Autocrine production of interleukin-6 confers ovarian cancer cells resistance to tamoxifen via ER isoforms and SRC-1

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\textbf{A B S T R A C T}

Although 40–60% of ovarian cancer (OVCA)s express estrogen receptor (ER)\textsuperscript{a}, only a minor proportion of patients respond to anti-estrogen treatment with ER antagonist tamoxifen (TAM). The mechanism underlying TAM resistance in the course of OVCA progression is incompletely understood. However, interleukin-6 (IL-6) plays a critical role in the development and progression of OVCA. Here we explore an association between IL-6 and TAM resistance. We demonstrate that both exogenous (a relatively short period of treatment with recombinant IL-6) and endogenous IL-6 (by transfecting with plasmid encoding for sense IL-6) induce TAM resistance in non-IL-6-expressing A2780 cells, while deleting of endogenous IL-6 expression in IL-6-overexpressing CAOV-3 cells (by transfecting with plasmid encoding for antisense IL-6) promotes the sensitivity of these cells to TAM. Further investigation indicates that TAM resistance caused by IL-6 is associated with the alteration of ER\textsuperscript{a}, ER\textsuperscript{b} and steroid hormone receptor coactivator (SRC)-1 expression levels, the protein interactions between SRC-1 and ER\textsuperscript{a}, but not ER\textsuperscript{b}, as well as blockage of estrogen-induced ER receptor nuclear translocation. These results show that IL-6 secreted by OVCA cells may contribute to the refractoriness of these cells to TAM via ER isoforms and SRC-1. Overexpression of IL-6 not only plays an important role in OVCA progression but also contributes to TAM resistance. Our studies suggest that TAM-IL-6-targeted adjunctive therapy may lead to a more effective intervention than TAM alone.

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1. Introduction

Ovarian cancer (OVCA) continues to be the most fatal gynecologic cancer, with an estimated 5-year survival of only 50% (Jemal et al., 2009). Ovarian carcinogenesis mechanisms have not yet been elucidated but appear to be different from those of breast tumor progression. Indeed, about two-thirds of breast cancer patients with estrogen receptor (ER)-positive tumors respond clinically to anti-estrogen treatment with ER antagonist tamoxifen (TAM). Though 40–60% of OVCA expresses ER\textsuperscript{a} (Rao and Slotman, 1991; Havrilesky et al., 2001), only a minor proportion of patients (ranging from 7% to 18%) respond to TAM therapy (Hatch et al., 1991; Scambia et al., 1995). Several studies have highlighted an increased risk of OVCA in patients receiving long-term estrogen replacement therapy (Beral et al., 2007; Glud et al., 2004; Lacey et al., 2006; Rossing et al., 2007). Estrogen exerts its effect through two receptors, ER\textsuperscript{a} and ER\textsuperscript{b}, which are responsible for different biological functions, as indicated by their specific expression patterns and different effects in gene knockout experiments (Merchanthaler and Shugrue, 1999; Couse et al., 2000). A loss of ER\textsuperscript{b} expression or a decreased in ER\textsuperscript{b}/ER\textsuperscript{a} ratio in epithelial OVCA as compared with normal tissues has been reported consistently by several groups (Brandenberger et al., 1998; Pujol et al., 1998; Rutherford et al., 2000; Bardin et al., 2004). Some studies have shown that ER\textsuperscript{b} expression might affect cellular proliferation, motility, and apoptosis of OVCA cells (Bardin et al., 2004; Treeck et al., 2007). Given that ER\textsuperscript{b} can counteract ER\textsuperscript{a} signaling in some settings, loss of ER\textsuperscript{b} is thought to enhance ER\textsuperscript{a}-mediated proliferation of hormone-dependent cancer cells (Lindberg et al., 2003). Bossard et al. have confirmed that ER\textsuperscript{b} can repress the expression, activity and signaling of ER\textsuperscript{a}, thusly blocking its proliferative action (Bossard et al., 2012). Additionally, they have shown ER\textsuperscript{b} to both strongly reduce orthotopic ovarian xenograft development and decrease the presence of tumor cells at sites of metastasis.
therefore increasing the mouse survival rate (Bossard et al., 2012). In particular, recent clinical observations have documented that the loss of ERα expression could correlate with a shorter overall survival of OVCA patients (Halon et al., 2011) and a metastatic lymph node status (Burges et al., 2010). A recently identified polymorphism (rs1275727) of the ERα gene has been associated with an increased risk of developing an OVCA (Lurie et al., 2011). However, it is still unknown whether this polymorphism affects ERα expression. The intracellular location of ERα in tumor cells seems to be important. Indeed, a recent study has shown ERα to localize in the cytoplasm of tumor cells, while nuclear localization was primarily observed in normal epithelial cells (De Stefano et al., 2011). Moreover, cytoplasmic expression of ERα was correlated to a poor outcome for patients with advanced serous OVCA (Drummond and Fuller, 2010). Combined with the aforementioned clinical correlations between ERα and patient survival, these findings lead us to hypothesize that ERα is a critical factor in ovarian tumor progression and to delineate the precise contribution of this receptor in the molecular pathways underlying OVCA carcinogenesis.

ERα is the marker of choice to decide endocrine treatment of breast cancer. However, despite an initial response to TAM therapy, one-third of patients will acquire resistance even though their ERα status remains unchanged (Osborne, 1998). ERα has also been considered a marker of endocrine response. Emerging data support different functions for ERα when it is expressed alone and when co-expressed with ERβ (Murphy and Watson, 2006). With regard to the latter group (ERα+/ERβ+), the vast majority of retrospective clinical outcome studies strongly support the hypothesis that increased expression of ERα is associated with an increased likelihood of response to endocrine therapy (Murphy and Watson, 2006). A more recent study has suggested a link between ERα expression and endocrine sensitivity by increasing phosphatase and tensin homologue deleted on chromosome 10 (PTEN) levels and decreasing proto-oncogene c-erbB-2 (HER2)/Receptor tyrosine protein kinase erbb-3 (HER3) signaling, thereby reducing Akt signaling with subsequent effects on proliferation, survival and TAM sensitivity of breast cancer cells (Lindberg et al., 2011).

Nuclear hormone receptor coactivators are involved in enhancing the ligand-dependent transcriptional signal of numerous nuclear hormone receptors, including ER. Perhaps the most important of these coactivators is the p160 family, steroid hormone receptor coactivator (SRC-1) (also called NCoA1), SRC-2 (also called TRAM1 or NCoA2), and SRC-3 (also called AIB1, ACTR, p/CIP RAC3, TRAM1 or NCoA3) (Xu and Li, 2003). Elevated levels of SRC-1 and SRC-3 have been associated with decreased response to endocrine therapy and poorer clinical outcome, which may ultimately result in TAM resistance through enhancing its agonist behavior (Smith et al., 1997; Xu et al., 1998; Osborne et al., 2003; Myers et al., 2004). Interleukin-6 (IL-6), a known mediator of immunological and inflammatory events, was elevated in serum and peritoneal fluid from patients with OVCA; high levels of IL-6 in body fluids were associated with poor prognosis and survival (Lane et al., 2011; Tempfer et al., 1997; Penton et al., 2006). IL-6 target cells express a low affinity receptor (IL-6Rα) devoid of transducing activity on their surface. The complex of IL-6 and IL-6Rα associates with the signal transducing membrane protein gp130, thereby inducing its dimerization and the initiation of signaling (Kishimoto et al., 1995). Multiple studies suggested a pathogenic role of this cytokine in the malignant transformation, progression and chemother-apy resistance of OVCA (Syed et al., 2002; Yang et al., 2009; Rabinovich et al., 2007; Nilsson et al., 2005; Wang et al., 2012, 2010). Our previous studies revealed that IL-6 was able to regulate two ER types expression and activate ER transcription through the mitogen activated protein kinase (MAPK) signaling pathway in the absence of estrogen. Human OVCA cell growth was also promoted, partly through activation of ER pathway (Yang et al., 2009).

In this study, we investigated the role of IL-6 expression in modulating cellular sensitivity to TAM in human OVCA cells. We also explored the potential mechanisms involved in IL-6-mediated TAM resistance.

2. Materials and methods

2.1. Cell lines and cell culture

Human OVCA cell lines A2780, CAOV-3 and ES-2 were obtained from the American Type Culture Collection. A2780 and ES-2 cells were cultured in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) containing 10% fetal bovine serum (FBS) (Life Technologies, Inc.), CAOV-3 cells were grown in DMEM (Life Technologies, Inc.) with 10% FBS. Reconstituant human IL-6 (R&D Systems, Minneapolis, MN) was used to pretreat A2780 cells. The cells were cultured in the presence of exogenous IL-6 (50 ng/ml) for 10 days. IL-6 was added to the culture every 2 days (Conze et al., 2001). After the pretreatment period, the cells (A2780/pref-L-6) were harvested, washed, and replated in the presence of IL-6, and their resistance to TAM (Sigma, St. Louis, MO, USA) was determined by the MTT assay.

2.2. Enzyme-Linked Immunosorbent Assay (ELISA)

The cells were cultured for 48 h in 1 ml of medium containing 1% charcoal-stripped FBS (sFBS) (Hyclone Laboratories, Inc., Logan, UT). The supernatants were collected and clarified by centrifugation. The level of IL-6 was determined by ELISA (R&D Systems) according to the manufacturer’s instructions.

2.3. Semiquantitative RT-PCR

Total RNA was isolated from cells with TRIzol (Invitrogen, San Diego, CA) according to the manufacturer’s instructions. Primer sequences were designed by Vector NTI 8 software and synthesized by TaKaRa Biotechnology Co., Ltd. (Dalian, China). The primer sequences were as follows: IL-6, 5’-TGGACAAACAAATCCTGCTACA-3’ (forward) and 5’-AGGGTGCCCATGTCATA-3’ (reverse); for ERα, 5’-AACAAAGCCGATGAGATCTGT-3’ (forward) and 5’-GTGATGGAATACTTTTCAAG-3’ (reverse); for ERβ, 5’-GGGCTCTGTCGAGGTTACG’ (forward) and 5’-CCACATTCCACCTTTGACAC-3’ (reverse), for SRC-1, 5’-CCACACTTGGCAGCTGTAATG-3’ (forward) and 5’-TGATGTAATCAATTTTCAAGG-3’ (reverse); for SRC-2, 5’-TGATTGGAATACTTTTCAAGG-3’ (forward) and 5’-TAAAGCCAGTCTGTCGAGGTTACG’ (forward) and 5’-CGGCTTGGGATGGATGACG’ (reverse), for β-actin, 5’-TGGAACTTGTTGGCTGATGACG’ (forward) and 5’-AACGCGTCTGTCGAGGTTACG’ (reverse). One Step RNA PCR Kit (AMV) (TaKaRa Biotechnology) was used to do RT-PCR. PCR products were fractionated on 1.5% agarose gel and analyzed with Quantity One 4.5.6 software (Bio-Rad, Hercules, CA). The results were normalized against β-actin, and presented as target mRNA: β-actin ratio.

2.4. Total cell lysate and nuclear extract preparation

Total cell lysates were obtained as previously described (Yang et al., 2009). To prepare nuclear extracts, cells were harvested, washed with PBS once, and resuspended in a hypotonic buffer (10 mM HEPES-KOH pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.4% NP-40, 0.5 mM PMSF, 0.5 mM DTT, 1 mM NaV, 20 mM NaF, and 1 μg/ml aprotinin) and incubated on ice for 20 min. Nuclei were precipitated by centrifugation at 6000 rpm at 4°C for 10 min. After washing once with hypotonic buffer, the nuclei were lysed in a high-salt buffer (10 mM HEPES-KOH pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 mM NaV, 20 mM NaF, 20%
whole cell protein extracts or nuclear extracts were subjected to 6–10% SDS–PAGE and analyzed by blotting with rabbit polyclonal anti-IL-6Rα (sc-661, 1:200), anti-pg130 (sc-655, 1:200), anti-ERα (sc-543, 1:400), mouse monoclonal anti-ERβ (sc-53494, 1:300) (Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit monoclonal anti-SRC-1 antibody (#2191, 1:1000) (Cell Signaling Technology, Beverly, MA). Membranes were stripped by incubating with stripping buffer at 50 °C for 30 min and then blotted with mouse monoclonal anti-β-actin (Sigma) or rabbit polyclonal anti-Lamin B1 (Epitomics, Inc., CA) antibody. Immunodetection was performed using the corresponding secondary HRP-conjugated antibody, and HRP activity was detected using chemiluminescent substrate kit (SuperSignal WestPico Trial Kit, Pierce Biochemicals).

2.6. Cytotoxicity assay

In vitro cytotoxicity assays were performed by MTT assay. MTT was obtained from Sigma. Briefly, 4 × 10^5 cells per well were plated in 96-well plates for 24 h, and then switched to medium containing 1% FBS and cultured for another 24 h. The cells were pretreated with different concentrations (0.1, 1, 10, 100 or 1000 nM) of TAM in DMSO (Sigma) for 30 min before 1 nM 17β-estradiol (E2, Sigma) was added into the medium. After culture for 48 h, MTT solution (0.5 mg/ml PBS) was added to each well and incubated for 4 h. After dissolving the resulting formazan product with acid–isopropanol, the absorbance was measured at 490 nm using ELISA microplate reader. Data represents the average absorbance of six wells in one experiment. The percentage of surviving cells was estimated by dividing the A490 nm of these cells by the A490 nm of control cells. Data were derived from at least three independent experiments.

2.7 Plasmids, RNA interference and transient transfection

pcDNA3.1(+)–ssIL-6 (i.e., sense IL-6 vector), pcDNA3.1(+)–asIL-6 (i.e., antisense IL-6 vector), pGenesil-ERβ small hairpin RNA (shRNA) and pGenesil-scramble shRNA plasmids were as described previously (Wang et al., 2010). The expression vector for ERβ (i.e., pSG5-ERβ, also called HG0) (Kumar et al., 1986) and the expression vector for SRC-1 (i.e., pcR3.1-SRC-1) (Rowan et al., 2000) were kindly provided by Prof. Sophie Doisneau-Sixou (INSERM U563 and Institut Claudius Regaud, Toulouse, France) and Dr. Nancy L. Weigel (Baylor College of Medicine, Houston, TX), respectively. The human SRC-1 small interfering double-stranded RNAs (siRNA) was purchased from Shanghai GenePharma Co., Ltd. For transfection, 4 × 10^5 cells were seeded into 12-well plates with medium containing 10% FBS. Cells were transfected with either SRC-1 plasmid (0.1, 0.2 µg) or ERβ siRNA (0, 10, and 20 µM) using Lipofectamine™ 2000 (Invitrogen, San Diego, CA) according to the manufacturer’s instructions. The total amount of DNA was kept constant with vector control at 0.2 µg/well.

2.8. Generation and selection of cells stably transfected with pcDNA3.1(+)–ssIL-6, pcDNA3.1(+)–asIL-6, pSG5-ERα or pGenesil-ERβ shRNA

A2780 cells were transfected with pcDNA3.1(+)–ssIL-6 by Lipofectamine™ 2000 and three A2780/ssIL-6 stable cell lines that produced low (A2780/ssIL-6L), middle (A2780/ssIL-6M) and high (A2780/ssIL-6H) levels of IL-6 were cloned as described previously (Wang et al., 2010). A2780 and CAOV-3 cells (4 × 10^5) were plated onto 6-well plates until 90–95% confluence before transfection. A2780 cells were cotransfected with 3 µg of pSG5-ERα and 1 µg of pcDNA3.1(+)–asIL-6 or pGenesil-ERβ shRNA. Selection for the neomycin gene was initiated 48 h after transfection by adding 500 µg (A2780 cells) or 400 µg (CAOV-3 cells) of G418 (Life Technologies, Inc.)/ml to the supplemented culture medium. This selection medium was changed every 2 days for 4 weeks, until all non-transfected cells died. Resistant cell clones were isolated and expanded for further characterization. The empty vector pSG5 and pcDNA3.1(+) were also cotransfected into A2780 cells served as negative control whereas pcDNA3.1(+) or pGenesil-scramble shRNA was transfected into CAOV-3 cells served as negative control or nonspecific shRNA control.

2.9. Apoptosis assay

Apoptosis was measured by Annexin-V-FITC apoptosis detection kit (KeyGEN) according to the manufacturer’s instructions. Briefly, cells were collected from six-well plates and washed in cold PBS. 5 × 10^5 cells were incubated for 15 min at room temperature in the presence of 5 µl Annexin V-FITC, 5 µl of propidium iodide (PI) and 500 µl of Binding Buffer. Staining was detected on a FACS Calibur flow cytometry (Becton Dickinson, Heidelberg, Germany). At least 10,000 cells per treatment condition were analyzed on CellQuest Pro software (Becton Dickinson, Heidelberg, Germany).

2.10. Immunoprecipitation

Endogenous ER isoforms, ERα and ERβ, or coactivator SRC-1 were immunoprecipitated from CAOV-3 or A2780 cells that were plated in 100 mm culture dishes with the density of 5 × 10^6 cells. The cells were incubated in medium containing 1% FBS for 24 h before treating with vehicle, E2 (10 nM), or IL-6 (50 ng/ml) for 1 h. Cells were harvested, and nuclear lysates were prepared as described above. 500 µg of protein was incubated with anti-SRC-1 (1:100), anti-ERα (2 µg), anti-ERβ (2 µg) or anti-rabbit IgG (1:300, Santa Cruz Biotechnology) antibody for 1 h at 4 °C, and then 20 µl of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) was added, and the mixture was incubated overnight at 4 °C. The immunoprecipitates were washed four times with PBS and resuspended in sample buffer. The immunocomplexes were subjected to SDS–PAGE and blotted with anti-ERα (1:400), anti-ERβ (1:400) or anti-SRC-1 (1:1000) antibody.

2.11. Statistical analysis

Multiple comparisons were performed using two-way analysis of variance (ANOVA) with multiple post hoc comparisons. All statistical tests were two-sided. For all tests, the level of significance was set at P < 0.05. Statistical analysis was done using the SPSS version 11.0 software.

3. Results

3.1. Comparing expression levels of IL-6 and its receptor (IL-6Rα and gp130) as well as sensitivity to TAM in three OVCA cell lines

In order to investigate the role of IL-6 expression in the acquisition of the TAM resistance phenotype in OVCA cells, we first analyzed the expression of IL-6 and its receptor (IL-6Rα and gp130) as well as the response to TAM in three OVCA cell lines. As shown...
in Fig. 1A, the secretion level of IL-6 was approximately 75-fold higher in CAOV-3 cells (15873.47 ± 620.52 pg/ml) than ES-2 cells (211.59 ± 11.98 pg/ml). However, no IL-6 was detected in the supernatant from A2780 cells. The mRNA levels of IL-6 resembled their respective protein levels in three OVCA cells (Fig. 1B). As determined by Western Blot analysis, these three cell lines were demonstrated to express IL-6Rα and gp130 (Fig. 1C). The sensitivity to TAM also varied among these cell lines as shown in Fig. 1D. A2780 cells were the most sensitive, then came ES-2 cells, whereas CAOV-3 cells were drug-resistant, indicating that autocrine production of IL-6 by OVCA cell lines were inversely associated with their sensitivity to TAM. Taken together, these results suggest that IL-6 receptor-bearing OVCA cell lines, A2780 (non-IL-6-expressing and drug-sensitive) and CAOV-3 (IL-6-overexpressing and drug-resistant) are suitable cell models to investigate the effect of IL-6 on TAM-mediated cytotoxicity in OVCA cells.

3.2. IL-6 confers TAM resistance in OVCA cells

Our previous reports have shown that IL-6 promoted human OVCA cell growth partly through activation of ER pathway (Yang et al., 2009). Accordingly, results given above indicate that autocrine production levels of IL-6 by OVCA cell lines were inversely associated with their responsiveness to the ER antagonist TAM. It suggests that IL-6 could play a role in the resistance of OVCA cells to the activities of anti-estrogen compounds. To address this hypothesis, A2780 cells were cultured in the presence or absence of IL-6 for 10 days. After the pretreatment period, cells were harvested, washed, and replated in the presence or absence of IL-6, and their resistance to TAM was determined by the MTT assay. Pretreatment of A2780 cells with IL-6 caused marked increase in the level of TAM resistance in OVCA cells.

To determine whether the endogenous production of IL-6 by tumor cells could provide self-protection against drug-induced cell death, we constitutively expressed IL-6 in A2780 cells and inhibited expression of IL-6 in CAOV-3 cells and examined the effect of IL-6 expression on the drug resistance of these cells. A2780 cells were transfected with plasmid encoding for sense IL-6 and CAOV-3 cells were transfected with plasmid encoding for antisense IL-6. Stably transfected A2780 (A2780/ssIL-6) and CAOV-3 (CAOV-3/asIL-6) clones were isolated and screened for their ability to produce IL-6. Three representative clones that produced low (A2780/ssIL-6L), intermediate (A2780/ssIL-6Mi) and high (A2780/ssIL-6Hi) levels of IL-6 as described previously (Wang et al., 2010) were selected. Two representative clones with intermediate (63.82%, CAOV-3/asIL-6Mi) and high (75.69%, CAOV-3/asIL-6Hi) inhibition of IL-6 production (Fig. 2B), compared with the corresponding parental (i.e., untransfected) and control vector-transfected CAOV-3 cells, were also selected, for subsequent studies. IL-6 mRNA expression levels in the stably transfected CAOV-3 clones were also examined by semiquantitative RT-PCR and found to be consistent with secreted IL-6 levels in stably transfected CAOV-3 clones (Fig. 2C).

To determine whether the endogenous production of IL-6 in A2780 cells can confer resistance to drug treatment, we examined the susceptibility or resistance of sense IL-6-transfected A2780 cells to TAM treatment using the MTT assay. As shown in Fig. 2D, A2780/ssIL-6M and A2780/ssIL-6H cells exhibited increased resistance to TAM as compared with parental A2780 cells (P < 0.05). Control A2780/pDNA3.1(+) cells that did not produce IL-6 exhibited similar drug sensitivity to parental A2780 cells (P > 0.05). These data suggest that overexpressing of IL-6 confers a moderate level of TAM resistance in OVCA cells.

To determine whether suppression of endogenous IL-6 in CAOV-3 cells could increase their responsiveness to drug treatment, we also examined the susceptibility or resistance of...
antisense IL-6-transfected CAOV-3 cells to TAM (Fig. 2E). In correlation with data from sense IL-6-transfected A2780 cells, CAOV-3/asIL-6Mi and CAOV-3/asIL-6Hi cells exhibited increased responsiveness to TAM as compared with parental CAOV-3 and those of ER\(_a\) expression and down-regulated ER\(_b\) expression (Fig. 4A and B), whereas the inverse was observed in antisense IL-6-transfected CAOV-3 clones (i.e., A2780/ER\(_b\)-specific shRNA) (Fig. 4C and D) in a dose-dependent manner. In sense IL-6-transfected A2780 cells, the mRNA and protein levels of ER\(_a\) were enhanced and those of ER\(_b\) reduced (Fig. 4A and B), whereas the inverse was observed in antisense IL-6-transfected CAOV-3 cells (Fig. 4C and D) compared with corresponding parental and control vector-transfected cells, which had no difference. Therefore, these data suggest that IL-6 can regulate both ER\(_a\) and ER\(_b\) expression levels of OVCA cells in the absence of estrogen.

Having shown that the protein level of ER\(_a\) is lower and that of ER\(_b\) is higher in non-IL-6-expressing and drug-sensitive A2780 cells, whereas the opposite situation was observed in IL-6-overexpressing drug-resistant CAOV-3 cells, we next determined whether changes in the ratio of ER isoforms expression are associated with the acquisition of resistance of OVCA cells to TAM. A2780 cells were stably transfected with constructs encoding for sense ER\(_a\)-specific and ER\(_b\)-specific shRNA. Stable A2780 transfected clones (i.e., A2780/ss ER\(_a\) and A2780/ER\(_b\) shRNA) were isolated and screened by Western Blot with anti-ER\(_a\) or anti-ER\(_b\) antibody. As illustrated in Fig. 5A and B, ER\(_a\) and ER\(_b\) protein levels in sense ER\(_a\)-transfected A2780 cells were significantly increased while ER\(_b\) protein levels in ER\(_b\)-shRNA-transfected A2780 cells were markedly decreased compared with parental (i.e., untransfected) and control vector-or scrambled shRNA control-transfected A2780 cells (i.e., A2780/pSg5 or A2780/scrambled shRNA), which did not vary. Maximum overexpression of ER\(_a\) protein 8.93-fold (Fig. 5A) and maximum knockdown of ER\(_b\) protein 82% (Fig. 5B) were confirmed in all analyzed samples, and were chosen for subsequent studies. It is
no different in ERβ protein levels in A2780/ssERα cells (Supplementary Fig. 1A) and ERα protein levels in A2780/ERβ shRNA cells (Supplementary Fig. 1B) by Western blot analysis.

Next we examined the susceptibility or resistance of sense ERα- or ERβ shRNA-transfected A2780 cells to TAM treatment using the MTT assay. As shown in Fig. 5C and D, both A2780/ss ERα cells and A2780/ERβ shRNA cells exhibited increased resistance to TAM as compared with parental A2780 cells and control A2780/pSG5 or A2780/scrambled shRNA cells (P < 0.05). Control A2780/pSG5 or A2780/scrambled shRNA cells exhibited similar
drug sensitivity to parental A2780 cells (P > 0.05). Interestingly, we found that TAM at concentrations of 100 nM caused an obvious increase in cell proliferation of A2780/ERα cells (P < 0.005). This is likely associated with the fact that ERα has no marked effect (Bardin et al., 2004), though further studies are warranted in this matter.

The increased resistance or cell growth to TAM observed in A2780 cells transfected with sense ERα or ERβ shRNA could result not only from cell cycle progression, but also from decreased apoptosis. To examine the apoptotic effect of ER isoform alterations on A2780 cells, double staining with Annexin V and PI was performed.

The percentage of apoptosis was 8.22 ± 0.87% (A2780/sense ERα, Fig. 5A) and 6.86 ± 0.12% (A2780/ERβ, Fig. 5B) respectively compared with the corresponding DMSO-treated cells. Taken together, these data suggest that alterations of ER isoforms expression may play an important role in IL-6-induced TAM resistance of OVCA cells by altering cell cycle distribution and inhibiting apoptosis. Further, alteration of ERβ expression seems to be more obvious than that of ERα.

3.4. SRC-1 expression is associated with IL-6-induced TAM resistance in OVCA cells

The p160 family coactivators interact with ER to enhance ligand-dependent transactivation of ER. Increased expression of SRC-1 and SRC-3 is associated with decreased response to endocrine therapy and poorer clinical outcome (Smith et al., 1997; Xu et al., 1998; Osborne et al., 2003; Myers et al., 2004), suggesting that SRC-1 and SRC-3 may be involved in TAM resistance through enhancing its agonist behavior. To determine another potential mechanism through which IL-6 causes TAM resistance in OVCA cells, we also examined the expression levels of SRC-1 and SRC-3 in the above three OVCA cell lines. The mRNA and protein levels of SRC-1 (Fig. 7A and B) and SRC-3 (Supplementary Fig. 2A and B) do vary with each cell lines. A2780 cells present the lowest expression, increasing expression in ES-2 cells, and the highest expression is observed in CAOV-3 cells, suggesting that autocrine mechanism through which IL-6 causes TAM resistance in OVCA cells.
significantly increased the mRNA and protein levels of SRC-1 (Fig. 7C and D) in a dose-dependent manner, but had no effect on those of SRC-3 (Supplementary Fig. 2C and D). The mRNA and protein levels of SRC-1, but not SRC-3 (Supplementary Fig. 2E–H), was up-regulated in sense IL-6-transfected A2780 cells (Fig. 7E and F), and down-regulated in antisense IL-6-transfected CAOV-3 cells (Fig. 7G and H) compared with the corresponding untransfected and control vector-transfected cells, which did not vary. Taken together, these results suggest that IL-6 can also enhance SRC-1 expression levels of OVCA cells in the absence of estrogen.

Having shown that the level of SRC-1 protein is elevated in IL-6-overexpressing and drug-resistant CAOV-3 cells compared with non-IL-6-expressing and drug-sensitive A2780 cells, we next determined whether SRC-1 expression is associated with the acquisition of resistance to TAM by CAOV-3 cells. CAOV-3 cells were transfected with shRNA specific to SRC-1, then treated with TAM and viability was determined by MTT assay. Knockdown of SRC-1 expression by SRC-1-specific shRNA in CAOV-3 cells sensitized their response to TAM (Fig. 8A), whereas overexpression of SRC-1 in A2780 cells by transfection of a plasmid encoding SRC-1 cDNA increased their resistance to TAM treatment (Fig. 8B). Collectively, these results indicate that SRC-1 expression also plays a critical role in IL-6-mediated TAM resistance in OVCA cells.

### 3.5. Effect of IL-6 on the protein interactions between endogenous ER and SRC-1 in OVCA cells in the absence of estrogen

We next determined whether IL-6 could affect the protein interactions between endogenous ER isoforms and SRC-1 in OVCA cells in the absence of estrogen. The protein interactions between ERα...
or ERβ and SRC-1 were investigated using endogenous complexes isolated from CAOV-3 cells naturally expressing high level of ERα protein or A2780 cells endogenously expressing high level of ERβ protein that were exposed to E2 (10 nM) or IL-6 (50 ng/ml). As illustrated in Fig. 9A and B, in addition to the alteration of ERα, ERβ and SRC-1 expression levels by IL-6 or E2, and the protein interactions between SRC-1 and both ERα and ERβ were seen in cells exposed to E2, while the protein interactions between SRC-1 and ERα, but not ERβ, were observed in the present of IL-6. This suggests that IL-6 also induces the protein interactions between

![Figure 7](image7.png)

**Fig. 7.** Various SRC-1 expression of mRNA and protein in three OVCA cell lines (A and B). Exogenous IL-6 increases SRC-1 expression in both mRNA and protein level in A2780 cells (C and D). The mRNA and protein levels of SRC-1 in three stable sIL-6-transfected A2780 clones (E and F), two aIL-6-transfected CAOV-3 clones (G and H), and the corresponding parental and vector control cells. The mRNA and protein levels of SRC-1 were detected by semiquantitative RT-PCR and Western blot, respectively, as described above. The data shown is representative of three independent experiments with similar results.

![Figure 8](image8.png)

**Fig. 8.** Effects of SRC-1 expression on the response of OVCA cells to TAM. (A) CAOV-3 cells were transfected with increasing doses of SRC-1 specific shRNA (0, 0.1, 0.2 μM). (B) A2780 cells were transfected with increasing doses of SRC-1 expression plasmid (0, 0.1, 0.2 μg). Different response of cells to TAM was determined by the MTT assay. Bottom, SRC-1 protein expression by Western Blot analysis. Levels of SRC-1 protein were normalized to those of β-actin and expressed as fold induction relative to the controls. Data are shown as the mean ± SD of three separate experiments with six iterations. *P < 0.05 compared with vehicle control.
SRC-1 and ER\(_a\), but not ER\(_\beta\) in OVCA cells in the absence of estrogen.

3.6. Effect of IL-6 on ER\(_a\) or ER\(_\beta\) nuclear translocation in OVCA cells

TAM (100 nM) treatment blocked ER\(_a\) or ER\(_\beta\) nuclear translocation induced by E\(_2\) (10 nM) in A2780/pcDNA3.1(+) cells (Fig. 9C) but failed to do so in IL-6-overexpressing A2780 cells (Fig. 9C). Collectively, these results suggest that persistent activation of ER signaling by IL-6 may contribute to TAM resistance.

4. Discussion

TAM and its active metabolite, 4-hydroxytamoxifen (OHT), have an ER\(_a\)-selective partial agonist/antagonist function but pure ER antagonist effect through ER\(_\beta\) (McDonnell et al., 1995; Barkhem et al., 1998; McInerney et al., 1998). Watanabe et al. showed that the agonistic effect of TAM depends on the cell type, ERE-promoter context, and ER subtypes, and that this action is ER\(_a\) specific (Watanabe et al., 1997). TAM is an ER\(_a\) antagonist in breast (Jordan, 1992) and ovaries (Kedar et al., 1994) but an agonist in bone (Love et al., 1992) and uterine tissues (Kedar et al., 1994). TAM and OHT can compete with E\(_2\) and other estrogens for binding to ER. Structural studies and chromatin immunoprecipitations show that OHT-ER induces an ER conformation that does not recruit coactivators to target genes and in many cell and promoter contexts recruits corepressors (Shang et al., 2000; Shang and Brown, 2002). The therapeutic effectiveness of TAM in treatment of hormone-dependent cancers and in preventing breast cancer in high risk women is thought to arise primarily from its ability to compete with estrogens for binding to the ER. It is thought that TAM-ER and OHT-ER are unable to effectively activate transcription of genes important for the growth and development of estrogen-dependent tumors.

TAM has been the primary line of therapy for ER\(_a\)-positive breast cancer patients for nearly three decades and continues to be the choice of therapy for pre-menopausal patients (Cuzick et al., 2003). The majority of OVCA also express ER\(_a\) (Rao and Slotman, 1991; Havrilesky et al., 2001), but only a minority of patients respond to TAM (Hatch et al., 1991; Scambia et al., 1995). Lack of response to estrogen and anti-estrogen can result from primary resistance (e.g., due to lack of ER expression or activity) or can develop as an acquired resistance, for example, from activation of alternative pathways. The mechanism underlying TAM resistance in the course of OVCA progression is incompletely understood. However, IL-6 plays a critical role in the development and progression of OVCA (Syed et al., 2002; Yang et al., 2009; Rabinovich et al., 2007; Nilsson et al., 2005; Wang et al., 2012). Previous work from our group showed that IL-6 promoted human OVCA cell growth partly through the activation of the ER pathway (Yang et al., 2009). In the current study, we first demonstrated that the autocrine production of IL-6 by OVCA cell lines, including A2780, CAOV-3, and ES-2, is inversely associated with their response to TAM. Notably, we also observed that A2780 cells expressed IL-6 receptor, though they did not secrete IL-6, suggesting that the expression of IL-6 receptor by OVCA cells could be not associated with their IL-6 production status. Therefore, IL-6 receptor-bearing OVCA cell lines, non-IL-6-expressing and TAM-responsive A2780,

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**Fig. 9.** Physical interactions between endogenous ER\(_a\) or ER\(_\beta\) and SRC-1 in CAOV-3 and A2780 cells exposed to IL-6 (A and B). Cells were cultured in medium containing 1% sFBS for 24 h and then exposed to E\(_2\) (10 nM), IL-6 (50 ng/ml), or vehicle for 1 h. Nuclear extracts were precleared with rabbit IgG, immunoprecipitated with anti-SRC-1, anti-ER\(_a\) (A) or anti-ER\(_\beta\) (B) antibody, and then analyzed by Western Blot using antibodies as indicated. Effect of IL-6 on ER\(_a\) or ER\(_\beta\) nuclear translocation (C). A2780/pcDNA3.1(+) and A2780/ssIL-6 H cells were cultured in 1% sFBS condition and treated with or without 100 nM TAM and 10 nM E\(_2\). Nuclear proteins were isolated after overnight treatment and subjected to Western Blot analysis using anti-ER\(_a\) or anti-ER\(_\beta\) antibody. Lamin B1 was used as protein loading control.
and IL-6-overexpression and TAM-resistant CAOV-3 were suitable cell model to study the effect of IL-6 on TAM resistance.

Recently, Feng et al. have reported that overexpression of IL-6 in androgen-responsive LNCaP prostate cancer cells increases resistance to anti-androgen treatment with bicalutamide (Feng et al., 2005). However, the effect of IL-6 expression on the response to anti-estrogen treatment with TAM in OVCA has not been investigated. Here we demonstrate that both exogenous (a relatively short period of treatment with recombinant IL-6) and endogenous IL-6 (by transfecting with plasmid encoding for sense IL-6) induce TAM resistance in non-IL-6-expressing A2780 cells, while deleting of endogenous IL-6 expression in IL-6-overexpressing CAOV-3 cells (by transfecting with plasmid encoding for antisense IL-6) promotes cell sensitivity to TAM. These findings suggest that the production of IL-6 protects the cells from cytotoxic agents and the expression level of IL-6 is directly correlated to the degree of TAM resistance in OVCA cells. Other studies have shown that IL-6 transfection of breast cancer cell line (Conze et al., 2001) causes multidrug resistance to chemotherapeutics. Furthermore, the inhibition of IL-6 secretion in OVCA cell lines by siltuximab, a monoclonal anti-IL-6 antibody (Guo et al., 2010), or in prostate cancer cell lines by either anti-IL-6 antisense (Borsellino et al., 1995) or antisense IL-6 oligonucleotide phosphorothioates (Pu et al., 2004) increases the sensitivity of these cells to anticancer drugs. Our recent study has demonstrated that IL-6 secreted by OVCA cells may contribute to the refractoriness of these cells to cisplatin and paclitaxel through down-regulation of proteolytic activation of caspase-3 (Wang et al., 2010). Thus, some tumor cells may acquire the ability to express and produce IL-6 as a protective mechanism against drug induced death. The stimuli responsible for the constitutive expression of IL-6 in drug resistant cells remain to be determined. One possibility is that TAM itself may initiate an inflammatory response to increase IL-6 production by OVCA cells.

To date, the precise mechanism(s) by which autocrine IL-6 regulates an OVCA response to TAM remains unclear. It is now established that anti-tumor activity of TAM is mediated by binding to either ER subtype. However, it is generally only patients whose tumors are ER+ that are treated with TAM (or other endocrine therapies), but ER+ status is determined only by ERα. In fact, the majority of tumors being assessed in previous studies are those co-expressing ERα with ERβ. These data support the hypothesis that assessment of ERβ together with ERα is a better predictor of endocrine responsiveness than ERα alone (Murphy and Watson, 2006). One possible mechanism for this is that in combination with EGFR, estrogen and anti-estrogen responsiveness in OVCA is the relative expression of ERα and ERβ (Havrilesky et al., 2001). ERα and ERβ form heterodimers that retain DNA-binding ability in vitro. Fluorescence resonance energy transfer (FRET) analyses showed formation of both ERα and ERβ homo- and hetero-dimers in situ in living cells in culture (Bai and Giguère, 2003). Hall and McDonnell (Hall and McDonnell, 1999) have shown that ERβ contains a repressor domain and might act as an inhibitor of ERα-mediated transcription, suggesting that differential levels of the two isoforms might determine cellular responses to estrogen and anti-estrogen. In two studies of ER isoforms in ovarian tissues, the relative levels of ERα and ERβ were altered in OVCA compared with normal ovaries and benign and borderline tumors (Brandenberger et al., 1998; Pujol et al., 1998). In this study, we first observed that IL-6 can up-regulate ERα expression and down-regulate ERβ expression in a dose-dependent manner in A2780 cells in the absence of estrogen, which are in agreement with our previous findings obtained with another OVCA cell line, OVCAR-3 cells expressing both ERα and ERβ (Yang et al., 2009). Moreover, the mRNA and protein levels of ERα were elevated and those of ERβ were reduced in IL-6 overexpressed A2780 cells compared with the corresponding control cells, while the inverse was observed in IL-6 depleted CAOV-3 cells.

Both overexpression of ERα by sense ERα and knockdown of ERβ expression by ERβ shRNA in low ERα and high ERβ expressing A2780 cells increased cellular resistance to TAM treatment. Thus, changes in the ratio of ER isoforms by IL-6 coming from OVCA cells are a potential mechanism responsible for the refractoriness of these cells to endocrine therapy. Additionally, IL-6 expression attenuated TAM-mediated blockage of estrogen-induced ERα or ERβ nuclear translocation, thus allowing ERα or ERβ to translocate to nucleus and activate estrogen-responsive genes even in the presence of TAM. These results provide evidence that IL-6 may directly participate in TAM resistance.

The magnitude of ER gene regulation is influenced not only by the ligand but also by the presence of specific coregulatory proteins, present at rate-limiting levels, which modulate transcription. Over the past 10 years a number of nuclear receptor–interacting proteins have been isolated using various screening strategies. These include the p160 family coactivator proteins–SRC-1, SRC-2, and SRC-3. The coactivator proteins drive nuclear receptor transcriptional activity by doing the significant reactions required for control of enhancer-dependent gene expression. These coactivator proteins modulate the entire transcriptional process, including chromatin modification, transcription initiation, chain elongation, and RNA splicing, through to termination of the transcriptional response (O’Malley, 2007). A previous study has shown that estrogen can induce SRC-1 recruitment to the ER response element (ERE) in breast cancer cell lines and in primary cell cultures derived from patient tumors (Fleming et al., 2004). In patients undergoing endocrine treatment, SRC-1 protein expression strongly associates with reduced disease-free survival on both univariate and multivariate analysis (Myers et al., 2004). These data are supported by prostate cancer studies, in which SRC-1 protein expression was associated with both clinical and pathologic variables of increased tumor aggression (Agoulnik et al., 2005). Scott et al. observed that SRC-1 was elevated in TAM-resistant MCF-7 breast cancer cells relative to TAM-sensitive MCF-7 cells (Scott et al., 2007). Shang and Brown (2002) concluded that high levels of SRC-1 were sufficient to support the agonist activity of TAM and showed increased expression of ER-regulated genes by TAM stimulation in SRC-1-transfected MCF-7 cells, but not SRC-1-transfected cells. This is in agreement with Smith et al. (1997) who demonstrated that overexpression of SRC-1 was associated with the enhanced agonist activity of TAM, and upheld by Xu et al. (1998), who used SRC-1 knockout mice to demonstrate the potential involvement of SRC-1 in TAM resistance. Our recent studies in this study we found that IL-6 increased SRC-1 expression in a dose-dependent manner in OVCA cells as a mechanism of TAM resistance. Knockdown of SRC-1 expression by SRC-1 shRNA in CAOV-3 cells sensitized the cells to TAM treatment, whereas overexpression of SRC-1 in A2780 cells increased cellular resistance to TAM.

Specific coactivator-nuclear receptor interactions are thought to allow selective regulation of target genes through distinct histone acetylation (Shang and Brown, 2002). Both ERα and ERβ have a strong affinity preference for particular coactivators, which may be mediated through isoform specific utilization of different LXXLL motifs for their interaction with p160 proteins (Li et al., 2003). Wong et al. have observed that SRC-1 from hypothalamus, but not hippocampus, interacts more with ERα than ERβ in a ligand-dependent manner (Wong et al., 2001). Recently, quantitative coassociation analysis in the breast cancer patient tissue micro-array revealed significantly stronger colocalization of SRC-1 with ERα in patients who have relapsed in comparison with those patients in whom cancers did not recur, suggesting that the interactions of the p160 proteins with ERα can predict the response of patients to TAM treatment (Molenda-Figueira et al., 2008). However, others have reported SRC-1 ligand-dependent interactions with both ERα and ERβ (Bai and Giguère, 2003; Fleming et al., 2004). One possible mechanism responsible for low or absent endocrine responsiveness than ERα in drug resistant cells remain to be determined.
In vitro studies have demonstrated that while estrogen enhances the affinity of ERα and ERβ for p160 coactivators, TAM and other ER modulators inhibit this interaction (Li et al., 2003). In this study, SRC-1 immunoprecipitates of CAOV-3 cells naturally expressing high level of ERα protein or A2780 cells endogenously expressing high level of ERβ show that estrogen induces protein interactions between SRC-1 and both ERα and ERβ, whereas IL-6 selectively enhances the protein interactions between SRC-1 and ERα, but not ERβ. These data suggest that IL-6 secreted by OVCA cells may promote endocrine resistance via increase of ERα/SRC-1 interactions.

Why is there different responsiveness to TAM therapy between breast cancer and OVCA? Several explanations could be proposed. OVCA that express ER may be lower than breast cancer and less than the original estimates of 60% obtained by biochemical assays (Redmond et al., 2009), as immunohistochemical studies of cancer epithelial cells indicate that only 38% of OVCA are positive for ER (Rao and Slotman, 1991). Moreover, the average magnitude of receptor concentration in OVCA cells is lower than in breast or endometrial cancer cells (Kommoss et al., 1992). Clinical studies of TAM therapy may not accurately represent efficacy since they were conducted on small numbers of OVCA patients heavily pre-disposed with refractory disease (Redmond et al., 2009). Also, trials of hormonal treatment in OVCA have been retrospective and lack-


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