Cisplatin is a platinum-containing chemotherapeutic drug that has been widely used to treat various human cancers. It acts by forming inter- and intracross-links of DNA, which is believed to be a major cause for its therapeutic efficacy. However, little attention has been paid to the effect of cisplatin on death ligand-induced cell death. Here we demonstrate that cisplatin inhibits death ligand-induced cell death in cell lines in a p53-independent manner. This inhibitory effect of cisplatin on cell death is direct, whereby cisplatin forms a complex with caspases leading to their inactivation. The cisplatin-caspase complex is reversed by the addition of reducing agent dithiothreitol, and caspase activity is regained. In addition, cisplatin shows a death-inhibition effect in in vivo animal models of fulminant liver damage induced by Fas activation and lipopolysaccharide-induced liver shock mediated by tumor necrosis factor-α. Together, we demonstrate that cisplatin inhibits cell death induced by death ligands in cell lines and in mice through caspase inactivation.

Cell death induced by death ligands, such as TNF-α, TRAIL, and FasL, has been shown to play a pivotal role in maintaining a homeostasis like the immune response (1, 2). However, death ligands have been shown to participate in many pathological processes. For instance, FasL, TNF-α, or TRAIL contributes to acute hepatic failure such that blocking of these death ligands does not occur unless the mitochondria-dependent signaling pathway is actively engaged (8).

Cisplatin was first identified as a growth suppressor of bacterial cells and later found to have a tumor-killing activity in mice, leading to its use as an anti-cancer agent in humans. It has been widely prescribed to treat the various human tumors including germ-cell tumors, advanced bladder carcinoma, breast cancer, and lung carcinoma. Cisplatin is believed to cause cross-linking in intra- and interstrands of DNA, eventually leading to cell death mediated through the activation of caspases (9, 10). Many studies have demonstrated that chemotherapeutic agents including cisplatin significantly increase the death-inducing activity of death ligands. Even at a subtherapeutic doses, which show no induction of cell death, chemotherapeutic agents are able to promote death ligand-induced killing (11, 12). Here, using in vitro and in vivo models, we show that cisplatin has the opposite effect. Rather than promoting death ligand-mediated cell killing, cisplatin inhibits cell death induced by death ligands, and this effect is achieved through direct association of cisplatin with caspases.

MATERIALS AND METHODS

Cell Culture and Measurement of Cell Viability—HeLa, PC-3, and HCT116 cells were cultured in Dulbecco's modified Eagle's medium or McCoy's 5A medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were plated on 48-well plates for cell death assay. Cell viability was determined by crystal violet staining as described previously (23). In brief, cells were stained with 0.4% crystal violet in methanol for 10 min at room temperature and then washed with tap water. Stained cells were extracted with 4% methanol, and dye extracts were measured at a 540 nm wavelength using a micro-plate reader.
Western Blotting—Cell lysates or recombinant proteins were separated by a 15% SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The membranes were hybridized with anti-caspase-8 antibody (Cell Signaling, Beverly, MA), anti-Bid antibody (a generous gift from Dr. Xiao-Ming Yin at the University of Pittsburgh), or anti-caspase-3 antibody (Santa Cruz, San Diego, CA).

Analysis of Bid Cleavage—Active recombinant caspase-8 protein (100 ng) was pretreated with cisplatin, etoposide, z-VAD-fmk, or cisplatin plus DTT in the reaction buffer (10 mM Pipes, pH 7.4, 100 mM NaCl, 0.1 mM EDTA, 10 mM DTT, 10% sucrose, 0.1% CHAPS), and then recombinant Bid protein (100 ng) or cytosolic lysate (200 μg) prepared from mouse liver was added to the reaction mixtures. The cleavage of Bid was analyzed by Western blotting with anti-Bid antibody.

In Vitro Analysis of Caspase Activities—Caspase-8 (Calbiochem) and caspase-3 assay kits (R&D System) were purchased, and the assays were carried out according to the manufacturer’s instructions. Ac-IETD-pNA and Ac-DEVD-pNA were used as the substrates for caspase-8 and caspase-3, respectively. Recombinant caspase-8 or caspase-3 protein was preincubated with cisplatin, etoposide, z-VAD-fmk, or cisplatin plus DTT for 10 min at 25 °C prior to the addition of substrates and then further incubated for 1 h at 37 °C upon the addition of substrates. Caspase activities were measured at 405 nm wavelength using a micro-plate reader.

Binding of Cisplatin to Caspases—Active recombinant caspase-8 (50 ng) or caspase-3 (60 ng) preincubated with cisplatin (25 μg) or etoposide (25 μg) in 10 μl of phosphate-buffered saline buffer and then further incubated on ice for 1 h. Reaction mixtures were subjected to SDS-PAGE in the presence or absence of 2-mercaptoethanol, and caspase-3 and -8 were visualized by Western blotting with anti-caspase-3 antibody or anti-caspase-8 antibody.

Animal Models—All animal studies followed the institutional guidelines. Adult BALB/c mice were purchased from Damool science (Daejeon, Korea) and injected intravenously with 1.5 mg of Jo-2 antibody/mouse (Pharmingen). Cisplatin (0.6 mg/mouse) was injected intravenously 2 h before LPS/gal-gal injection. After treatment, the mice were sacrificed by cervical dislocation. The photographs of the gross liver were taken with an Olympus digital camera, and the liver tissues were collected and processed for hematoxylin and eosin staining.

RESULTS

Protective Effect of Cisplatin on Death Ligand-induced Cell Death in Cell Lines—Chemotherapeutic agents substantially increase cell death induced by TRAIL, FasL, or TNF-α and even induce cell death in cells resistant to the individual agent alone (12–15). Thus, we first evaluated the death-enhancing activity of various chemotherapeutic agents on TRAIL-induced cell death. HeLa cells were treated with TRAIL for 6 h in the presence of cisplatin or etoposide and analyzed for cell death. Cisplatin or etoposide alone did not show any significant cell death-inducing activity at 6 h of treatment, whereas TRAIL effectively induced cell death in HeLa cells (Fig. 1A). When co-treated with TRAIL, etoposide increased TRAIL-induced cell death as previously observed (Fig. 1A). Similarly, other chemotherapeutic agents, such as actinomycin D, doxorubicin, cycloheximide, and MG132, also promoted TRAIL-induced cell death (data not shown). However, cisplatin inhibited TRAIL-induced cell death in HeLa cells. Moreover, cisplatin attenuated the death-enhancing activity of etoposide in TRAIL-induced cell death (Fig. 1, C–E), indicating that the death-inhibitory activity of cisplatin is dominant over the death-enhancing activity of etoposide. These findings prompted us to investigate the detailed mechanism of the death-inhibitory activity of cisplatin.

First of all, we asked the question whether the death-inhibitory activity of cisplatin is only for TRAIL or whether it extends to other death ligands. To answer the question, we examined the effects of cisplatin and etoposide on FasL-induced cell death in HeLa cells. Similar to the results observed with TRAIL, cisplatin prevents agonistic anti-Fas (CH11)-induced cell death in HeLa cells (Fig. 1B). Our data showed that cisplatin effectively inhibits the cell death-inducing activity of both TRAIL and FasL, indicating that cisplatin may block a common signaling pathway activated by TRAIL and FasL.

The death-induction activity of cisplatin has been thought to be mainly because of the formation of intra- and interstrand DNA cross-links, which leads to the stabilization of the p53 tumor suppressor protein and subsequently the induction of either cell cycle arrest or apoptosis (16, 17). Thus, we examined whether or not the inhibitory effect of cisplatin on TRAIL-induced cell death is associated with p53 function. We first utilized a PC-3 prostate tumor cell line that has a p53-null mutation and is susceptible to TRAIL-induced cell death. As shown in Fig. 1C, cisplatin inhibited TRAIL-induced cell death and the death-enhancing activity of etoposide in PC-3 cells, as observed in HeLa cells. This indicates that p53 is not associated with the death-inhibitory activity of cisplatin. To further confirm that p53 is not associated with the inhibitory effect of cisplatin on TRAIL-induced cell death, we next utilized a human colon cancer HCT116 p53-deficient cell line in which the p53 gene was specifically deleted by homologous recombination. HCT116 p53+/− or HCT116 p53−/− cells were treated with cisplatin or etoposide in the presence or absence of TRAIL and analyzed for cell death. As shown in Fig. 2, D and E, cisplatin inhibited TRAIL-induced cell death regardless of p53 status, whereas etoposide did not show any inhibitory effect. Our data confirm that the death inhibition of cisplatin does not depend on the p53 status.

Cisplatin Inhibition of Caspase Activation and Bid Cleavage—Caspase activation is a key mechanism for the induction of cell death by various death stimuli, including death ligands and chemotherapeutic agents. Studies have shown that upon engagement of TRAIL and FasL, caspase-8 activation is required for their induction of cell death, because caspase-8-deficient cells are resistant to these death ligands (18). Thus, to elucidate the mechanism of the death-inhibitory activity of cisplatin, we investigated the upstream and downstream components of the mitochondrial-dependent death pathway comprising caspase-8 activation, Bid cleavage, and caspase-3 activation. HeLa cells were pretreated with cisplatin or etoposide for 15–120 min and incubated for 2 more hours following TRAIL addition. Cisplatin clearly inhibited TRAIL-induced caspase-8 activation and Bid cleavage in a time-dependent manner; however, etoposide enhanced TRAIL-induced caspase-8 activation (Fig. 2A). These results agree with our observation of the death-inhibitory effect of cisplatin and the death-enhancing effect of etoposide on TRAIL addition. Because caspase-8 has been known to activate caspase-3 both directly and indirectly, we examined whether TRAIL-induced caspase-3 activation was inhibited by cisplatin. Caspase-3 activation was inhibited by cisplatin but not by etoposide in a similar fashion as caspase-8 activation (Fig. 2A).

Caspase-8-cleaved Bid is known as a key factor for the initiation of the mitochondria-dependent death signal pathway, leading to the release of mitochondrial death factors from the mitochondria to the cytosol (7, 19). Because we observed the cisplatin inhibition of caspase-8 activation and Bid cleavage, we next examined whether cisplatin inhibits the release of cytochrome c in response to TRAIL. HeLa cells were pretreated with cisplatin or etoposide for 1–2 h and treated with TRAIL. Then, we detected the release of cytochrome c to the cytosol. The amount of the released cytochrome c was decreased by cisplatin but not etoposide treatment in agreement with the attenuated caspase-8 activation and the decreased Bid cleavage seen with cisplatin (Fig. 2B). Our data strongly suggest that cisplatin inhibits TRAIL-induced cell death by suppressing the activation and/or activity of caspases including caspase-3 and -8, the inhibition of which abrogates both the
mitochondria-dependent and the mitochondria-independent signal pathways.

**Cisplatin Inactivates Caspases**—Although it has been well recognized that cisplatin forms DNA adducts, several studies have reported that cisplatin is also able to form a complex with proteins, as observed in high mobility group box protein 1, albumin, TATA box-binding protein, and histone H1 (9, 20, 21). It is worth noting that cisplatin has been shown to interact with the sulfur group of the cysteine residue, forming a cisplatin-protein complex (22). Notably, caspases are cysteine proteases with a cysteine residue at the active site, and modification or change of the cysteine residue to another amino acid abrogates their activities. Thus, we examined whether cisplatin directly inactivates caspases by interacting with the sulfur group of their cysteine residues. Active recombinant caspase-8 was preincubated with cisplatin, etoposide, or a pan caspase inhibitor z-VAD-fmk in the presence or absence of a reducing agent, DTT, and then recombinant full-length Bid protein or cytosolic lysates from mouse liver were added. Bid cleavage was analyzed by Western blotting. Bid cleavage by caspase-8 was inhibited by cisplatin and z-VAD-fmk but not by etoposide (Fig. 2, C and D). DTT eliminated the inhibitory effect of cisplatin on the Bid cleavage activity of caspase-8 (Fig. 2D). Our data indicate that cisplatin directly inactivates caspase-8, which is reversed by DTT.

To further confirm that caspases are inactivated by cisplatin, active recombinant caspases were preincubated with cisplatin, etoposide, or their inhibitors, and their activities were subsequently measured using specific colorimetric substrates. As shown in Fig. 2E, cisplatin inhibited the activity of caspase-8, and this inhibitory effect of cisplatin was eliminated by the addition of DTT. Cisplatin also inhibited the activity of caspase-3 in a way similar to that of caspase-8 (Fig. 2F).

**Cisplatin Directly Binds to Caspases, Leading to Caspase Inactivation**—Nitric oxide has been shown to effectively inhibit the cell death of hepatocytes by inactivating caspases via formation of S-nitrosylation with caspases at the cysteine residue of their active sites, and a reducing agent, DTT, reverses...
Caspase inhibition activities by eliminating S-nitrosylation from caspases (23, 24). Moreover, cisplatin is known to form a cisplatin-protein complex with several proteins by binding to the sulfur group of cysteine residues as mentioned (22). Together, the evidence suggests that cisplatin may inactivate caspases by binding to the sulfur group of their cysteine residues. To examine this possibility, active recombinant caspase-3 or -8 was incubated with cisplatin or etoposide and analyzed by SDS-PAGE in the presence or absence of the reducing agent 2-mercaptoethanol. Cisplatin, but not etoposide, treatment caused mobility shifts of caspase-3 and -8 in the absence of 2-mercaptoethanol. These cisplatin-induced mobility shifts were eliminated by the addition of 2-mercaptoethanol (Fig. 3, A and B). Our data indicate that cisplatin inactivates caspases by directly forming a cisplatin-caspase complex at the sulfur group of cysteine residues of caspases.

Inhibition of TNF-α-mediated Hepatic Cell Death in Mice—
To examine whether cisplatin inhibits cell death induced by death ligands in animal models, we adopted the model of TNF-α-mediated cell death of hepatocytes in mice induced by LPS and d-galactosamine (LPS/d-gal) (25, 26). BALB/c mice injected with 1.5 μg of LPS/mouse and 20 mg of d-galactosamine/mouse, which causes death of hepatic cells within 20–24 h. Massive liver damage was observed grossly (Fig. 4C) and histologically
**FIG. 3. Cisplatin directly binds to caspase-3 and -8.** A, active recombinant caspase-8 protein (50 ng) was treated with cisplatin (25 μg) or etoposide (25 μg) for 1 h on ice. Reaction mixtures were subjected to reducing or non-reducing SDS-PAGE followed by Western blotting with anti-caspase-8 antibody. B, active recombinant caspase-3 protein (50 ng) was treated with cisplatin (25 μg) or etoposide (25 μg) for 1 h on ice. Reaction mixtures were analyzed as described above. Asterisks indicate the mobility shifts of caspase-8 or caspase-3. 2-ME, 2-mercaptoethanol.

**FIG. 4. Cisplatin protects mice from LPS-induced hepatic cell death in mice.** Adult BALB/c mice were injected intravenously with phosphate-buffered saline (A), cisplatin (B), LPS/D-gal (C), or LPS/D-gal and cisplatin (D). Cisplatin was injected intravenously at 2 h before LPS/D-gal injection. After treatment, mice were sacrificed by cervical dislocation. The gross liver appearances were captured with an Olympus digital camera. Caspase-8 activities in lysates from the liver tissues as indicated were measured using the colorimetric substrate Ac-IETD-pNA (E). The liver tissues were collected and processed for H/E staining (200× magnification) (F) and for Western blotting with anti-caspase-8 and anti-caspase-3 antibody (G). BALB/c mice were injected intravenously with cisplatin alone (n = 8), with LPS/D-gal (n = 8) alone, or with LPS/D-gal and cisplatin (n = 8). Survival rates (%) were observed for 50 h (H).
Pretreatment of 0.6 mg of cisplatin/mouse at 2 h before injection of LPS/d-gal tremendously reduced hepatic cell death in the liver as judged by the gross appearance of the liver (Fig. 4D) and hemotoxylin and eosin staining (Fig. 4F). Caspase-8 activity was significantly decreased (Fig. 4E) and procaspase-3 and -8 were found to be cleaved to a lesser degree (Fig. 4G) as compared with that observed in mice without cisplatin treatment. An intravenous injection of LPS/d-gal into mice caused 80% mortality; however, cisplatin significantly reduced the mortality to 25% (Fig. 4H). Together, these data indicate that the reduction of mouse mortality was attributable to a reduction in liver damage due to the caspase inactivation by cisplatin.

Inhibition of FasL-induced Fulminant Liver Damage in Mice—To further confirm that cisplatin inhibits death ligand-induced cell death in mice, we utilized another animal model of FasL-induced fulminant liver damage in mice. FasL has been shown to induce fulminant liver damage in mice through a massive cell death of hepatocytes (5, 8, 26). BALB/c mice were intravenously injected with Jo-2 antibody, an agonistic antibody to mouse Fas. Mice injected with 7.5 μg of Jo-2 antibody/mouse were dead of fulminant liver failure within 6–7 h. The liver damage was readily observed grossly (Fig. 5C) and microscopically by H/E staining (Fig. 5G). Caspase-8 activity measured by a colorimetric assay (Fig. 5E) and cleavage of procaspase-8 and -3 (Fig. 5F) were evident. Consistent with the LPS/d-gal-injected mice, mice pretreated with cisplatin 2 h before Jo-2 injection were evidently protected from the liver damage as judged by the reduction in liver damage seen grossly (Fig. 5D) and at the histological level (Fig. 5G) by H/E staining of liver. Caspase-8 activity (Fig. 5E) and the production of cleaved caspase-8 and -3 were substantially reduced (Fig. 5F) as well. These data indicate that cisplatin inhibits FasL-induced hepatic cell death in mice mediated through caspase inactivation.

**DISCUSSION**

Cisplatin is one of the most widely used chemotherapeutic agents for treatment of cancer patients because of its potent death-inducing activity against various human cancers derived from testicular, ovarian, lung, and head and neck cancer. A number of studies have demonstrated that the tumor suppressing activity of cisplatin mainly depends upon DNA damage, leading to apoptotic cell death through p53 stabilization (9, 10,
21). Surprisingly, here we demonstrated that cisplatin inhibits death ligand (TRAIL, FasL, or TNF-α)-induced cell death in cancer cells irrespective of p53 status (Fig. 2) and death ligand-induced hepatic injury in mice (Figs. 4 and 5). Our studies revealed that the mechanism by which cisplatin inhibits death ligand-induced cell death is through direct association of cisplatin with caspases. This association most likely occurs between the cysteine residues in caspases and cisplatin leading to abrogation of caspase activity (Figs. 2 and 3).

Many studies, however, have demonstrated that cisplatin promotes cell death in various tumor cells (11, 12, 14). This discrepancy between the previous reports and our data on the action of cisplatin leads us to speculate that two possible mechanisms for the action of cisplatin exist, which depend on the duration of treatment. The immediate early response appears to be associated with inactivation of pre-existing caspases resulting from the prompt binding of cisplatin to caspases. This inactivation of the pre-existing caspases by cisplatin is likely to be a major determinant for inhibiting death ligand-induced cell death.

On the other hand, the late responses possibly initiated by cisplatin-DNA adducts are believed to involve several cellular processes like gene expression or repression, modulation of protein activity, and/or other unknown events, which require more than 48 h to be observed, which is the time cisplatin alone can induce cell death in HeLa cells. These cellular processes finally activate caspases, which may be newly synthesized, to induce cell death. This process of caspase activation appears to be a secondary event rather than a direct interaction of cisplatin with caspases. Thus, the caspase activation by cisplatin observed in majority of previous studies results from the secondary cellular processes. In this study, in contrast, we have utilized both cisplatin and death ligands such that we had a chance to observe the direct interaction between cisplatin and caspase activation caused by death ligands. This is possible mainly because of the different activation time points of caspases induced by cisplatin and TRAIL in that caspase activation by TRAIL occurs within 2–4 h in HeLa cells, the time when cisplatin alone cannot activate the caspases, whereas caspase activation by cisplatin takes 24–48 h. Thus, we are able to distinguish the caspase activation by death ligands or cisplatin alone. This allows us to see the new aspect of cisplatin that is inactivation of caspases. Together, we suggest that cisplatin has the dual function of caspase inactivation through direct association at the early responses and caspase activation through cellular processes at the late responses.

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