Decursin Prevents Cisplatin-Induced Apoptosis via the Enhancement of Antioxidant Enzymes in Human Renal Epithelial Cells

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Adverse effects, nephrotoxicity and hepatotoxicity, of anticancer drugs such as cisplatin have limited the usage for cancer therapy. Therefore, development or identification of supplement agents in anticancer drugs is attractive to reduce side effects and enhance antitumor activity. Here, we found that decursin isolated from Angelica gigas showed protective effects of cisplatin-induced damage in normal human primary renal epithelial cells (HRCs). We found that decursin significantly blocked cisplatin-induced cytotoxicity by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay in HRCs. Further, we found that decursin inhibited sub-G1 and cell death by suppression of cleavage of caspase-3, -9 and poly(ADP-ribose) polymerase (PARP) induced by cisplatin treatment in HRCs. Importantly, decursin effectively restored the activities of Cu/Zn superoxide dismutase (SOD), catalase and glutathione peroxidase in cisplatin-treated HRCs. Taken together, our findings demonstrate that decursin prevents cisplatin-induced cytotoxicity and apoptosis through the activation of antioxidant enzymes in HRCs and suggest further that combination of decursin might suppressed adverse effects of anticancer drugs in cancer patients.

Key words decursin; cisplatin; renal epithelial cell; apoptosis; antioxidant enzyme

Materials and Methods

Chemicals Decursin (Fig. 1A) was extracted and purified as described previously. cisplatin (cis-diamminedichloroplatinum(II)) (Fig. 1B) was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Cell Culture Primary HRCs isolated from the urine of a healthy, non-stone forming male using the method of Dorrenhaus.12) were gratefully obtained from Dr. John C. Lieske.13) Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Welgene, Daegu, South Korea) supplemented with fetal bovine serum (FBS) and 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

Cytotoxicity Assay The cytotoxicity of decursin and cisplatin was measured by a tetrazolium salt, 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) colorimetric assay. HRCs were seeded onto 96-well microplates at a density of 1×104 cells per well in 100 μl of growth medium and treated with decursin and/or cisplatin for 24 or 48 h. After incubation, 50 μl of the XTT working solution was added to each well. The XTT working solution (1 ml of XTT stock solution (1 mg/ml) in phosphate...
buffered saline (PBS)) was added to the cells and incubated at 37 °C for 2 h and the optical density (OD) was measured using a microplate reader (Sunrise, TECAN, Männedorf, Switzerland) at 450 nm. Cell viability was calculated as a percentage of viable cells in drug-treated group versus an untreated control by employing the following equation.

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\text{cell viability (%) = \left\{ \frac{\text{OD (treatment)} - \text{OD (blank)}}{\text{OD (control)}} \right\} \times 100}
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**Cell Cycle Analysis** To determine apoptosis, cell cycle analysis was performed as previously described. HRCs (4 × 10^5 cells) treated with decursin and/or cisplatin were harvested, washed twice with cold PBS and fixed in 75% ethanol at −20 °C. The cells were resuspended in 100 μl of PBS containing 10 μl of RNase A (10 mg/ml) and incubated for 1 h at 37 °C. The cells were stained by adding 400 μl of propidium iodide (PI) (50 μg/ml) for 30 min at room temperature in the dark. The DNA contents of the stained cells were analyzed using CellQuest Software with the FACSCalibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

**Measurement of Mitochondrial Membrane Potential** Mitochondrial membrane potential (MMP) was determined as previously described. HRCs were pretreated with decursin for 1 h and then treated with cisplatin for 24 h. After washing twice with cold PBS, the pellets were resuspended in 1 ml of 150 μM tetramethylrhodamine ethyl ester (TMRE) (St. Louis, MO, U.S.A.) and incubated for 30 min at 37 °C in the dark. The fluorescent intensities of the cells were analyzed by FACSCalibur flow cytometry (BD Biosciences, San Jose, CA, USA.) at 582 nm.

**Live and Dead Assay** To measure apoptosis, the Live and Dead assay kit (Molecular Probes, Carlsbad, CA, U.S.A.) was utilized to determine intracellular esterase activity and plasma membrane integrity. Briefly, HRCs were pretreated with decursin for 1 h and treated with cisplatin for 24 h. Then, the cells were stained with the Live and Dead reagent (2 μM ethidium homodimer and 5 μM calcine-AM) and then incubated at 37 °C for 30 min in the dark. Cells were analyzed under an Axio vision 4.0 fluorescence microscopy (Carl Zeiss Inc., Weimar, Germany).

**TdT-Mediated dUTP Nick End Labeling (TUNEL) Assay** TUNEL assay was performed using an in situ cell death detection reagent (Roche Molecular Biochemicals, Mannheim, Germany) as described by the manufacturer’s instructions. DNA end (3’-OH) generated by DNA fragmentation was nick end-labeled with fluorescein isothiocyanate (FITC)-conjugated deoxyuridine 5’-triphosphate (dUTP), introduced by terminal deoxynucleotidyl transferase (TdT) using a TUNEL label mix and TUNEL enzyme and analyzed by flow cytometry. HRCs (4 × 10^5 cells) were treated with decursin and/or cisplatin for 24 h. The cells were fixed in 4% paraformaldehyde (PFA) in PBS at room temperature for 1 h, then washed in PBS and treated with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. The cells were washed twice in PBS and resuspended in the TUNEL reaction mixture with the enzyme and incubated for 1 h at 37 °C in a humidified atmosphere in the dark. The cells were then washed three times with PBS and analyzed by FACSCalibur flow cytometry (BD Biosciences, San Jose, CA, U.S.A.).

**Western Blotting Analysis** HRCs (4 × 10^5 cells) treated with decursin and/or cisplatin were harvested and washed with cold PBS. The cell pellets were lysed in 30 μl of lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 1 mM Na_2VO_4, 1 mM NaF and protease inhibitor cocktail) for 30 min on ice. The lysates were centrifuged at 13000×g for 20 min at 4 °C and the protein contents in the supernatants were measured using a Bio-Rad DC protein assay kit II (Bio-Rad, Hercules, CA, U.S.A.). The lysates containing 20 μg of total protein were separated on 4—12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, U.S.A.) with 1×NuPAGE 2-(N-morpholino)ethanesulfonic acid (MES) SDS running buffer and then electro-transferred onto a Hybond ECL transfer membrane (GE Health Care Bio-Sciences, Piscataway, NJ, U.S.A.) in the transfer buffer (25 mM Tris, 250 mM glycin, 20% methanol) at 300 mA for 2 h. The membranes were blocked with 5% non-fat dry milk in a Tris buffered saline (TBS) buffer containing 0.1% Tween 20 (TBST) for 2 h at room temperature. The membranes were hybridized with primary antibodies caspase-3, -9, PARP (Cell signaling, Danvers, MA, U.S.A.), catalase, glutathione peroxidase (Young In Frontier, Seoul, South Korea) and Cu/Zn SOD (Assay Designs, Ann Arbor, MI, U.S.A.) at 4 °C overnight and incubated with a secondary antibody for 2 h at room temperature. The proteins were developed using an ECL Western blotting detection kit and exposed to X-ray film. The protein contents were normalized by probing the same membrane with β-actin.

**Statistical Analysis** Statistical analysis of the data was conducted using SigmaPlot version 12 software (Systat Software Inc., San Jose, CA, U.S.A.). All data were expressed as means ± standard deviation (S.D.). The statistically significant differences between control and drug-treated cells were calculated by the Student’s t test.

**RESULTS**

**Decursin Protected Cisplatin-Induced Cytotoxicity in HRCs** To examine the cytotoxicity of decursin or cisplatin in HRCs, we performed the XTT assay. HRC cells were treated with decursin (0, 20, 40 or 80 μM) or cisplatin (0, 20, 40 or 80 μM) for 24 h and the cell viability was evaluated. As shown in Fig. 2A, cisplatin reduced the viability in a dose-dependent manner (IC_{50} ca. 85 μM) in HRC cells, while decursin did not show any significant cytotoxicity in the cells. However, the combined treatment with decursin and cisplatin significantly protected cisplatin-induced cytotoxicity in HRCs (Fig. 2B). Consistently, cisplatin-induced apoptotic features including cell shrinkage and apoptotic body formation were suppressed by the co-treatment with decursin and cisplatin (Fig. 2C). These results indicated that decursin inhibited cisplatin-induced cell death.

**Decursin Inhibited Apoptotic Cell Death Induced by Cisplatin Treatment in HRCs** To assess whether decursin protects cisplatin-induced apoptosis in HRCs, we performed the Live and Dead assay, TUNEL assay and cell cycle analysis in cells treated with combination of decursin and/or cisplatin. In the Live and Dead assay, which detects membrane permeability, decursin showed no significant effect on cell death compared with control (Figs. 3A, B). Consistent with previous studies, cisplatin increased over 80% of cells stained with ethidium homodimer, indicator of the dead cells,
in HRCs (Figs. 3A, B). In contrast, decursin reduced ethidium stained cells to 10—20% (Figs. 3A, B), indicated that decursin prevent cell death induced by cisplatin. Furthermore, TUNEL assay confirmed that decursin significantly reduced TUNEL positive cells accounting for DNA fragmentation, one of apoptosis indicator, compared with cisplatin-treated HRCs (Figs. 4A, B). Consistently, cell cycle analysis also demonstrated that cisplatin significantly increased the sub-G1 DNA contents undergoing apoptosis compared with untreated control (Fig. 5). In contrast, the combined treatment with decursin and cisplatin reduced cisplatin-induced increase of the sub-G1 DNA in HRCs (Fig. 5).

**Decursin Suppressed the Loss of Mitochondrial Membrane Potential (MMP) in Cisplatin-Treated HRCs**

Loss of mitochondrial membrane potential is one of the apoptosis indicators. Thus, MMP was measured by flow cytometry using tetramethylrhodamine (TMRE), a cell-permeant red-orange fluorescent dye that is easily sequestered by active mitochondria. As shown in Figs. 6A—C, cisplatin decreased the fluorescence intensity compared with untreated control, indicating cisplatin-induced the loss of MMP. In contrast, decursin blocked cisplatin-induced MMP loss when HRCs were preincubated with decursin for 1 h. However, decursin alone did not have any effect on the MMP loss in HRCs. In addition, Western blotting revealed that cisplatin induced activation of caspase-3 and -9 and the cleavage of poly(ADP-ribose) polymerase (PARP) whereas decursin blocked the
effect of cisplatin on the induction of caspase-3 and -9 activation and PARP cleavage in HRC cells. However, decursin did not show any significant effect on caspase activation or PARP cleavage in HRCs (Figs. 6D, E). Rescue rate of decursin against cisplatin-induced apoptosis was 85% and 100% at 40 μM and 80 μM, respectively. These results demonstrated that decursin inhibited mitochondrial damages, results in inhibition of activation of apoptotic signaling such as cleavages of the caspase-3, cleaved caspase-9 and PARP. Protein contents were normalized by probing the same membrane with anti-β-actin antibody.

Decursin Impaired Cisplatin-Induced Apoptosis via Activation of Antioxidant Enzymes in HRC Cells Mitochondria frequently increases the reactive oxygen species (ROS) generation during apoptosis that results in oxidative stress or damage.19) Cisplatin also has been known to generate ROS and inhibit the activities of antioxidant enzymes.20,21) To examine the hypothesis, we investigated whether decursin can restore cisplatin-mediated downregulation of antioxidant enzymes such as catalase, Cu/Zn SOD and glutathione peroxidase (Fig. 7). However, a known antioxidant N-acetyl-L-cystein (NAC) did not restore antioxidant enzyme levels inhibited by cisplatin treatment in HRCs. Taken together, these results indicated that the antioxidant activity of decursin mediates the protective effects on cisplatin induced cytotoxicity and apoptosis in HRCs.

DISCUSSION

Cisplatin is a chemotherapy agent most commonly used to treat various types of cancer with serious side effects.1—4) Recently, several studies provided the evidences that natural compounds or herbal medicines can be used for preventing the side effects such as cisplatin-induced cytotoxicity in normal cells. Our group reported the protective effect of Rhus verniciflua Stokes on cisplatin-induced nephrotoxicity in Madin–Darby canine kidney (MDCK)-1 renal cells.20) Also, several oriental medicinal herbs such as bojungbankdocktang, Ginkgo biloba extract and ethanol extract of Brassica rapa have been reported to have the protective effects on cisplatin-induced toxicity in vitro or in vivo.16,22,23)
Decursin is a natural compound isolated from Korean Danggui (Angelica gigas) root that has been known for its antitumor effects through growth inhibition, cell cycle arrest and apoptosis in various cancer cells. On the other hand, the protective effect of decursin was reported on glutamate-induced neurotoxicity in primary cultures of rat cortical cells. In the current study, we demonstrated that decursin contained protective effect induced by cisplatin treatment in human primary renal cells. We found that decursin blocked the cytotoxic effect of cisplatin in HRCs. Consistently, decursin prevented cisplatin-induced apoptosis in HRCs. Decursin significantly reduced the number of TUNEL positive cells and sub-G1 population of cell cycle induced by cisplatin in HRCs, indicating that decursin is a natural supplemental agent to protect normal kidney cells from the nephrotoxicity by cisplatin treatment.

Mitochondrial dysfunction is a key event during apoptosis. Cytochrome c is commonly localized in the mitochondrial inner membrane and released from the mitochondria to the cytosol during apoptosis. The release of cytochrome c that precedes the loss of mitochondrial membrane potential (MMP) is required for energy (ATP) production and cellular homeostasis. Previous reports indicate that cisplatin induced the loss of MMP in normal MCF-10A breast endothelial cells and HEI-OC1 auditory cells. Cytochrome c release that results from the mitochondrial membrane barrier disruption precedes the activation of caspase cascade signaling including caspase-3, -9 and PARP. Similarly, we also observed that cisplatin significantly induced the loss of MMP and pretreatment of decursin inhibited MMP loss and reduction of activation of apoptotic signaling molecules such as cleavage of caspase-3, -9 and PARP.

Important, recent studies have demonstrated that nephrotoxicity induced by cisplatin treatment is caused by oxidative stress. Because oxidative stress is a well known apoptosis mediator, many apoptosis inhibitors have an anti-oxidant activity or increase antioxidant defense mechanisms. For instance, vitamin E is the best known antioxidant for cardiovascular disease, eye disorder, cognitive decline as well as cancer and selenium can modulate oxidative stress-induced cell apoptosis in human myeloid HL-60 cells through the regulation of calcium release and caspase-3 and...
-9 activities. Our study indicated that decursin inhibited apoptosis induced by cisplatin treatment in HRCs. We found that decursin treatment restored antioxidant enzymes including Cu/Zn SOD, catalase and glutathione peroxidase downregulated by cisplatin treatment. However, antioxidant enzyme levels were not restored by treatment of N-acetyl-L-cysteine (NAC), a known antioxidant to be able to prevent cisplatin-induced nephrotoxicity, hepatotoxicity, and gastrointestinal toxicity through caspase-dependent pathway. These results indicated that decursin can be more effectively used for prevention of cisplatin-induced cytotoxic damage than NAC in HRCs.

In this study, we investigated the role of decursin as a supplemental agent to prevent adverse effects, such as cytotoxicity and apoptosis, of the anticancer drugs including cisplatin in normal renal cells. Although other previous studies also indicated that several natural products can protect normal kidney cells against cisplatin- or glutamate-induced cytotoxicity from rat or dog, our results clearly demonstrated that decursin protected human kidney primary cells from cisplatin-induced cytotoxicity and apoptosis through restore of antioxidant enzymes. Taken together, these findings suggest that decursin is a supplemental agent to be usefully applied to reduce normal tissue damage by treatment of anticancer drugs such as cisplatin and combination treatment of decursin with cisplatin might improve therapeutic effects of the cancer in human.

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REFERENCES