Knock-down of Superoxide Dismutase 1 Sensitizes Cisplatin-resistant Human Ovarian Cancer Cells

JONG WON KIM[1], HEATHER SAHM[1], JINSAM YOU[2] and MU WANG[1,2]

[1]*Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN 46202, U.S.A.;*
[2]*Monarch LifeSciences, LLC., Indianapolis, IN 46202, U.S.A.*

Abstract. *Background: Overexpression of superoxide dismutase 1 (SOD1) has been shown to be one of the factors involved in causing cisplatin resistance in ovarian cancer. Reduction of SOD1 expression is expected to restore, at least partially, cisplatin sensitivity in ovarian cancer chemotherapy. Here, we explored the potential of RNAi as a therapy for reversal of cisplatin resistance. Materials and Methods: SOD1-specific small-interfering RNA (siRNA) was synthesized and transfected into cisplatin-resistant cell line A2780/CP prior to treatment with 15 μM cisplatin. Cell survival was assessed by clonogenic assay. Results: An enhanced cisplatin sensitivity was observed in the A2780/CP cells treated with SOD1-specific siRNA, compared to non-siRNA-treated or scrambled-siRNA-treated control cells. Conclusion: Specifically targeting SOD1 could lead to sensitization of cisplatin-resistant ovarian cancer cells, and SOD1 may be used as a potential target for chemosensitizers.*

Ovarian cancer is the fourth leading cause of death in women (1). Currently, the most effective treatment is surgery, followed by platinum-based chemotherapy (2). About 90% of patients with the disease are initially very responsive to the chemotherapy, but the majority eventually relapse and become refractory to additional treatment (3, 4). This drug resistance has become a major obstacle for the successful treatment of ovarian cancer patients today. To understand the mechanism of drug resistance and factors involved in inducing this phenotype, a number of genomic and proteomic studies have been carried out (5-9). However, to date, no conclusive key factor(s) involved in platinum-based drug resistance has been identified. In our recent attempt to search for protein biomarkers of cisplatin resistance using a pair of cisplatin-sensitive and cisplatin-resistant ovarian cancer cell lines (A2780 and its counterpart A2780/CP) (7), a novel redox regulated pathway involving superoxide dismutase 1 (SOD1) has been identified as being one of the key pathways promoting cisplatin resistance (7). Inhibition of SOD1 activity in the cisplatin-resistant cells led to partial chemosensitization (10).

In humans, three known SODs have been discovered, and they are ubiquitously present in different organelles within the cell (11). SOD1 is present in the cytosol, nucleus and the intermembrane space of mitochondria; SOD2 is a manganese-containing enzyme present in the mitochondrial matrix; and SOD3 is a secreted copper-containing protein found in the extracellular matrix of tissues. The major physiological role of SOD proteins is to protect the cells against reactive oxygen species (ROS) toxicity, which can cause oxidative stress such as DNA damage, leading to cell death (12, 13). In mice studies, *SOD2* knock-out is lethal (14); while the *SOD1* and *SOD3* knock-out phenotypes are less severe (15-19). Mice lacking *SOD1* develop a wide range of pathologies, including hepatocellular carcinoma, an acceleration of age-related muscle mass loss, an earlier incidence of cataracts and a reduced lifespan (15-18). More recently, $SOD1^{-/-}$ mice showed an elevated susceptibility to liver tumors (17). *SOD1* knock-down by siRNA has also been shown to induce senescence in fibroblasts (20).

In one of our recent studies, we showed that the copper and zinc-chelating agent triethylenetetramine dihydrochloride (TETA) successfully inhibited SOD1 activity and was able to partially sensitize cisplatin-resistant ovarian cancer cells (10). However, it is important to note that SOD1 activity may not be the only cellular activity altered by TETA. This is the primary rationale that has led to the current study described in this report in which a more specific RNAi technology is used to knock-down *SOD1* expression levels in cisplatin-resistant ovarian cancer cells. In addition, we also evaluated the effects of *SOD1* knock-down in restoring cisplatin sensitivity.

Correspondence to: Mu Wang, Ph.D., Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, 635 Barnhill Drive, MS 4053, Indianapolis, IN 46202, U.S.A. Tel: +1 3172780296, Fax: +1 3172744686, e-mail: muwang@indiana.edu

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Materials and Methods

Materials. Urea (99.5%), dithiothreitol (DTT), iodoacetamide, acetonitrile, and ammonium bicarbonate were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Modified trypsin was obtained from Promega (Madison, WI, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA, USA). Anti-SOD1 (Cu^{2+}/Zn^{2+}), anti-SOD2 (Mn^{2+}), and anti-GAPDH antibodies were purchased from Millipore (Billerica, MA, USA).

Cell culture. A pair of human ovarian cancer lines, A2780 (cisplatin-sensitive) and A2780/CP (cisplatin-resistant), were used in this study. They were obtained from Dr. Stephen B. Howell of the University of California-San Diego, La Jolla, CA, USA. All cell lines were handled under identical conditions and maintained at 37˚C in a humidified incubator containing 5% CO_2 in RPMI-1640 media supplemented with 15% fetal bovine serum as described previously (7).

SOD1 knock-down experiment by siRNA. The specific oligonucleotides were synthesized by Integrated DNA Technologies (San Diego, CA, USA). The sequences used for *SOD1* and *SOD2* were 5'-TTC GAG CAG AAG GAA AGT AAT GGA CCA-3' (siSOD1) and 5'-GGA GAA GGA GGA TGT TTA TTT GCA-3' (siSOD2), respectively. The sequences 5'-GUC ACA CGG GAA GAG AGU UAA AGA CUA-3' (SCR_siSOD1) and 5'-GGA UAU GGG AAG AGC GUA GUU AAU U-3' (SCR_siSOD2) were used as scrambled, non-silencing control siRNAs for *SOD1* and *SOD2*, respectively.

Cells (3×10^5 cells) were prepared one day before transfection in growth medium. One nanomolar of each siRNA was transfected into the cells using Oligofectamine™ Reagent from Invitrogen (Carlsbad, CA, USA). Cells were allowed to grow for an additional 24, 48 or 72 h. RNAi-induced down-regulation of *SOD1* expression was measured by both immunoblotting and a selected-reaction-monitoring (SRM)-based assay (21).

Immunoblot analysis. Crude cell extracts (20 μg) were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membrane and probed with the indicated antibodies. The signal from horseradish peroxidase-conjugated secondary anti-immunoglobulin G was visualized by the enhanced chemiluminescence detection system (ECL) from Bio-Rad (Hercules, CA, USA).

SRM assay. Cells were homogenized using 100 μl of freshly made 8 M urea. Protein concentration was determined by the Bradford Protein Assay (Bio-Rad) (22). The same lysis buffer was used as the background reference for the protein assay and as the buffer for making the protein standards (bovine serum albumin). The resulting cell lysates (100 μg) supplemented with 0.5 μg of chicken lysozyme (used as external standard for relative quantification) were reduced and alkylated by 10 mM DTT and 55 mM iodoacetamide and then digested by trypsin (1:50). The resulting solutions were filtered through Durapore PVDF 0.45 μm centrifugal tubes (Millipore) before mass spectrometric measurements.

As described in our most recent work (21), all mass spectrometric analyses were performed on a Thermo-Fisher Scientific LTQ linear ion-trap mass spectrometer (Thermo-Fisher Scientific, Waltham, MA, USA) interfaced with a Surveyor high performance liquid chromatography (HPLC) system containing a binary pump and thermostated autosampler. Liquid chromatography (LC) was performed on an X-Bridge C18 column (Waters, 2.1 mm ×100 mm). Peptides were eluted with a linear gradient from 5 to 45% acetonitrile developed over 60 min at a flow rate of 200 μl/min, and effluent was electro-sprayed into the LTQ mass spectrometer. The parameters for the electrospray ionization (ESI) setup were as follows: capillary temperature of 225˚C, ESI spray voltage of 4 kV, source collision-induced dissociation (CID) voltage of 35 V and exclusion width of 4.0 m/z, tube lens voltage of 125 V, and sheath and auxiliary gas flow rates at 28 and 0 arbitrary units, respectively. The source lenses were set by maximizing the ion current for the $M+2H^+$ charge state of angiotensin. Chromatographic data acquisition, peak integration and quantification were carried out using the Xcalibur 2.0 package from Thermo-Fisher Scientific. Three SRM transitions for the SOD1 target peptide ^{11}GDGPVQGIINFEQK^{24} were monitored: (SOD1_A) m/z 751.3 ($M+2H^+$) → m/z 665.5, (SOD1_B) m/z 751.3 ($M+2H^+$) → m/z 778.5, and (SOD1_C) m/z 751.3 ($M+2H^+$) → m/z 948.5, respectively, as described previously (21). We also monitored two other transitions for internal standard [40S ribosomal protein S12, target peptide: ^{85}LGEWVGLCK^{93}, m/z 524.24 ($M+2H^+$) → m/z 878.44 and m/z 524.24 ($M+2H^+$) → m/z 935.47] and three other transitions for a spiked external standard [chicken lysozyme, target peptide: ^{64}NTDGSTDYGILQINSR^{79}, m/z 877.5 ($M+2H^+$) → m/z 730.4, m/z 877.5 ($M+2H^+$) → m/z 900.5, and m/z 877.5 ($M+2H^+$) → m/z 1063.5, respectively] to ensure the accuracy of the relative measurements.

Clonogenic assay. Cells (1×10^6 cells/10 ml PBS) were treated for 1 h with 15 μM cisplatin prepared in phosphate-buffered saline (PBS), washed with PBS three times, and then plated in 4 60-mm plates (1×10^4 cells/plate). Colonies were allowed to form over 8 days, followed by fixing and staining with 0.1% crystal violet dissolved in methanol/acetic acid (10:10:80, MeOH:AcOH:H_2O) (10). Colonies with fewer than 50 cells were disregarded. The percentage of cells surviving treatment was calculated by setting the survival of the control cells at 100%.

Results

Selective suppression of SOD1 expression. Small-interfering RNA targeting *SOD1* (siSOD1) was designed using the online software available from the Integrated DNA Technologies (IDT) website (http://www.idtdna.com/Scitools/Applications/RNAi). Figure 1 shows the ability of siSOD1 to specifically knock-down *SOD1* expression as measured by Western blot (Figure 1A) and SRM assay (Figure 1B), respectively. The transfection of siSOD1 dramatically reduced the expression level of SOD1 protein in A2780/CP (cisplatin-resistant) cells 24 h after transfection, whereas scrambled control siRNA (SCR_siSOD1) did not affect the expression level of SOD1 protein (data not shown). SRM analysis of SOD1 showed that the protein expression level was reduced by 43% after 24 h, 74% after 48 h, and 80% after 72 h (Figure 1B), which is consistent with the results obtained from the Western blot analysis (Figure 1A).

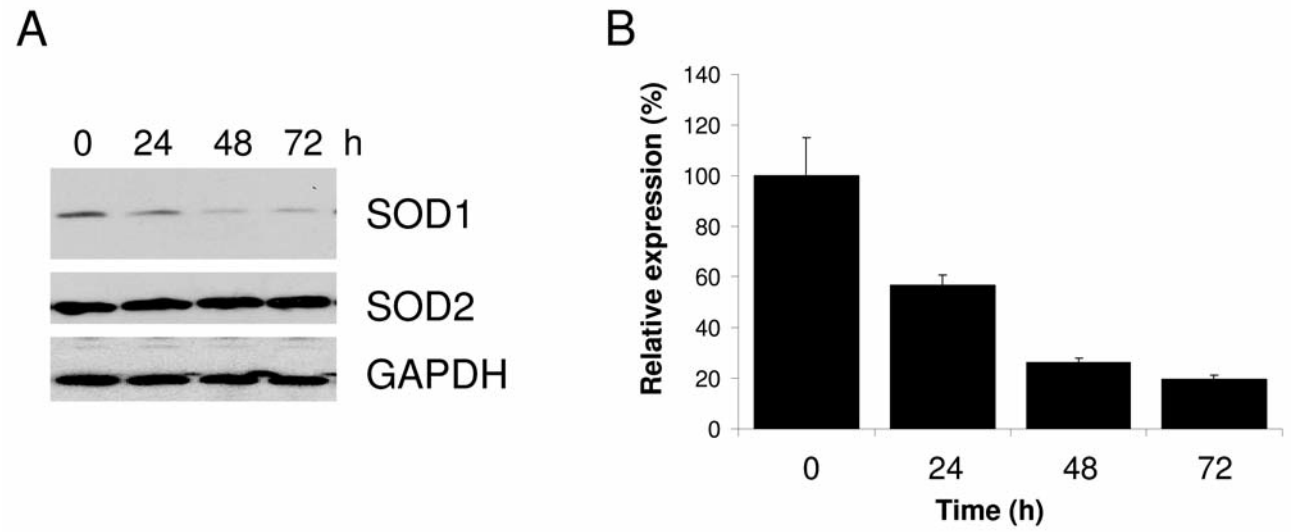

Figure 1. *RNAi-induced down-regulation of SOD1 expression measured by immunoblotting and SRM assay. A: Twenty micrograms of cell extract from cells transiently transfected with siSOD1 or SCR_siSOD1 (scrambled control) were prepared at 24, 48, and 72 h after transfection and then analyzed by Western blot. Anti-SOD2 and anti-GAPDH antibodies were used as loading controls. B: Relative SOD1 expression measured by SRM assay. The transitions for SOD1 target peptide (^{11}GDGPVQGIINFEQK^{24}), internal standard 40S ribosomal protein S12 target peptide (^{85}LGEWVGLCK^{93}), and external standard chicken lysozyme peptide (^{64}NTDGSTDYGILQINSR^{79}) were monitored, and the extracted ion chromatograms (XICs) were used for relative quantification. The data were normalized based on a constant amount of both internal and external standards in all samples studied.*

Effect of siRNA-directed knock-down of SOD1 on the cytotoxicity of cisplatin in the cisplatin-resistant cells. To evaluate the effect of siRNA-directed knock-down of *SOD1* on sensitizating the cisplatin-resistant ovarian cancer cells, a cell survival clonogenic assay was performed. A2780/CP (cisplatin-resistant) ovarian cancer cells were transfected with either siSOD1 or SCR_siSOD1 prior to cisplatin treatment. Non-siRNA transfected A2780/CP (mock) and A2780 (cisplatin-sensitive) cells were used as controls. The half maximal inhibitory concentration (IC_{50}) values for both the cisplatin-resistant and cisplatin-sensitive cells were 15 μM and 1 μM, respectively, determined by the same method as in our previous study (10). Figure 2 shows the percentage cell survival of cisplatin-sensitive cells and of siRNA-treated and untreated cisplatin-resistant cells after 1 h incubation with 15 μM cisplatin, followed by 8 days in culture. In the presence of 15 μM cisplatin, there was little survival for the cisplatin-sensitive cell line A2780 (<3%). Consistent with previous observations (10), roughly 50% of the non-siRNA-treated (mock) or SCR_siSOD1-treated A2780/CP (cisplatin-resistant) cells survived under the same conditions. However, the siSOD1-treated A2780/CP cells were approximately four times more sensitive than non-siSOD1-treated cells (~25% survival after normalization, as shown in Figure 2, or ~12% *vs*. 50% survival without normalization). It was noted that when the A2780/CP cells were treated with siSOD1 alone, an approximately 50%

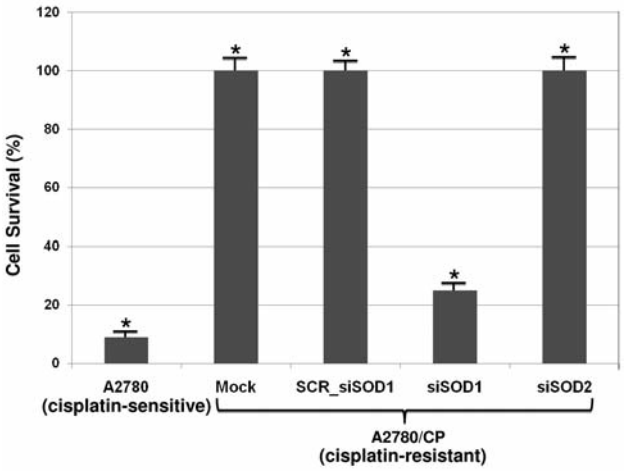

Figure 2. *Effects of siSOD1 on the clonogenecity of A2780/CP (cisplatin-resistant) cells treated with cisplatin. Cells were treated with SCR_siSOD1, siSOD1, or siSOD2 for 48 h before incubating with 15 μM of cisplatin for 1 h. After the treatment, cells were then rinsed with fresh media and plated in 5 60-mm tissue culture dishes per condition. Colony counting was performed after 8 days to determine clonogenecity. A2780 (cisplatin-sensitive) and non-siRNA-treated A2780/CP (cisplatin-resistant) (mock) cells were used as controls. The percentage of cell survival under different conditions was normalized to 100% based on the percentage of survival of mock cells. *p<0.05, t-test, siRNA treated compared with untreated (mock) cells.*

decrease in cell survival was observed compared to the untreated A2780/CP cells. Conversely, when the same A2780/CP cells were treated with siSOD2 prior to 15 μM cisplatin treatment, the percentage cell survival did not differ from that of the non-siSOD1-treated cells (Figure 2), demonstrating that SOD1 is specifically involved in maintaining cisplatin resistance.

Discussion

Drug-resistance is a major hindrance to the successful treatment of ovarian cancer. In order to understand the mechanism of drug-resistance, we previously conducted a global quantitative proteomic analysis of cisplatin-sensitive (A2780) and cisplatin-resistant (A2780/CP) ovarian cancer cells in the presence or absence of cisplatin with the hope of identifying a biomarker(s) of cisplatin resistance (7). This biomarker discovery study revealed that one of the novel pathways involved in the drug-resistance is the redox-regulated pathway involving SOD1. A subsequent study on the role of SOD1 in cisplatin resistance was performed by inhibiting its activity using a small-molecule inhibitor TETA. The results of that study supported SOD1 as a potential target for a novel anticancer chemosensitizer (10). The inhibition of SOD1 activity enhanced the cisplatin sensitivity in the resistant cells (10), supporting the hypothesis that a redox-regulated pathway involving SOD1 may play a key role in acquiring cisplatin resistance and that inhibition of SOD1 activity or down-regulation of its expression may sensitize the cisplatin resistant cells. Here, we demonstrate that selective inhibition of SOD1 expression can be achieved by RNAi technology, and knock-down of *SOD1* expression sensitizes the cells to cisplatin. Combination therapy consisting of siRNA knock-down of *SOD1* and cisplatin administration may be a potential strategy to enhance the efficacy of anticancer chemotherapy in a variety of drug-resistant human tumors.

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