Heat Shock Protein 27 Protects L929 Cells from Cisplatin-Induced Apoptosis by Enhancing Akt Activation and Abating Suppression of Thioredoxin Reductase Activity

Yong Zhang[1] and Xun Shen[1,2]

Abstract

Purpose: Heat shock protein 27 (Hsp27) is up-regulated in multiple malignancies and implicated in cisplatin resistance. It is attempted to know how Hsp27 endues cell with cisplatin resistance by interfering with upstream of both apoptosis signal–regulating kinase 1 (ASK1)/p38 mitogen-activated protein kinase–activated apoptotic signaling and serine/threonine kinase Akt-dependent survival signaling.

Experimental Design: The mouse L929 cells stably transfected with human Hsp27 or its dominant-negative mutant and the human cervical cancer HeLa cells transfected with Hsp27 siRNA were used. The cisplatin-induced apoptosis and activation of ASK1, p38, and Akt were compared in control cells, cells overexpressing Hsp27, and cells with their endogenous Hsp27 knocked down.

Results: Hsp27 effectively protected the cells from cisplatin-induced DNA fragmentation. The p38 inhibitors obviously decreased whereas Akt inhibitors markedly increased the apoptotic fraction in cisplatin-treated cells. Overexpression of Hsp27 doubly enhanced the drug-induced Akt activation while substantially depressing the drug-induced activation of ASK1 and p38. Knockdown of the endogenous Hsp27 in HeLa cells resulted in the effects opposite to that observed in the Hsp27-overexpressing cells. Enhancement of Akt activation is associated with complex formation between Akt and Hsp27, whereas depression of ASK1/p38 activation is attributed to a reversion of the drug-induced inhibition of thioredoxin reductase activity and subsequent oxidation of thioredoxin.

Conclusions: Hsp27 endues cells with cisplatin resistance via depression of the drug-induced ASK1/p38 activation and enhancement of the drug-induced Akt activation. This study revealed the intervention of Hsp27 in upstream of both ASK1/p38 apoptotic signaling and phosphatidylinositol 3-kinase/Akt survival signaling. Therefore, the inhibition of Hsp27 may be a novel strategy of cancer chemotherapy.

Cisplatin (*cis*-diammine-dichloroplatinum) is a potent inducer of growth arrest or apoptosis in various types of cell and is among the most effective and widely used chemotherapeutic drugs used for treatment of human cancers. However, a major limitation of cisplatin chemotherapy is serious drug resistance.

Multiple mechanisms have been implicated in the development of cisplatin resistance, including reduced drug accumulation, increased levels of glutathione, enhanced expression of metallothionein, enhanced DNA repair, increased expression levels of Bcl-2–related antiapoptotic genes, and alterations in signal transduction pathways involved in apoptosis (1–3).

Several heat shock proteins, most notably heat shock protein 27 (Hsp27) and Hsp70, are implicated in oncogenesis or in chemotherapy resistance (4, 5). Up-regulation of small heat shock protein 27 (Hsp27) has been observed in multiple malignancies including squamous cell carcinoma (6), gastric carcinoma (7), ovarian carcinoma (8), and acute myeloid leukemia (9, 10). Furthermore, the up-regulation of Hsp27 was found in cisplatin-resistant human ovarian tumor cells (11) and even in the Photofrin-resistant human colon carcinoma HT29 Cells (12). High expression of Hsp70 has been reported in high-grade malignant tumors such as pancreatic cancer (13), colorectal carcinoma (14), breast cancer (15), endometrial cancer (16), osteosarcoma (17), and renal cell tumors (18). The up-regulation of Hsp70 was also found in cisplatin-resistant human ovarian tumor cells (11), 17-allylamino-demethoxy-geldanamycin–resistant lung cancer A549 cell line (19), vincristine-resistant childhood leukemia (20), and in

Note: Present address for Y. Zhang: Department of Life Science, Laiyang Agricultural College, Tsingtao, China.
Requests for reprints: Xun Shen, Institute of Biophysics, Chinese Academy of Sciences, 15# Datun Road, Chaoyang District, Beijing 100101, P.R. China. Phone: 86-10-6488-8574; Fax: 86-10-6487-1293; E-mail: shenxun@sun5.ibp.ac.cn.

breast cancers treated with combination chemotherapies (21). In addition, higher expression levels of Hsp27 and Hsp70 were also found to be correlated with metastasis and tumorigenicity of various cancers such as mammary and prostate tumors (22), human colon cancer cells (23), and pancreatic adenocarcinoma (13). Therefore, it seems that Hsp27 and Hsp70 may play an important role in the development of resistance to cisplatin and other anticancer drugs. However, the mechanisms that underlie the Hsp27- or Hsp70-induced drug resistance in chemotherapy are still poorly understood. This study focused on the mechanisms involved in the Hsp27-induced cisplatin resistance.

It is generally accepted that cytotoxicity of cisplatin is mediated through induction of apoptosis and arrest of cell cycle resulting from its interaction with DNA, such as the formation of cisplatin-DNA adducts, which activates multiple signaling pathways including those involving ataxia telangiectasia mutated and Rad3-related, p53, p73, Bcl-2 family, caspases, cyclins, cyclin-dependent kinases, pRb, protein kinase C, mitogen-activated protein kinase, and phosphatidylinositol 3-kinase/Akt, and culminate in the activation of apoptosis (24, 25). In view of signal transduction, c-jun NH$_2$-terminal kinase and p38 are predominantly activated through environmental stresses including osmotic shock, UV radiation, heat shock, oxidative stress, protein synthesis inhibitors, stimulation by Fas, and inflammatory cytokines such as tumor necrosis factor α and interleukin-1 (26). Specific inhibition of c-jun NH$_2$-terminal kinase/p38 through inhibitors, dominant negative mutants, or knockout of their expression suppresses various types of stress-induced apoptosis (27). It has been also reported that activation of c-jun NH$_2$-terminal kinase/p38 is involved in the cisplatin-induced apoptosis in some cancer cells, such as A431 epidermoid carcinoma cells, HT29 colon cancer cells (28), and human ovarian carcinoma cell line (29). As upstream activator of c-jun NH$_2$-terminal kinase and p38, apoptosis signal–regulating kinase 1 (ASK1), a member of the mitogen-activated protein kinase kinase kinase family that activates both the c-jun NH$_2$-terminal kinase and p38 signaling cascades, plays a critical role in cisplatin-induced apoptosis in various cell types including human kidney cells and human ovarian cancer cells (30, 31). Although it was recently reported that Hsp27 inhibited etoposide-mediated phosphorylation of p38 and c-Jun, cytochrome c release, and subsequent apoptosis in acute myeloid leukemia cells (32), there has been no report on possible modulation of the ASK1-p38 signaling by Hsp27 in cisplatin-induced apoptosis.

Accumulated evidence shows that Akt, the family of phosphatidylinositol 3-kinase-regulated serine/threonine kinases, and its downstream targets constitute a major cell survival pathway. Akt is frequently activated and/or overexpressed in ovarian cancer (33, 34). Akt promotes cell survival; suppresses apoptotic death in a number of cell types induced by a variety of stimuli including growth factor withdrawal, cell cycle discordance, and loss of cell adhesion (35); and regulates the sensitivity of ovarian cancer cells toward cisplatin treatment (33, 34, 36). However, there has been no report on the possible participation of Hsp27 in Akt activation induced by cisplatin and by other anticancer drugs.

Here, we report the alteration of upstream events (i.e., activation of Akt and ASK1/p38 kinases in both the apoptotic and survival signaling pathways) by Hsp27 in the cisplatin-treated mouse fibroblast L929 cells and human cervical cancer cells (HeLa cell line). It may reveal some new mechanisms involved in Hsp27-induced cisplatin resistance in tumor treatment.

Materials and Methods

Reagents. Cisplatin, wortmannin, LY294002, SB203580, SKF86002, propidium iodide, myelin basic protein, and insulin were purchased from Sigma. Rabbit anti-Hsp27, rabbit anti-thioredoxin, and goat anti-Akt antibodies were obtained from Santa Cruz Biotech. Rabbit anti-Akt, mouse anti-p38, rabbit anti–phospho-Akt (Ser^{473}), and rabbit anti–phospho-p38 (Thr^{180}/Try^{182}) antibodies were obtained from Cell Signaling. The plasmids pKS2711 and pKS27-3G, which contain wild-type human Hsp27 gene and mutated Hsp27 gene (Ser^{15}, Ser^{78}, and Ser^{82} were substituted with glycines), respectively, were gifts from Dr. L.A. Weber (Department of Biology, University of Nevada, Reno, NV). Recombinant human thioredoxin was kindly provided by Dr. A. Holmgren (Karolinska Institute, Stockholm, Sweden). [γ-^{32}P]ATP was obtained from Furui Biotech Company. Enhanced chemiluminescence Western blotting detection reagents were purchased from Pierce.

Cell culture and treatments. The mouse fibroblast L929 cells were grown in DMEM containing 10% FCS, 100 units/mL penicillin, and 100 µg/mL streptomycin at 37°C in 5% CO$_2$. For cisplatin treatment, the cells were cultured in fresh DMEM containing 0.5% FCS for 12 h before treatment. Kinase inhibitor was added 1 h before cisplatin treatment.

Cell growth curve. Cells were seeded in 25-cm^2 culture bottles (1 × 10^5 per culture bottle). At 1, 2, 3, and 4 days, adherent cells were detached by 0.25% trypsin, collected, and total cell numbers were counted. Cell numbers were plotted against the time points, giving rise to the growth curve of cells.

Plasmid construction and transfection. The cDNAs for human Hsp27 and its mutant Hsp27-3G were obtained by PCR amplification from pKS2711 and pKS2711-3G plasmids, respectively. The vectors pcDNA3-Hsp27 and pcDNA3-Hsp27-3G, which express human Hsp27 and Hsp27-3G, respectively, were constructed by subcloning Hsp27 cDNA or Hsp-3G cDNA into pcDNA3 in *Eco*RI/*Bam*HI sites. The expression vectors pcDNA3, pcDNA3-Hsp27, and pcDNA3-Hsp27-3G were transfected into L929 cells with Lipofectamine 2000. After selection with 400 µg/mL G418 for 3 weeks and screening by immunoblotting analysis of Hsp27 expression, three monoclonal cell lines (i.e., cells expressing only neomycin-resistant gene, cells overexpressing Hsp27, and dominant-negative mutant Hsp27-3G) were established.

RNA interference. The siRNA for Hsp27 and the scrambled oligonucleotide (mock RNA) are 5′-AAGCTGCAAAATCCGATGAGA-3′ and 5′-UUCUCCGAACGUGUCACGU-3′, respectively (32). They were chemically synthesized by GenePharma Company. Cells were seeded in six-well plates and incubated overnight, then transfected with 100 nmol/L siRNA for 6 h using 2 µL of VigoFect (Vigorous Biotechnology) per well. The expression of Hsp27 in the cells was assayed 48 h after transfection by Western blotting with anti-Hsp27 antibody.

Detection of apoptotic DNA fragmentation. Cells (1 × 10^6) were seeded in 25-cm^2 culture bottles and treated with 5 µg/mL cisplatin for 12 h, then washed twice with PBS and incubated in fresh medium for 18 h. Both attached and detached cells were harvested, washed, and lysed with 20-µL lysis buffer (100 mmol/L Tris-HCl, 20 mmol/L EDTA, 0.8% SDS, pH 7.8). Following incubation at 37°C for 30 min in the presence of 10-µL RNase A (10 mg/mL), the lysate was further incubated for 2 h at 50°C after addition of 10-µL proteinase K (1 mg/mL). Eight microliters of cell lysate were subjected to gel electrophoresis in 2% agarose in Tris-borate EDTA buffer (45 mmol/L Tris, 45 mmol/L boric acid and 1 mmol/L EDTA, pH 8.0). The DNA bands were stained with ethidium bromide and visualized under UV illumination.

Flow cytometric analysis of apoptotic cells. After treatment with 5 µg/mL cisplatin for 12 h and culturing in fresh culture medium containing 0.5% FCS for additional 18 h, both attached and detached

cells were harvested and washed with PBS buffer containing 5 mmol/L EDTA. After fixation in 70% ethanol at 4°C for 2 h, the cells were collected, resuspended in PBS containing 250 µg/mL RNase A, and incubated at 37°C for 30 min. After propidium iodide staining, 3×10^4 cells of each sample were analyzed on a BD FACSCalibur flow cytometer. The portion of the apoptotic cells was calculated according to the apoptotic peak in the cell distribution against the propidium iodide fluorescence per cell.

Immunoblotting. Cells were lysed in lysis buffer (20 mmol/L Tris, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerolphosphate, 1 mmol/L Na_3VO_4, 1 µg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride, pH 7.5) on ice for 15 min. Cell debris was removed by centrifugation at $18,000 \times g$ for 15 min at 4°C. The protein content of the lysate was determined using the Bio-Rad protein assay kit. The cell lysates with equal protein content were loaded and separated by SDS-PAGE. The protein bands were electrotransferred onto nitrocellulose membrane and blocked with 5% nonfat milk in TBS/T buffer (20 mmol/L Tris base, 135 mmol/L NaCl, 0.1% Tween 20, pH adjusted to 7.6 with HCl) for 1 h. After incubation of the membrane with the appropriate antibodies for at least 4 h, specific protein bands were visualized with SuperSignal West Pico Chemiluminescent Substrate.

Assay of ASK1 activity by myelin basic protein phosphorylation. The cells, either untreated or treated with cisplatin for 3 h, were lysed according to the same procedure described in the preceding section. After centrifugation at $10,000 \times g$ at 4°C for 15 min, the cell lysate supernatant, which contained 200-µg total proteins, was incubated with 2 µg of anti-ASK1 antibody overnight at 4°C with gentle rocking, then with 20-µL protein A-sepharose (50%, v/v) for an additional 3 h. Afterwards, the mixture was centrifuged at $4,000 \times g$ at 4°C for 3 min; the pellet was washed twice with lysis buffer and twice with kinase buffer [25 mmol/L Tris (pH 7.5), 5 mmol/L β-glycerolphosphate, 2 mmol/L DTT, 0.1 mmol/L Na_3VO_4, 10 mmol/L $MgCl_2$]. The precipitate was solved in 50-µL kinase buffer supplemented with 200 µmol/L ATP, 2 µCi of [γ-^{32}P]ATP, and 4 µg of myelin basic protein (the substrate of ASK1); incubated at 30°C for 30 min with gentle rocking; and the reaction was terminated with 25 µL of 3× SDS sample buffer [187.5 mmol/L Tris-HCl (pH 6.8), 6% SDS, 30% glycerol, 150 mmol/L DTT, 0.03% bromphenol blue]. After boiling for 5 min, the reaction mixture was separated by 15% SDS-PAGE. The gel was dried in vacuum and the band of the ^{32}P-incorporated myelin basic protein was autoradiographed on Kodak X-Omat BT Film. The ASK1 activity was estimated as the incorporated radioactive ^{32}P in myelin basic protein.

Measurement of thioredoxin reductase activity. The cells were lysed with a new lysis buffer (50 mmol/L potassium phosphate, 1% Triton X-100, 2 mmol/L EDTA and 1 mmol/L phenylmethylsulfonyl fluoride, pH 7.5). After sonication in ice bath, the cell lysate was centrifuged at $15,000 \times g$ for 30 min. The supernatant fraction was heated at 55°C for 5 min and filtered with a centrifugal filter of 30-kDa cutoff. The retentate was washed thrice with PE buffer (50 mmol/L potassium phosphate and 2 mmol/L EDTA, pH 7.5) and used as crude cell extract. Thioredoxin reductase activity in the cell extract was measured by the modified 5,5'-dithiobis(2-nitrobenzoic acid) reduction assay (37). Protein levels of the cell extracts were determined using the Bio-Rad Protein Assay kit. To inhibit thioredoxin reductase, the cell extracts (0.3 mg protein/mL) were incubated with 20 µmol/L gold thioglucose at room temperature for 20 min before assay. In all assays, 50 µL of the cell extracts were added into 750 µL of the assay mixture [5 mmol/L 5,5'-dithiobis(2-nitrobenzoic acid), 0.2 mmol/L NADPH, 0.1 mol/L potassium phosphate, 10 mmol/L EDTA, and 0.2 mg/mL bovine serum albumin, pH 7.5] and the changes of absorbance at 412 nm were recorded. The thioredoxin reductase activity in the cell extract was determined by subtracting the 5,5'-dithiobis(2-nitrobenzoic acid) reduction rate in the presence of gold thioglucose from that in the absence of the inhibitor.

Redox Western blot analysis of thioredoxin. Separation of the reduced and oxidized thioredoxin was done according the procedure

reported in literature (38). Briefly, cells were lysed in a buffer containing 6 mol/L guanidine-HCl, 50 mmol/L Tris (pH 8.3), 3 mmol/L EDTA, 0.5% Triton X-100, and 50 mmol/L iodoacetic acid. After incubation at 37°C for 30 min, excess iodoacetic acid was removed by MicroSpin G-25 columns (Amersham Biosciences). The eluate was diluted in 5× sample buffer [0.1 mol/L Tris-HCl (pH 6.8), 50% glycerol, 0.05% bromphenol blue], separated on a discontinuous native polyacrylamide gel (5% stacking gel, 15% resolving gel), and electrotransferred onto nitrocellulose membrane. The protein bands on the membrane were probed with rabbit anti-thioredoxin antibody and visualized with horseradish peroxidase–conjugated antirabbit immunoglobulin G antibody by chemiluminescence detection.

Immunoprecipitation analysis. The cells were lysed for 10 min on ice with the same lysis buffer used for immunoblotting. Following centrifugation at $14,000 \times g$ for 10 min at 4°C, 1 mL of clear lysates was incubated with 5-µL goat anti-Akt antibody or 5-µL normal goat immunoglobulin G overnight with continuous rotation at 4°C. Protein A-sepharose beads (30 µL) were then added and the samples were gently rocked 4°C for 3 h. After five washes with lysis buffer, the beads were recovered and resuspended in 40 µL of 2× SDS sample buffer (4% SDS, 0.125 mol/L Tris-HCl, 20% glycerol, and 0.04% bromphenol
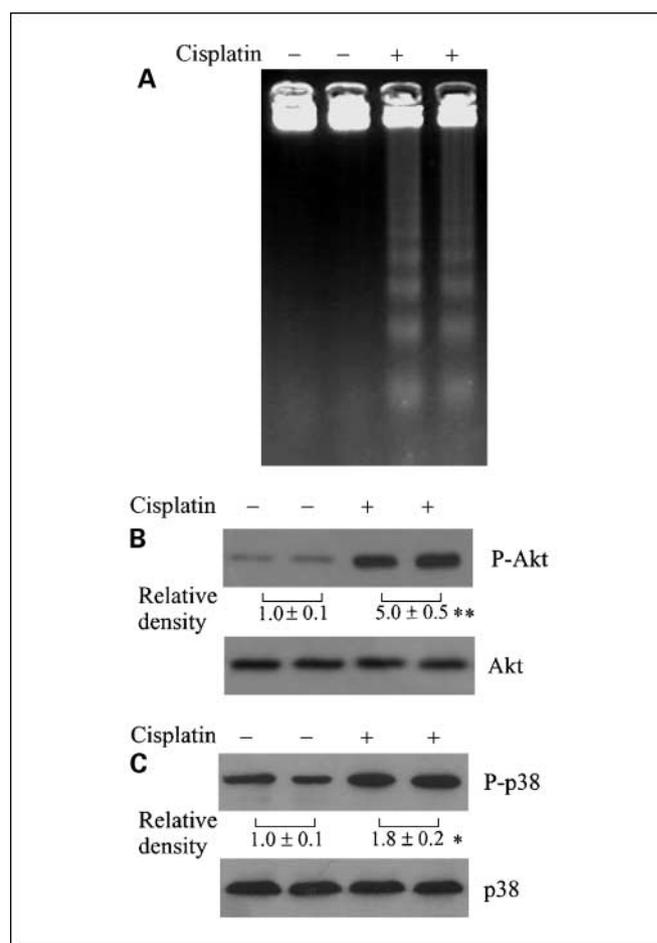

Fig. 1. Cisplatin induces apoptosis and activation of Akt and p38 kinase in L929 cells. *A,* apoptotic DNA fragmentation patterns in the cells without cisplatin treatment (*two left lanes*) or exposed to 5 µg/mL cisplatin for 12 h and then incubated in cisplatin-free medium for 18 h (*two right lanes*). *B,* upper immunoblot shows the phosphorylated Akt in the lysates of the cells either untreated or exposed to 5 µg/mL cisplatin for 3 h. The Akt contents in the lysates are shown in lower immunoblot as control. *C,* the phosphorylated and total p38 in the cells either untreated or under the same treatment as specified in (*B*). The numbers indicated below each immunoblot represent mean relative blot densities ± SD.

blue, pH 6.8) and then boiled for 5 min. The proteins dissociated from the beads were separated by 10% SDS-PAGE, electrotransferred onto nitrocellulose membrane, and blocked with 5% nonfat milk in TBS/T buffer for 1 h. The blots were probed sequentially with rabbit anti-Hsp27 and rabbit anti–phospho-Akt antibody, followed by peroxidase-conjugated secondary antibodies, and visualized by chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate. After striping, the same blots were probed with rabbit anti-Akt antibody as control.

Statistics. All data obtained in this study are mean of three independent measurements and subjected to Student's t test. In the figures showing statistical analysis of the data, single asterisk indicates statistical significance at $P < 0.05$ and double asterisks indicates $P < 0.01$.

Results

Cisplatin induces apoptosis and activation of Akt and p38 mitogen-activated protein kinase in L929 cells. As previously reported in human ovarian cancer cell lines (39), treatment with 5 μg/mL cisplatin for 12 h resulted in marked apoptotic DNA fragmentation in the cells (Fig. 1A). It was also observed that Akt and p38 kinase were significantly activated in the cells after exposure to cisplatin at the same concentration (Fig. 1B and C). Cisplatin treatment resulted in a 5-fold increase of Akt activity and a 1.8-fold increase of p38 activity ($P = 0.012$). To confirm the involvement of p38 and Akt activation in the cisplatin-induced apoptosis, flow cytometric analysis was done in the cells exposed to 5 μg/mL cisplatin for 12 h in the presence of either the selective inhibitors of p38, SB203580 (40), or SKF86002 (41) or the inhibitors of Akt, wortmannin (42, 43), or LY294002 (44). The results are shown in Fig. 2. It was observed that the apoptotic fraction in the cisplatin-treated cells was reduced from 26% to ~ 14% and 12% in the presence of 2 μmol/L SB203580 and 10 μmol/L SKF86002, respectively, whereas the apoptotic fraction increased to ~ 43% or 49% in the presence of 100 nmol/L wortmannin or 5 μmol/L LY294002. The results clearly indicate that inhibition of p38 activation prevents, whereas inhibition of Akt activation promotes, cisplatin-induced cell apoptosis.
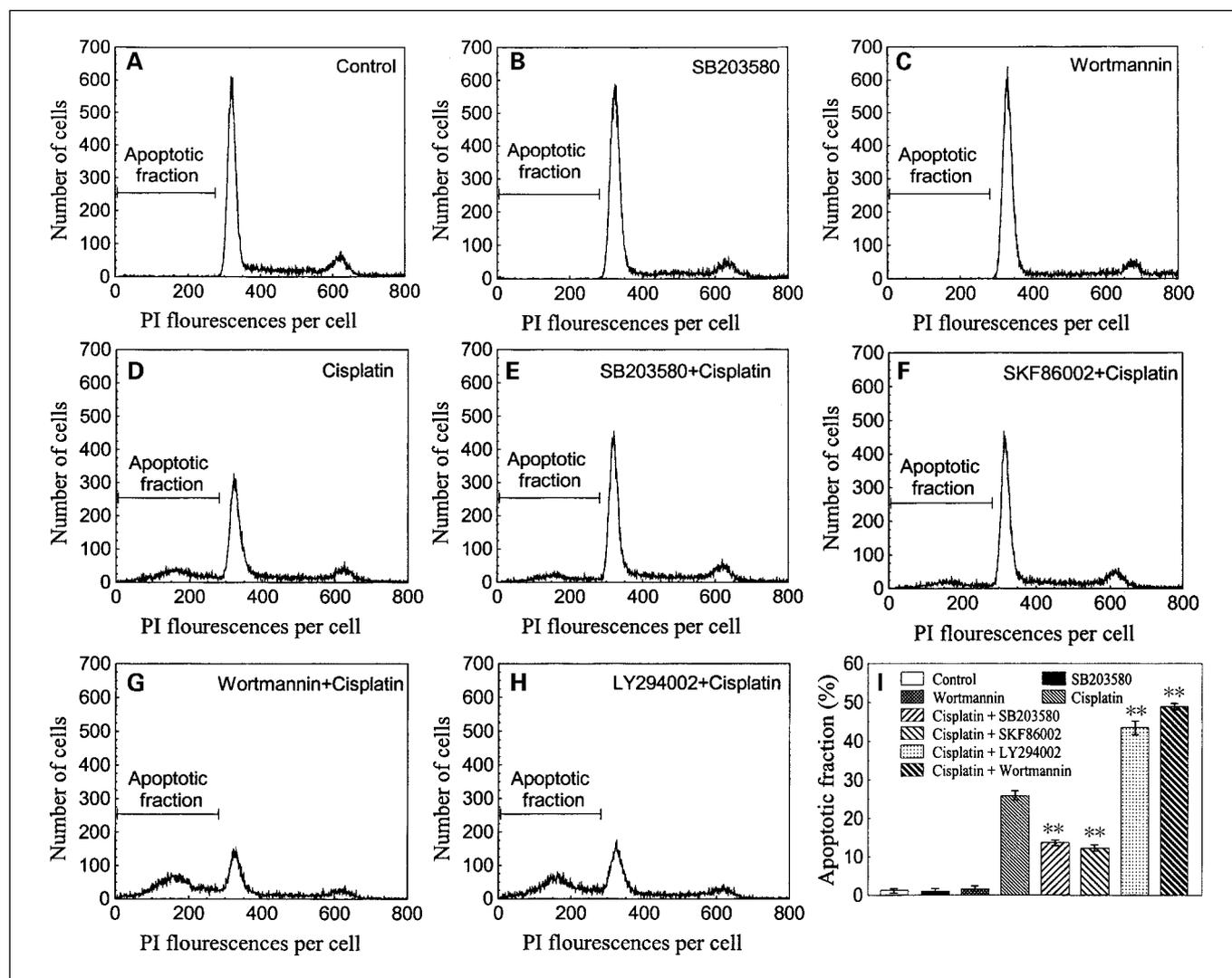

Fig. 2. The effects of p38 and Akt inhibitors on apoptosis in the L929 cells exposed to 5 μg/mL cisplatin for 12 h. The typical flow cytometric analysis of the apoptotic fraction in untreated cells in the absence and presence of 2 μmol/L SB203580 or 100 nmol/L wortmannin (*A-C*), the cells exposed to cisplatin in the absence any inhibitor (*D*), and the cisplatin-treated cells in the presence of 2 μmol/L SB203580 (*E*), 10 μmol/L SKF86002 (*F*), 100 nmol/L wortmannin (*G*), and 5 μmol/L LY294002 (*H*). *I,* the apoptotic fractions in the cells under various conditions are statistically analyzed based on three independent flow cytometric measurements; bars, SD.

Hsp27 protects L929 cells from cisplatin-induced apoptosis by reducing the cisplatin-induced p38 activation and enhancing the cisplatin-induced Akt activation. The L929 cells stably transfected with human Hsp27 and its dominant-negative mutant Hsp27-3G were used to investigate the effects of Hsp27 on the cisplatin-induced apoptosis. Because a neomycin-resistant gene was also transfected with Hsp27 or Hsp27-3G in the cells, the cells stably transfected only with the neomycin-resistant gene were used as control and referred to as neo cells. The expression level of human Hsp27 in the three cell lines was determined by immunoblotting. As shown in Fig. 3A, the protein recognized by anti-Hsp27 antibody was detected only in the cells transfected with Hsp27 or Hsp27-3G but not in the neo cells. Undetectable level of Hsp27 in the neo cells indicates that the L929 cells contain extremely low endogenous mouse Hsp27.

The growth curve for wild-type L929 cells and cells over-expressing human Hsp27 in the DMEM containing 10% FCS was determined. As Fig. 3B shows, overexpression of Hsp27 does not change the growth rate of the cells. To examine if the transfected human Hsp27 still functions normally, the cells stably transfected with Hsp27, Hsp27-3G, and neomycin-resistant gene were subjected to heat shock at 44°C for 30 min, and then imaged on a microscope 30 h later. As Fig. 3C shows, heat shock resulted in a large fraction of death in the neo cells and the cells transfected with dominant-negative mutant of Hsp27, whereas much less dead cells were observed in the heat-shocked Hsp27-overexpressing cells. These experiments indicate that the transfected human Hsp27 functions normally, and the overexpression of Hsp27 did not change the phenotype of L929 cells.

The DNA fragmentation pattern and the apoptotic fraction in the neo cells and the cells overexpressing Hsp27 or Hsp27-3G were assayed by gel electrophoresis and flow cytometry, respectively. As shown in Fig. 4A and B, both assays showed that cisplatin treatment caused significant apoptosis in the neo cells and the cells expressing dominant-negative mutant Hsp27, but not in the cells expressing wild-type human Hsp27, indicating that overexpression of Hsp27 protects the cells from cisplatin-induced apoptosis.

To understand how Hsp27 prevents the drug-induced cell death, activation of p38 and Akt was assayed by immunoblotting whereas ASK1 activity was determined by its ability to phosphorylate its substrate myelin basic protein in the three transfected cell lines. As Fig. 4C shows, after treatment with cisplatin at the same concentration (5 μg/mL) for the same time period (3 h), obviously higher phosphorylation of Akt, lower phosphorylation of p38, and lower activation of ASK1 were found in the cells overexpressing Hsp27 in comparison with those in the neo cells. However, the elevated Akt activation and reduced activation of p38 and ASK1 were not observed in the cells overexpressing dominant-negative mutant Hsp27. The results reasonably suggest that Hsp27 protects cells from cisplatin-induced apoptosis through enhancement of the cisplatin-induced Akt activation and depression of the cisplatin-induced activation of ASK1 and p38. Statistical analyses of three independent estimates on the cisplatin-induced activation of these kinases in the three cell lines are shown as a histogram in Fig. 4D. It clearly shows that overexpression of Hsp27 resulted in 2-fold increase of Akt activation in the cells treated with 5 μg/mL cisplatin. In contrast, the overexpression of Hsp27 reduced the cisplatin-induced activation of p38 and
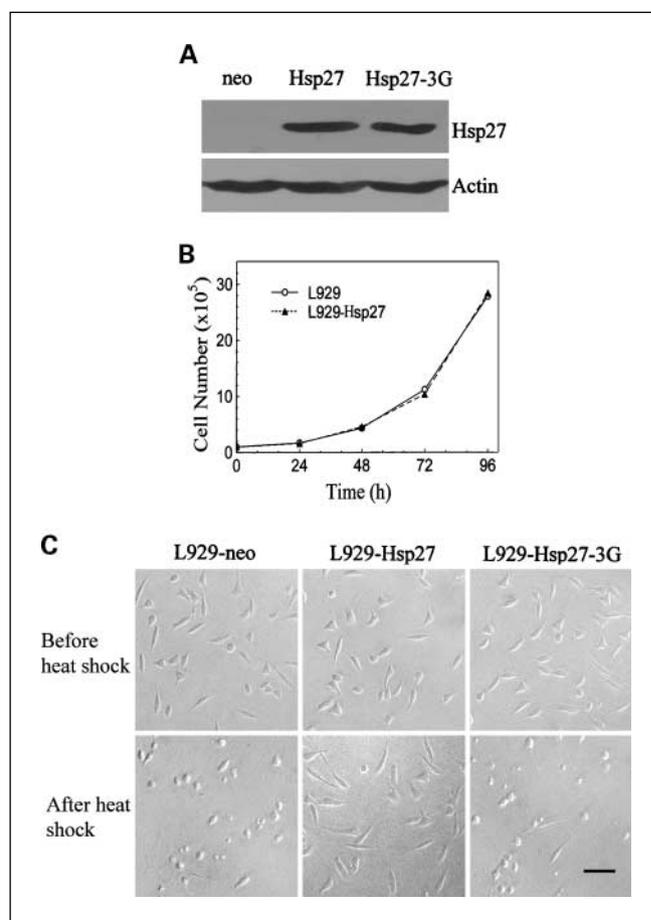

Fig. 3. Expression and function of the transfected Hsp27 in L929 cells. *A*, immunoblot of Hsp27 or Hsp27-3G detected by rabbit anti–human/mouse Hsp27 antibody in the neo cells and the cells transfected with human Hsp27 or its mutant Hsp27-3G. The actin bands are used as indication of equal loading. *B*, growth curves of wild-type and Hsp27-overexpressing cells. *C*, protection of the cells from heat shock at 44°C for 30 min by transfected Hsp27. Microscopic images of the neo cells and the cells transfected with human Hsp27 or its dominant-negative mutant Hsp27-3G, before (*top*) and 30 h after (*bottom*) the heat shock, respectively. Bar, 40 μm.

ASK1 only by ~ 30% and ~ 20%, respectively. These data imply that the enhancement of Akt activation by overexpression of Hsp27 might dominate the protective effect of Hsp27 on the cisplatin-induced apoptosis.

Knockdown of the endogenous Hsp27 resulted in more apoptosis, enhanced activation of ASK1 and p38, and less activation of Akt in the cisplatin-treated HeLa cells. The previous results showed that overexpressing exogenous human Hsp27 effectively protects L929 cells from apoptosis. However, one may question if the endogenous Hsp27 plays the same role. To avoid any artificial effect caused by transfecting cells with a exogenous gene, knockdown of the endogenous human Hsp27 was done in the human cervical cancer cells (HeLa cells) and to see if depletion or reduction of endogenous Hsp27 results in effects just opposite to the effect observed in the L929 cells overexpressing human Hsp27. For this reason, the cisplatin-induced apoptosis and the cisplatin-induced activation of p38, ASK1, and Akt were determined in wild-type cells and the cells transfected either with Hsp27 siRNA or a scrambled oligonucleotide. As shown in Fig. 5A, the expression
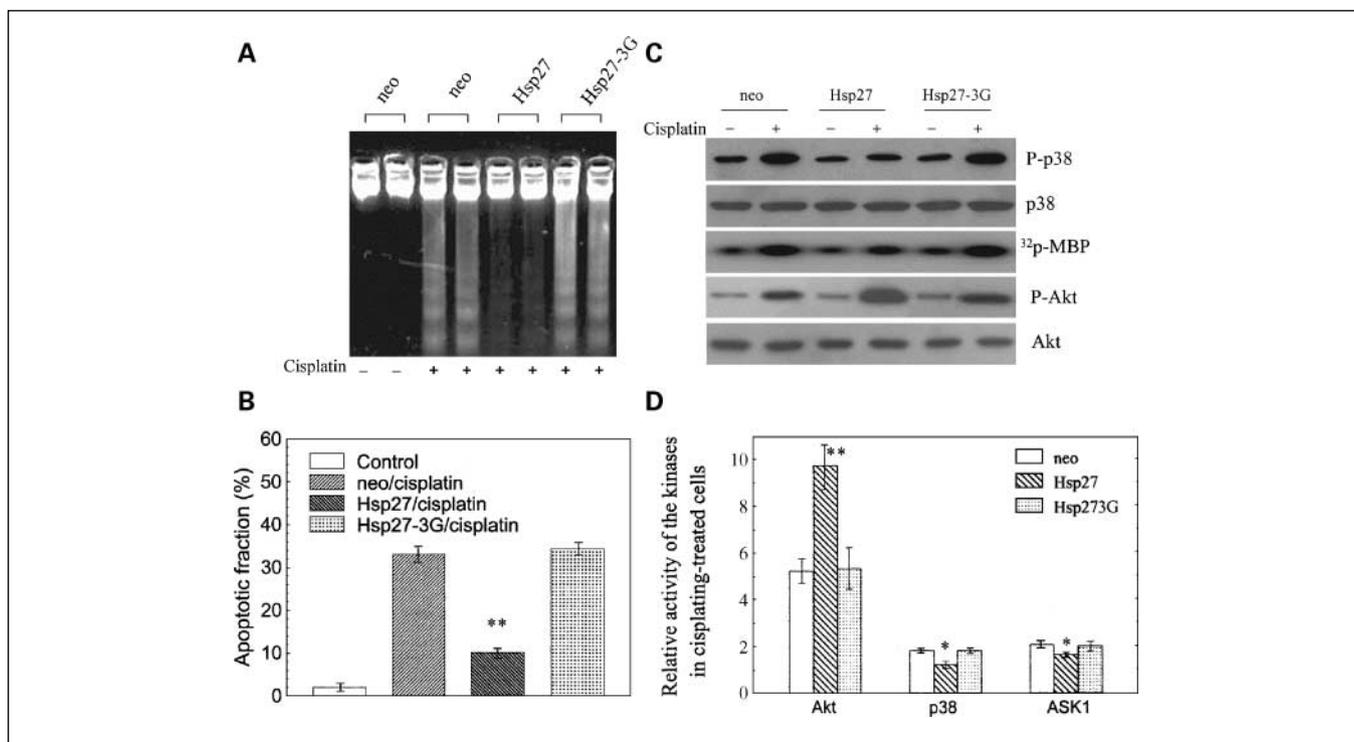
Fig. 4. Effects of overexpressed Hsp27 on the apoptosis and the activation of p38, ASK1, and Akt in cisplatin-treated cells. *A,* the apoptotic DNA fragmentation pattern in the untreated neo cells and in the neo, Hsp27-overexpressing, and Hsp27-3G – overexpressing cells after exposure to 5 μg/mL cisplatin for 12 h and further incubation for 18 h. *B,* the apoptotic fractions in the cells under various conditions are statistically analyzed based on three independent flow cytometric measurements. Bars, SD. *C,* immunoblot analysis of phosphorylated p38, phosphorylated Akt, and [2]P-labeled ASK1-phosphorylated myelin basic protein (*MBP*) in the neo cells and Hsp27-overexpressing and Hsp27-3G – overexpressing cells, either untreated or exposed to 5 μg/mL cisplatin for 3 h. The total p38 and Akt in the lysates from the corresponding cells were assayed as loading control. *D,* basal and cisplatin-induced activities of Akt, p38, and ASK1 in the three types of cells. The data were statistically analyzed based on three independent measurements. Bars, SD.

level of the endogenous Hsp27 in the HeLa cells transfected with 100 nmol/L Hsp27 siRNA for 48 h was reduced by ∼ 55% in comparison with that in wild-type cells or cells transfected with mock RNA. The flow cytometric analysis of apoptotic fraction in wild-type cells, cells with Hsp27 knocked down, and cells transfected with mock RNA after exposure to 5 μg/mL cisplatin for 12 h and further incubation for 36 h is shown in Fig. 5B. The statistical analysis of three independent flow cytometric measurements of apoptotic fraction in each cell type is shown in Fig. 5C. It clearly shows that the knockdown of 55% endogenous Hsp27 resulted in an increase of the apoptotic fraction from ∼ 22% to 31% in the cells exposed to cisplatin.

To verify if the increase of cisplatin-induced apoptosis in the cells with their endogenous Hsp27 knocked down corresponded to any enhancement of the drug-induced ASK1/p38 activation and depression of the drug-induced Akt activation, the activation of p38, ASK1, and Akt was also assayed in the wild-type cells and cells transfected with Hsp27 siRNA or scrambled oligonucleotide after cisplatin treatment. As Fig. 5D shows, after treatment with cisplatin at the same concentration (5 μg/mL) for the same time period (3 h), notably higher phosphorylation of p38 and activation of ASK1 and lower phosphorylation of Akt were found in the Hsp27-knockdown cells in comparison with those in either wild-type or mock RNA – transfected cells. The statistical analysis of three independent activity assays of the three kinases shows that knockdown of 55% endogenous Hsp27 resulted in an increase

of p38 and activation by ∼ 40% and 23%, respectively. In contrast, the drug-induced Akt activation was ∼ 20% lower in the cells transfected with Hsp27 siRNA than those in controls (see Fig. 5E). The results confirmed the role of endogenous Hsp27 in protecting cells from cisplatin-induced apoptosis. Both experiments with overexpression of an exogenous Hsp27 in L929 mouse fibroblast cells and depletion of the endogenous Hsp27 in HeLa cells show that Hsp27 protects cell from apoptosis through two signaling pathways: enhancing the cisplatin-induced Akt activation and depressing the cisplatin-induced activation of ASK1 and p38.

Hsp27 inhibits the cisplatin-induced oxidation of thioredoxin by reversing the drug-induced inhibition of thioredoxin reductase activity. We have shown depression of the cisplatin-induced activation of p38 and its upstream activator ASK1 in the Hsp27-overexpressing cells. What is the mechanism behind? For this reason, the effects of Hsp27 on the activity of thioredoxin, the inhibitor of ASK1 (45), in neo, Hsp27-overexpressing, and Hsp27-3G – expressing cells exposed to cisplatin were investigated to know if Hsp27 suppressed the drug-induced ASK1 activation by altering the activity of its inhibitor. Because only reduced form of thioredoxin can bind to ASK1 and inhibit its activation, the reduced and oxidized forms of thioredoxin, thioredoxin-$(SH)_2$ and thioredoxin-S_2, were analyzed by redox immunoblotting in these stably transformed cell lines after exposure to 5 μg/mL cisplatin for 3 h. As shown in Fig. 6A, the redox immunoblotting clearly shows that cisplatin oxidized thioredoxin to its oxidized form

(thioredoxin-S$_2$); Hsp27 but not its mutant reduced the cisplatin-induced thioredoxin oxidation. The relative contents of the reduced thioredoxin in these three different cell lines were estimated as the mean densities of the corresponding blots on three independent immunoblots and shown in Fig. 6B. It can be seen that the relative content of thioredoxin-(SH)$_2$ was ~85% in all three cell lines without cisplatin treatment, whereas overexpression of Hsp27 increased the reduced form of thioredoxin from ~54% to ~66% in the cells exposed to the drug. Inhibition of thioredoxin oxidation by Hsp27 may account for lightening the cisplatin-induced ASK1 activation and subsequent p38 activation.

Because thioredoxin reductase is the primary enzyme that catalyzes the NADPH-dependent reduction of the oxidized

thioredoxin, we investigated whether reduction of the cisplatin-induced thioredoxin oxidation by Hsp27 was attributed to the alteration of thioredoxin reductase activity in the cells over-expressing Hsp27. As shown in Fig. 6C, cisplatin significantly suppressed the thioredoxin reductase activities in all three cell lines. However, overexpression of the functional Hsp27 markedly abated the suppression. Reversing the cisplatin-induced inhibition of thioredoxin reductase activity by Hsp27 may account for the reduction of the cisplatin-induced thioredoxin oxidation.

Hsp27 may enhance Akt activation through association with Akt. To know the mechanism involved in the Hsp27-enhanced cisplatin-induced Akt activation, possible interaction of Hsp27 with Akt was studied by immunoprecipitation
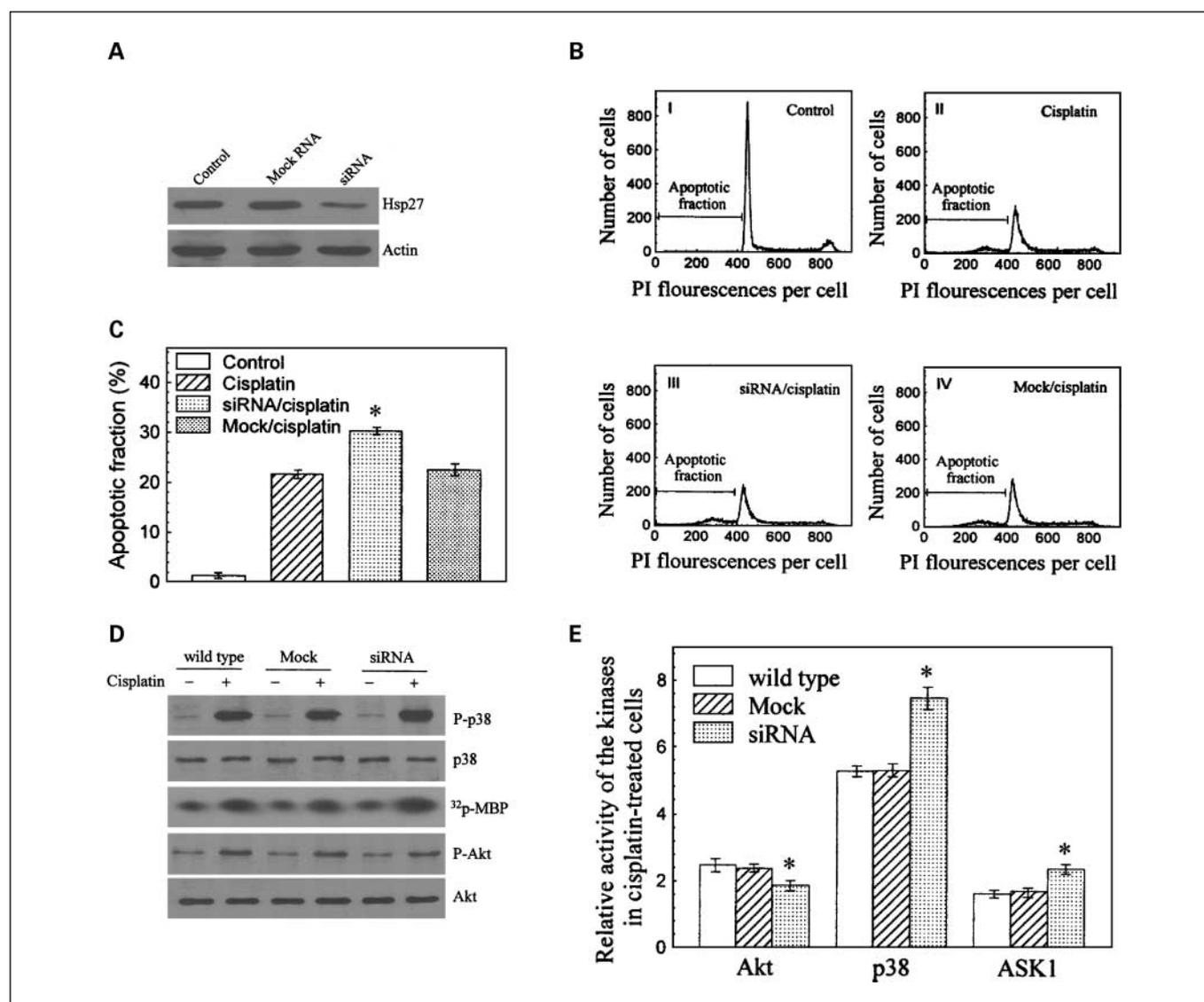

Fig. 5. The cisplatin-induced apoptosis and activation of p38, ASK1, and Akt in Hsp27-knockdown HeLa cells. *A,* expression of the endogenous Hsp27 in wild-type cells and the cells transfected with either 100 nmol/L Hsp27 siRNA or 100 nmol/L scrambled oligonucleotide (*Mock RNA*) for 48 h. The actin bands are used as indication of equal loading. *B,* a typical flow cytometric analysis of the apoptotic fraction in untreated cells (*I*), wild-type cells (*II*), Hsp27-knockdown cells (*III*), and cells transfected with mock RNA (*IV*) after exposure to 5 μg/mL cisplatin for 12 h and subsequent incubation for 36 h. *C,* statistical analysis of the apoptotic fractions in untreated cells and three different types of cisplatin-treated cells based on three independent flow cytometric measurements. Bars, SD. *D,* immunoblot analysis of phosphorylated p38, phosphorylated Akt, and ^{32}P-labeled ASK1-phosphorylated myelin basic protein in wild-type cells and cells transfected with either Hsp27 siRNA or mock RNA after exposure to 5 μg/mL cisplatin for 3 h. Total p38 and Akt in the lysates from the corresponding cells were assayed as loading control. *E,* statistical analysis of the cisplatin-induced activities of Akt, p38, and ASK1 in the three types of cells based on three independent measurements. Bars, SD.
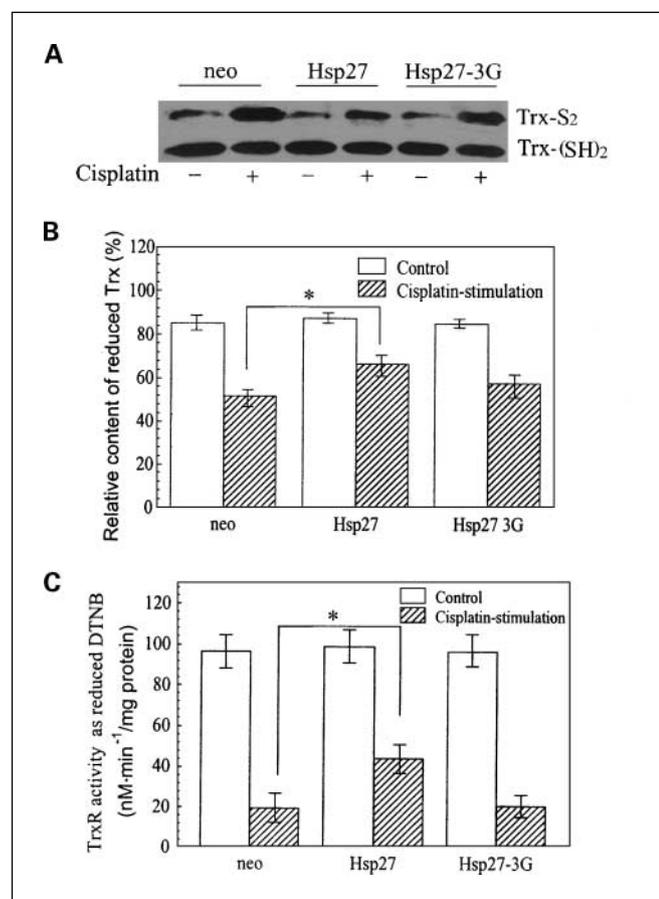
Fig. 6. Reversal of the cisplatin-induced oxidation of thioredoxin and suppression of the thioredoxin reductase activity by overexpression of Hsp27. *A,* a typical redox immunoblot of thioredoxin in the neo cells and the Hsp27-overexpressing and Hsp27-3G – overexpressing cells either untreated or exposed to 5 μg/mL cisplatin for 3 h. *B,* statistical analysis of the cisplatin-induced decrease of the reduced form of thioredoxin [*Trx-(SH)_2*] in the above three cell lines based on three independent redox-immunoblot analyses. *C,* thioredoxin reductase activities measured in neo cells, Hsp27-overexpressing cells, and Hsp27-3G – overexpressing cells either untreated or exposed to 5 μg/mL cisplatin for 3 h. Columns, mean of three independent measurements; bars, SD.

detected in the lysate of Hsp27-3G–overexpressing cells but not in the immunoprecipitate with anti-Akt antibody from the same lysate suggests that the Hsp27 mutant, in which residues Ser[15], Ser[78], and Ser[82] at its phosphorylation site were mutated to glycines, cannot form complex with Akt. The enhancement of the cisplatin-induced Akt phosphorylation in the cells having their Akt complexed with Hsp27 may suggest that more associated Hsp27 lead to more phosphorylation of Akt. It is noticed that the amount of detected Hsp27 in the lysate of the Hsp27-overexpressing cells did not change after exposure to cisplatin (Fig. 7B, *lanes 5-8*) but obviously decreased in the precipitate with anti-Akt antibody from the same lysate (comparing *lanes 8* and *9* with *lanes 6* and *7* in Fig. 7A). This indicates that a considerable amount of Hsp27 is dissociated from Akt after activation of Akt by cisplatin. It seems that Hsp27 sensitizes Akt activation by association with Akt and dissociates from Akt after the associated Akt is phosphorylated.

Discussion

Besides higher expression level of Hsp27 in multiple malignancies including gastric carcinoma, ovarian carcinoma,
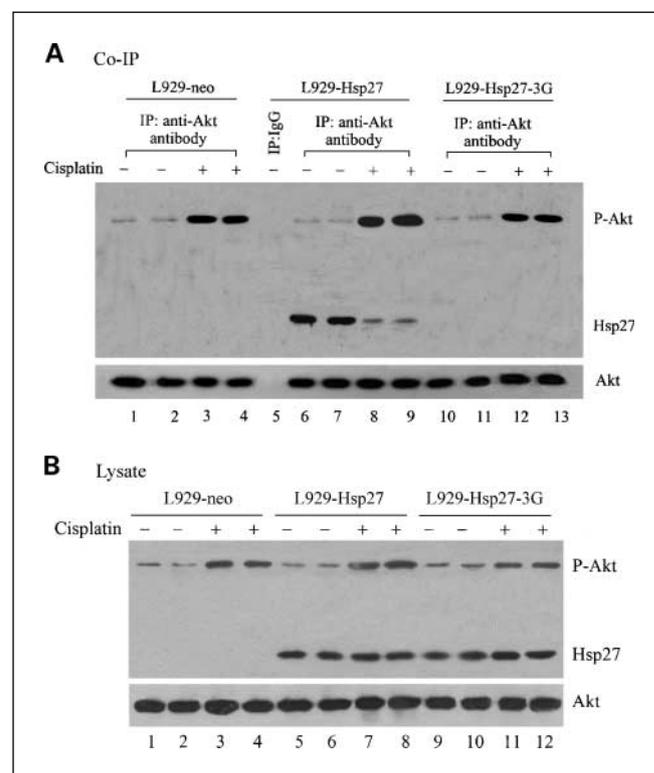

Fig. 7. Dependence of the cisplatin-induced Akt activation on the complexed Hsp27. *A,* immunoblot of Hsp27 and phosphorylated Akt probed sequentially by rabbit anti-Hsp27 and rabbit anti – phospho-Akt (Ser[473]) antibodies in the precipitate with goat anti-Akt antibody in the lysates from the neo cells and the Hsp27-overexpressing and Hsp27-3G – overexpressing cells, either untreated or exposed to 5 μg/mL cisplatin for 3 h. The immunoblot of Akt in the precipitate was done as control for equal loading of the samples. The precipitation of the untreated Hsp27-overexpressing cells with goat immunoglobulin G revealed no interaction of phospho-Akt and Hsp27 with the normal immunoglobulin G (*lane 5*). *B,* the same immunoblot of Akt, phosphorylated Akt, and Hsp27 in the lysates from the neo, Hsp27-overexpressing, and Hsp27-3G – overexpressing cells, respectively, either untreated or exposed to 5 μg/mL cisplatin for 3 h.

analysis. As shown in Fig. 7A, Hsp27 was detected in the precipitate with anti-Akt antibody in the lysate of the Hsp27-overexpressing cells but not in the lysates from the neo cells and the cells overexpressing Hsp27-3G. This unique property of the Hsp27-overexpressing cells suggests that wild-type human Hsp27 but not its dominant-negative mutant binds to Akt as complex in the cells. Although increased phosphorylation of Akt was observed in all three cell lines after exposure to cisplatin, the highest phosphorylation of Akt was detected only in the cells where Akt was associated with Hsp27 in a complex (see *lanes 8* and *9* in Fig. 7A). The immunoprecipitation with normal immunoglobulin G followed by Western blot analysis with anti-Akt antibody revealed no detectable association of phospho-Akt (see *lane 5* in Fig. 7A), indicating the specificity of phospho-Akt interactions with anti-Akt antibody. As control, the immunoblot of Hsp27 and phosphorylated Akt was also done in the lysates of the three cell lines. As shown in Fig. 7B, no Hsp27 was detected in the lysate of neo cells, suggesting that the cell expresses extremely low level of Hsp27. The observation that the protein recognized by the anti-Hsp27 antibody was

and acute myeloid leukemia (7–10), there has been quite a number of reports on the Hsp27-induced cisplatin resistance (11, 46–48). However, the underlying mechanisms still remain to be elucidated. What has been established for the Hsp27 protection from cell apoptosis induced by cisplatin and other chemotherapeutic drugs may be summarized as decreasing reactive oxygen species levels (46), restoring protein homeostasis and promoting cell survival either by repairing damaged proteins (protein refolding) or by degrading them (49), stabilizing actin-cytoskeleton (50), delaying the release of cytochrome *c* from mitochondria and Bid intracellular redistribution (51), and inhibiting activation of caspase-3 (52). However, there has been no report on the role of Hsp27 in the development of cisplatin resistance through interference with the events upstream of either the ASK1/p38 activation-initiated apoptotic signaling or the phosphatidylinositol 3-kinase/Akt activation–initiated survival pathway. In this study, we showed that overexpression of human Hsp27 in L929 cells protects cells from cisplatin-induced apoptosis by depressing the cisplatin-induced activation of ASK1/p38 kinase and enhancing the cisplatin-induced Akt activation, whereas knockdown of the endogenous Hsp27 in HeLa cells promotes apoptosis by enhancing the drug-induced activation of ASK1/p38 and depressing the drug-induced Akt activation. In the former case, the reduced activation of ASK1 and subsequent p38 activation by overexpression of Hsp27 are achieved by lightening the inhibitory effect of cisplatin on the activity of thioredoxin reductase, which leads to less oxidation of thioredoxin. Because only reduced form of thioredoxin is the inhibitor of ASK1, the less oxidation of thioredoxin results in less activation of ASK1 and subsequently less activation of p38. In latter case, the enhanced Akt activation by overexpression of Hsp27 is attributed to more Hsp27 associated to Akt in the cells overexpressing Hsp27. The observation that lack of association of Hsp27 with Akt in the cells overexpressing the dominant-negative mutant of Hsp27 may provide further evidence for the correlation between the greater association of Hsp27 and the higher activation of Akt. Stabilizing Akt and protecting Akt from dephosphorylation by Hsp27 have been suggested as one of the reasons for Hsp27-enhanced Akt activation (53).

There have been only a few reports on the inhibition of ASK1 by Hsp27. Charette et al. (54, 55) reported that phosphorylated dimers of Hsp27 blocked Daxx-mediated apoptosis by interacting with Daxx, a mediator of Fas-induced apoptosis, and preventing the interaction of Daxx with both ASK1 and Fas. Very recently, Schepers et al. (32) reported that Hsp27 protects acute myeloid leukemia cells against etoposide-induced apoptosis through modulation of p38 and c-Jun. These reports suggest a Daxx-mediated mechanism by which Hsp27 reduced the Fast- or etoposide-induced ASK1/p38 activation. The present study may reveal a different mechanism in which Hsp27 depressed cisplatin-induced activation of ASK1 and p38 by reversing the drug-induced inhibition of thioredoxin reductase activity and oxidation of thioredoxin. In contrast to the depression of cisplatin-activated ASK1/p38 activation, the significant increase of the drug-induced apoptosis in the presence of Akt inhibitors and double enhancement of the drug-induced Akt activation in the cells overexpressing Hsp27 may suggest that enhancement of Akt activation by Hsp27 may dominate the protective role against cisplatin-induced cell death.

The L929 cells used in this study are characterized by extremely low expression of the endogenous mouse Hsp25. The GI$_{50}$ of cisplatin for the mouse fibroblast cells was determined as 2.2 μmol/L (data not shown). It was found that cisplatin was unable to induce any notable expression of the endogenous Hsp25, although heat shock could do it (data are not shown). This cell property prevents the observed effect of overexpressed human Hsp27 from a possible covering up by cisplatin-induced mouse Hsp25. The experiments with knockdown of endogenous Hsp27 in HeLa cells may prove that the observed protective role of Hsp27 in mouse fibroblast cells might be extended to tumor cells. This study further suggests the importance of human Hsp27 as a targeting molecule in the strategy to overcome cisplatin resistance in cisplatin-based chemotherapy.

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