Cancer Research

Increased Expression of Annexin A3 Is a Mechanism of Platinum Resistance in Ovarian Cancer

Xuedong Yan[1], Jie Yin[1], Huiyu Yao[2], Ning Mao[2], Yili Yang[3], and Lingya Pan[1]

Abstract

Resistance to platinum drugs has emerged as a major obstacle in the treatment of ovarian cancers. Through proteomic analysis, we have found that the expression of annexin A3, a member of the Ca^{2+} and phospholipid-binding annexin family, is significantly increased in platinum-resistant ovarian cell lines. Anti–annexin A3 immunostaining indicated that cancers from platinum-resistant patients also possess higher levels of annexin A3 than those from platinum-sensitive patients. Although expression of annexin A3 made susceptible ovarian cancer cells more resistant to platinum, expression of antisense annexin A3 downregulated its expression and rendered the resistant cells more sensitive to platinum. In athymic mice, the growth of tumors from inoculated SKOV3 cells was inhibited by the administration of platinum, whereas tumors from annexin A3–expressing SKOV3/Ann were resistant to platinum treatment. Interestingly, the intracellular platinum concentration and platinum-DNA binding are significantly lower in annexin A3–overexpressing cells than those in parental cells. The lower cisplatin concentration was also accompanied by reduced induction of p53, which could be restored by downregulation of annexin A3. These results indicate that increased expression of annexin A3 is a mechanism of platinum resistance in ovarian cancer. It seems to act by preventing uptake or accumulation of platinum in cells. Therefore, it is conceivable that annexin A3 could be a target for therapeutic intervention and may also serve as a biomarker for drug resistance in ovarian cancer patients. *Cancer Res; 70(4); 1616–24. ©2010 AACR.*

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Introduction

Ovarian cancer is the most common cause of cancer death from gynecologic tumors in the world. Cytoreductive surgery followed by a platinum-based chemotherapy is the main treatment strategy at present. However, initial responses to the drugs are often followed by the eventual evolution of fatal drug resistance. There are also no biomarkers that have achieved the necessary specificity to predict the development of platinum resistance in ovarian cancer patients. This has prompted extensive studies to investigate the cellular and molecular mechanisms of platinum resistance in various cancer cells. Development of platinum resistance has been attributed to decreased drug accumulation, enhanced detoxification capability, aberrant apoptosis pathway, and increased repair of drug-induced DNA damage (1–8). It has been reported that metallothioneins (9), glutathione *S*-transferase π (10), p53 (11), ERCC1 (12), copper transporters (13), and XIAP (14) can be responsible for these changes in cells. However, it is still not fully understood how these genes render cells resistant to platinum, and there are conflicting data regarding their roles in platinum resistance in patients (15–18).

Annexins belong to the protein family that includes five groups (A–E); among which, those in group A are the human and vertebrate orthologues and are identified numerically (19). As Ca^{2+}-dependent phospholipid-binding proteins, annexins often function as organizers of membrane domains and membrane-recruitment platforms for proteins in response to Ca^{2+} flux in cells (20). Therefore, they have diversified activities that include mediating the anti-inflammation action of glucocorticoids, participating in the regulation of blood coagulation, modulating ion channels, and participating in membrane fusion and exocytosis (19). The ability of annexin A5 to bind phosphatidylserine also makes it a valuable tool to detect apoptosis (21).

We have found that annexin A3 is one of the proteins upregulated in platinum-resistant ovarian cancer cell lines (22). In present study, the role of annexin A3 in platinum resistance is investigated through examining primary tumors and their gene overexpression and downregulation in culture and in athymic mice. Our data indicate that increased expression of annexin A3 is a specific mechanism for platinum resistance in ovarian cancers.

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doi: 10.1158/0008-5472.CAN-09-3215

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Materials and Methods

Cell lines and culture conditions. Human epithelial ovarian cancer cell lines SKOV3, SKOV3/Cis, SKOV3/Car, A2780, A2780/Cis, and A2780/Car were described previously (16, 23). They were maintained in DMEM supplemented with 10% FCS, 100 units/mL penicillin, and 150 μg/mL streptomycin.

Western blot analysis. The level of annexin A3 and p53 were analyzed using standard Western blot procedures described previously (23) with the rabbit anti-human annexin A3 primary antibody (24) and mouse anti-human p53 antibody (Santa Cruz Biotechnology), respectively.

Fluorescence microscopy. The cells were fixed with 4% paraformaldehyde for 20 min and made permeable with 0.2% Triton X-100. After blocking with 2% bovine serum albumin in PBS for 60 min, they were incubated at 4°C overnight with anti–annexin A3 antibody. Following incubation with goat anti-rabbit IgG antibodies conjugated with fluorescein (Santa Cruz Biotechnology), cell nuclei were stained with propidium iodide for 20 min. The specimens were examined under a Radiance 2100 confocal laser-scanning microscope (400×; Bio-Rad).

Patients, tissue specimens, and pathologic data. Tumor tissues were collected from 42 patients with epithelial ovarian cancer from 2000 to 2005 at the Department of Obstetrics and Gynecology, Peking Union Medical College Hospital, Beijing, China. The patients gave informed consent according to the criteria used at Peking Union Medical College Hospital. Relevant clinical information was gathered, and tissue samples and clinical data were anonymized. The tissue specimens were taken intraoperatively, immediately frozen in liquid nitrogen, and stored at −80°C.

The 42 patients were consecutively selected as follows: (*a*) patients who suffered from primary epithelial ovarian cancer; (*b*) patients who obtained the primary treatment in our hospital; (*c*) patients who nearly harbored no residual tumors after initial debulking surgery and comprehensive staging surgery; (*d*) patients who received a standard platinum combined adjuvant chemotherapy in at least six courses; and (*e*) patients who were in a routine follow-up for more than 3 years or till death.

According to the definition, all tissue specimens of the patients were divided into two groups, the platinum-sensitive group and the platinum-resistant group. Patients who responded to initial platinum-based therapy and experienced a treatment-free interval of 6 months or more are considered to be clinically platinum sensitive. Those who progress while on initial platinum-based therapy, whose best response to initial therapy is stable disease, or who relapse after initial response within 6 mo of completion of platinum-based therapy are considered to be clinically platinum resistant (25).

Follow-up. Patients were seen for follow-up 1 to 5 y after surgery; every 1 mo thereafter during the first year, every 3 mo in the second year, and then 6 mo or annually during the subsequent years. At each visit, a serum CA125 analysis and a pelvic examination were performed. Imaging studies or biopsies were performed as appropriate.

Immunohistochemistry. Immunostaining of tissue sections was performed using Dako LASB system (DakoCytomation) according to the manufacturer's instruction. The sections of normal hepatic tissue were used as positive controls for annexin A3. As a negative control, the sections were incubated with rabbit IgG at the same dilution. The tissue sections were evaluated under a light microscope (400×) and scored as follows: 1, no detectable immunostaining; 2, immunostaining <25%; 3, immunostaining between 25% and 50%; 4, immunostaining between 50% and 75%; 5, immunostaining >75%. The immunostaining intensity was evaluated by two independent observers who were blinded to clinical and annexin A3 data.

Construction of sense and antisense annexin A3–expressing plasmids and gene transfection. Annexin A3 cDNA (Genbank accession number gi: 4826642) was cloned through reverse transcriptase-PCR from SKOV3 cells and inserted into the *Eco*RI site of pcDNA3.1(+) (Invitrogen). The sequence and orientation of the constructs were confirmed by sequencing. Lipofectamine 2000 (Invitrogen) was used to transfect the cells according to manufacturer's instruction. Stable transfectants were obtained by selecting with medium containing 800 μg/mL G418.

Drug sensitivity assay. Single-cell suspensions were prepared and dispersed in 96-well plates. After incubation for 72 h with the platinum compounds, the MTT (Sigma) solution (0.5 mg/mL) was added. Following incubation for 4 h, 100 μL of extraction buffer were added to each well. After an overnight incubation, absorbance at 540 nm was measured using an Immunoskan 340 (Labsystems).

Intracellular content of cisplatin. Cells were seeded in triplicate at a density of 2×10^6/mL in complete medium and incubated with 10 μmol/L of cisplatin for 24 h. After washing three times with PBS, cells were scraped free in 1 mL of PBS. Following centrifugation at 4°C, the pellets were digested in 70% nitric acid at 65°C for 2 h and diluted to 5% nitric acid by adding an appropriate volume of double-distilled deionized water. Platinum in the hydrolysate was quantified using Agilent 7500ce inductively coupled plasma mass spectrometry (ICP-MS; Agilent).

Cisplatin-DNA binding. After being treated with 10 μmol/L of cisplatin for 24 h, the cells were washed three times with ice-cold PBS and harvested. A FlexiGene DNA Kit (Qiagen) was used for isolation of DNA. Aliquots of the DNA were digested in 70% nitric acid at 65°C for 2 h and diluted to 5% nitric acid. Platinum content was measured using Agilent 7500ce ICP-MS (Agilent).

In vivo *tumor growth inhibition studies.* All of the procedures involving animals in this study were approved by the animal care committee of Peking Union Medical College Hospital in accordance with institutional and Chinese government guidelines for animal experiments. SKOV3 and SKOV3/Ann cells were harvested during exponential growth of the cell culture. Five-week-old female nu/nu athymic mice were randomly divided into each group (n = 5) before inoculation. Five million cells were implanted s.c. in the flank of each mouse. I.p. cisplatin treatment (3 mg/kg/d) was initiated just after the inoculation in the treatment group and was

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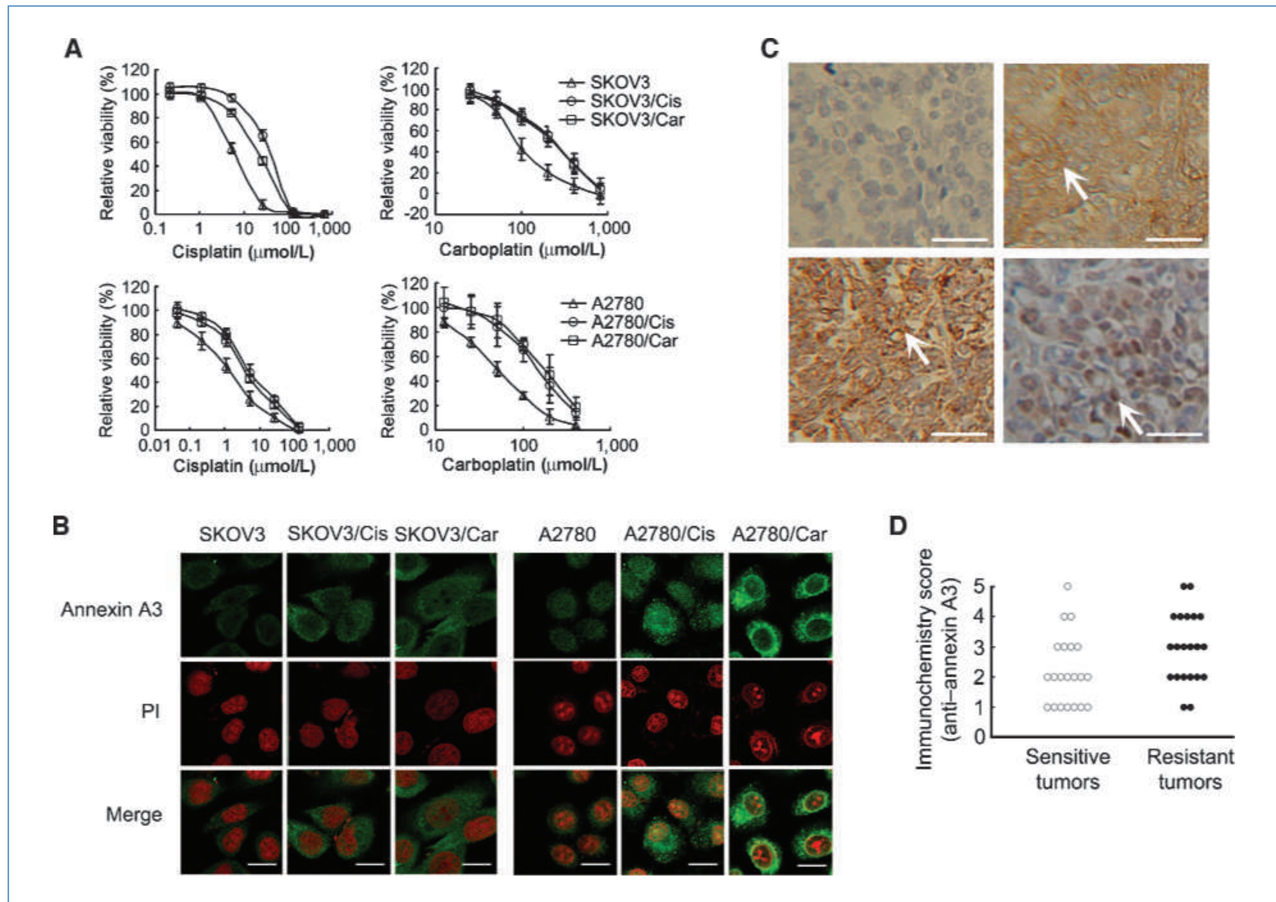

Figure 1. Annexin A3 expression is increased in platinum-resistant ovarian cancer cell lines and tumors from patients. A, the relative viability of the sensitive and resistant cell lines in the presence of cisplatin or carboplatin. Compared with their parent human ovarian cancer cell lines SKOV3 and A2780, platinum-selected SKOV3/Cis, A2780/Cis, SKOV3/Car, and A2780/Car are relatively resistant to cisplatin and carboplatin. B, intracellular staining of annexin A3 in SKOV3, A2780, and derived resistant cell lines. Anti–annexin A3 antibody and fluorescein-conjugated second antibody were used to visualize annexin A3 (green), and propidium iodide (PI) was used to stain cell nuclei (red). Annexin A3 is largely localized in cytoplasm, particularly the perinuclear area. The level of annexin A3 is increased in all the resistant cell lines. Bar, 20 μm. C, immunohistochemical staining of annexin A3 in various ovarian cancers. Top left, negative staining of endometrioid carcinoma; top right, positive staining of serous papillary cystadenocarcinoma. Bottom left, positive staining of transitional cell carcinoma; bottom right, positive staining of clear cell carcinoma. Bar, 100 μm. D, scores of annexin A3 immunohistochemical staining in ovarian cancer sections. Tumor sections were obtained from 21 chemosensitive and 21 chemoresistant patients. Analysis with Mann-Whitney *U* test shows that there is a significant difference between the two groups.

given every 2 d for a total of four times. The mice in control groups were treated with saline. Tumors were measured across three dimensions twice a week, and the tumor volume (mm^3) was calculated using the following formula: tumor volume = π (length × width × thickness) / 6. Inhibition of tumor growth was calculated according to the following formula: (1– the mean tumor volume of the treated group / the mean tumor volume of the control group at the time point of observation) × 100%.

Statistical analysis. Results were expressed as the mean ± SD of three to five independent experiments. Statistical analyses were carried out using one-way ANOVA and Student's *t* test to evaluate the continuous variables. To determine the relative significance between annexin A3 immunohistochemical staining and other factors such as drug resistance, patient age, pathologic type, pathologic stage, grade, and CA125 serum concentration, a nonparametric Mann-Whitney *U* test was used. All statistical tests were two-sided. Differences between groups were considered significant at $P < 0.05$. Data analyses were carried out using SPSS11.5 statistical software package.

Results

Expression of annexin A3 is increased in platinum-resistant ovarian cancer cells and tumors from patients. Between the two antitumor platinum compounds, cisplatin is commonly used for *in vitro* studies to investigate the mechanisms of platinum resistance, whereas carboplatin is widely used in the clinic due to its lower toxicity to the peripheral nervous system and kidneys. To determine whether the resistant cell line selected by cisplatin is also resistant to

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carboplatin, and vice versa, we assessed the viability of parental cell lines (SKOV3 and A2780), cisplatin-selected cell lines (SKOV3/Cis and A2780/Cis), and carboplatin-selected cell lines (SKOV3/Car and A2780/Car) following treatment with different concentrations of cisplatin or carboplatin. As shown in Fig. 1A, all the selected cell lines are more resistant to both cisplatin and carboplatin, suggesting that these cells developed similar mechanisms that make them resistant to the platinum compounds.

We have compared the mass spectrum profiles of the two sensitive cell lines (SKOV3 and A2780) with that of the four resistant cell lines (SKOV3/Cis, SKOV3/Cis, A2780/Car, and A2780/Car). Thirty-six differentially expressed proteins were identified (22). Among them, the altered expressions of annexin A3, destrin, cofilin, glutathione *S*-transferase, and cytoplasmic NADP$^+$-dependent isocitrate dehydrogenase have been validated using quantitative PCR and immunoblotting. In particular, the level of annexin A3 increased by 3- to 20-fold in all of the resistant cell lines (22). We also examined the expression of annexin A3 in the platinum-resistant cell lines by immunofluorescent staining with anti–annexin A3 antibody. In all the resistant cell lines, there is a significantly increased expression of annexin A3 that is mainly located in the cytoplasm, particularly in the perinuclear area and likely on the nuclear membrane (Fig. 1B).

To determine whether the expression of annexin A3 is related to platinum resistance, a total of 42 eligible patients, 50% of whom had ovarian cancers that were resistant to carboplatin, were enrolled and anti–annexin A3 immunostaining was performed on tumor tissue sections. The characteristics of 42 patients are shown in Table 1. There was no statistical difference among characteristics of patient data between the sensitive and the resistant groups. As observed in cultured cell lines, annexin A3 is mostly present in the perinuclear area in annexin A3–positive cancers (Fig. 1C). Compared with that of platinum-sensitive tumors, the immunostaining scores are significantly higher in platinum-resistant tumors ($P = 0.035$; Fig. 1D), indicating that the expression of annexin A3 is increased in tumors that are resistant to the platinum compounds.

Annexin A3 is a specific protein associated with platinum resistance. To further understand the role of annexin A3 in platinum resistance, SKOV3 and A2780 cells were transfected with an annexin A3–expressing plasmid and selected with G418 to generate stable transfectants. Compared with parental and vector-transfected cells, the stable transfectants of SKOV3 and A2780 (named SKOV3/Ann and

Table 1. Patient data from the ovarian cancer cohort

Characteristics	Sensitive *n* = 21 No. (%)	Resistant *n* = 21 No. (%)	Total *n* = 42 No. (%)	*P*
Age (y)				
<60	18 (86)	15 (71)	33 (79)	
≥60	3 (14)	6 (29)	9 (21)	0.265
Histology				
Serous papillary cystadenocarcinoma	12 (57)	11 (53)	23 (55)	
Mucinous cystadenoma	1 (5)	1 (5)	2 (5)	
Endometrioid carcinoma	6 (29)	3 (14)	9 (21)	
Transitional cell carcinoma	0 (0)	3 (14)	3 (7)	
Clear cell carcinoma	2 (9)	3 (14)	5 (12)	0.524
Grade				
G 1/2	5 (24)	4 (19)	9 (21)	
G 3	16 (76)	17 (81)	33 (79)	0.710
Stage				
II	5 (24)	1 (5)	6 (14)	
III	15 (71)	19 (90)	34 (81)	
IV	1 (5)	1 (5)	2 (5)	0.141
CA125 (units/mL)				
<35	0 (0)	4 (19)	4 (10)	
>35 to ≤200	6 (29)	7 (33)	13 (31)	
>200 to ≤1,000	7 (33)	4 (19)	11 (26)	
>1,000 to ≤3,000	5 (24)	4 (19)	9 (21)	
>3,000	3 (14)	2 (10)	5 (12)	0.204

NOTE: These patients had histologically confirmed epithelial ovarian cancer and received the postoperative chemotherapies based on carboplatin or cisplatin for at least six courses. They were classified as sensitive ($n = 21$) and resistant groups ($n = 21$) by the standards described in Materials and Methods. *P* values (two-sided) were calculated using the Mann-Whitney *U* test.

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A2780/Ann) express significantly higher levels of annexin A3 (Fig. 2A). We were also able to obtain stable transfectants by transfecting the four resistant cell lines with an antisense annexin A3–expressing construct and selecting with G418. The levels of annexin A3 were dramatically decreased in these transfectants (named SKOV3/Cis/R, SKOV3/Car/R, A2780/Cis/R, and A2780/Car/R; Fig. 2A). The sensitivities of these transfected cell lines to cisplatin and carboplatin were then assessed by the MTT assay. As shown in Fig. 2B, SKOV3/Ann and A2780/Ann exhibited a right-shifted dose-survival curve and become more resistant to cisplatin and carboplatin (Fig. 2B; Table 2). Compared with corresponding vector-transfected cells (SKOV3/Vec and A2780/Vec), the IC_{50} for cisplatin increased by 3.7- and 2.9-fold. Similar increases of IC_{50} were observed for carboplatin. Consistent with these results, SKOV3/Cis/R, SKOV3/Car/R, A2780/Cis/R, and A2780/Car/R all exhibited a left-shifted dose-survival curve when treated with the platinum compounds. The IC_{50} for them had a 1.6- to 2.4-fold decrease (Fig. 2C; Table 2).

It has been shown that tumor cells may develop cross-resistance toward various chemotherapeutic drugs in a single-step selection (16). We asked whether these cell lines

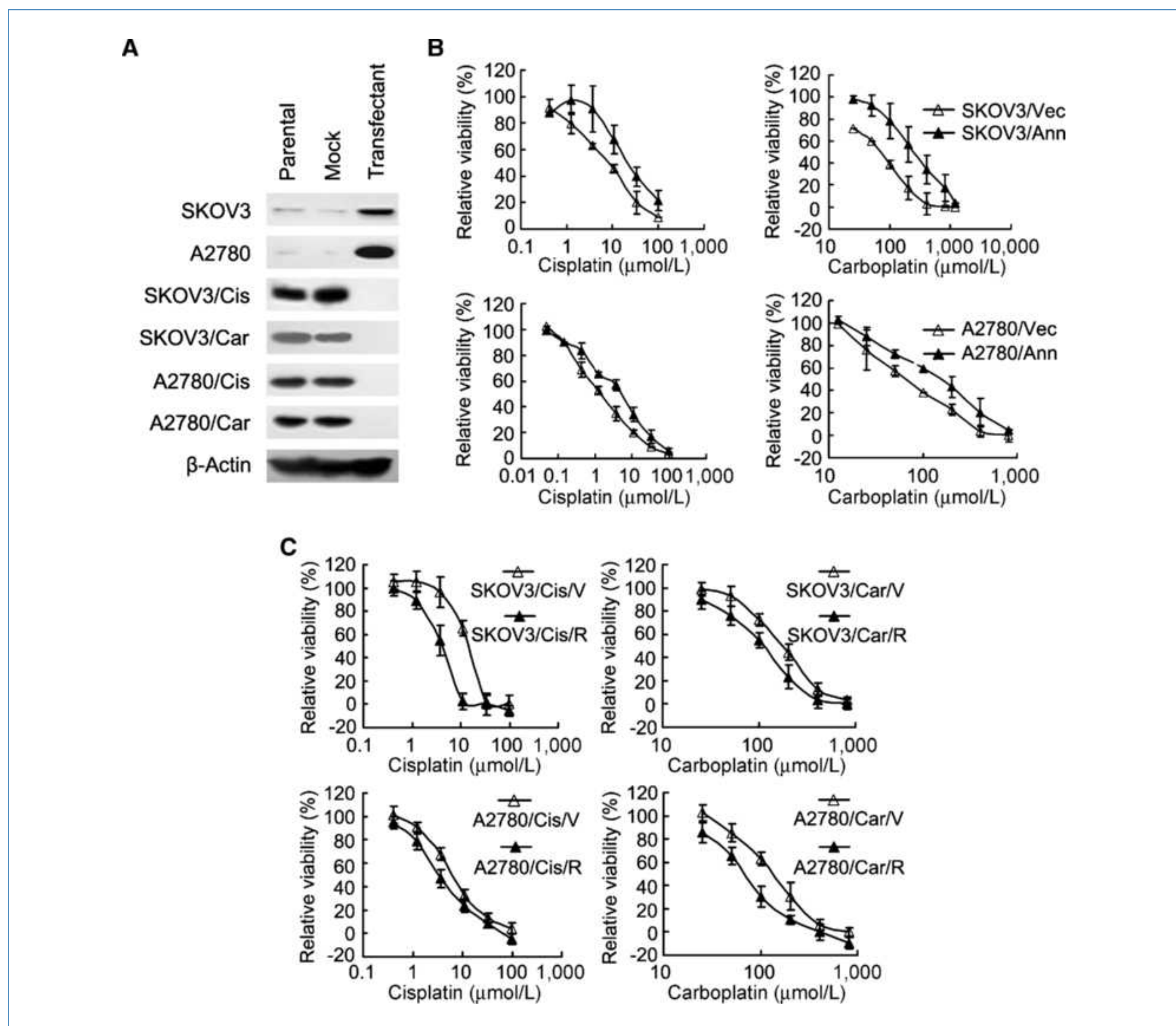

Figure 2. The level of annexin A3 correlates with the sensitivity of ovarian cancer cell lines to the platinum compounds. A, expression of annexin A3 in transfected cells. Transfection of SKOV3 and A2780 with annexin A3–expressing construct generated SKOV3/Ann and A2780/Ann, which express high levels of annexin A3. The resistant cell lines, SKOV3/Cis, SKOV3/Car, A2780/Cis, and A2780/Car, were transfected with antisense annexin A3–expressing plasmid. The transfectants, SKOV3/Cis/R, SKOV3/Car/R, A2780/Cis/R, and A2780/Car/R, express little annexin A3. Transfection of control vector to these cells generate SKOV3/Vec, A2780/Vec, SKOV3/Cis/V, SKOV3/Car/V, A2780/Cis/V, and A2780/Car/V, which had similar levels of annexin A3 with that of the parental cells. B, expression of annexin A3 made SKOV3 and A2780 cells more resistant to cisplatin and carboplatin. C, downregulation of annexin A3 made platinum-resistant cell lines more sensitive to cisplatin and carboplatin. Data represent mean ± SD from three independent experiments.

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Table 2. Sensitivity of cell lines to platinum, Taxol, and epirubicin

Cell lines	Cisplatin		Carboplatin		Taxol		Epirubicin	
	IC_{50} (μmol/L)	RI	IC_{50} (μmol/L)	RI	IC_{50} (nmol/L)	RI	IC_{50} (nmol/L)	RI
SKOV3/Vec	6.3 ± 1.4	1.0	77.6 ± 13.5	1.0	69.8 ± 9.0	1.0	282.9 ± 35.7	1.0
SKOV3/Ann	23.4 ± 3.2*	3.7	180.8 ± 36.0*	2.3	73.3 ± 14.5	1.1	242.5 ± 37.7	0.9
SKOV3/Cis/V	15.2 ± 3.5	1.0	256.9 ± 10.7	1.0	318.6 ± 41.8	1.0	571.0 ± 15.2	1.0
SKOV3/Cis/R	6.8 ± 3.4†	0.4	118.6 ± 35.4*	0.5	277.0 ± 30.0	0.9	544.7 ± 114.0	1.0
SKOV3/Car/V	10.5 ± 1.9	1.0	171.0 ± 20.1	1.0	150.2 ± 6.9	1.0	457.0 ± 53.7	1.0
SKOV3/Car/R	6.2 ± 1.1†	0.6	104.4 ± 17.5†	0.6	162.2 ± 73.1	1.1	379.0 ± 23.2	0.8
A2780/Vec	1.5 ± 0.3	1.0	67.5 ± 8.8	1.0	26.1 ± 5.6	1.0	358.2 ± 74.0	1.0
A2780/Ann	4.3 ± 0.8*	2.8	129.8 ± 19.1*	1.9	27.1 ± 2.4	1.0	333.2 ± 43.8	0.9
A2780/Cis/V	6.7 ± 1.2	1.0	148.3 ± 28.5	1.0	61.8 ± 13.2	1.0	1098.0 ± 87.4	1.0
A2780/Cis/R	3.0 ± 1.6†	0.4	70.3 ± 16.2†	0.5	50.1 ± 2.7	0.8	927.0 ± 19.2	0.8
A2780/Car/V	13.1 ± 4.1	1.0	132.9 ± 24.5	1.0	54.9 ± 9.0	1.0	665.4 ± 141.4	1.0
A2780/Car/R	5.5 ± 0.6†	0.4	64.7 ± 11.9†	0.5	43.8 ± 9.1	0.8	663.6 ± 136.2	1.0

Abbreviations: IC_{50}, 50% inhibitory concentration; RI, resistance index.
*$P < 0.01$ (two-sided Student's *t* test) compared with the cells transfected with mock plasmid.
†$P < 0.05$ (two-sided Student's *t* test) compared with the cells transfected with mock plasmid.

have also become resistant to Taxol and epirubicin. Whereas the platinum compounds kill tumor cells by causing cross-linking of DNA, Taxol and epirubicin act by preventing the breakdown of microtubules during cell division and intercalating DNA strands, respectively. Interestingly, the four resistant cell lines, SKOV3/Cis, SKOV3/Car, A2780/Cis, and A2780/Car, are also more resistant to Taxol and epirubicin, with a 1.6- to 4.5-fold increase of IC_{50}. However, resistance to these two compounds is not affected by enforced expression or downregulation of annexin A3 (Table 2), suggesting that other mechanisms, such as increased expression of glutathione *S*-transferase, may be responsible for their resistance in these cells. Taken together, these results indicate that an elevated level of annexin A3 is a specific mechanism for the increased tolerance to the platinum compounds in ovarian cancer cells.

Increase in expression of Annexin A3 reduces the accumulation of platinum in cells and decreases cisplatin-induced p53. One of the commonly accepted cellular mechanisms that contribute to resistance against various chemotherapeutic agents is reduced cellular accumulation of the drugs by a number of proposed methods (26). To determine whether this mechanism is related to the acquired resistance to the platinum compounds in ovarian cancer cells, cisplatin cellular accumulation and DNA binding were determined in the cell lines. After incubating with 10 μmol/L of cisplatin for 24 hours, the intracellular concentrations of cisplatin in SKOV3/Ann and A2780/Ann were significantly lower than those in SKOV3/Vec and A2780/Vec (Fig. 3A). The intracellular concentration of cisplatin in antisense annexin A3–transfected cells (SKOV3/Cis/R and A2780/Cis/R) was also significantly higher than in SKOV3/Cis and

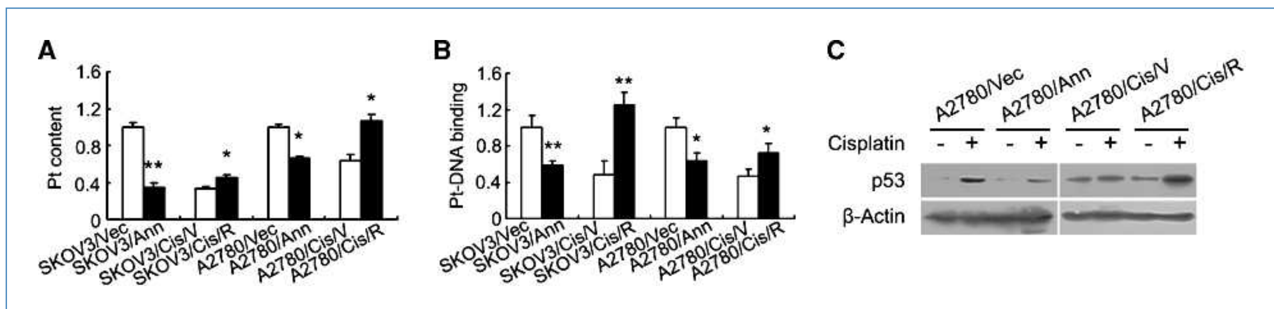

Figure 3. Increased expression of annexin A3 reduces the accumulation of platinum- and cisplatin-induced p53. A, intracellular platinum content of various cell lines after incubating with cisplatin for 24 h. There are significant differences between each pair of cell lines (*, $P < 0.01$; **, $P < 0.001$, two-sided Student's *t* test). B, the amount of DNA-bound platinum in various cell lines after incubating with cisplatin for 24 h. There are significant differences between each pair of cell lines (*, $P < 0.01$; **, $P < 0.001$, two-sided Student's *t* test). Data represent mean ± SD of three to four independent experiments. C, expression of p53 after cisplatin treatment in A2780 and derived cell lines. Upregulation or enforced expression of annexin A3 reduced cisplatin-induced p53, whereas downregulation of annexin A3 enhanced cisplatin-induced p53.

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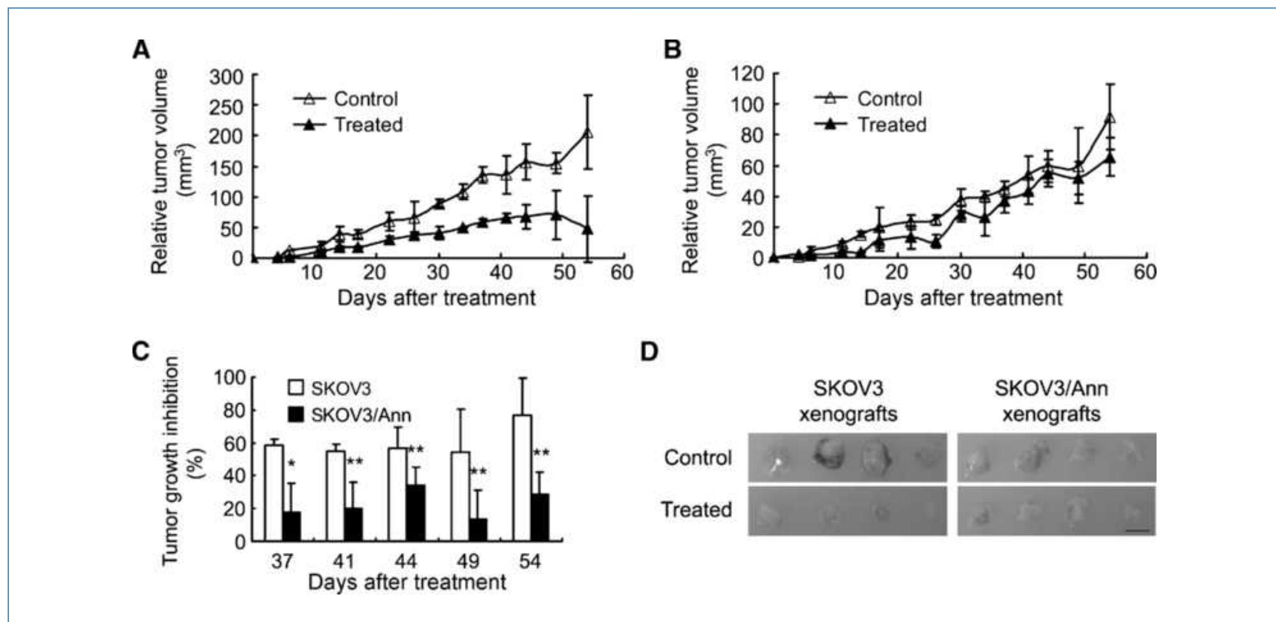

Figure 4. Annexin A3–expressing ovarian cancer cells are resistant to cisplatin *in vivo*. Athymic mice were inoculated with SKOV3 or SKOV3/Ann cells and given saline (control group) or cisplatin i.p. (3 mg/kg/d) every 2 d for four times (treatment group). A, growth curves of tumors from SKOV3 cells in the treatment and control groups. B, growth curves of tumors from SKOV3/Ann cells in the treatment and control groups. C, comparison of the cisplatin-induced growth inhibition of tumors from SKOV3 and SKOV3/Ann cells (*, $P < 0.05$; **, $P < 0.01$, two-sided Student's t test, $n = 5$). Data represent mean ± SD. D, photographs of tumors from SKOV3 and SKOV3/Ann cells in the control and treatment groups.

A2780/Cis cells transfected with control vector (Fig. 3A). As shown in Fig. 3B, similar results were observed when the levels of DNA-bound platinum were measured in various cell lines treated with cisplatin. These results indicated that overexpression of annexin A3 causes the decrease of intracellular platinum levels. Given the association of annexin family proteins with the membranes, it is conceivable annexin A3 may affect the transmembrane transportation of cisplatin. This may be particularly relevant to the nuclear uptake of cisplatin, as overexpressed annexin A3 is concentrated around the nucleus.

As a critical guardian of the genome, the intracellular level of tumor suppressor p53 is tightly controlled, primarily through Hdm2-mediated ubiquitination and proteasomal degradation (27, 28). In response to DNA damage, p53 level is increased to induce a cell cycle block, senescence, or apoptosis (29). It plays an important role in the cytotoxic action of chemotherapeutic agents in tumors retaining a wild-type p53 gene and may also be used as readout for the cellular effects of DNA-damaging agents. We asked whether overexpression of annexin A3 in A2780 cells, which contain wild-type p53, could affect cisplatin-induced increase of p53. As shown in Fig. 3C, expression of annexin A3 reduced the p53 response induced by cisplatin (compare lanes 2 and 4). Although the high–annexin A3 cell line A2780/Cis showed little p53 upregulation in response to cisplatin (compare lanes 5 and 6), downregulation of annexin A3 caused the cell line (A2780/Cis/R) to respond to cisplatin with a significant increase of p53 (compare lanes 7 and 8). These results are consistent with the observation that overexpression of annexin A3 reduces the amount of platinum bound to DNA and indicate that increased annexin A3 is likely the major mechanism responsible for cisplatin resistance.

***Enforced expression of annexin A3 confers platinum resistance* in vivo.** To determine whether overexpression of annexin A3 influences the sensitivity of ovarian cancer cells to platinum *in vivo*, athymic nude mice were inoculated with either SKOV3 or SKOV3/Ann cells. Both cell lines gave rise to tumors whose volume increased similarly postinoculation (Fig. 4A and B). Mice in the treatment group received cisplatin at 3 mg/kg/d i.p. every 2 days for a total of four times. Whereas cisplatin significantly inhibited the increase of the mean volumes of tumors from SKOV3 cells (Fig. 4A), it had much less of an effect on the growth of tumors from SKOV3/Ann cells (Fig. 4B). As shown in Fig. 4C, there are significant differences in mean tumor volumes between these two treated groups from day 27 to day 54 postinoculation. By day 54, treatment with cisplatin resulted in a reduction of tumor volume by 76.9 ± 22.5% compared with the controls, whereas the drug only reduced the size of SKOV3/Ann tumor by 28.5 ± 13.7% ($P = 0.002$; Fig. 4D). These results show that overexpression of annexin A3 in ovarian cancer cells is sufficient to confer resistance to cisplatin *in vivo*.

Discussion

Our studies found that annexin A3 is upregulated in ovarian cancer cells that are more resistant to cisplatin and carboplatin. There is also a significant increase of annexin A3 in platinum-resistant cancers from patients. Overexpression of

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annexin alone is sufficient to make the cells more resistant to the drugs *in vitro* and *in vivo*, whereas downregulation of annexin A3 makes the cells more sensitive to the platinum compounds. Furthermore, we have shown that annexin A3 selectively confers resistance to the platinum compounds through decreasing their intracellular concentration, which leads to a reduced p53 response to the drugs. The role of annexins in drug resistance was also supported by the findings that an increased expression of annexin A1 made cultured cells resistant to multiple drugs, and increased expression of annexin A4 is associated with resistance to paclitaxel (30, 31). These observations are also consistent with the role of annexins in many membrane-related events and the realization that one of the critical mechanisms in developing drug resistance is altered membrane protein trafficking.

Alterations of annexin expression have been found in a variety of cancers. The expression of annexin A1 was found to be increased in gastric, colorectal, pancreatic, and lung adenocarcinoma as well as in hepatocellular carcinoma, clear cell renal carcinoma, and hairy cell leukemia (32). However, annexins A1, A2, A7, and A11 were reported to be downregulated in a number of tumors (32, 33). In particular, expression of annexin A3 is increased in colorectal cancer (34) and prostate cancer (35). It was also found that colorectal cancer had increased expression of multiple annexins, including A1, A2, A4, and A11 (36). These studies clearly illustrate that there are complicated connections between members of the annexin family and cancers. Given the association between annexin A3 and platinum resistance we reported here, it is likely that the variations of annexins in tumors may be significantly affected by their histories of chemotherapies.

Although annexins are known as intracellular proteins, it has been shown that some annexins, such as A1 and A2, can be secreted and detected in culture medium or bodily fluids (37, 38). Therefore, it would be interesting to examine whether annexin A3 could be secreted by cultured ovarian cancer cells, in particular, those that are resistant to platinum compounds and express high levels of annexin A3. Given the increased expression of annexin A3 in tumors from platinum-resistant patients, it is also conceivable that examining the level of annexin A3 in serum might provide important information regarding the sensitivity of ovarian cancers to platinum treatment. Interestingly, it has been found recently that annexin A3 could be detected in urine sample of patients with prostate cancers, indicating that it is a hopeful candidate for noninvasive biomarker (39).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Joel D. Ernst (University of California, San Francisco, San Francisco, CA) for the anti–annexin A3 antibody; Dr. Alan O. Perantoni (National Cancer Institute, Frederick, MD) and Sheng Zhou (St. Jude Children's Research Hospital, Memphis, TN) for critical reading and comments on the manuscript; and our colleagues in the Department of Cell Biology, Institute of Basic Medical Sciences, for support and technical assistance.

Grant Support

Key Foundation of Peking Union Medical College Hospital grant 200203*.

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Received 8/28/09; revised 11/15/09; accepted 12/1/09; published OnlineFirst 1/26/10.

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