per cell was increased, especially in taxol-resistant SKOV3/TR cells (Fig. 6B). We also found that apigenin treatment had no effect on STAT3 phosphorylation in these cells (Fig. 6C). Taken together, the results indicate that apigenin has no effect on IL-6 production and concomitant STAT3 phosphorylation and its anti-proliferative effect does not result from suppression of IL-6/STAT3 axis.

Discussion

Cisplatin- and taxol-based chemotherapy is still the first-line therapeutic choice for ovarian cancer. However, the intrinsic and acquired resistance to these drugs have major limitations leading to the failure of treatment (30-33). Therefore, it is urgent to elucidate characteristics and underlying molecular mechanisms of the resistance to improve final outcomes.

We found that in taxol-resistant SKOV3/TR cells, the levels of IL-6, IL-6 receptor, STAT3 and its phosphorylated form were significantly increased compared to those in parental SKOV3 cells. Moreover, intervention of this IL-6/STAT3 signaling via silencing of IL-6 and a STAT3 inhibitor, stattic, were found to exert anti-proliferative effect on SKOV3/TR cells. These results indicate that activation of IL-6/STAT3 axis resulted from the long-term exposure of cells to taxol and need a strategy or a compensation to survive under the pressure of taxol. Because of dual function of STAT3 as a downstream effector of IL-6 and a transcription factor to induce IL-6 expression, a positive feedback loop between STAT3 and IL-6 is established, which results in autocrine production of IL-6 and constitutive activation of STAT3. Consistent with our data, the anti-apoptotic effect of IL-6 and the involvement in drug resistance have been reported in various cancers including myeloma (34), prostate (35) and breast cancers (36), which supports the idea that combination of IL-6/STAT3 pathway inhibitor with chemotherapeutic agents could be effective, in patients with acquired chemoresistance.

A clinical significance of TAM receptor tyrosine kinases (RTKs), Tyro3, Axl and Mer, as well as their ligands has been demonstrated. For example, in 48.3% of ovarian adenocarcinoma tissues, Axl protein level was elevated and reflected in disease stage and lymph node metastasis. In lung cancer cases, overexpression of Axl, Mer, and their ligands was also found in more than half of non-small cell lung cancer (NSCLC) cell lines (37,38) and RNA interference or monoclonal antibodies against Axl have been reported to reduce NSCLC proliferation, metastasis and xenograft tumor growth (39). In accordance with these studies, we recently demonstrated that anti-proliferative effect on apigenin, a dietary phytochemical derived from various fruits and vegetables resulted from downregulation of Axl expression in NSCLC cells, suggesting that Axl is a novel target of apigenin. Since the initial report showed the inhibitory effect of apigenin on mutagenesis and tumor promotion (40), many follow-up studies further demonstrated its anti-oxidant, anti-inflammatory, anti-angiogenic and anti-proliferative activities. Based on the above evidence, apigenin has received considerable attention as a chemotherapeutic and chemopreventive agent. In the present study, apigenin was further found to suppress the expression of Axl and Tyro3, incurring decreased proliferation of both parental and taxol-resistant SKOV3 cancer cells. Of note, Tyro3 induction contrary to downregulation of Axl in SKOV3/TR cells seems to be a compensation or another strategy for survival, which resulted from long-term treatment of taxol. However, IL-6 and STAT3 expression and STAT3 phosphorylation were not affected by apigenin, IL-6 production per cell was increased, suggesting that IL-6/STAT3 signaling pathway is not involved in the anti-proliferative effect of apigenin.

In summary, our data demonstrated that silencing of IL-6 and STAT3 inhibition intervened IL-6/STAT3 signaling pathway and apigenin caused downregulation of expression...
in all TAM RTKs, which eventually restricted in proliferation of taxol-resistant ovarian cancer cells, suggesting that inhibition of IL-6/STAT3 axis and targeting TAM RTKs might be feasible approaches to overcome taxol resistance in ovarian cancer cells.

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References