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Autocrine production of interleukin-8 confers cisplatin and paclitaxel resistance in ovarian cancer cells

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ABSTRACT

It has been widely reported that interleukin-8 (IL-8) is overexpressed in ovarian cyst fluid, ascites, serum, and tumor tissue from ovarian cancer (OVCA) patients, and elevated IL-8 expression correlates with a poor final outcome and chemosensitivity. However, the role of IL-8 expression in the acquisition of the chemoresistance phenotype and the underlining mechanisms of drug resistance in OVCA cells are not yet fully understood. Here we show that both exogenous (a relatively short period of treatment with recombination IL-8) and endogenous IL-8 (by transfecting with plasmid encoding for sense IL-8) induce cisplatin and paclitaxel resistance in non-IL-8-expressing A2780 cells, while deleting of endogenous IL-8 expression in IL-8-overexpressing SKOV-3 cells (by transfecting with plasmid encoding for antisense IL-8) promotes the sensitivity of these cells to anticancer drugs. IL-8-mediated resistance of OVCA cells exhibits decreased proteolytic activation of caspase-3. Meanwhile, the further study demonstrates that the chemoresistance caused by IL-8 is associated with increased expression of both multidrug resistance-related genes (MDR1) and apoptosis inhibitory proteins (Bcl-2, Bcl-xL, and XIAP), as well as activation of PI3 K/Akt and Ras/MEK/ERK signaling. Therefore, modulation of IL-8 expression or its related signaling pathway may be a promising strategy of treatment for drug-resistant OVCA.

1. Introduction

Ovarian cancer (OVCA) is the second most common and the most deadly malignancy of the female reproductive tract [1]. Although tumor-reductive surgery and carboplatin- and paclitaxel-based chemotherapy regimens are effective treatments for primary disease in the majority of OVCA patients, recurrence is common and often leads to death. As a consequence, the overall 5-year survival is only 30% [2]. Thus, there is a pressing need to either identify novel therapies for OVCA or to discover drugs which (re)sensitize tumor cells to existing chemotherapy. Several factors have previously been implicated in drug resistance, including genes which regulate drug influx and efflux, drug metabolism, damage repair, and the apoptotic response to drug-induced damage. Indeed, it is possible that numerous resistance mechanisms could contribute to a drug-resistant phenotype and these mechanisms might be coordinately regulated [3].

Interleukin-8 (IL-8) is a multifunctional chemokine that is secreted by multiple cell types, including monocytes, neutrophils, endothelial and mesothelial cells, and tumor cells. As a member of the Cysteine-X-Cysteine (CXC) motif chemokines, IL-8 is responsible for recruiting neutrophils, T cells, and basophils during immune system activation [4–7]. Induction of IL-8 expression is mediated primarily by activator protein and/or nuclear factor kappa B (NF-κB), although additional hormone response elements and nuclear factor IL-6 (NF-IL-6) consensus sites have been characterized on the IL-8 gene promote [8]. Studies have shown that tumor progression and metastasis may be associated with overexpression of IL-8 [9,10].

IL-8 was elevated in ovarian cyst fluid, ascites, serum, and tumor tissue from OVCA patients [11–22], and elevated IL-8 expression was associated with poor prognosis [15,22] and chemosensitivity [19–22]. Our previous study demonstrated that IL-8 may contribute to OVCA cell growth partly through the activation of androgen receptor (AR) and estrogen receptor (ER) pathways [23,24]. *In vitro* studies with OVCA cell lines show that generation of paclitaxel-resistant sublines is often associated with increased IL-8 mRNA

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expression using cDNA array technology [25]. However, the role of IL-8 expression in the acquisition of the chemoresistance phenotype and the underlining mechanisms of drug resistance in OVCA cells remain unclear.

IL-8 exerts its effects by binding to two cell-surface G protein-coupled receptors (GPCR), IL-8 receptor A and IL-8 receptor B or CXCR1 and CXCR2, respectively. Both receptors are expressed on most tumor cells as well as on endothelial cells [26,27]. Signals are transmitted across the membrane through ligand-induced conformational changes, exposing epitopes on the intracellular loops and carboxy-terminal tail of the receptor that promote coupling to functional heterotrimeric G proteins. After activation of heterotrimeric small G proteins, IL-8 signaling promotes activation of the primary effectors PI3 K (phosphotidylinositol 3 kinase) or phospholipase C, promoting the activation of Akt, PKC (protein kinase C), calcium mobilization and/or Ras/Raf/MEK(mitogen-activated protein or extracellular signal-regulated kinase kinase)/ERK(extracellular signal regulated kinase) signaling cascades [8]. In addition, IL-8 signaling activates members of the RhoGTPase family and activates a number of nonreceptor tyrosine kinases [e.g., Src family kinases and focal adhesion kinase (FAK)] that regulate the architecture of the cell cytoskeleton and its interaction with the surrounding extracellular environment [8]. Recently, growing evidence suggests activation of PI3 K/Akt [28–31] and Ras/Raf/MEK/ERK [32–34] signaling pathways play an important role in chemoresistance of OVCA. Therefore, we hypothesized that one potential mechanism that IL-8 induces chemoresistance of OVCA cells by triggering activation of PI3 K/Akt and Ras/MEK/ERK signaling.

In this study, we investigated the role of IL-8 expression in modulating cellular sensitivity to chemotherapeutic drugs in OVCA cells. Furthermore, we also explored possible underlying mechanisms involved in drug resistance induced by IL-8. Our data suggest that the autocrine production of IL-8 by OVCA cells promotes resistance of these cells to chemotherapy through decrease of proteolytic activation of caspase-3. The further study demonstrates that IL-8-induced resistance of OVCA cells may be associated with up-regulation of multidrug resistance-related genes [multidrug resistance gene 1 (MDR1)] and apoptosis inhibitory proteins [Bcl-2, Bcl-xL and X-linked inhibitor of apoptosis (XIAP)], as well as activation of Ras/MEK/ERK and PI3 K/Akt signaling.

2. Materials and methods

2.1. Cell lines and cell culture

Human OVCA cell lines A2780, CAOV-3 and SKOV-3 were obtained from the American Type Culture Collection. A2780 and SKOV-3 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 15% FBS.

Recombinant human IL-8 (R&D Systems, Minneapolis, MN) was used to pretreat A2780 cells. The cells were cultured in the presence of exogenous IL-8 (50 ng/ml) for 10 days. IL-8 was added to the culture every 2 days [35]. After the pretreatment period, the cells (A2780/preIL-8) were harvested, washed, and replated in the presence of IL-8, and their resistance to cisplatin or paclitaxel was determined by the MTT assay.

2.2. pcDNA3.1(+)-ssIL-8 (i.e., sense IL-8 vector) and pcDNA3.1(+)-asIL-8 (i.e., antisense IL-8 vector) expression vector construction

A 318 base pair cDNA fragment containing the full ORF of human IL-8 was amplified by RT-PCR from the RNA of SKOV-3 cell line that overexpresses IL-8. PCR primers for sense IL-8 were: forward 5′-CTC*GGATCC*ATGACTTCCAAGCTGGCCGTG-3′ to introduce a BamHI site as italicized, and reverse 5′-AGA*CTCGAG*TTATGAATTCTCA GCCCTCTT-3′ to introduce a XhoI site as italicized. PCR primers for antisense IL-8 were: forward 5′-CTC*GGATCC*ATGTGAATTCT-CAGCCCTCTT-3′ to introduce a BamHI site as italicized, and reverse 5′-AGA*CTCGAG*TTAACTTCCAAGCTGGCCGTG-3′ to introduce a XhoI site as italicized. The resulting sense or antisense IL-8 PCR product was cloned to pCR® 2.1 vector using Invitrogen's Original TA Cloning Kit (Carlsbad, CA, USA). After sequence confirmation, sense or antisense IL-8 was cut from the pCR® 2.1 vector, purified, subcloned to the BamHI and EcoRI (Life Technologies) sites of the pcDNA3.1(+) expression vectors (Invitrogen, San Diego, CA). We used DNA sequence analysis and restriction enzyme mapping to distinguish between the resulting plasmids, pcDNA3.1(+)-ssIL-8 (i.e., sense IL-8 vector) and pcDNA3.1(+)-asIL-8 (i.e., antisense IL-8 vector), which constitutively expressed the IL-8 cDNA in the sense and antisense orientations, respectively, from a cytomegalovirus promoter. We used the pcDNA3.1(+) vector without IL-8 inserts as negative controls in subsequent experiments.

2.3. Generation and selection of cells stably transfected with pcDNA3.1(+)-ssIL-8 and pcDNA3.1(+)-asIL-8

Transfection was done using Lipofectamine™ 2000 (Invitrogen, San Diego, CA) as recommended by the manufacturer's instructions. A2780 and SKOV-3 cells (4×10^5) were plated onto 6-well plates until 90–95% confluence before transfection. A2780 cells were transfected with 4 μg of pcDNA3.1(+)-ssIL-8, and SKOV-3 cells were transfected with 4 μg of pcDNA3.1(+)-asIL-8. Selection for the neomycin gene was initiated 48 h after transfection by adding 500 μg (A2780 cells) or 600 μg (SKOV-3 cells) of G418 (Life Technologies)/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 pcDNA3.1(+) was also transfected into A2780 or SKOV-3 cells and served as negative controls.

2.4. 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 follow: IL-8, 5′-AACATGACTTCCAAGCTGGCCG-3′ (forward) and 5′-CAGTTTT CCTTGGGGTCCAGAC-3′ (reverse), for MDR1, 5′-TGACTACCAGGCTCGCCAATGA T-3′ (forward) and 5′-TGT GCCACCAAGTAGGCTCCAAA-3′ (reverse), for Bcl-2, 5′-TGC ACCTGAC GCCCTTCAC-3′ (forward) and 5′-AGACAGCCAGGAGAAATCAAACAG-3′ (reverse), for Bcl-xL, 5′-ATGTCTCAGAGCAACCGGGAGC-3′ (forward) and 5′-GCGATC CGACTCACCAATACCT-3′ (reverse), for XIAP, 5′-ATGATACCATCTTCCAAAATC C-3′ (forward) and 5′-TTTCTGTAATG AAGTCTGACTT-3′ (reverse), for β-actin, 5′-TGGAATCCTGTGGCATC-CATGAAAC-3′ (forward) and 5′-TAAAACGCAGCTCAGTAACAGTCC-3′ (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.

2.5. Enzyme-linked immunosorbent assay (ELISA)

The cells were cultured for 48 h in 1 ml of medium containing 5% charcoal-stripped FBS (sFBS) (Life Technologies, Inc.). The supernatants were collected and clarified by centrifugation. The

level of IL-8 was measured using ELISA Kits (R&D Systems) according to the manufacturer's instructions.

2.6. Western blot analysis

Analysis and quantitation were performed as previously described [35]. In brief, cell lysates were subjected to 8–12 % SDS–PAGE and analyzed by blotting with rabbit polyclonal anti-IL-8RA, anti-IL-8RB, anti-MDR1, anti-Bcl-2, anti-Bcl-xL, or anti-XIAP antibody (Santa Cruz Biotechnology, Santa Cruz, CA), respectively. Membranes were stripped by incubating with stripping buffer at 50 °C for 30 min and then blotted with mouse monoclonal anti-β-actin antibody (Sigma). 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).

A2780 cells were plated in 100-mm culture dishes with the density of 4×10^6 cells for 24 h, and then incubated in 5% sFBS with vehicle DMSO, or wortmannin (100 or 200 nM), or PD98059 (25 or 50 μM) for 30 min prior to IL-8 (50 ng/ml) for 6 h. Total cell lysates were isolated and quantified. The phosphorylation status of Akt and ERK was analyzed by Western blot as described above, except that the filters were probed with anti-phospho-Akt or anti-phospho-ERK antibodies (Cell Signaling Technology, Beverly, MA) to detect phosphorylated Akt or phosphorylated ERK, visualized by chemiluminescent substrate Kit. The filters were subsequently stripped and then reprobed with anti-Akt antibodies (Cell Signaling Technology, Beverly, MA) or anti-ERK (BD Biosciences, San Diego, CA) to detect both the phosphorylated and unphosphorylated forms of Akt or ERK.

2.7. Cytotoxicity assay

In vitro cytotoxicity assays were performed by MTT assay as previously described [23,24]. MTT was obtained from Sigma (St. Louis, MO, USA). Briefly, 4×10^4 cells per well were plated in 96-well plates. Culture medium was RPMI 1640 containing increasing concentrations of cisplatin or paclitaxel (all obtained from commercial sources). 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 A_{490} nm of treated cells by the A_{490} nm of control cells. The IC_{50} is defined as the drug concentration required to inhibit A_{490} to 50% of the control value. IC_{50} values were estimated from the dose–response curve. Data were derived from at least three independent experiments.

A2780 cells pretreated with IL-8 (A2780/preIL-8) were plated in 96-well plates at 4×10^3 cells per well for 24 h, and then incubated in 5% sFBS with vehicle DMSO, or wortmannin (100 or 200 nM), or PD98059 (25 or 50 μM) for 30 min prior to IL-8 (50 ng/ml) and cisplatin (1 or 10 μM) or paclitaxel (0.01 or 0.1 μM) or control for 48 h. MTT assay was performed as described above. Data are shown as the mean ± SD of two separate experiments with sextuple samples.

2.8. Caspase-3 activation assay

Caspase-3 activation assay was performed as previously described [35]. Briefly, cells left untreated or treated with 10 μM cisplatin or 0.1 μM paclitaxel for 24 h and then lysed in lysis buffer for 10 min on ice. The lysed cells were centrifuged at 14,000 rpm for 5 min, and 100 μg of protein was incubated with 20 μl of reaction buffer and 10 μl of caspase-3 substrate at 37 °C for 1 h, and absorbance was measured at a wavelength of 405 nm on a plate reader. Blank values were subtracted, and fold increase in activity was calculated based on activity measured from untreated cells. Each sample was measured in triplicates.

2.9. Statistical analysis

Data are expressed as the mean of three experiments, each in triplicate or sextuple samples for individual treatments or dosage regimens. Statistical analysis was carried out using a one-way ANOVA, followed by Tukey's *post hoc* test. Values are presented as the mean ± SD. All statistical tests were two-sided and were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Comparing expression levels of IL-8 and its receptor (IL-8RA and IL-8RB) as well as sensitivity to cisplatin and paclitaxel in three OVCA cell lines

In order to investigate the role of IL-8 expression in the acquisition of the chemoresistance phenotype in OVCA cells, we first analyzed the expression of IL-8 and its receptor (IL-8RA and IL-8RB) as well as the response to cisplatin and paclitaxel in three OVCA cell lines. The secretion levels of IL-8 were significant various in three OVCA as shown in Fig. 1A. High and middle levels of IL-8 secretion were observed in CAOV-3 (12298.40 ± 305.47 pg/ml) and SKOV-3 (715.92 ± 35.28 pg/ml), respectively. However, no IL-8 was detected in the supernatant from A2780 cells. The mRNA levels of IL-8 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-8RA and IL-8RB (Fig. 1C). The sensitivity to cisplatin and paclitaxel also varied among these cell lines as shown in Fig. 1D and E. A2780 cells were the most sensitive (IC_{50} for cisplatin and paclitaxel were 8.06 ± 0.49 μM and 0.74 ± 0.08 μM, respectively), whereas CAOV-3 (IC_{50} for cisplatin and paclitaxel were 74.40 ± 0.82 μM and 9.65 ± 0.35 μM, respectively) and SKOV-3 cells (IC_{50} for cisplatin and paclitaxel were 66.19 ± 3.42 μM and 6.67 ± 0.17 μM, respectively), were drug-resistant ($P < 0.001$), indicating that autocrine production level of IL-8 by OVCA cell lines were inversely associated with their sensitivity to cisplatin and paclitaxel. Taken together, these results suggest that IL-8 receptor-bearing OVCA cell lines, A2780 (non-IL-8-expressing and drug-sensitive) and CAOV-3 or SKOV-3 (IL-8-over-expressing and drug-resistant) are the suitable cell models to investigate the effect of IL-8 on cisplatin- or paclitaxel-mediated cytotoxicity in OVCA cells.

3.2. IL-8 confers cisplatin and paclitaxel resistance in OVCA cells

Previous reports have suggested that elevated IL-8 expression correlates with poor prognosis [15,22] and chemosensitivity [19–22]. In correlation, our above results showed that autocrine production level of IL-8 by OVCA cell lines were inversely associated with their responsiveness to cisplatin and paclitaxel. It suggests that IL-8 could play a role in the resistance of OVCA cells to the cytotoxic activities of anticancer compounds. To address this hypothesis, A2780 cells were cultured in the presence or absence of IL-8 for 10 days. After the pretreatment period, the cells were harvested, washed, and replated in the presence or absence of IL-8, and their resistance to cisplatin or paclitaxel was determined by the MTT assay [23,24]. Pretreatment of A2780 cells with IL-8 caused 6.07-fold and 7.23-fold increase in resistance to cisplatin and paclitaxel, respectively ($P < 0.001$, Fig. 2), indicating that the presence of exogenous IL-8 increased the resistance of OVCA cells to cisplatin or paclitaxel treatment.

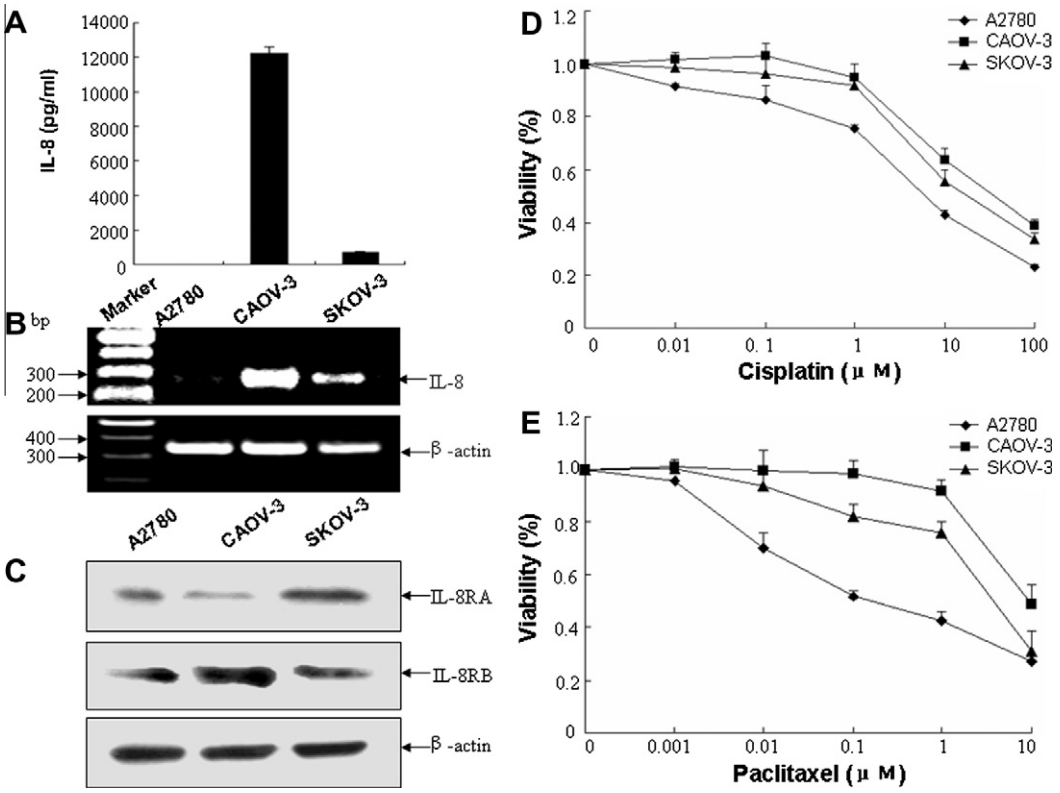

Fig. 1. Expression pattern of IL-8 and its receptor (IL-8RA and IL-8RB) as well as different sensitivity to cisplatin and paclitaxel in three OVCA cell lines. (A and B) Expression of IL-8 protein and mRNA in OVCA cell lines. Secreted IL-8 production in three cell lines were analyzed by ELISA. Data are shown as the mean of three separate experiments with triplicate samples and represent the mean ± SD. The mRNA level of IL-8 was detected by semiquantitative RT-PCR. Target fragment levels were normalized against β-actin. (C) Expression of IL-8RA and IL-8RB protein in OVCA cell lines. The same amount of total cell lysate of A2780, CAOV-3 and SKOV-3 cells were separated by 10% SDS–PAGE gel, followed by Western blot with anti-IL-8RA antibody, anti-IL-8RB antibody or anti-β-actin antibody. Target protein levels were normalized against β-actin to control for variance in sample loading and transfer. (D and E) Different responsiveness to cisplatin or paclitaxel in A2780, CAOV-3 and SKOV-3 cells was assessed by the MTT assay. The experiment shown is representative of three independent experiments with similar results.

To determine whether the endogenous production of IL-8 by tumor cells could provide self-protection against drug-induced cell death, we constitutively expressed IL-8 in A2780 cells and inhibited expression of IL-8 in SKOV-3 cells and examined the effect of IL-8 expression on the drug resistance of these cells. A2780 cells were transfected with plasmid encoding for sense IL-8 and SKOV-3 cells were transfected with plasmid encoding for antisense IL-8. Stable A2780 (A2780/ssIL-8) and SKOV-3 (SKOV-3/asIL-8) transfected clones were isolated and screened for their ability to produce IL-8. Three representative clones that produced low (19.86 ± 6.24 pg/ml, A2780/ssIL-8L), middle (73.44 ± 9.02 pg/ml, A2780/ssIL-8 M) and high (101.81 ± 13.01 pg/ml, A2780/ssIL-8H) levels of IL-8 ($P < 0.001$, Fig. 3A) and two representative clones that were middle (64.13%, SKOV-3/asIL-8 Mi) and high (77.52%, SKOV-3/asIL-8Hi) inhibition of IL-8 production ($P < 0.001$, Fig. 3C) compared with the corresponding parental (i.e., untransfected) and control vector-transfected A2780 and SKOV-3 cells were chosen for subsequent studies. The levels of IL-8 gene expression in the stable transfected clones were also examined by semiquantitative RT-PCR analysis. The levels of IL-8 mRNA were consistent with the secreted IL-8 levels in these stable transfected clones (Fig. 3B and D).

To determine whether the endogenous production of IL-8 in A2780 cells can confer resistance to drug treatment, we examined the susceptibility or resistance of ssIL-8-transfected A2780 cells to cisplatin or paclitaxel treatment using the MTT assay. As shown in Fig.4A and B, A2780/ssIL-8L, A2780/ssIL-8M and A2780/ssIL-8H cells exhibited increased resistance to both cisplatin (6.49-fold, 7.39-fold and 8.53-fold, respectively) and paclitaxel (6.36-fold, 7.59-fold and 8.08-fold, respectively), as compared with parental A2780 cells ($P < 0.001$). Control A2780/pcDNA3.1(+) cells that did not produce IL-8 exhibited similar drug sensitivity to parental A2780 cells ($P > 0.05$). These data suggest that overexpressing of IL-8 confers a moderate level of drug resistance in OVCA cells.

To determine whether deleting of endogenous IL-8 in SKOV-3 cells could increase their responsiveness to drug treatment, we also examined the susceptibility or resistance of asIL-8-transfected SKOV-3 cells to cisplatin and paclitaxel. In correlation with data from ssIL-8-transfected A2780 cells, SKOV-3/asIL-8Mi and SKOV-3/asIL-8Hi cells exhibited increased responsiveness to both cisplatin (IC_{50} was 10.58 ± 1.67 and 7.75 ± 0.88 μM, respectively, Fig. 4C) and paclitaxel (IC_{50} was 0.89 ± 0.05 and 0.76 ± 0.04 μM, respectively, Fig. 4D) as compared with parental SKOV-3 (IC_{50} for cisplatin and paclitaxel were 66.50 ± 3.42 and 6.79 ± 0.45 μM, respectively) and control SKOV-3/pcDNA3.1(+) cells (IC_{50} for cisplatin and paclitaxel were 65.32 ± 4.18 and 6.74 ± 0.33 μM, respectively) ($P < 0.001$), which did not vary ($P > 0.05$). These results indicate that deleting of endogenous IL-8 by OVCA cells restores their response to chemotherapy.

Caspase-3 plays a direct role in proteolytic cleavage of cellular proteins responsible for progression to apoptosis. To test whether cisplatin and paclitaxel resistance or susceptibility in ss/asIL-8-transfected cells may have affected caspase-3 activity with cisplatin or paclitaxel exposure, we measured caspase-3 activities in these cells after exposure to two drugs. As shown in Fig. 5, there was a significantly reduced level of caspase-3 in ssIL-8-transfected A2780 cells ($P < 0.001$, Fig. 5A and B), while there was a markedly increased in asIL-8-transfected SKOV-3 cells ($P < 0.001$, Fig. 5C and

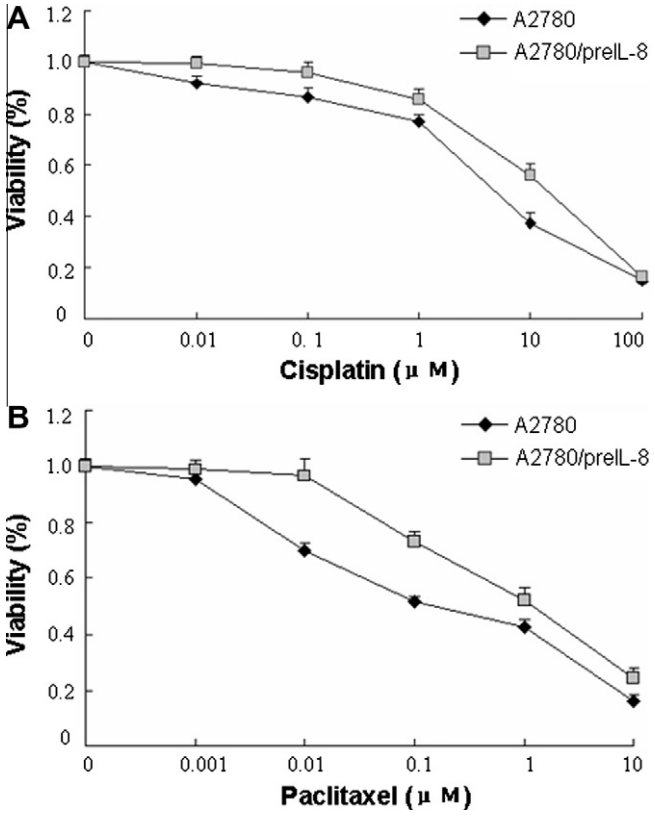

Fig. 2. Treatment with IL-8 increased the resistance of A2780 cells. A2780 cells were cultured in the presence (A2780/preIL-8) or absence (A2780) of exogenous IL-8 for 10 days. After the pretreatment period, the cells were replated in the presence or absence of IL-8, and their resistance to cisplatin (A) or paclitaxel (B) was determined by the MTT assay. The experiment shown is representative of three independent experiments with similar results.

D) as compared with the corresponding parental and control vector-transfected cells.

3.3. IL-8 up-regulates MDR1 expression in OVCA cells

Cross-resistance to both cisplatin and paclitaxel suggests a multidrug resistant phenotype possibly explained by drug transport or metabolism, cellular repair or detoxification mechanisms. To evaluate this possibility the expression of several genes already known to be involved in the mutidrug resistance phenomenon [MDR1, glutathione S transferase pi (GSTpi), multidrug resistance-associated protein (MRP), lung resistance-related protein (LRP) and topoisomerase I (TopoI)] in above several OVCA cell lines was first measured by semiquantitative RT-PCR and Western blot analysis. This analysis demonstrates that the mRNA and protein levels of MDR1, GSTpi, MRP, LRP and TopoI (data not shown) are lower in A2780 cells, while those are higher in CAOV-3 and SKOV-3 cells, indicating that autocrine production levels of IL-8 by OVCA cell lines were consistent with the expression levels of above several putative resistance factors in these cells. To determine the effects of exogenous and endogenous IL-8 on mutidrug resistance-related genes, we further studied IL-8-induced above several putative resistance factors expression of mRNA and protein in A2780 cells treated with IL-8, ss/asIL-8-transfected cells and the corresponding untransfected and control vector-transfected cells by semiquantitative RT-PCR and Western blot analysis. IL-8 significantly up-regulated the mRNA and protein levels of MDR1 (Fig. 6A and B) but not GSTpi, MRP, LRP and TopoI (data not shown) in a dose-dependent manner in A2780 cells. The mRNA and protein levels of MDR1 (Fig. 7A and B) but not GSTpi, MRP, LRP and TopoI (data not shown) enhanced in ssIL-8-transfected A2780 cells (Fig. 7A and B), and reduced in asIL-8-transfected SKOV-3 cells (Fig. 7C and D) compared with the corresponding parental and control vector-transfected cells, which had no difference. Therefore, these data suggest that IL-8 may confer cisplatin and paclitaxel resistance in OVCA cells by increasing MDR1 expression.

3.4. IL-8 up-regulates expression of Bcl-2, Bcl-xL and XIAP in OVCA cells

Previous study demonstrated that the expression of apoptosis inhibitory proteins [36–39] may be an important mechanism

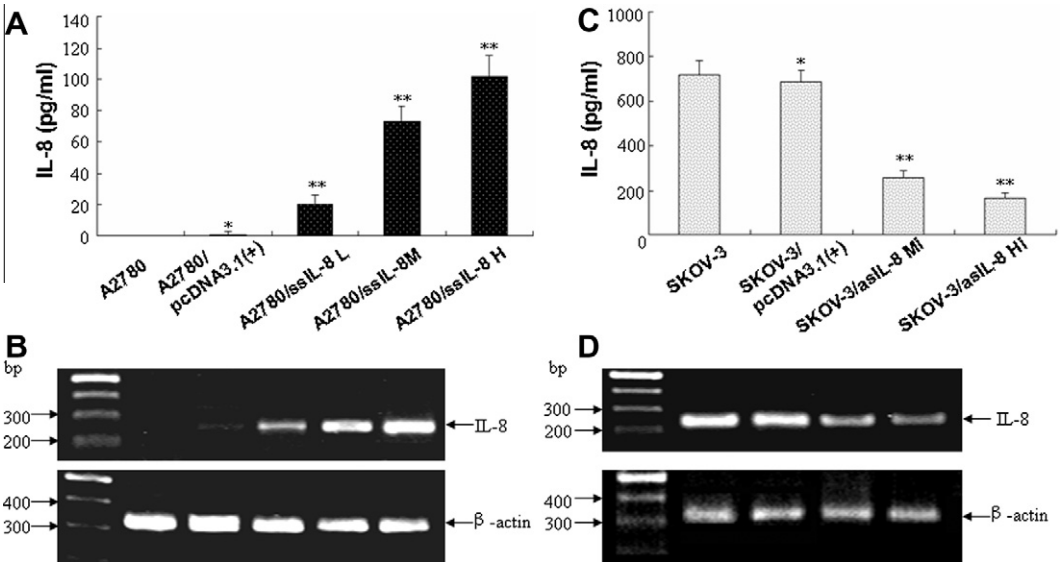

Fig. 3. Representative ELISA and RT-PCR demonstrating levels of IL-8 expression in A2780 and SKOV-3 cells and their transfectants. A2780 cells were stably transfected with empty vector or with vector encoding for sense IL-8, and SKOV-3 cells were stably transfected with empty vector or with vector encoding for antisense IL-8. Three stable sense IL-8-transfected A2780 clones that produced low (A2780/ssIL-8L), middle (A2780/ssIL-8 M) and high (A2780/ssIL-8H) levels of IL-8 (A and B), two stable antisense IL-8-transfected SKOV-3 clones that were middle (SKOV-3/asIL-8Mi) and high (SKOV-3/asIL-8Hi) inhibition of IL-8 production (C and D), and the corresponding control vector-transfected A2780 and SKOV-3 cells were isolated for further studies. $^{*}P > 0.05$, $^{**}P < 0.001$, compared with A2780 or SKOV-3 cells.

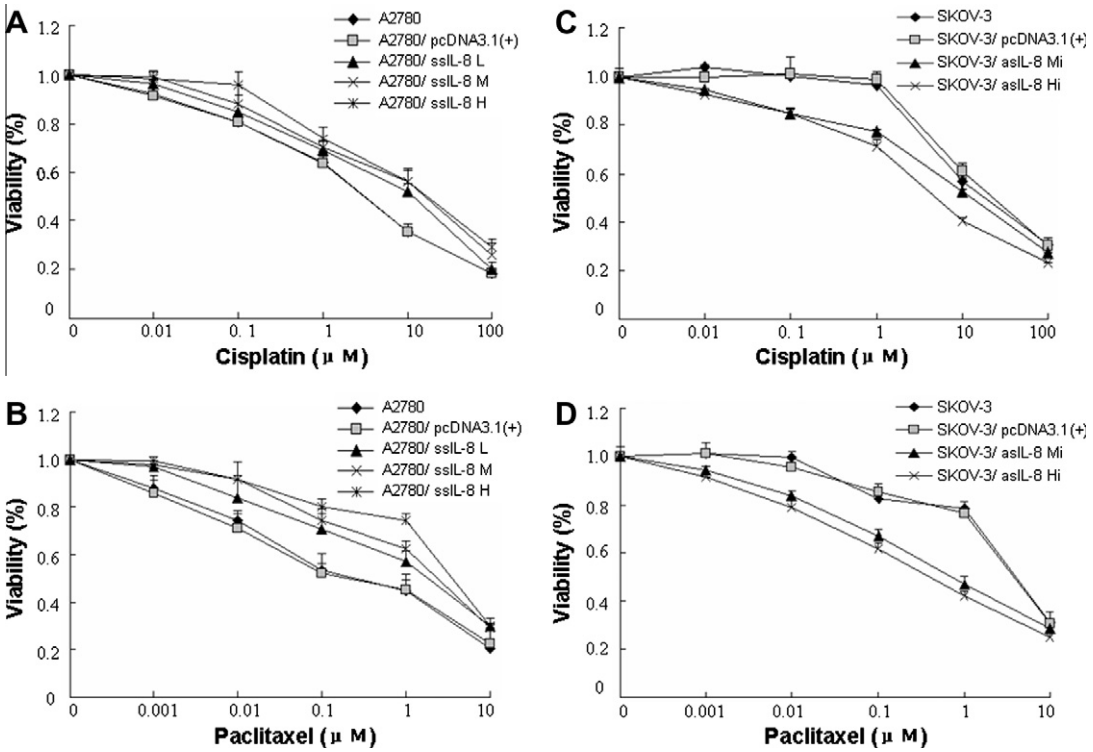

Fig. 4. Effect of IL-8 expression on the responsiveness of OVCA cells to cisplatin and paclitaxel. Three stable ssIL-8-transfected A2780 clones and their parental and control vector-transfected cells were plated out, and the sensitivity of the cells to cisplatin (A) and paclitaxel (B) was determined by the MTT assay. Similarly, the responsiveness of two asIL-8-transfected SKOV-3 clones and their parental and control vector-transfected cells to cisplatin (C) and paclitaxel (D) was examined by the MTT assay.

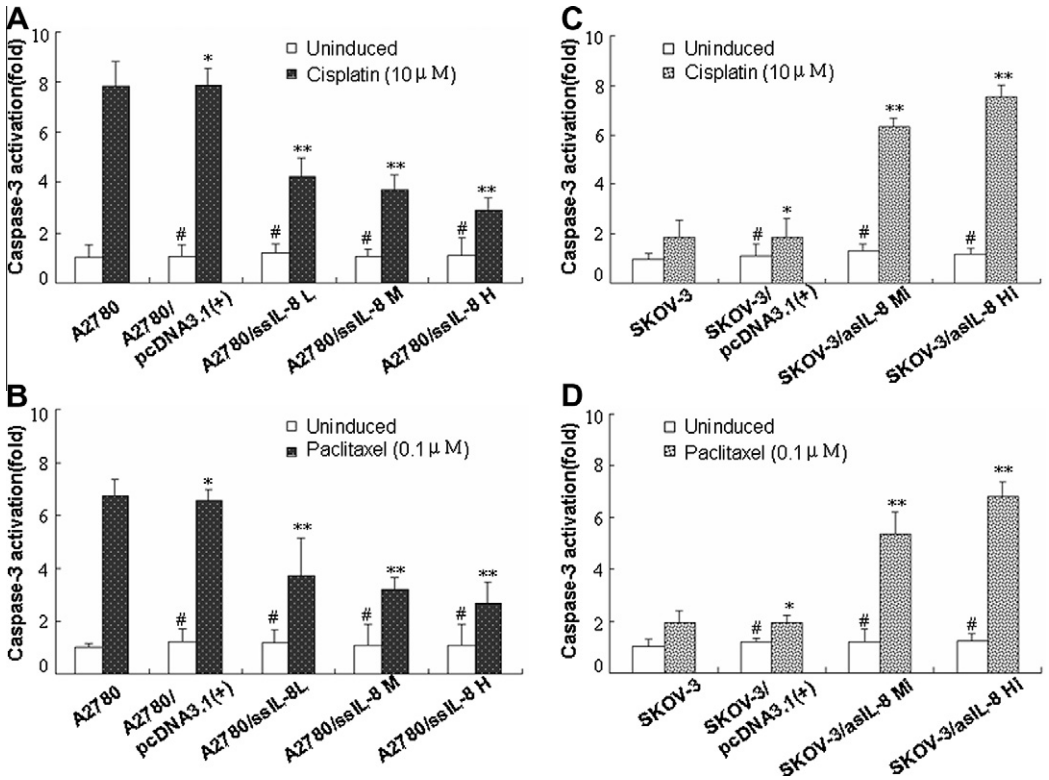

Fig. 5. Caspase-3 activity in ssIL-8-transfected A2780 cells (A and B), asIL-8-transfected SKOV-3 cells (C and D) and the corresponding parental and control vector-transfected cells at baseline and with cisplatin or paclitaxel treatment. The cells were treated with 10 μM cisplatin or 0.1 μM paclitaxel for 24 h. Caspase-3 activity was measured using the caspase-3 colorimetric assay. Data are shown as the mean of three separate experiments with triplicate samples and represent the mean ± SD. $^{\#}P > 0.05$, compared with uninduced A2780 or SKOV-3 cells; $^{*}P > 0.05$, $^{**}P < 0.001$, compared with cisplatin- or paclitaxel-induced A2780 or SKOV-3 cells.

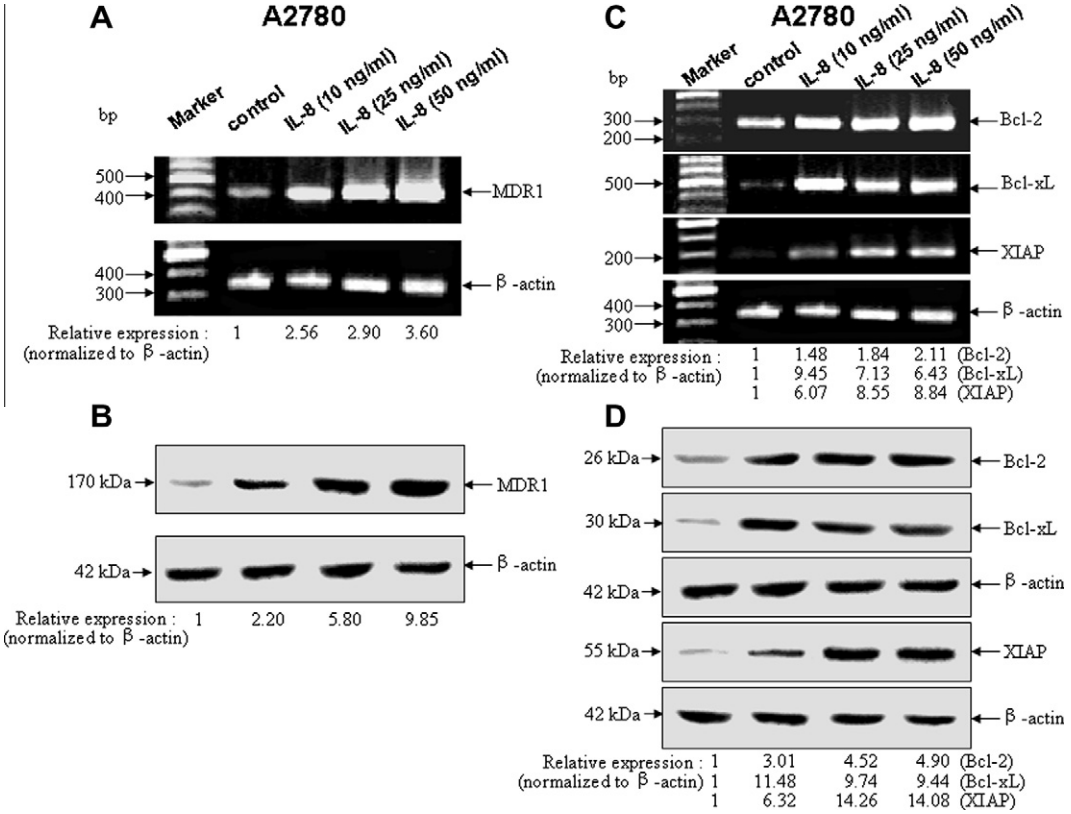

Fig. 6. Exogenous IL-8 increases MDR1 (A and B), Bcl-2, Bcl-xL and XIAP (C and D) expression in both mRNA and protein levels in A2780 cells. The mRNA and protein levels of MDR1, Bcl-2, Bcl-xL and XIAP were measured with semiquantitative RT-PCR and Western blot, respectively, as described above. The experiment shown is representative of three independent experiments with similar results.

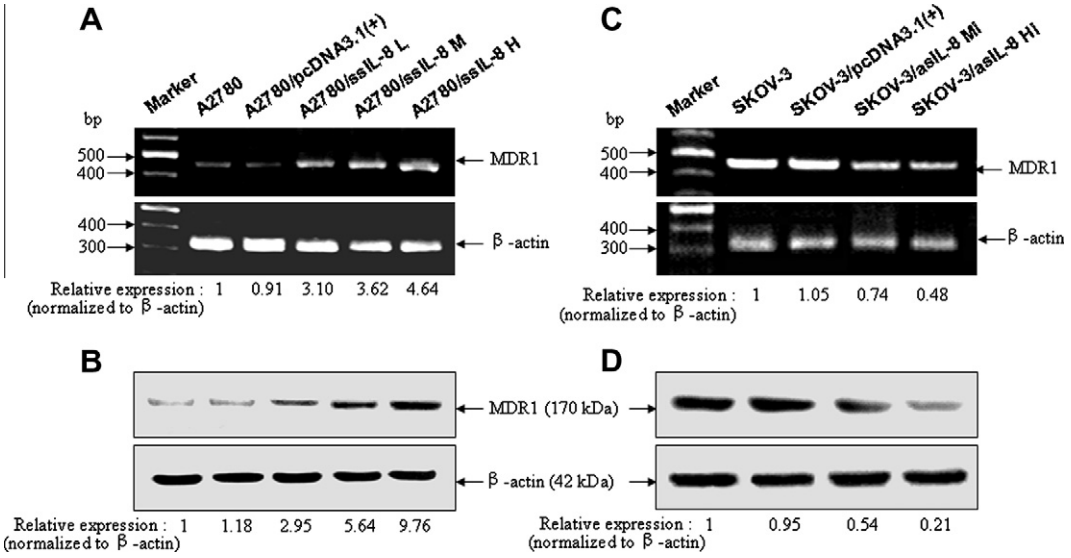

Fig. 7. Expression of MDR1 mRNA and protein in three stable ssIL-8-transfected A2780 clones, vector control A2780/pcDNA3.1(+) and parental A2780 cells (A and B). The mRNA and protein levels of MDR1 in two asIL-8-transfected SKOV-3 clones, vector control SKOV-3/pcDNA3.1(+) and parental SKOV-3 cells (C and D). The mRNA and protein levels of MDR1 was detected by semiquantitative RT-PCR and Western blot, respectively, as described above. The experiment shown is representative of three independent experiments with similar results.

responsible for chemotherapy resistance in OVCA. To determine the another potential mechanism through which IL-8 causes chemotherapy resistance in OVCA cells, we also examined the expression levels of several apoptosis inhibitory proteins in above several OVCA cell lines. The mRNA and protein levels of Bcl-2, Bcl-xL, XIAP and survivin (data not shown), are lower in A2780 cells, whereas those are higher in CAOV-3 and SKOV-3 cells, suggesting that autocrine production levels of IL-8 by OVCA cell lines were also in agreement with the expression levels of four apoptosis inhibitory proteins studied in these cells. To determine the effects of IL-8 on these apoptosis inhibitory proteins, we further studied IL-8-mediated mRNA and protein expression of Bcl-2, Bcl-xL, XIAP

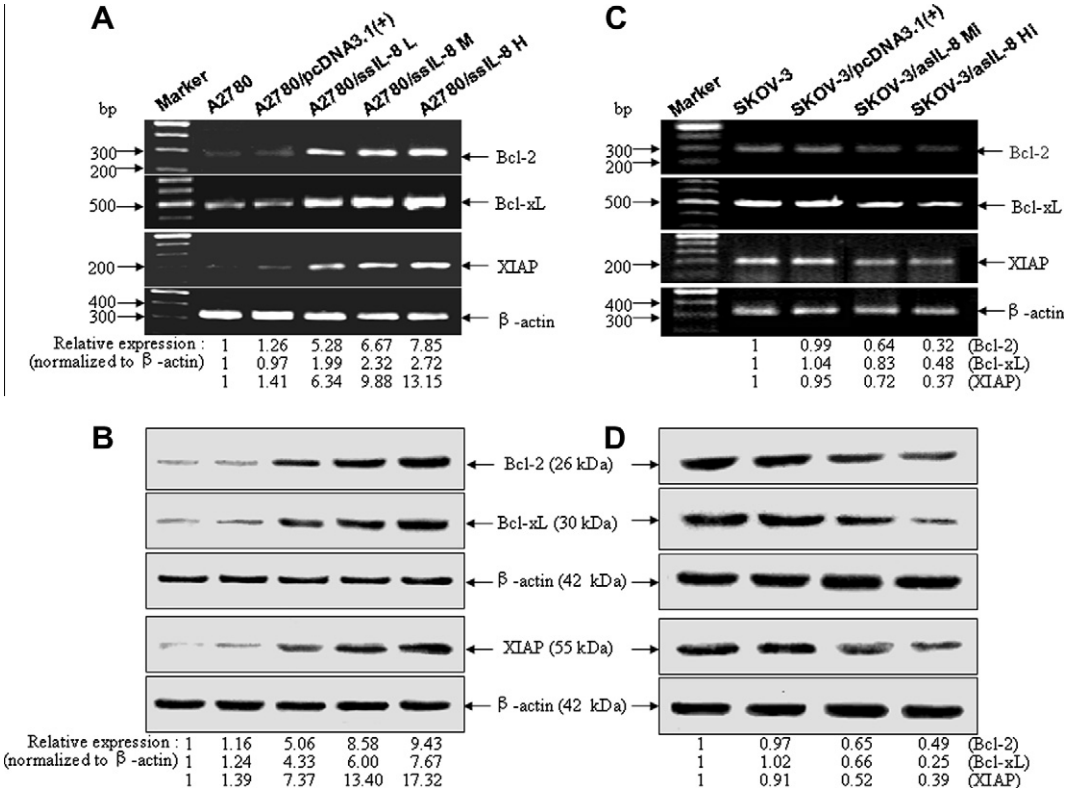

Fig. 8. The mRNA and protein levels of Bcl-2, Bcl-xL and XIAP in three stable ssIL-8-transfected A2780 clones (A and B), two asIL-8-transfected SKOV-3 clones (C and D), and the corresponding parental and vector control cells. The mRNA and protein levels of Bcl-2, Bcl-xL and XIAP were detected by semiquantitative RT-PCR and Western blot, respectively, as described above. The experiment shown is representative of three independent experiments with similar results.

and survivin in A2780 cells treated with IL-8, ss/asIL-8-transfected cells and the corresponding untransfected and control vector-transfected cells by semiquantitative RT-PCR and Western blot analysis. IL-8 significantly increased the mRNA and protein levels of Bcl-2, Bcl-xL and XIAP (Fig. 6C and D) in a dose-dependent manner, but had no effect on the mRNA and protein levels of survivin (data not shown) in A2780 cells. The mRNA and protein levels of Bcl-2, Bcl-xL and XIAP (Fig. 8) but not survivin (data not shown) up-regulated in ssIL-8-transfected A2780 cells (Fig. 8A and B), and down-regulated in as IL-8-transfected SKOV3 cells (Fig. 8C and D) compared with the corresponding untransfected and control vector-transfected cells, which did not vary. Taken together, these results suggest that IL-8 may cause chemoresistance in OVCA cells by enhancing Bcl-2, Bcl-xL and XIAP expression.

3.5. IL-8-induced chemoresistance to OVCA cells is through PI3K/Akt and Ras/MEK/ERK activation

To investigate what role PI3 K/Akt and Ras/MEK/ERK pathways play in the signal transduction of IL-8 in OVCA cells, we determined the effects of wortmannin, a PI3 K specific inhibitor at 100 or 200 nmol/L, and PD98059, a MEK1/2 specific inhibitor at 25 or 50 μmol/L, on IL-8-induced phosphorylation of Akt and ERK and IL-8-induced cisplatin and paclitaxel resistance of A2780 cells. It was found that wortmannin and PD98059 significantly antagonized IL-8-induced phosphorylation of Akt and ERK, respectively (Fig. 9A and B), and both of them blocked IL-8-induced cisplatin and paclitaxel resistance (Fig. 9C and D) and the inhibitory effects of wortmannin and PD98059 were dependent on its concentration. These data confirm that activation of Akt and ERK are mediated by PI3 K- and MEK1/2-dependent mechanism, respectively, and suggest that IL-8-induced cisplatin or paclitaxel resistance to OVCA cells is through activation of PI3 K/Akt and Ras/MEK/ERK.

In addition to detecting increased phosphorylation of Akt, increase in Akt expression in A2780 cells was also indicated by densitometry analysis after stimulation with IL-8 (Fig. 9B). Therefore, our experiments indicate that IL-8 signaling increases both the activation and the expression level of Akt in OVCA cells.

4. Discussion

It has been widely reported that IL-8 is overexpressed in ovarian cyst fluid, ascites, serum, and tumor tissue from OVCA patients [11–22], and elevated IL-8 expression correlates with a poor final outcome [15,22] and chemosensitivity [19–22]. Previous work from our group and others has shown that IL-8 promotes OVCA cell growth [22–24,27]. In the present study, we first demonstrated that autocrine production level of IL-8 by OVCA cell lines, including A2780, CAOV-3 and SKOV-3, is inversely associated with their response to cisplatin and paclitaxel. Some studies have consistently demonstrated that CAOV-3 and SKOV-3 cells are resistant to cisplatin, while A2780 cells are responsive [40,41]. Our data further demonstrated that CAOV-3 and SKOV-3 cells were also paclitaxel-resistant and A2780 cells were paclitaxel-sensitive. Furthermore, we found that CAOV-3 and SKOV-3 cells produced higher levels of IL-8, while A2780 cells do not. Notably, we also observed that A2780 cells expressed IL-8 receptor, though they did not secrete IL-8, suggesting that the expression of IL-8 receptor by OVCA cells could be not associated with their IL-8 production status. Therefore, IL-8 receptor-bearing OVCA cell lines, non-IL-8-expressing and cisplatin/paclitaxel-responsive A2780, and IL-8-overexpressing and cisplatin/paclitaxel-resistant CAOV-3 or SKOV-3 were used to study the effect of IL-8 on multidrug resistance, but in our study, A2780 and SKOV-3 cell lines were chosen as suitable cell models.

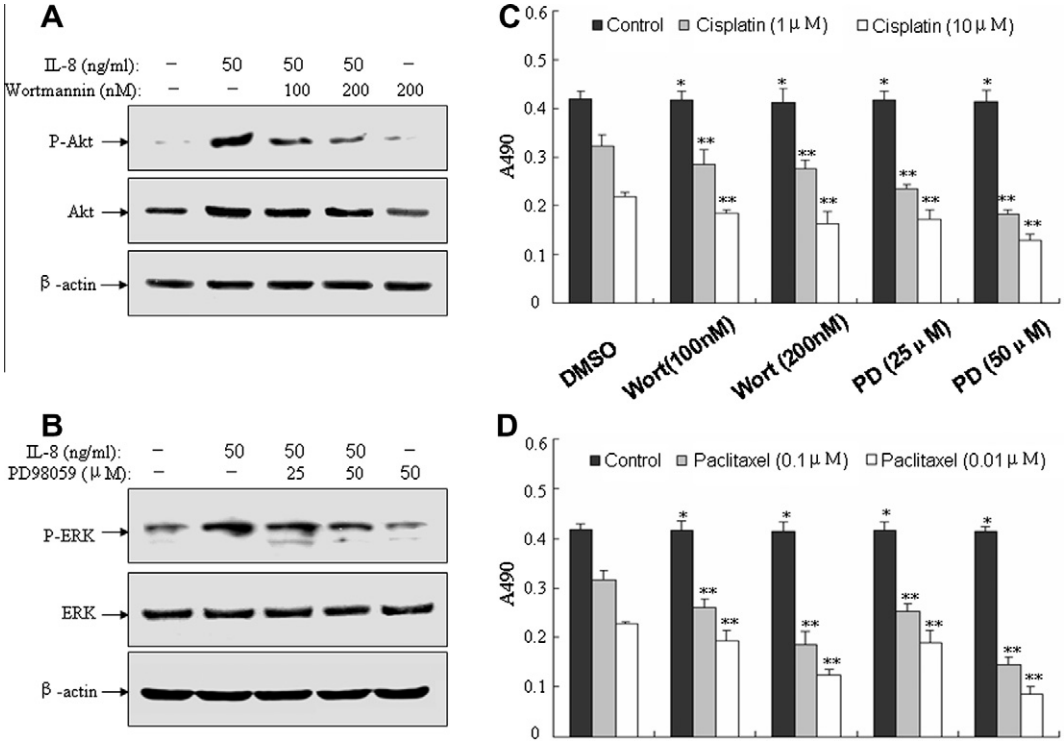

Fig. 9. Effects of wortmannin or PD98059 on IL-8-induced phosphorylation of Akt or ERK in A2780 cells and IL-8-mediated cisplatin and paclitaxel resistance to A2780 cells pretreated with IL-8 (A2780/preIL-8). The cells were pretreated with wortmannin (100 or 200 nM), PD98059 (25 or 50 μM) or DMSO of equal volume for 30 min at 37 °C before IL-8 was added into the medium. (A and B) A2780 cells were cultured for 6 h in the presence of IL-8. After the cells were collected and washed, whole-cell extracts were prepared and subjected to Western blot assay. (C and D) The cells were seeded into 96-well plate (4×10^4 cells per well) and cultured in the presence of IL-8 (50 ng/ml) and cisplatin (1 or 10 μM) or paclitaxel (0.01 or 0.1 μM) for 48 h. Afterwards, MTT assay was performed to determine the effect of wortmannin and PD98059 on IL-8-induced cisplatin or paclitaxe resistance to A2780 cells pretreated with IL-8 (A2780/preIL-8). The experiment shown is the average data of three independent experiments. $^{*}P > 0.05$, compared with control; $^{**}P < 0.001$, compared with cisplatin or paclitaxel (no inhibitors).

Several recent studies have addressed the role of IL-8 in tumor cell chemoresistance, including in melanoma [42], colorectal cancer [43], renal cell carcinoma [44], and prostate cancer [45,46]. However, the role of IL-8 expression in the acquisition of the multidrug resistance phenotype in OVCA has not been investigated. Here we show that both exogenous (a relatively short period of treatment with recombination IL-8) and endogenous IL-8 (by transfecting with plasmid encoding for sense IL-8) induce cisplatin and paclitaxel resistance in non-IL-8-producing A2780 cells, whereas deleting of endogenous IL-8 expression in IL-8-overexpressing SKOV-3 cells (by transfecting with plasmid encoding for antisense IL-8) promotes the sensitivity of these cells to anticancer drugs. Meanwhile, we confirm that IL-8-mediated resistance of OVCA cells exhibits decreased proteolytic activation of caspase-3. These findings suggest that the production of IL-8 protects the cells from cytotoxic agents through down-regulation of proteolytic activation of caspase-3 and expression level of IL-8 is positively associated with their degree of chemoresistance in OVCA cells. Other studies have shown that transfection of IL-8 into colorectal cancer [43] or prostate cancer cell line [45] causes drug resistance and inhibition of IL-8 overexpression in colorectal cancer [43] or prostate cancer [45,46] cell lines with small interfering RNA (siRNA) increases the sensitivity of these cells to anticancer drugs. Thus, some tumor cells may acquire the ability to express and produce IL-8 as a protective mechanism against drug induced death. The stimuli responsible for the constitutive expression of IL-8 in chemoresistant cells are not yet fully understood. Previous findings have suggested that expression of IL-8 is regulated by a number of different stimuli including inflammatory signals (e.g., tumor necrosis factor α, IL-1β), chemical and environmental stresses (e.g., exposure to chemotherapy agents and hypoxia), and steroid hormones (e.g., androgens, estrogens, and dexamethasone; reviewed in Ref. [47]). These stimuli may be associated with the constitutive expression of IL-8 in chemoresistant cells, but further experiments need to be done to determine this presumption.

In OVCA, up to two-thirds tumor specimens have been found to overexpress MDR1 (also known as ABCB1, which encodes the P-glycoprotein) on immunohistochemistry [48–50], and this overexpression has been shown in some cases to correlate with poor overall survival and chemotherapy resistance. Recently studies have shown that inhibition of MDR1 expression by siRNA in human multidrug resistant OVCA cell lines enhances the intracellular accumulation of and restored sensitivity to cisplatin [51]. The results of the many studies cited above suggests that MDR1 play an important role in the mechanisms responsible for chemoresistance of OVCA cells. Here we demonstrate that IL-8 regulates expression of MDR1 but not GSTpi, MRP, LRP and TopoI in OVCA cells, in correlation, increases the resistance of the cells to cisplatin/paclitaxel treatment. Thus, the regulation of MDR1 gene expression is a potential mechanism by which IL-8 provides drug protection. In this study, we first demonstrate that IL-8 up-regulates MDR1 gene expression in a dose-dependent manner in OVCA cells.

A number of studies have shown that the anti-apoptotic ability of IL-8 was associated with expression of the Bcl-2 and Inhibitor of Apoptosis (IAP) families proteins such as Bcl-2, Bcl-xL and survivin [46,52]. Bcl-2 [36], Bcl-xL [37], survivin [38] and XIAP [39] have been shown in OVCA to be associated with resistance to chemotherapy. Thus, we investigated whether IL-8 alters the expression of apoptosis inhibitory proteins as a mechanism of drug resistance. We found that IL-8 increased expression of Bcl-2, Bcl-xL and XIAP but not survivin in a dose-dependent manner in OVCA cells as a

mechanism of drug resistance. The enhancement of Bcl-2 and Bcl-xL expression by IL-8 in OVCA cells is in accordance with the results of Singh et al. [46] in prostate cancer cell line. While our findings that survivin levels are not altered by IL-8 in OVCA cells is different from the results of Wilson et al. [52], who have reported that stimulation of androgen-independent prostate cancer cells with IL-8 increases the transcription and expression of survivin. Here we provide the first evidence that IL-8 may up-regulates XIAP expression in a dose-dependent manner in OVCA cells.

Several lines of evidence implicated that the activation of PI3 K/Akt [28–31] and Ras/Raf/MEK/ERK [32–34], the most important cell survival signalings, protects OVCA cells from chemotherapy. It has reported that Akt inactivation sensitizes human OVCA cells to cisplatin [28] and paclitaxel [29]. Moreover, the inactivation of a downstream targets of the PI3 K/Akt pathway, such as BAD [28] and the transcription factors of Forkhead [53] and NFκB [54], also sensitize human OVCA cells to cisplatin *in vitro*. Finally, Akt inactivation by a PI3 K inhibitor also enhances the sensitivity of OVCA to cisplatin [28] and paclitaxel [29] *in vivo*. It has been shown that cisplatin treatment activates ERK in OVCA cells and that activation of ERK protects OVCA cells from cisplatin-induced death [32–34]. Furthermore, inhibition of ERK signaling by the MEK1/2 inhibitor PD98059 blocked ERK activation and increased cisplatin sensitivity in SKOV-3 cells [33]. In this study, IL-8-induced activation of ERK and Akt in OVCA cells is blocked by their specific inhibitors to signal transducers, which inhibit IL-8-induced cisplatin and paclitaxel resistance of OVCA cells. Also, we interestingly find that IL-8 increases not only phosphorylation of Akt but expression of Akt in OVCA cells, which is in accordance with the result of MacManus et al. [55] in prostate cancer cells. Taken together, our data suggest that IL-8 promotes chemoresistance of OVCA cells via activation of multiple signal transduction pathways including PI3 K/Akt pathway and ERK cascade. In toto, these results provide support for these signal transduction pathways as a strategy for reversing drug resistance.

In summary, we conclude that IL-8 secreted by OVCA cells may contribute to the refractoriness of these cells to conventional chemotherapy through down-regulation of proteolytic activation of caspase-3. Furthermore, IL-8-induced chemoresistance may be associated with increase of both multidrug resistance-related genes (MDR1) and apoptosis inhibitory proteins (Bcl-2, Bcl-xL and XIAP), as well as activation of PI3 K/Akt and Ras/MEK/ERK. Therefore, modulation of IL-8 expression or its related signaling pathway may be a promising strategy of treatment for drug-resistant OVCA.

Acknowledgments

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