p53 restoration can overcome cisplatin resistance through inhibition of Akt as well as induction of Bax

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Abstract. Cisplatin (CDDP) is a chemotherapeutic agent that is widely used to treat many cancers. However, initial resistance to CDDP is a serious problem in treating cancers. In this study, in order to develop an approach to overcome resistance to CDDP, we investigated the difference in apoptotic processes between CDDP-sensitive cells and CDDP-resistant cells. By screening with CDDP sensitivity tests, we chose SNU-16 cells which are relatively resistant to CDDP, and SNU-1 cells which are sensitive to CDDP. We compared the difference between the two cell lines focusing on apoptosis. CDDP-induced reactive oxygen species (ROS) generation significantly induced loss of mitochondrial membrane potential (MMP, $\Delta\Psi_m$) in SNU-1 cells, but not in SNU-16 cells. In addition, the ratio of Bax to Bcl-2 was increased by CDDP treatment in SNU-1 cells, but not in SNU-16 cells. To augment the loss of MMP, $\Delta\Psi_m$ in SNU-16, we inhibited Akt activity of SNU-16 cells to suppress their anti-apoptotic activity. The inhibition of Akt activity led to suppression of the anti-apoptotic protein XIAP. Akt inhibition slightly enhanced CDDP-induced apoptosis in SNU-16 cells. In addition, we enhanced pro-apoptotic activity by transfecting the cells with the wild-type p53 gene. The induction of wild-type p53 can enhance CDDP-induced apoptosis not only by inducing Bax protein but also by suppressing anti-apoptotic proteins through inhibition of Akt. In conclusion, this study suggests that the primary contributor to resistance to CDDP in SNU-16 cells may well be a failure of induction of apoptosis due to a lack of induction of pro-apoptotic proteins rather than suppression of anti-apoptotic proteins, and that restoration of p53 function can overcome the resistance to CDDP not only by augmenting the pro-apoptotic drive through p53-mediated transcriptional activation but also by inhibiting the anti-apoptotic drive through inhibition of Akt activity.

Introduction

Cisplatin (CDDP, cis-diaminedichloroplatinum) is a widely used chemotherapeutic agent for the management of gastric cancers. Resistance to CDDP based chemotherapy is a major cause of treatment failure. Chemotherapy resistance is a multifactorial phenomenon of the molecular mechanisms, many of which are poorly understood. One mechanism of resistance may be mediated through enhanced anti-apoptotic activity (1). In general, the target for CDDP is DNA, to which it binds efficiently to form a variety of monoadducts and cross links, either between adjacent bases on the same strand of DNA or on opposing strands (2,3). These DNA lesions contribute to the cytotoxicity of CDDP through blocking DNA replication and stimulating signals for apoptosis (4). In the CDDP-induced apoptosis, mitochondria play an important role. CDDP can induce MMP ($\Delta\Psi_m$), which leads to cytochrome c release to the cytoplasm and subsequent activation of caspases (5,6). In this process, pro-apoptotic and anti-apoptotic proteins serve as determinants of the cell fate. In the induction of pro-apoptotic proteins by DNA-damage-induced signaling, the role of tumor suppressor p53 is indisputable. Upregulation of proapoptotic proteins such as Bax and PUMA is mediated by post-translational modifications, such as phosphorylation and acetylation of the p53 protein.
However, the wild-type p53 alone is not a direct predictor of the chemotherapeutic response (6). Regarding CDDP resistance, activation of the phosphatidylinositol-3-kinase/Akt pathway also plays an important role in chemotherapy resistance by inducing anti-apoptotic proteins. We are still confronting this resistance problem in treating cancer patients even though much effort has been devoted to solve it. In this study, we screen the sensitivity of cancer cells to CDDP and then looked into the CDDP-induced apoptotic process to investigate CDDP resistance.

Materials and methods

Cell line and cell culture. Three of the gastric cancer cell lines, SNU-1, SNU-5, SNU-16 cells were obtained from the Laboratory of Cell Biology at the Cancer Research Institute in Seoul National University College of Medicine. They were cultured in RPMI-1640 supplemented with 10% FBS (Gibco-BRL, Carlsbad, CA, USA), 100 units of penicillin and 100 µg/ml of streptomycin at 37°C in the humidified atmosphere of 95% air and 5% CO₂ in an incubator. Molecular mass markers for proteins were obtained from Pharmacia Biotech (Saclay, France). Antibodies against phospho-Akt (Ser473), Akt 1/2/3, XIAP, Bel-2 (N-19), p53, phospho-p70 S6 kinase α (Thr389), and GFP were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibodies against phospho-Akt (Thr308), and phospho-p53 (ser15) were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Antibody against β-actin was from Sigma (Beverly, MA, USA). Peroxidase-labeled donkey anti-rabbit and sheep antimmunoglobulin, and an enhanced chemiluminescence (ECL) kit were purchased from Amersham (Arlington Heights, IL, USA). All other chemicals not specifically cited here were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

tdT-mediated dUTP nick end labeling staining. TdT-mediated dUTP nick end labeling (TUNEL) staining was conducted using an in situ cell death detection kit, TMR Red, according to the protocol supplied by the manufacturer (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, cells were plated in 25-cm² flasks at a density of 2x10⁵ cells/ml. The following day cells were treated with 0-50 µg/ml of CDDP, harvested and fixed with 2% paraformaldehyde solution and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing twice with PBS, cells were incubated in a TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and tetramethyl-rhodamine-dUTP. Cells were analyzed for fluorescence intensity using a FACS flow cytometer (Becton-Dickinson, San Jose, CA, USA). SNU-1 and SNU-16 cells were treated with the same condition with CDDP. After exposure to CDDP, the cells were incubated with 10 µM 2',7'-dichlorofluorescein diacetate (DCF-DA) for ROS levels and 30 nM 3',3'-dihexyloxacarbocyanine iodide [DiOC₆(3)] (Sigma Chemical Co.) for MMP (∆Ψₘ) at 37°C for 30 min. The cells were then washed with ice-cold PBS and harvested by trypsinization. Fluorescence was determined using a FACS flow cytometer.

Western blot analysis. Cells were washed twice with cold PBS, and total cell lysates were obtained using lysis buffer containing 0.5% SDS, 1% NP-40, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5), and protease inhibitors. The concentrations of cell lysate proteins were determined by means of the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA, USA) using bovine serum albumin as the standard. Molecular mass markers for proteins were obtained from Pharmacia Biotech. For western blot analysis, protein (30 µg) was resolved by electrophoresis, electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA), and then incubated with primary antibodies followed by incubation with a secondary antibody conjugated to peroxidase. Blots were developed with an ECL detection system. Autoradiography film was exposed at multiple time points to obtain the best images.

Results

SNU-16 cells are relatively resistant to CDDP whereas SNU-1 cells are sensitive and the difference is derived from the difference in apoptosis. To investigate CDDP-induced apoptosis in gastric cancer cells, we examined the CDDP sensitivity through MTT assay of the three cell lines (SNU-1, SNU-5, SNU-16 cells). As shown in Fig. 1A and B, the growth of the three cell lines was inhibited by CDDP treatment in a dose-dependent manner, and IC₅₀ for the 24 h CDDP treatment was less than 10 µg/ml in SNU-1 cells whereas in SNU-16 cells IC₅₀ was greater than 20 µg/ml. SNU-16 was the most resistant to CDDP. To determine whether the decrease in viability was related to apoptosis, we performed a TUNEL assay. The discrepancy in the degree of apoptosis became apparent after 12 h of treatment (Fig. 1C). These findings suggest that SNU-16 cells are relatively resistant to CDDP whereas SNU-1 cells are sensitive and that the difference is derived from the difference in apoptosis between the two cell lines.
CDDP treatment induces caspase-dependent apoptosis in SNU-1 cells. To confirm that CDDP-induced apoptosis was caspase-dependent, we performed MTT assay with caspase inhibitors (z-VAD-FMK, Z-LEHD-FMK, Z-IETD-FMK and Z-DEVDFMK). The SNU-1 cells were exposed to only 12 µg/ml of CDDP for 20 h which caused 70% apoptosis in the SNU-1 cells. These inhibitors significantly suppressed the apoptosis induced by CDDP (Fig. 2). This result suggests that CDDP-induced apoptosis is caspase-dependent.

Loss of mitochondrial membrane potential \([\text{MMP}(\Delta \Psi_m)]\) is critical in CDDP-induced apoptosis in SNU-1 and SNU-16 cells. Oxidative damage plays an important role in CDDP-induced apoptosis (7). To investigate the causes of the difference in sensitivity to CDDP between SNU-1 cells and SNU-16 cells, we compared intracellular ROS level in SNU-1 and SNU-16 cells after CDDP treatment. CDDP (12 µg/ml for 20 h) increased the ROS level in SNU-16 cells (data not shown). Many SNU-1 cells were dead at the time of measurement (data not shown). Hence we assessed the effects of the ROS scavenger, N-acetyl-L-cysteine (NAC) on CDDP-treated cells. The effects were different; NAC treatment significantly reduced apoptotic cell death of SNU-1 cells, but not in SNU-16 cells (Fig. 3A). We also compared the loss of MMP \((\Delta \Psi_m)\) induced by CDDP between SNU-1 and SNU-16 cells. The loss of MMP level was significantly higher in SNU-1 cells than in SNU-16 cells (Fig. 3B). These findings indicated that CDDP-induced reactive oxygen species (ROS) generation significantly induced loss of MMP \((\Delta \Psi_m)\) in SNU-1 cells, but not in SNU-16 cells, suggesting that the loss of MMP \((\Delta \Psi_m)\) may determine the level of apoptotic cell death.

CDDP increased Akt phosphorylation but the induced Akt activity did not prevent phosphorylation of p53 in SNU-16 cells. Increased Akt activity promotes CDDP resistance by inhibiting pro-apoptotic drive as well as augmenting anti-apoptotic drive (8-10). First, we assessed Akt expression in SNU16 cells treated with CDDP over the time frame. Western blot analysis revealed...
that CDDP increased Akt activity (Fig. 5A). Increased Akt activity promotes CDDP resistance in cancer cells through inhibition of p53 phosphorylation and transcriptional activity (10). In p53-functioning cells, the phosphorylation of p53 has also been reported to be an independent determinant of transcriptional upregulation of pro-apoptotic proteins such as...
Bax and PUMA in CDDP-induced apoptosis. However, Bax proteins can be upregulated by other transcriptional factors (11). Hence, we examined the expression of p53 or p-p53 levels. CDDP increased the expression of p53 and p-p53 (Ser15) in both SNU-1 and SNU-16 cells (Fig. 5B). This result suggests that phosphorylated p53 should not induce Bax protein in SNU-16 cells, which means that SNU-16 cells should be p53-mutant or p53-non-functioning cancer cells; the results also confirmed that phosphorylated p53 (Ser15) is an independent determinant for CDDP-induced apoptosis only in p53 functioning cancer cells. These findings suggest that Bax induction by CDDP in SNU-1 cells may be derived from p53 activation, and that increased Akt may not significantly suppress CDDP-induced phosphorylation of p53 in SNU-16 cells.

PI3K/Akt inhibition enhanced CDDP-induced apoptosis by suppression of anti-apoptotic proteins, but the efficacy was minimal. To augment loss of MMP, ΔΨm in SNU-16 by suppressing anti-apoptotic activity, and to investigate the role of PI3K/Akt pathway in CDDP-induced apoptosis in SNU-16 cells we inhibited the Akt activity of SNU-16 cells using LY294002, a representative PI3k/Akt inhibitor. As shown in Fig. 6, LY294002 slightly accentuated the cytotoxicity of CDDP and LY294002.

Figure 5. CDDP increased Akt phosphorylation but the induced Akt activity may not be related to phosphorylation and expression of p53 in SNU-16 cells. Western blot analysis for the effects of CDDP on the activity of Akt and p53 in SNU-1 and SNU-16 cells. The cells were treated with 12 µg/ml of CDDP. Equal amounts of the cell lysate were separated by SDS-polyacrylamide gels and then transferred to nitrocellulose membranes and probed with the indicated antibodies and detected by an ECL detection system. (B lower panel) The expression levels of total p53 were quantified by densitometry, normalized to the internal control β-actin. The expression of the indicated proteins was measured by densitometry, and the values represent means ± SD. *P<0.05 versus control.

Figure 6. Akt pathway is not critical for the resistance of SNU-16 cells to CDDP treatment. (A) Viability determined by MTT assay. SNU-16 cells were seeded at 1x10⁵ cells/ml and then treated with LY294002 (10 µg/ml) 1 h prior to the treatment with CDDP (12 µg/ml) for 24 h. The values represent means ± SD. *P<0.05 versus control. (B) Western blot analysis of the effects of LY294002 on CDDP-induced cell death in SNU-16 cells. The cells were treated according to the above time schedule. Equal amounts of the cell lysate were separated by SDS-polyacrylamide gels and then transferred to nitrocellulose membranes and probed with the indicated antibodies and detected by an ECL detection system.
KIM et al: p53 RESTORATION OVERCOMES CISPLATIN RESISTANCE

CDDP in MMT assay and no synergism was observed between LY294002 (PI3k/Akt inhibitor) and CDDP in SNU-16 cells. To confirm this finding at the molecular level, we performed western blot analysis for apoptosis-related factors and p-Akt. The suppression of Akt phosphorylation led to inhibition of XIAP and activation of apoptosis-related enzyme (PARP and caspase 3). We found that LY294002 enhanced the cytotoxicity of CDDP by suppressing XIAP. This result suggests that inhibition of Akt may not significantly enhance the sensitivity of CDDP in SNU-16 cells even though CDDP induces Akt activation.

The resistance to CDDP of SNU-16 cells can be overcome by p53 augmentation through inhibition of Akt as well as induction of Bax. We assumed that the reason why PI3k/Akt inhibitor did not induce synergism with CDDP is the lack of the proapoptotic drive due to loss of p53 function. Therefore, we transfected SNU-16 cells with wild-type p53, and then tested the sensitivity to CDDP. MTT assay revealed that in the SNU-16 cells transfected with wild-type p53, the sensitivity to CDDP was significantly enhanced (Fig. 6A). To confirm this finding at the molecular level, we performed western blot analysis for p53 and downstream molecules of PI3k/Akt pathway. Successful transfection with wild-type p53 was confirmed with GFP. Interestingly, the transfection of wild-type p53 alone did not significantly influence phosphorylation of p70S6K, or of Akt as well as Bax induction. However, addition of CDDP on the p53-trasfected cells not only augmented Bax induction but also suppressed Bcl-2 through inhibition of Akt phosphorylation. These results suggest that restoration of p53 function can overcome the resistance to CDDP by augmenting the proapoptotic drive through p53-mediated transcriptional activation and by inhibiting the anti-apoptotic drive through inhibition of Akt activity (Figs. 7 and 8).

Discussion

Our study was designed to demonstrate the difference in apoptotic processes in CDDP-induced apoptosis between constitutively CDDP-sensitive and CDDP-resistant gastric cancer cells in vitro, and to find a method to overcome the resistance to CDDP. SNU-16 cells are the most resistant to CDDP and SNU-1 the most sensitive among the 3 cell lines (SNU-1, SNU-5 and SNU-16). The major contributor to the big difference in CDDP-induced cell death between SNU-1 cells and SNU-16 cells was loss of MMP (ΔΨm). The loss of MMP (ΔΨm) is one of the main events of the apoptotic process induced by chemotherapeutic drugs (12,13), and this results in either caspase-dependent or independent apoptosis (14,15). In this study, significant loss of MMP (ΔΨm) by CDDP treatment was observed in SNU-1 cells, but not in SNU-16 cells. This finding indicates that lack of the proapoptotic drive or a...
surplus of anti-apoptotic drive induced the failure of CDDP in inducing loss of MMP ($\Delta \Psi_m$) in SNU-16 cells. Our data indicated that SNU-16 cells were p53-non-functioning cells. Actually, SNU-16 cells have a missense mutation of codon 205, TAT to TTT, Tyr to Phe (16). This area belongs to the p53 DNA binding domain (17). This is consistent with our findings. Here, we also tested whether an Akt inhibitor can enhance the CDDP sensitivity in p53 mutation cancer cells by suppressing the anti-apoptotic proteins because a previous study suggested that the mutation status of p53 might not predict the chemo-response, and that Akt activation might be involved in CDDP resistance. Unlike the previous report, inhibition of PI3K/Akt pathway was not adequate to overcome CDDP resistance in SNU-16 cells. We also tested whether the anthocyanins enhanced CDDP sensitivity, because it has been reported that anthocyanins isolated from Vitis coignetiae Pulliat can enhance apoptosis by suppressing anti-apoptotic proteins such as Bcl-2, and XIAP through the inhibition of Akt and NF-κB that are involved in drug resistance (18,19). Similar to the results of LY294002, the anthocyanins also slightly enhanced the CDDP sensitivity of SNU 16 cells, but they did not show a clear synergism (data not shown).

Here, we demonstrated that the restoration of p53 functions in SNU-16 cells enhancing CDDP-induced apoptosis not only by inducing apoptotic factors through p53-mediated transcriptional activation but also by inhibiting anti-apoptotic proteins through inhibition of Akt activity. This finding was also observed in wild-type SNU-1 cells (Fig. 4); CDDP not only augmented Bax expression but also suppressed Bcl-2 expression in SNU-1 cells. This can be explained by previous studies that suggested that DNA damage can activate pTEN through p53 activation followed by inhibition of Akt (20,21). Our data encourage the use of gene therapy with wild-type p53 in cancer treatment. This result is supported by the successful results of p53 gene therapy in combination with CDDP in in vitro and xenograft cancer models, and in the patients with small cell lung cancer (22,23). However, there is still controversy surrounding p53 gene therapy because there are also negative results showing no additional benefit with combination therapy (24).

The limitation of this study is that we only compared three gastric cell lines and validated the role of p53 restoration only in SNU-16 cells. In addition, there may be many other ways to enhance CDDP sensitivity. Therefore, these issues will require investigation. To reveal the clinical significance, an in vivo study followed by a clinical trial is warranted. In conclusion, this study suggests that the primary contributor to resistance to CDDP in SNU-16 cells may well be a failure of induction of apoptosis due to lack of induction of proapoptotic activities rather than an increase in anti-apoptotic activity, and that restoration of p53 function can overcome the resistance to CDDP not only by augmenting the proapoptotic drive through p53-mediated transcriptional activation but also by inhibiting the anti-apoptotic drive through inhibition of Akt activity. This study supports that the restoration of p53 is still important in CDDP-induced apoptosis in p53 mutant SNU-16 human gastric cancer cells.

**Figure 8.** Schematic representation of CDDP-induced apoptosis in SNU 16 human gastric carcinoma cells. (A) SNU-16 cells showed resistance to CDDP treatment due to increased Akt activity as well as p53 mutation. Increased Akt induced XIAP. (B) Inhibition of Akt by LY294002 enhanced the cytotoxicity of CDDP by suppressing XIAP in p53 mutant SNU-16 cells, but the efficacy was minimal due to lack of apoptotic drive. (C) When we restored p53 gene function by transfecting wild type p53 gene into SNU16 cells, the wild type p53 can enhance CDDP-induced apoptosis by induction of Bax as well as suppression of anti-apoptotic proteins through the inhibition of Akt in SNU-16 cells. Taken together, this study suggests that the restoration of p53 is still critical in CDDP-induced apoptosis in p53 mutant SNU-16 human gastric cancer cells.
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