Role of reactive oxygen species in p53 activation during cisplatin-induced apoptosis of rat mesangial cells


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Abstract. – BACKGROUND AND OBJECTIVES: Nephrotoxicity is one of the main side effects of the anticancer drug cisplatin, and one of its main therapeutic limitations. It has been suggested that p53 activation plays important roles in renal cell injury by cisplatin. However, the mechanism of p53 activation by cisplatin is unclear. This study examined whether reactive oxygen species (ROS) production by cisplatin would be linked to p53 activation in rat mesangial cells.

MATERIALS AND METHODS: Renal cells were incubated with cisplatin in the absence or presence of pifithrin-α (PFT), N-acetyl-cysteine (NAC), or dimethylthiourea (DMT). Cell viability was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Apoptosis was evaluated by caspase-3 activity and cleavage of poly (ADP-ribose) polymerase (PARP). The relative levels of ROS and p53 phosphorylation were determined by fluorometric assay and Western blot analysis, respectively.

RESULTS: Cisplatin induced apoptotic cell death via caspase-3 activation and PARP cleavage, and also increased p53 activation and ROS production. The p53 inhibitor PFT inhibited cisplatin-induced apoptosis. NAC and DMT, two antioxidants, also inhibited cisplatin-induced apoptosis. Interestingly, NAC and DMT reduced ROS production and suppressed p53 activation in renal cells exposed to cisplatin.

CONCLUSIONS: Our results suggest that the ability of cisplatin to induce apoptosis of rat mesangial cells requires ROS-dependent p53 activation, thus, supporting the potential therapeutic role of antioxidants in preventing the cisplatin nephrotoxicity.

Key words:
Cisplatin, Nephrotoxicity, p53, Reactive oxygen species.

Introduction

Cisplatin (cisplatinum or cis-diamminedichloroplatinum (II)) is one of the most effective and widely used chemotherapeutic agents employed for treatment of human cancers. However, clinical use of this anticancer drug is limited by severe side effects, such as nephrotoxicity, neurotoxicity, ototoxicity, and emetogenicity. Among them, nephrotoxicity has been reported as the major limiter in cisplatin chemotherapy. Because of the importance of cisplatin chemotherapy, many studies have focused on protective strategies targeting the main molecular mechanisms of cisplatin nephrotoxicity.

Apoptosis is considered as an ongoing normal event in the control of cell populations. However, apoptosis can also be induced by a variety of toxic stimuli, resulting in loss of affected cell populations. Whilst the initiation of apoptosis by toxic stimuli may be seen initially to have a physiological role in maintaining whole body homeostasis, the eventual loss of cells by this process will have a devastating outcome in organs like the kidney. Apoptosis occurs primarily through two well-recognized pathways; the intrinsic or mitochondrial-mediated pathway, and the extrinsic or death receptor-mediated pathway. Both pathways subsequently activate certain cascades of factors that ultimately lead to cell death through activation of caspases, including caspase-3, and cleavage of poly (ADP-ribose) polymerase (PARP).

Cisplatin induces two models of cell death: apoptosis and necrosis. Necrosis has been mainly associated with high doses of cisplatin, whereas apoptosis is associated with therapeutic...
The apoptosis triggered by DNA damage is mediated by the tumor suppressor protein p53 that activates pro-apoptotic proteins and inactivates anti-apoptotic proteins. The dividing tumor cells are particularly susceptible to DNA damage, and the anticancer activity of cisplatin has been mainly attributed to DNA adducts formation. Unlike tumor cells, healthy renal cells are non-dividing; therefore, the formation of adducts with DNA might not play a key role in cisplatin nephrotoxicity. Several studies indicate that cisplatin nephrotoxicity is mainly associated with oxygen reactive species (ROS), which leads to the activation of apoptotic pathways. Interestingly, ROS have been shown to induce p53 activation, presumably by causing oxidative DNA damage. Thus, one may assume that cisplatin increases ROS production, which in turn contributes to p53 activation and, ultimately, apoptosis of renal cells.

In this study, in order to better understand the multiple interconnected pathways of cisplatin nephrotoxicity, we have investigated the possible involvement of ROS in p53 activation during cisplatin-induced apoptosis of rat mesangial cells.

**Material and Methods**

**Reagents and Antibodies**

Cisplatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ribonuclease A (RNase A), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), pifithrin-α (PFT), N-acetylcysteine (NAC), 1,3-dimethyl-2-thiourea (DMT), and protease inhibitor cocktail were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). N-Acetyl-Asp-Glu-Val-Asp-pNA (Ac-DEVD-pNA) and Z-Val-Ala-Asp(OMe)-CH₂F (VAD) were purchased from Calbiochem (La Jolla, CA, USA). Anti-cleaved caspase-3 (p20), anti-PARP, anti-p53 and anti-phospho (p)-p53 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

**Cell culture**

Immortalized rat mesangial cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco’s Modified Eagle’s medium (DMEM) (Invitrogen, Burlington, ON, Canada) supplemented with 0.4 mg/ml G418, 15% fetal bovine serum (FBS) (Invitrogen, Burlington, ON, Canada), 100 U/ml penicillin, 100 mg/ml streptomycin at 37 °C in humidified 95% air and 5% CO₂ incubator. Cells were propagated for at least five passages in medium supplemented with G418 to verify stable growth characteristics and morphological features, the subsequent cells were cultured on culture plates in DMEM containing 15% FBS, 100 mg/ml penicillin. When the cells reached confluence, subcultures were prepared using 0.25% trypsin-0.03% EDTA solution. Cells were plated in cell culture dishes (or plates) 1 day prior to cisplatin treatment.

**MTT assay**

Cell viability was determined using the MTT. The MTT assay is based on the reduction of MTT to formazan by the mitochondrial enzyme succinate dehydrogenase. Exponentially growing cells were seeded into a 12 well plate at 5 x 10⁴ cells/well. After pre-incubation for 24 h, cells were treated with cisplatin for 24 h. After the incubation period, culture medium was removed and 500 µl of MTT (0.5 mg/ml in phosphate buffered saline: PBS, pH 7.4) solution was added to each wells and the cells were incubated for 3 h at 37°C. The supernatant was removed and 500 µl of DMSO (dimethyl sulfoxide) was added to each well to solubilize the water-insoluble purple formazan crystals. The amount of formazan was determined at 560 nm using SpectraMAX 250 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The percent of cell viability was calculated using the equation: (mean optical density (OD) of treated cells/mean OD of control cells) x 100%.

**Caspase-3 activity assay**

Cells were resuspended in lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM dithiothreitol (DTT), 0.1 mM EDTA) and lysed by freezing/thawing. Cells lysates were obtained after centrifugation (10,000 rpm) for 1 min at 4°C. Caspase-3 activity assay was performed following the manufacturer’s instructions. In brief, the whole reaction contained 10 µl cell lysates (30 mg total protein), 88 µl reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% glycerol), and 2 µl fluorogenic Ac-DEVD-pNA (caspase-3) substrate (200 µM final concentration). Samples were incubated for 1 h at 37°C and enzyme-catalyzed release of p-nitroanilide was monitored at 405 nm in SpectraMAX 250 microplate spectrophotometer.
**Reactive oxygen species assay**

Reactive oxygen species (ROS) were determined by 2’,7’-Dichlorofluorescein diacetate (DCFH-DA) fluorescence assay. The cells were washed twice with PBS (pH 7.4) and incubated in PBS (pH 7.4) solution containing 10 µM DCFH-DA at 37°C for 30 min. The fluorescence intensity was determined with excitation wavelength of 485 nm and emission wavelength of 524 nm.

**Western blot analysis**

Cells were washed with ice-cold PBS (pH 7.4) and gently resuspended in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 1 mM Na3VO4) with freshly added 1% protease inhibitor cocktail, incubated on ice for 30 min. Cell lysates were centrifuged at 14,000 rpm for 15 min at 4°C, and the protein concentration was determined using a Bradford assay. Samples containing 30 µg of total protein were resolved by SDS-PAGE gel, and transferred onto a nitrocellulose membrane for 3 h at 40 V. The membranes were blocked with Tris-buffered saline with Tween-20 (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20) containing 5% non-fat dry milk and probed with primary antibodies (all 1:1000 in 3% BSA (bovine serum albumin) in Tris-buffered saline with Tween-20) overnight at 4°C with gentle shaking. Immunoreactivity was detected using anti-rabbit peroxidase-conjugated secondary immunoglobulin G antibody followed by SuperSignal West Pico Chemiluminescent (Pierce, Rockford, IL, USA), visualized on the Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA).

**Statistical analysis**

Statistical analysis was performed using Microsoft Office Excel 2003 (Microsoft, Redmond, WA, USA). The data were expressed as means ± standard deviation (SD). The statistically significant differences between two groups were calculated by Student’s t-test.

**Results**

**Induction of apoptosis by cisplatin**

Incubation of rat mesangial cells with cisplatin resulted in a dose- and time-dependent reduction of cell viability (Figure 1); a marked reduction of cell viability was observed at 30 µM of cisplatin for 24 h. As shown in Figure 2, cisplatin (30 µM) also increased the activity of caspase-3, which plays a key role in execution of apoptotic cell death[^19^], and the levels of cleaved caspase-3 form, which is observed only when caspase-3 is activated[^19^]. Moreover, cisplatin induced proteolytic cleavage of PARP with accumulation of its cleaved form (Figure 2B), which is cleavage by caspase-3 and thus recognized as a hallmark of apoptosis[^9^]. These results support the previous studies demonstrating that cisplatin can induce apoptosis of renal cells[^20^].

![Figure 1. Effects of cisplatin on cell viability.](image)

A. Renal cells were incubated with 30 µM of cisplatin for indicated times. B. Renal cells were incubated for 24 h with indicated concentrations of cisplatin. Cell viability was measured by MTT assay, as described under Materials and methods. Data are expressed as means ± SD from 3 to 4 experiments. *p < 0.05 versus untreated control group (Con).
Activation of p53 by cisplatin

Activation of p53 by cisplatin has been shown to play an important role in induction of apoptosis of tumor cells\(^1\). Thus, we examined whether cisplatin could also induce apoptosis of rat mesangial cells. When renal cells were exposed to 30 \(\mu\)M cisplatin, the levels of p53 phosphorylation, which is directly associated with p53 activation, was increased in a time-dependent manner, and reached the peak at 12 h without significant changes in p53 protein expression (Figure 3). We next examined whether p53 activation was associated with cisplatin-induced apoptosis. As shown in Figure 3, cisplatin-induced activation of caspase-3 and cleavage of PARP were almost completely inhibited by VAD, a general caspase inhibitor, suggesting caspase-3-dependent apoptosis, and significantly by PFT, a selective inhibitor of p53, suggesting the possible involvement of p53 activation in induction of apoptosis by cisplatin.

Production of ROS by cisplatin

ROS production has been suggested as a part of mechanism of cisplatin-induced apoptosis\(^2\). Thus, we studied the effect of cisplatin on ROS production by using DCFH-DA. As shown in Figure 5, treatment of rat mesangial cells with 30 \(\mu\)M cisplatin showed a significant increase in intracellular ROS production. We next examined whether ROS production was involved in cisplatin-induced apoptosis. As shown in Figure 6, cisplatin-induced activation of caspase-3 and cleavage of PARP were significantly inhibited by NAC, a general antioxidant, and DMT, a specific antioxidant to hydroxyl radical, indicating the involvement of ROS production in induction of apoptosis by cisplatin. It should be noted that inhibitory effect of the antioxidant DMT was more potent than that of the p53 inhibitor PFT, presumably implying that ROS production might be the major mechanism of cisplatin-induced apoptosis.

Activation of p53 via ROS production by cisplatin

ROS production has been shown to activate various protein kinases that may result in p53 activation\(^3\). Moreover, a previous study has suggested that oxidative stress induced by ROS production is involved in p53 activation in renal cells\(^4\). Thus, we finally tested our hypothesis that cisplatin might increase ROS production, which could contribute to p53 activation. For this end, the effects of antioxidants on p53 activation were examined in rat

**Figure 3.** Effects of cisplatin on p53 activation. Renal cells were incubated with 30 \(\mu\)M of cisplatin for indicated times. Phosphorylation and expression of p53 were measured by Western blot analysis, as described under Materials and methods. Blots shown are representative of three independent experiments.
mesangial cells exposed to cisplatin. As shown in Figure 7, NAC and DMT significantly reduced ROS production and suppressed p53 activation.

**Discussion**

Nephrotoxicity is one of the main side effects of the anticancer drug cisplatin, and also one of its main therapeutic limitations. In this study, the mechanisms of cisplatin-induced apoptosis were investigated in rat mesangial cells. The hypothesis that cisplatin might increase ROS production, which could contribute to p53 activation and apoptosis of renal cells, was tested for evaluating the potential therapeutic role of antioxidants in preventing the cisplatin nephrotoxicity.

Cisplatin is known to induce cell death in different cell types. Apoptosis was the predominant mode of cell death caused by cisplatin in renal cells. Caspases are cysteine proteases and activated by cleavage after aspartic acid residues. Many studies have showed that cisplatin induced apoptotic injury to renal cells by enhancing caspase-3 activation. In agreement with these studies, we showed that cisplatin induced apoptosis of rat mesangial cells by activation of caspase-3 and subsequent cleavage of PARP, which is a DNA repair protein that can be activated by DNA strand break. Cisplatin has been shown to induce apoptosis in renal cells mainly by mechanisms depending on p53 and/or ROS. However, the relevance of these signaling pathways and the precise mechanism of activation appear to be different, depending on the cellular context and the dose of cisplatin, and it has not been fully characterized, especially in mesangial cells that play an important role in regulating glomerular structure and function of the kidney as a whole.

The tumor suppressor protein p53 is a universal sensor of genotoxic stress and plays a critical role in induction of apoptosis by the genotoxic cisplatin in various cell types. Indeed, cisplatin induced apoptosis of rat mesangial cells via p53 activation, because apoptotic cell death caused by cisplatin was significantly reduced in the presence of the p53 inhibitor.

**Figure 4.** Effects of VAD and PFT on cisplatin-induced activity of caspase-3 and cleavages of caspase-3 and PARP. Renal cells were pre-incubated with 100 µM of VAD or 20 µM of PFT for 3 h, and then exposed to 30 µM of cisplatin for 24 h. A, Caspase-3 activity was measured by a caspase-3 activity assay kit, as described under Materials and methods. Data are expressed as means ± SD from 3 to 4 experiments. *p < 0.05 versus untreated control group. **p < 0.01 versus group treated with cisplatin alone. B, Cleavages of caspase-3 and PARP were measured by Western blot analysis, as described under Materials and methods. Blots shown are representative of three independent experiments.

**Figure 5.** Effects of cisplatin on ROS production. Renal cells were incubated with 30 µM of cisplatin for indicated times. ROS production was measured by DCFH-DA fluorescence assay, as described under Materials and methods. Data are expressed as means ± SD from 3 to 4 experiments. *p < 0.05 versus untreated control group (Con).
Besides its activation of p53, cisplatin also increased the levels of intracellular ROS in rat mesangial cells. Interestingly, two well-known antioxidants, NAC and DMT, significantly reduced cisplatin-induced apoptosis, suggesting that ROS production, along with p53 activation, was also involved in induction of apoptosis by cisplatin. Considering that both ROS and p53 participated in cisplatin-induced apoptosis of rat mesangial cells, there should be interactions between ROS and p53. While ROS have long been established as a mediator of p53-induced apoptosis, recent evidence has revealed that ROS can also act as an upstream signal that triggers p53 activation. In other words, it is still unclear whether p53 activation is a cause, result, or epiphenomenon of ROS production.

A large number of p53 target genes have been implicated in mediating its apoptotic effects.
They include Bax, a pro-apoptotic Bcl-2 family member, several mitochondrial proteins, Noxa, p53-regulated-apoptosis-inducing protein 1, and p53-upregulated modulator of apoptosis, and several genes associated with death receptor-mediated apoptosis, Fas, Killer/DR5, and PIDD\textsuperscript{26}. There does not appear to be a single gene that is the principal mediator of p53-induced apoptosis, but rather the response involves the activation of several apoptotic genes.

Conclusions

We have established that the ability of cisplatin to induce apoptosis requires ROS-dependent p53 activation in rat mesangial cells, thus, supporting the potential therapeutic role of antioxidants in preventing the cisplatin nephrotoxicity.

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Conflict of interest

The Authors declare that they have no conflict of interests.

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