Phosphorylation of glycogen synthase kinase-3 ß at serine 9 confers cisplatin resistance in ovarian cancer cells

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Abstract. Cisplatin is commonly used in the treatment of advanced ovarian carcinoma. A major limitation of the use of cisplatin is the development of resistance in tumors. Glycogen synthase kinase-3 ß (GSK-3ß) is a multi-functional serine/threonine kinase. Its activity is regulated negatively by the phosphorylation of serine 9 (pGSK-3ß-ser-9) and positively by the phosphorylation of tyrosine 216 (pGSK-3ß-tyr-216). We compared the expression/phosphorylation of GSK-3ß between the cisplatin-sensitive ovarian carcinoma cell line A2780 and its cisplatin-resistant derivative CP70. The expression levels of total GSK-3ß and pGSK-3ß-tyr-216 were similar in these cells; however, CP70 cells had a much higher expression of pGSK-3ß-ser-9 than A2780 cells. Lithium chloride, which is a GSK-3ß inhibitor and stimulates pGSK-3ß-ser-9, significantly increased the IC_{50} of cisplatin and counteracted cisplatin-induced apoptosis of A2780 and CP70 cells. In contrast, overexpression of a constitutively active S9A GSK-3ß mutant increased the sensitivity of CP70 cells to cisplatin and significantly enhanced cisplatin-mediated apoptosis. It is suggested that the cisplatin-resistance of CP70 cells is mediated by stabilizing p53. We demonstrated that GSK-3ß negatively regulated the expression of p53. Therefore, pGSK-3ß-ser-9 may confer the cisplatin resistance of ovarian carcinomas through the stabilization of p53 expression. Our study establishes a potential role of GSK-3ß in the development of cisplatin resistance in initially sensitive tumors.

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Introduction

A major problem with current cancer chemotherapy is the emergence of drug resistance. Ovarian carcinoma is known to be one of the most chemoresponsive tumors. Cisplatin is commonly used in the treatment of advanced ovarian carcinoma (1,2). However, cisplatin chemotherapy is often limited by the development of resistance in initially sensitive ovarian cancers (1,2). The mechanisms of cisplatin resistance in ovarian cancers remain incompletely elucidated. Glycogen synthase kinase-3 ß (GSK-3ß) is a serine/threonine kinase that was first identified as a critical mediator in glycogen metabolism and insulin signaling. GSK-3ß is a multifunctional kinase; more than 40 proteins are substrates of GSK-3ß, including transcription factors, cell cycle/survival regulators and oncogenic/proto-oncogenic proteins (3,4). Unlike most protein kinases, GSK-3ß is constitutively active in resting cells and undergoes a rapid and transient inhibition in response to a number of external signals. GSK-3ß activity is regulated by site-specific phosphorylation. Full activity of GSK-3ß generally requires phosphorylation on tyrosine 216, and conversely, phosphorylation at serine 9 inhibits GSK-3ß activity (3,4). GSK-3ß is a negative regulator of Wnt/ß-catenin signaling (3). Dysregulation of GSK-3ß has been implicated in tumorigenesis (5). The involvement of GSK-3ß in chemotherapy resistance in cancer cells, however, has not been investigated. Multiple mechanisms of cisplatin resistance have been described in ovarian carcinomas; one mechanism is resistance to cisplatin-induced apoptosis (6). Since GSK-3ß is a critical regulator of cell survival, we hypothesized that GSK-3ß plays a role in cisplatin resistance. In this study, we demonstrated that the cisplatin-resistant ovarian carcinoma cell line expressed much higher levels of serine 9-phosphorylated GSK-3ß (pGSK-3ß-ser-9) than cisplatin-sensitive counterparts. Inhibition of GSK-3ß activity by lithium chloride enhanced resistance to cisplatin; in contrast, expression of the constitutively active S9A GSK-3ß mutant increased the sensitivity of CP70 cells to cisplatin.

Materials and methods

Reagents. Antibodies against ß-actin, GSK-3ß, phospho-GSK-3ß (tyr-216) and phospho-GSK-3ß (ser-9) were obtained from

Cell Signaling Technology (Beverley, MA, USA). Antibodies against p53, p21 and GADD45 were obtained from Santa Cruz (Santa Cruz, CA, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

Cell culture and treatment. Human ovarian epithelial adenocarcinoma cell lines (A2780 and CP70) obtained from ATCC (Rockville, MD, USA) were grown in Eagle's MEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine and 25 μg/ml gentamicin, 100 units/ml penicillin and 100 μg/ml streptomycin at 37˚C with 5% CO_2. For determination of the inhibitive concentration 50 (IC_{50}) of cisplatin, cells were plated into tissue culture wells for 24 h, then exposed to different cisplatin concentrations for 72 h. The number of viable cells was determined by MTT assay (7). For analysis of apoptosis, A2780 and CP70 cells were treated with 20 μM or 40 μM cisplatin, respectively; the concentrations induced similar initial DNA damage in these cells (8).

Cell transfection. pcDNA3 carrying V5-tagged GSK-3ß (S9A) mutant was a kind gift from Dr Thilo Hagen (University Hospital Nottingham, Nottingham, UK). Cells were transfected with an S9A GSK-3ß mutant construct or empty pcDNA3 using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Assay for cell viability and apoptosis. Cell viability was measured by MTT assay as previously described (7). Apoptotic cells were determined by nuclear morphology using DAPI staining as previously described (9). In addition, apoptosis was quantified by the expression of Annexin-V using an Annexin-V FITC apoptosis detection kit according to the manufacturer's instructions. Briefly, cells ($1x10^6$) were suspended in 500 μl binding buffer (10 mM HEPES/NaOH, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 1.8 mM $CaCl_2$) and treated with 10 μl of FITC-conjugated anti-Annexin V-antibody and 10 μl of propidium iodide (PI) for 10 min in the dark at room temperature. The samples were analyzed by flow cytometry (Ex = 488 nm, Em = 530 nm).

Sample preparation and immunoblotting. Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% Na-deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 mM Na_3VO_4; 1 mM NaF; 1 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin). Cellular proteins were extracted and protein concentrations were determined as previously described (7). The immunoblotting procedure has been previously described (7). Briefly, aliquots of the protein samples (20 μg) were loaded into the lanes of an SDS-polyacrylamide gel. The protein samples were separated by electrophoresis, and the separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with either 5% BSA or 5% nonfat milk in TBST (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tween-20) at room temperature for 1 h. Subsequently, the membranes were probed with primary antibodies directed against target proteins for 2 h at room temperature or overnight at 4˚C. The dilutions for the primary antibodies were as follows: anti-GSK-3ß, 1:1500; anti-phospho-GSK-3ß-ser-9, 1:1000; anti-phospho-GSK-3ß-tyr-216, 1:500; anti-ß-actin, 1:5000; anti-p53, 1:1000; anti-p21, 1:250; and anti-GADD45, 1:200. After three quick washes in TPBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL, USA) diluted at 1:2000 in TPBS for 1 h. The immune complexes were detected by the enhanced chemiluminescence method. The expression of target proteins was normalized to the levels of actin. The density of immunoblotting was quantified with the software of Quantity One (Bio-Rad Laboratories, Hercules, CA, USA).

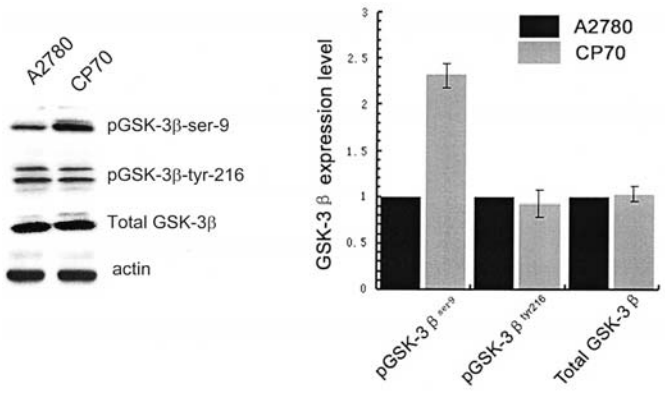

Figure 1. Expression of GSK-3ß in A2780 and CP70 cells. The expression of GSK-3ß and its phosphorylated forms (pGSK-3ß-ser-9 and pGSK-3ß-tyr-216) in A2780 and CP70 cells was examined with immunoblotting. The expression of ß-actin served as a loading control. The relative expression levels were quantified microdensitometrically and normalized to the expression of actin. Each data point (± SEM; bar) is the mean of three experiments.

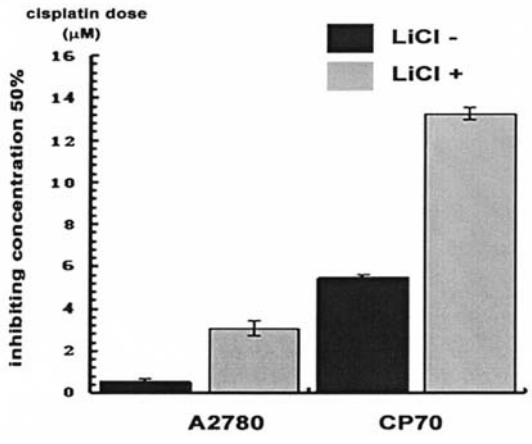

Figure 2. Effect of LiCl on the IC_{50} of cisplatin. A2780 and CP70 cells were pretreated with LiCl (0 or 20 μM) for 6 h, then exposed to cisplatin at a range of concentrations for 72 h. Cell viability was determined by MTT assay as described under the Materials and methods. The IC_{50} of cisplatin for A2780 and CP70 cells was calculated. Each data point (± SEM; bar) is the mean of three experiments.

Statistical analysis. The paired Student's t-test was used to determine the difference between treatment groups.

Results

First, we compared the expression of GSK-3ß and its two phosphorylated forms (pGSK-3ß-ser-9 and pGSK-3ß-tyr-216) in cisplatin-sensitive ovarian carcinoma cells (A2780) and

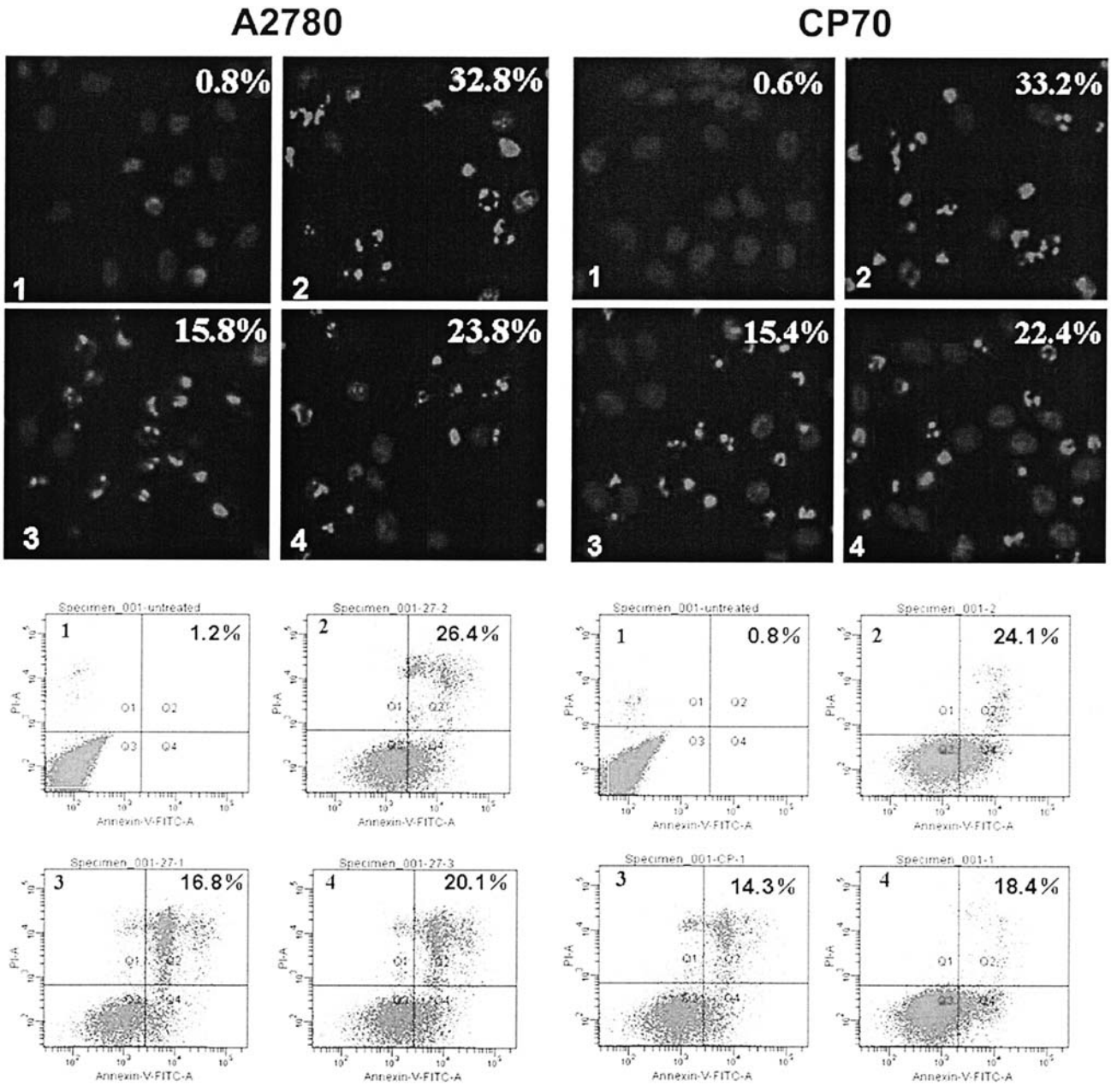

Figure 3. Effect of LiCl on cell apoptosis. A2780 and CP70 cells were treated with LiCl (0 or 20 mM) at either 6 h prior to or 6 h following the initiation of cisplatin exposure. Cells were then exposed to cisplatin (20 μM for A2780 cells and 40 μM for CP70 cells) for 18 h. Top panel, after treatment, nuclear morphology was visualized with DAPI staining. Cells with condensed or fragmented nuclei were counted. 1, control; 2, cisplatin-treated; 3, LiCl treatment 6 h prior to cisplatin exposure; 4, LiCl treatment 6 h after cisplatin exposure. Bottom panel, cells were stained with Annexin V and Annexin V-positive cells were analyzed via flow cytometry as described under the Materials and methods. 1, control; 2, cisplatin-treated; 3, LiCl treatment 6 h prior to cisplatin exposure; 4, LiCl treatment 6 h after cisplatin exposure. The experiment was replicated three times.

their cisplatin-resistant derivative cells (CP70). Cisplatin-resistant CP70 cells were screened from A2780 cells after prolonged exposure to cisplatin (10). As shown in Fig. 1, the expression levels of GSK-3ß and pGSK-3ß-tyr-216 were similar in A2780 and CP70 cells; the expression of pGSK-3ß-ser-9 in CP70 cells, however, was much higher than in A2780 cells.

The activity of GSK-3ß was negatively regulated by its phosphorylation at serine 9. Since cisplatin-resistant CP70 cells expressed higher levels of pGSK-3ß-ser-9, we sought to determine whether inhibition of GSK-3ß activation could confer A2780 cells resistant to cisplatin. LiCl is a potent inhibitor of GSK-3ß and also stimulates GSK-3ß phosphorylation at serine 9 (11,12). As shown in Fig. 2, treatment of LiCl significantly enhanced the cisplatin resistance in both A2780 and CP70 cells. The IC_{50} of cisplatin was increased from 0.51±0.13 μM to 3.04±0.28 μM in A2780 cells ($p<0.01$) and from 5.43±0.15 μM to 13.28±0.34 μM in CP70 cells ($p<0.01$) following treatment of LiCl. In addition, cisplatin induced apoptosis of A2780 and CP70 cells, which was characterized by an increase in cells with condensed/fragmented nuclei as well as Annexin V-positive cells (Fig. 3). For example, cisplatin treatment increases the percentage of A2780 cells with condensed/fragmented nuclei from

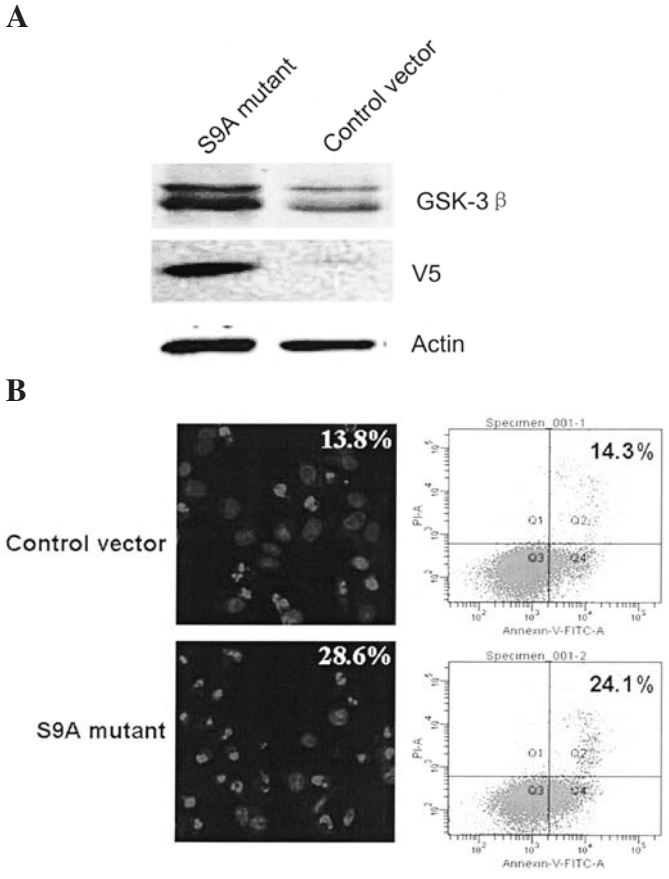

Figure 4. Effect of the GSK-3ß S9A mutant on CP70 cells. (A) CP70 cells were transfected with either a control vector or pcDNA3-V5-GSK3ß S9A for 48 h. The expression of exogenous GSK-3ß was identified by immunoblotting using an anti-V5 antibody. The overexpression of GSK-3ß was verified by immunoblotting using an anti-GSK-3ß antibody. (B) CP70 cells were transfected with either a control vector or pcDNA3-V5-GSK3ß S9A for 48 h, then treated with cisplatin (0 or 40 μM) for 18 h. After treatment, apoptotic cells were determined by counting cells with condensed or fragmented nuclei, or the expression of Annexin V by flow cytometry as described above. The percentage of apoptotic cells was calculated. The experiment was replicated three times.

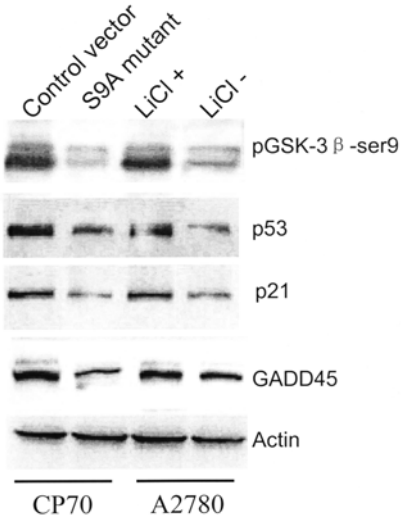

Figure 5. Effect of the GSK-3ß S9A mutant and LiCl on the expression of p53. CP70 cells were transfected with either a control vector or pcDNA3-V5-GSK3ß S9A for 48 h. A2780 cells were treated with LiCl (0 or 20 mM) for 24 h. The expression of pGSK-3ß-ser-9, p53, p21 and GADD45 was examined with immunoblotting. The expression of ß-actin served as a loading control. The experiment was replicated three times.

0.8±0.037% to 32.8±3.49%. The protective effect of LiCl against apoptosis was evaluated at 6 h prior or 6 h post exposure to cisplatin. LiCl pretreatment (6 h prior to cisplatin exposure) significantly decreased the percentage of A2780 cells with condensed/fragmented nuclei to 15.8±2.38% ($p<0.01$). LiCl treatment after initiation also offered a significant protection, but to a lesser extent; cisplatin induced 23.8±3.83% of A2780 cells with condensed/fragmented nuclei when LiCl was administered 6 h after cisplatin exposure ($p<0.01$). A similar observation was made in CP70 cells; LiCl pretreatment (6 h prior to cisplatin exposure) significantly decreased the percentage of CP70 cells with condensed/fragmented nuclei from 33.2±3.42% to 15.4±3.36% ($p<0.01$). Cisplatin caused 22.8±3.28% of CP70 cells with condensed/fragmented nuclei when LiCl was administered 6 h after cisplatin exposure ($p<0.01$). Similar results were obtained from Annexin V staining (Fig. 3).

To verify the involvement of pGSK-3ß-ser-9 in cisplatin resistance, we transfected CP70 cells with an S9A GSK-3ß mutant that is unable to be phosphorylated at serine 9. The expression of exogenous S9A GSK-3ß was confirmed by the expression of V5 tag as well as an increase in GSK-3ß protein levels (Fig. 4A). Overexpression of the S9A GSK-3ß mutant enhanced the sensitivity of CP70 cells to cisplatin. As shown in Fig. 4B, overexpression of the S9A GSK-3ß mutant increased the cisplatin-induced apoptosis of CP70 cells. For example, in response to the treatment of cisplatin, overexpression of the S9A GSK-3ß mutant significantly increased the cells with nuclear morphology characteristic of apoptosis from 13.8±2.39% to 28.6±1.52% ($p<0.01$). Consistently, overexpression of the S9A GSK-3ß mutant enhanced the cisplatin-mediated expression of Annexin V.

It has been demonstrated that differential sensitivity of A2780 and CP70 cells to cisplatin correlates with the status of p53 expression; stabilized p53 expression in CP70 cells may contribute toward their resistance to cisplatin (13). We therefore sought to determine whether overexpression of the S9A GSK-3ß mutant affected the levels of p53 expression. As shown in Fig. 5, overexpression of the S9A GSK-3ß mutant diminished the levels of pGSK-3ß^{ser9} in CP70 cells. Furthermore, overexpression of the S9A GSK-3ß mutant down-regulated the expression of p53 as well as two target genes of p53 (p21 and GADD45). Reversely, LiCl increased pGSK-3ß-ser-9 and inhibited GSK-3ß activity in A2780 cells; it up-regulated the expression of p53 and its target genes (Fig. 5).

Discussion

GSK-3ß is a multifunctional kinase that is involved in many cellular processes. This is the first report revealing a potential role of GSK-3ß in cisplatin resistance of ovarian carcinomas. Here, we demonstrate an increased expression of pGSK-3ß-ser-9 in cisplatin-resistant CP70 cells compared to their cisplatin-sensitive counterpart A2780 cells. High pGSK-3ß-ser-9 levels in CP70 cells suggest that a suppressed GSK-3ß activity may account for their resistance to cisplatin. This hypothesis is supported by our finding showing that LiCl, a

GSK-3ß inhibitor significantly raises the IC_{50} of cisplatin for both CP70 and A2780 cells. It has been reported that LiCl increases pGSK-3ß-ser-9 (12,14). Pretreatment of LiCl reduces cisplatin-induced apoptosis. Interestingly, LiCl treatment even after initiation of cisplatin exposure also offered a significant protection, but to a lesser extent. If down-regulation of GSK-3ß activity confers cisplatin resistance in ovarian carcinomas, activation of GSK-3ß should reverse cisplatin resistance and enhance the sensitivity of CP70 to cisplatin. Our results indicate this is indeed the case. Expression of a constitutively active S9A GSK-3ß mutant significantly sensitizes CP70 cells to cisplatin and counteracts cisplatin-induced apoptosis. Taken together, the evidence indicates that pGSK-3ß-ser-9 confers resistance to cisplatin in ovarian carcinomas.

Similar to our findings, a previous report shows that LiCl treatment confers the resistance of hepatoblastoma cells to two chemotherapy drugs, etoposide and camptothecin, both induce apoptosis (15). Their subsequent study demonstrates that GSK-3ß reactivation by exogenous expression of the S9A GSK-3ß mutant or treatment with LY294002 sensitizes hepatoma cells to etoposide- and camptothecin-induced apoptosis (16). Therefore, modulation of resistance to chemotherapy by GSK-3ß activity may be a general phenomenon in cancer cells.

Multiple mechanisms of cisplatin resistance have been described in ovarian carcinomas, among them resistance to cisplatin-induced apoptosis (6). GSK-3ß-mediated resistance to chemotherapy may be mediated by its regulatory effect on cell survival. Evidence obtained from neuronal cells indicates that GSK-3ß is a proapoptotic kinase. Thus, overexpression/activation of GSK-3ß sensitizes neuronal cells to apoptosis; contrarily, inhibition of GSK-3ß offers protection against apoptosis (14,17,18). One of the important functions of GSK-3ß is the regulation of p53; results obtained in different cell contexts indicate that GSK-3ß can act as a positive or negative physiological regulator of the p53 protein (19-21). In general, activation of GSK-3ß results in destabilization of p53 (22). Inhibition of GSK-3ß causes an increase in p53 levels (22,23). GSK-3ß can phosphorylate Mdm2, a regulator of p53 degradation; p53 stabilization caused by a GSK-3ß inhibitor is probably due to the hypophosphorylation of Mdm2 which loses its ability to degrade p53 (22). Yazlovitskaya *et al* (13) demonstrate that resistance of CP70 cells to cisplatin correlates with prolonged p53 protein stabilization and accumulation. Our results indicate that LiCl increases the expression of p53 in A2780 cells while the expression of the S9A GSK-3ß mutant down-regulates p53 levels in CP70 cells. Thus, these results suggest that pGSK-3ß-ser-9-induced ciplatin resistance may be mediated by stabilization of p53.

Dysregulation of the expression and activity of GSK-3ß has been observed in various human tumors (5). Recent studies suggest that sustained GSK-3ß phosphorylation at serine 9 and suppressed GSK-3ß activity could contribute to tumorigenesis (15,24-26). However, controversial observation exists in favor of GSK-3ß as a positive regulator of survival and proliferation in cancers cells. For example, high expression/activity of GSK-3ß was observed in colorectal cancers; inhibition of GSK-3ß resulted in apoptosis and attenuated proliferation of colon cancer cells (23,27). In particular, it has been reported that the expression level of GSK-3ß is significantly higher in ovarian carcinoma tissues (28). GSK-3ß positively regulates the proliferation of ovarian carcinoma cell line SKOV3 and lithium inhibits the proliferation of these cells (29). Because of its negative regulation of survival and proliferation of some cancer cells, the GSK-3ß inhibitor has been suggested to be useful for the treatment of certain cancers (5). The present study suggests that using a GSK-3ß inhibitor as a novel strategy to treat cancers needs to be carefully evaluated because inhibition of GSK-3ß may confer resistance to chemotherapy.

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