Antioxidant ameliorates cisplatin-induced renal tubular cell death through inhibition of death receptor-mediated pathways

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CISPLATIN IS ONE OF THE MOST potent anticancer drugs used in the treatment of solid tumors (46). The cytotoxicity of cisplatin is considered to be due to a combination of factors, including peroxidation of the cell membrane (52), mitochondrial dysfunction (8), inhibition of protein synthesis (32), and DNA injury (27). However, the most common adverse effect limiting the use of cisplatin is nephrotoxicity that develops primarily in the S3 segment of the proximal tubule (4). Although reactive oxygen species (ROS) have been considered to play a central role in this injury (30, 35, 36), the exact roles of free radicals and the mechanisms underlying the beneficial effects of free radical scavengers have not been fully evaluated.

On the other hand, cellular damage during acute renal failure (ARF) induced by ischemia-reperfusion injury or nephrotoxic agents in the kidney is considered to consist mainly of proximal tubular necrosis (56). Recent studies have demonstrated that apoptosis, a form of cell death characterized by DNA fragmentation, as well as necrosis might play a crucial role in the pathogenesis of ARF (15, 43, 50). A number of protein systems regulate the apoptotic events. One of these systems operates through the TNF receptor family, including Fas antigen (Fas, CD95, APO-1) and TNF receptor 1 (TNFR1). Both Fas and TNF receptor 1 (TNFR1) contain conserved death domains in their cytoplasmic tails, which mediate defined protein-protein interactions (24, 54), allowing recruitment of other death domain-containing proteins such as Fas-associated death domain protein (FADD). The association of FADD with Fas or TNFR1 results in recruitment of caspase-8, activation of which consequently leads to cell death (6, 41). Although a number of reports have documented Fas expression on renal tubular cells (RTCs) and up-regulation of Fas expression during acute and chronic renal failure (7, 16, 19, 26, 43, 45, 47), whether RTC apoptosis under those conditions depends on the Fas-Fas ligand (FasL) pathway remains controversial. In our studies, the role of the Fas-FasL pathway remains controversial. In our studies, the role of the Fas-FasL pathway remains controversial.
strated that ablation of Fas or TNFR1 protects RTCs against cisplatin toxicity (57).

Although ROS-mediated damage has been commonly considered as necrosis, recent observations suggest the potential role of apoptosis under these conditions. Thus excessive formation of ROS as well as depletion of cellular antioxidants resulted in apoptosis of different cell types (21, 49, 51). With regard to the association between ROS and death receptor-mediated apoptotic pathways, recent studies have shown that ROS modulate the expression of Fas or FasL in various cells, such as endothelial cells (53), hepatoma cells (22), microglial cells (59), and astrocytoma cells (31). Therefore, it is thought that ROS may influence apoptotic cell death by regulating the expression of receptors and ligands involved in the cell death process.

Thus the involvement of ROS and the death receptor-mediated apoptotic pathways in cisplatin-induced RTC death is evident; however, little is known about the association of ROS and these pathways in RTC death during ARF. Based on these findings, we postulated that ROS might induce RTC death through the activation of death receptor-mediated apoptotic pathways in cisplatin-induced RTC death. In this study, we examined gene expressions of the death receptors and ligands and activity of caspase-8 after cisplatin administration with or without a ROS scavenger. Our results suggest that the mechanism through which antioxidants ameliorate cisplatin-induced ARF may involve inactivation of the death receptor-mediated apoptotic pathways.

MATERIALS AND METHODS

In Vitro Studies

Cell culture. NRK52E cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI-1640 medium (GIBCO BRL, Grand Island, NY) containing 1% fetal bovine serum (GIBCO BRL) and were subcultured using a 0.05% trypsin-EDTA solution consisting of (in mM) 137 NaCl, 5.4 KCl, 5.5 glucose, 4 NaHCO3, 0.5 EDTA, and 5 HEPES, pH 7.2 (GIBCO BRL). The cells were incubated with various concentrations of cisplatin or vehicle for 72 h with vehicle or 10 mM DMTU. Caspase-8 activity was then measured in these cells using a ApoAlert Caspase-8 Colorimetric Assay Kit according to the protocol recommended by the manufacturer (Clontech, La Jolla, CA) thermal cycler according to the instructions provided by the manufacturer. The published sequences of the rat and mouse Fas, FasL, TNF-α, TNFR1, and GAPDH mRNA were retrieved from the GenBank database, and primer pairs for each were designed with oligonucleotide software (Oligo Primer Analysis Software, version 5.0; National Biosciences, Plymouth, MN). Primer sequences and PCR conditions are shown in Table 1. We determined the number of cycles for PCR of each gene by checking whether the amount of PCR product increased linearly in proportion to the increase in number of cycles and was directly proportional to the number of cycles in the exponential range of amplification. The resulting amplification products were sequenced, and the identities of the fragments were confirmed. Equal volumes of the amplification products were analyzed by agarose gel (1.5%) electrophoresis with ethidium bromide (0.5 mg/ml) staining.

Activity of caspase-8. NRK52E cells were exposed for 24, 48, or 72 h to 8 μM cisplatin or vehicle in the presence of vehicle or 10 mM DMTU. Caspase-8 activity was then measured in these cells using a Caspase-8 Colorimetric Assay Kit according to the protocol recommended by the manufacturer (Clontech, La Jolla, CA).

Cell viability assay. Cell viability was determined by the WST-1 assay, as described by Kawahara et al. (25). In brief, NRK52E cells (5 × 10^4 cells in 100 μl) were treated with various concentrations of cisplatin or vehicle for 72 h with various concentrations (0.1, 0.5, 2.0, or 10 mM) of DMTU, which was administered simultaneously with or 4, 8, 12, or 24 h after the initiation of cisplatin treatment. WST-1 reagents, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (Dojin Laboratories, Kumamoto, Japan), and 1-methoxy-5-methylphenazinium methylsulfate (Dojin Laboratories), were added to the cells at final concentrations of 5.0 and 0.2 mM, respectively, and incubated for 20 min, and at 95 °C for 5 min. The reaction products (2.0 μl) were subjected to PCR amplification using the Taq DNA polymerase (Promega) in a 50-μl reaction volume with 4 μM of each dNTP, 0.4 μM of each specific primer, and 2 mM MgCl2. PCR was performed using the PerkinElmer (Foster City, CA) thermal cycler according to the instructions provided by the manufacturer. The published sequences of the rat and mouse Fas, FasL, TNF-α, TNFR1, and GAPDH mRNA were retrieved from the GenBank database, and primer pairs for each were designed with oligonucleotide software (Oligo Primer Analysis Software, version 5.0; National Biosciences, Plymouth, MN). Primer sequences and PCR conditions are shown in Table 1. We determined the number of cycles for PCR of each gene by checking whether the amount of PCR product increased linearly in proportion to the increase in number of cycles and was directly proportional to the number of cycles in the exponential range of amplification. The resulting amplification products were sequenced, and the identities of the fragments were confirmed. Equal volumes of the amplification products were analyzed by agarose gel (1.5%) electrophoresis with ethidium bromide (0.5 mg/ml) staining.

Table 1. Sequences of upstream and downstream rat primers for RT-PCR analysis

<table>
<thead>
<tr>
<th>Primers</th>
<th>Annealing Temperature, °C</th>
<th>No. of Cycles</th>
<th>No. of Base Pairs</th>
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<tr>
<td>FasL 5'-GCA ACA GCC AGC CCC TAA GC</td>
<td>55</td>
<td>30</td>
<td>472</td>
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<tr>
<td>3'-CCA CAC AGC AGC CCA AAA CT</td>
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<tr>
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<td>30</td>
<td>404</td>
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<tr>
<td>3'-ATT TGG TGT TGC TGG TTT GT</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α 5'-GAC TGG TTT CCT TCT GTG C</td>
<td>59</td>
<td>32</td>
<td>295</td>
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<tr>
<td>3'-CAG CCT TGT CCC TGT TGG GTA ACC</td>
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</tr>
<tr>
<td>TNFR1 5'-GAC TGG TTT CCT TCT GTG TTT GT</td>
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<tr>
<td>GAPDH 5'-TCC CTC AAG ATT GTC AGC AA</td>
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<tr>
<td>3'-AGA TCC ACA AGC GAT ACA TT</td>
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FasL, Fas ligand; TNFR1, TNF receptor 1.

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incubated for 1 h at 37°C. Using an enzyme-linked immunoabsorbent assay autoreader, the cell viability was determined by measuring the difference between absorbance at 450 and 620 nm.

FACS analysis. NRK52E cells were cultured with 8 μM cisplatin or vehicle for 72 h with vehicle or 10 mM DMTU. Nonattached cells in the medium were collected, attached cells were trypsinized and washed, and both cell populations were incubated with FITC-conjugated annexin V and propidium iodide (PI) in binding buffer at concentrations suggested by the manufacturer (PharMingen, San Diego, CA) on ice in the dark for 15 min. Stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). Data were analyzed using CELL-QUEST software (Becton Dickinson).

TUNEL assay. The TUNEL technique (ApopTag Plus kit, Intergen, Gaithersburg, MD) was used to detect DNA fragmentation of the cells in situ according to the instructions provided by the manufacturer. Briefly, adherent cultured cells were fixed in 1% paraformaldehyde for 10 min at room temperature and ethanol for 5 min at −20°C, and endogenous peroxidase was quenched with 3% H2O2 for 5 min. The sections were incubated with terminal deoxynucleotidyl transferase (TdT) and a mixture of digoxigenin-labeled nucleotides for 60 min. This was followed by incubation with anti-digoxigenin-peroxidase for 30 min and color development with H2O2-diaminobenzidine for 3–6 min. Then, the slides werecounterstained with hematoxylin. For positive controls, specimens of thyroid tissue were provided by Intergen. Negative controls were prepared by omission of terminal deoxynucleotidyl transferase enzyme from the incubation buffers.

In Vivo Studies

Rat model of cisplatin-induced ARF. Male Sprague-Dawley rats weighing 200–250 g were obtained from Kyudo (Kumamoto, Japan). Animals were housed individually in standard laboratory cages and treated with the principles outlined in the Guide for the Care and Use of Laboratory Animals (National Academy Press, 1986). Cisplatin was administered by a single intravenous injection to rats at 8 mg/kg body wt. In addition to the single cisplatin injection, DMTU was intraperitoneally injected at 500 mg (4.8 mmol)/kg body wt 1 h before the injection of cisplatin, followed by intraperitoneal injection of 125 mg (1.2 mmol)/kg body wt twice a day (CP + DMTU group). DMTU was substituted by saline in the other rats (CP group). These rats were killed before control group (n = 6 rats) or 6 or 12 h (n = 4 rats/group) or 1, 2, 3, or 5 days (n = 6 rats/group) after cisplatin injection by exsanguination, blood samples were centrifuged (3,500 g for 5 min), and plasma samples were collected for measurement of plasma blood urea nitrogen (BUN) and creatinine (Cr). The kidneys were perfused in situ via the aorta with PBS, pH 7.4, and then excised for further analysis.

Measurement of plasma BUN and Cr. Plasma BUN and Cr levels were measured using the urease-UV and the alkaline picrate method, respectively. Measurements were conducted by SRL Laboratories (Tokyo, Japan).

Morphological assessment. Excised kidneys were immersed in Bouin’s solution and fixed for 12–24 h, embedded in paraffin, and 3-μm sections were mounted on silicate-coated glass slides. After deparaffinization and rehydration, the tissues were stained with periodic acid–Schiff, and the degree of morphological involvement in renal failure was determined using light microscopy, as described by Megyesi et al. (37) with some modifications. Briefly, the following parameters were chosen as indicative of morphological damage to the kidney after cisplatin injection: brush border loss, tubule dilatation, tubule degeneration, tubule necrosis, and tubular cast formation. These parameters were evaluated on a scale of 0–4, which ranged from absent (0), mild (1), moderate (2), severe (3), to very severe (4). Each parameter was determined in six different rats.

RT-PCR. Total RNA was extracted by the TRIzol method from rat kidneys according to the protocol provided by the manufacturer, followed by the manipulations described above in the in vitro study. Equal volumes of the amplification products were analyzed by agarose gel (1.5%) electrophoresis with ethidium bromide (0.5 mg/ml) staining. Gels were photographed and analyzed using the public domain National Institutes of Health (NIH) Image program (http://rsb.info.nih.gov/nih-image/). The results were normalized to the intensity of GAPDH bands.

Platinum contents of NRK52E cells and rat kidneys. Renal tissue was weighed and digested with 5 ml of 16 M HNO3 overnight at room temperature and then heated in a heating block for 4–5 h at 140°C. The digested sample was adjusted to 10 ml with 0.1 M HCl. The cultured cells were removed by scraping with a plastic scraper in ice-cold PBS and centrifuged at 1,000 g for 5 min. The pellet was resuspended with PBS and disrupted by sonication. After centrifugation at 13,400 g for 5 min, the protein content of the supernatant was determined using the Lowry method (34). The platinum contents in the samples were measured using a spectrometer (model 1100 flameless atomic absorption spectrometer, PerkinElmer, Norwalk, CT) monitoring at 265.9 nm. The latter measurements were conducted by SBS (Sagamihara, Japan).

Statistical analysis. Data are expressed as means ± SE. One-way ANOVA followed by Bonferroni’s t-test was used to compare the values in the CP group with control values, and an unpaired t-test was used to compare the values in the CP group with those of the CP + DMTU group. One-way ANOVA followed by Bonferroni’s t-test was used for comparison between the same cisplatin concentrations for cell viability assays and comparison between the same times after cisplatin administration for caspase-8 assays. Statistical significance was accepted at P < 0.05.

RESULTS

Effect of DMTU on Cisplatin-Induced Upregulation of FasL, Fas, and TNF-α mRNA Expression in NRK52E Cells and Rat Kidneys

In the in vitro study, low expression levels of FasL, Fas, and TNF-α mRNAs were observed in NRK52E cells in the absence of cisplatin stimulation. The mRNA levels of all these genes increased in the cells after incubation with 8 μM cisplatin for over 24 h and this increase was inhibited by treatment with 10 mM DMTU. In contrast, the level of TNFR1 mRNA was substantial and constant irrespective of stimulation of cisplatin and treatment with DMTU (Fig. 1A).

In the in vivo study, low expression levels of Fas, FasL, and TNF-α mRNAs were also observed in the kidneys of control rats. The mRNA levels of these genes were upregulated 12 h after cisplatin injection and peaked and were maintained at a high level of expression from 1–5 days after injection (Fig. 1B). Such an increase was inhibited by treatment with DMTU (Fig.
1C). Similar to the findings of the in vitro study, the TNFR1 mRNA level was substantial and did not change after cisplatin injection irrespective of DMTU treatment (Fig. 1, B and C). The signal densities were also quantified using the NIH Image program, and results were expressed relative to the intensity of GAPDH bands. Mean mRNA levels of these genes in control kidneys and in insulted kidneys 3 and 5 days after injection of cisplatin with or without DMTU are shown in Fig. 1D relative to the mean mRNA levels in control kidneys.

**Effect of DMTU on Cisplatin-Induced Increase in Caspase-8 Activity in NRK52E Cells**

Incubation of NRK52E cells with 8 μM cisplatin for over 24 h resulted in augmentation of caspase-8 activity. Caspase-8 activity was significantly higher in cisplatin-stimulated cells than in cells incubated with vehicle at each time interval after 24 h. However, in cells incubated with 10 mM DMTU, only a slight increase was observed despite stimulation with cisplatin (Fig. 2).

**In Vitro Effect of DMTU on Cisplatin-Induced RTC Apoptosis and Necrosis**

A significant dose-dependent loss of cell viability was noted after 72-h incubation with various concentrations (2, 4, or 8 μM) of cisplatin. The loss of cell viability was significantly inhibited by treatment with

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Fig. 1. Effects of cisplatin and dimethylthiourea (DMTU) on mRNA expression of death receptors and their ligands in NRK52E cells and rat kidneys determined by RT-PCR. A: representative data of Fas-L, Fas ligand (FasL), Fas, TNF-α, and TNF receptor 1 (TNFR1) mRNA expression in NRK52E cells after incubation with vehicle (control), 8 μM cisplatin (CP), or 8 μM and 10 mM DMTU (CP+DMTU) for the indicated time intervals. B: representative data of FasL, Fas, TNF-α, and TNFRI mRNA expressions in control rat kidneys and in kidneys 6 h, 12 h, and 1 (1 d), 2 (2 d), 3 (3 d), and 5 days (5 d) after CP. C: representative data of FasL, Fas, TNF-α, and TNFRI mRNA expression in control rat kidneys and in kidneys 3 and 5 days after CP or CP+DMTU. D: mean mRNA levels of these genes, quantified using National Institutes of Health Image software and normalized to the intensity of GAPDH bands, in kidneys before (C; open bars, n = 6) and 3 or 5 days after CP (closed bars, n = 6) or CP+DMTU (hatched bars, n = 6) are shown as a ratio of mean mRNA levels in control kidneys. Values are means ± SE. *P < 0.05 compared with control. **P < 0.01 compared with CP.

Fig. 2. Effects of cisplatin and DMTU on caspase-8 activity in NRK52E cells. Cells were incubated as described in Fig. 1A for 24, 48, or 72 h. Caspase-8 activity is shown as percent change from control cells, which were incubated with vehicle for 24 h. The experiments were performed in triplicate on 3 different occasions, and representative data are shown. Values are means ± SE. **P < 0.01 compared with control. #P < 0.05, ##P < 0.01 compared with CP.
various concentrations (0.1, 0.5, 2, or 10 mM) of DMTU in a dose-dependent manner (Fig. 3A). Delayed treatment with DMTU also provided protection against cisplatin-induced cell death, although the protective effect became weaker with longer delays in the treatment (Fig. 3B).

To confirm that the loss of viable cells was due to an increased population of nonviable cells, NRK52E cells, incubated with vehicle (control), 8 μM cisplatin and vehicle (CP), or 8 μM cisplatin and 10 mM DMTU (CP+DMTU) for 72 h, were stained with both PI and FITC-labeled annexin V and then analyzed by flow cytometry. PI can penetrate into necrotic or late apoptotic cells but not viable or early apoptotic cells. Annexin V, a protein with high affinity for phosphatidylserine, can bind to exposed phospholipids in apoptotic cells. Phosphatidylserine externalization is a feature of apoptosis induced by various drugs (28). In Fig. 4, the

Fig. 3. Effects of cisplatin and DMTU on viability of NRK52E cells determined by WST-1 assay. The results are expressed as the percentage of the value obtained without cisplatin. The experiments were performed in triplicate on 3 different occasions, and the average values ± SE were plotted. A: dose-dependent killing of NRK52E cells by 72-h exposure to cisplatin and a dose-dependent inhibition of the killing by DMTU, which was added simultaneously with cisplatin, are shown. B: time indicates delayed time (h) of DMTU treatment after the initiation of cisplatin exposure. Note the time-dependent attenuation of the effect of DMTU on inhibition of the killing of NRK52E cells by 72-h exposure to 8 μM cisplatin. Values are means ± SE. *P < 0.05, **P < 0.01 compared with data without DMTU.
bottom left cell population in each plot represents viable cells, which did not contain PI-stained cells and annexin V-binding cells. The top left population comprises necrotic cells, representing cells stained for PI but not FITC-labeled annexin V. The bottom and top right populations correspond to apoptotic and late apoptotic cells, respectively. Compared with cells unstimulated by cisplatin, both annexin V-positive cells and PI-positive cells were increased after 72-h exposure to 8 μM cisplatin (Fig. 4, B and C). Treatment with 10 mM DMTU markedly suppressed the increase in annexin V-positive cells and PI-positive cells (Fig. 4 D).

DNA fragmentation determined by TUNEL assay was not seen in cells that were not treated with cisplatin (Fig. 5 A). After 72-h exposure to 8 μM cisplatin, TUNEL-positive nuclei were seen in the cells (Fig. 5 B). Combination treatment of 10 mM DMTU and cisplatin prevented the cells from undergoing apoptosis (Fig. 5 C).

In Vivo Effect of DMTU on Cisplatin-Induced RTC Damage and Renal Dysfunction

As shown in Fig. 6, plasma levels of BUN and Cr were significantly increased after 3 and 5 days of cisplatin injection, whereas treatment with DMTU significantly inhibited such increases in these parameters. Histological examination of the kidneys 3 days after cisplatin injection showed severe RTC damage such as brush border loss, degeneration, necrosis, and cast formation in the outer medulla (data not shown). The damage was more severe 5 days after the injection and was associated with tubular dilatation (Fig. 7 A). These RTC injuries were also seen in the kidneys of rats treated with DMTU but were less severe compared with rats that were not treated with DMTU (Fig. 7 B). Semi-quantitative analyses of the histopathological changes confirmed the aforementioned injury (Fig. 7 C). Furthermore, tubular necrosis was observed not only in the S3 segment but also in S1 and S2 segments in the CP group, whereas such damage was restricted to the S3 segment in the CP + DMTU group.

DMTU Does Not Alter Cisplatin Incorporation into RTCs

To exclude the possibility that DMTU might hinder cisplatin incorporation into RTCs, we measured platinum levels in cultured RTCs treated with 8 μM cisplatin or 8 μM cisplatin plus 10 mM DMTU and kidneys of rats treated with 8 mg/kg cisplatin or cisplatin plus DMTU. After exposure to cisplatin, the platinum contents of both cultured RTCs and rat kidneys were similar irrespective of DMTU treatment, indicating that DMTU does not hinder cisplatin incorporation into RTCs (Fig. 8).

DISCUSSION

The major findings of the present study were 1) Fas, FasL, and TNF-α mRNA levels and caspase-8 activity increased after cisplatin administration; 2) these cisplatin-induced changes were inhibited by treatment with an antioxidant, DMTU; and 3) treatment with DMTU suppressed cisplatin-induced apoptosis and necrosis of RTCs and prevented renal dysfunction.
DMTU is known as a powerful scavenger of hydroxyl radicals (61). Matsushima et al. (36) demonstrated that DMTU is beneficial in preventing the accumulation of malondialdehyde, tubular damage, and renal dysfunction through scavenging the hydroxyl radicals in cisplatin-induced ARF. Later, the same group reported that the attenuation of such injuries by treatment with DMTU was associated with less apoptotic cell death (62). Similarly, our data revealed the beneficial effects of DMTU in the prevention of tubular damage and renal dysfunction. We showed not only the protective effect but also the therapeutic effect of DMTU; i.e., DMTU was also effective even when such treatment was delayed, although the protective effect became weaker with longer delays in treatment. These findings indicate that if there is inadvertent exposure to cisplatin, DMTU treatment should be considered whenever the treatment is delayed, although the effect is weak. We also demonstrated that such effects were associated with reductions in Fas, FasL, and TNF-α mRNA levels and caspase-8 activity, which were increased by cisplatin. The involvement of ROS in death receptor-mediated apoptotic pathways has been reported in various cells recently (22, 31, 53, 59); however, there is no documentation of such role in RTCs. Therefore, this is the first report that shows the association between ROS and death receptor-mediated apoptotic pathways in RTCs.

Although it has been well documented that apoptosis plays a crucial role in the pathogenesis of cisplatin-induced ARF, intracellular pathways of cisplatin-induced RTC apoptosis remain unknown. In this study, it is not clear whether the effect of DMTU on the inhibition of RTC death is directly due to reduced levels of Fas, FasL, and TNF-α. Therefore, one can argue that decreased expression levels of these genes by DMTU were merely secondary phenomena related to amelioration of RTC injury. However, it has been reported that upregulation of Fas/FasL system is involved in ischemia- or cisplatin-induced apoptosis in rodent kidneys and cultured human proximal tubular cells (43, 45). We also recently demonstrated the direct involvement of the death receptor-mediated apoptotic pathways in the pathogenesis of cisplatin-induced RTC death, in which *Fas* or *TNFRI*-knockout mice were resistant to cisplatin (57). In addition, we also showed in this study that cisplatin activated caspase-8 and DMTU suppressed such activation. On the basis of these findings, we consider that downregulation of Fas, FasL, and TNF-α mRNAs by the antioxidant is not a byproduct but rather the underlying mechanism of action of DMTU in preventing cisplatin nephrotoxicity.

If DMTU has a suppressive effect on cisplatin incorporation into RTCs, our argument in this study becomes questionable. To exclude such a possibility, we measured platinum levels in cultured RTCs and rat kidneys treated with cisplatin with or without DMTU. After exposure to cisplatin, the platinum contents of both cultured RTCs and rat kidneys were similar irrespective of DMTU treatment, indicating that DMTU does not hinder cisplatin incorporation into RTCs. On
the basis of these data, we can exclude the role of other alternative pathways in mediating the effect of DMTU, ones through which DMTU interacts with cisplatin in the incubation medium and the blood or peritoneum to form an inactive compound that limited cisplatin access to the cell.

Recently, Choi et al. (13) and Cummings and Schnellmann (14) emphasized the role of the tumor-suppressor protein p53 in RTC apoptosis in chronic obstructive uropathy and cisplatin-induced RTC apoptosis, respectively. Both reports suggested that RTC apoptosis is mediated by p53-dependent and -independent pathways. p53 Can be a possible candidate as a transcriptional regulator of Fas. The involvement of p53 in initiating apoptosis after exposure to a pleiotropic array of stimuli results in upregulation of proapoptotic members of the bcl-2 family of proteins such as Bax or cell cycle inhibitor p21 (9). Recent data suggest that p53-induced apoptosis occurs also through mechanisms other than Bax. For example, several laboratories have suggested the involvement of p53 in Fas/FasL-induced apoptosis (3, 39, 40). On the other hand, there are several lines of evidence indicating that p53 expression is increased by ROS (42, 60). Thus it is possible that cisplatin-induced upregulation of Fas in RTCs observed in this study might be mediated, in part, by p53, which could be activated by cisplatin-induced DNA damage and ROS.

On the other hand, Park et al. (44) reported recently that cisplatin can induce RTC apoptosis via translocation of cytochrome c from the mitochondria to the cytosol and via activation and translocation of the proapoptotic molecule Bax. They also demonstrated a sixfold upregulation of caspase-9 activity by cisplatin, but not of caspase-8. Their results indicate the involvement of a mitochondrial pathway in cisplatin-induced RTC apoptosis in view of the perspective that activation of Bax can trigger a sequence of events leading to alterations in mitochondrial permeability transition, release of mitochondrial cytochrome c into the cytosol, and activation of caspase-9. It seems that our results contradict those of the above study; however, we consider this not to be the case. That is, we suggest that the death receptor-mediated pathway and mitochondrial pathway are both involved in cisplatin-induced RTC apoptosis. However, with regard to caspase-8, different results were observed between these two studies. We suspect that these different data are due to the use of different methodologies, i.e., different cells and different concentrations of cisplatin used. In particular, we believe that the concentration of cisplatin is of critical importance. In support of this argument, Lieberthal et al. (33) demonstrated that at a high concentration (800 μM), cisplatin caused necrotic cell death that occurred over a few hours, whereas at low concentration (8 μM) it resulted in apoptosis occurring over several days, after incubation of mouse proximal tubular cells with cisplatin. We used cisplatin at a low concentration, whereas Park et al. (44) used moderate concentrations of the drug (25–100 μM). On the basis of these findings, we suggest that low concentrations of cisplatin mainly activate the death receptor-mediated pathway, whereas moderate concentrations mainly activate the mitochondrial pathway, although no direct evidence was obtained in support of this conclusion. Alternatively, it is possible that the mitochondrial pathway might be activated in part via the death receptor-mediated pathway. This is based on previous findings demonstrating that activated caspase-8 propagates the apoptotic signal, as well as directly cleaving and activating downstream caspases, by cleaving the BH3 Bcl2-interacting protein, which leads to the release of cytochrome c from mitochondria, triggering activation of caspase-9 in a complex with dATP and Apaf-1 (29). It is considered that activated caspase-9 then activates further “downstream caspases,” including caspase-8, indicating that the death receptor pathway and mitochondrial pathway might in part be correlated with each other.

Hughes and Johnson (23) demonstrated, using TUNEL assay, that the absence of functional Fas protected RTC from undergoing apoptosis in the murine kidneys injured by unilateral ureteric ligation and that such an effect was localized to the distal tubular cell compartment. In contrast, we recently demonstrated that the absence of Fas protected proximal tubular cells from undergoing apoptosis in the murine kidneys injured by cisplatin administration (57). In this respect, Hughes and Johnson (23) argued that their results correlated with those of a previous study (55), in which Fas was found to be most strongly expressed in distal tubular epithelium in obstructed murine kidneys. We also demonstrated using RT-PCR and FACS analysis that cisplatin markedly induced Fas expres-

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**Fig. 6. Effects of cisplatin and DMTU on renal function of rats.** Plasma levels of blood urea nitrogen (BUN) and creatinine before (Con) and at 1, 2, 3, and 5 days after injection of cisplatin with vehicle (CP) or CP+DMTU. Values are means ± SE; n = 6 rats. *P < 0.05, **P < 0.01 compared with control rats. #P < 0.05, ##P < 0.01 compared with CP group.
sion in cultured proximal tubular cells, and absence of Fas protected proximal tubular cells from undergoing apoptosis in the murine kidneys injured by cisplatin administration (57). Thus Fas/FasL seems to play a critical role in apoptosis of proximal tubular cells rather than distal tubular cells in cisplatin-induced RTC death. Therefore, it is considered that our data...
are reasonable and do not contradict the results of Hughes and Johnson (23).

Whereas death receptor-mediated pathways can be activated by ROS, several studies have documented that such cascades can also generate ROS (1, 2, 10, 11, 17, 20, 48, 58). That is, ROS can act as second messengers during death receptor-mediated apoptosis (5). Thus it is considered that ROS and death receptor-mediated apoptotic cascades can interact synergistically with each other. We propose that such interaction provides the best mechanism for the remarkable effect of antioxidants on cisplatin-induced RTC death.

In conclusion, we have demonstrated in the present study that treatment with DMTU, an antioxidant, inhibited upregulation of Fas, FasL, and TNF-α mRNA expression and ameliorated cisplatin-induced RTC death and renal dysfunction. Our findings suggest that ROS play a crucial role in the pathogenesis of RTC death through the activation of death receptor-mediated apoptotic cascades during cisplatin-induced ARF.

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