Kidney International, Vol. 63 (2003), pp. 72–82

Direct involvement of the receptor-mediated apoptotic pathways in cisplatin-induced renal tubular cell death

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Direct involvement of the receptor-mediated apoptotic pathways in cisplatin-induced renal tubular cell death.

Background. Tumor necrosis factor (TNF) receptor family members, such as Fas and TNF receptor 1 (TNFR1), are thought to induce apoptosis in a variety of cells and organs. Although a number of potential scenarios have been postulated for the involvement of these receptors in the pathogenesis of acute renal failure (ARF), direct evidence for their involvement in death of renal tubular cells (RTCs) and renal dysfunction is preliminary.

Methods. This study examined the roles of these receptors in RTC death in two systems: (*1*) in vivo murine and rat models of cisplatin-induced ARF, and (*2*) murine proximal tubular cells (PTCs), which were isolated from C57BL/6 (B6) mice, *Fas*-mutant B6-*lpr/lpr* mice and *TNFR1*-deficient mice, and normal rat kidney (NRK52E) cells in vitro.

Results. Reverse transcription-polymerase chain reaction indicated cisplatin-induced up-regulation of Fas, Fas ligand and TNF-α mRNAs in the kidney in vivo and in RTCs in vitro, both in mice and rats. In contrast, the level of TNFR1 mRNA was substantial but did not change in response to cisplatin. TNF-α production in cell culture medium determined by enzyme-linked immunosorbent assay (ELISA) and Fas expression determined by fluorescence-activated cell sorter (FACS) analysis increased following incubation with cisplatin in B6 PTCs. In order to examine whether Fas and TNFR1 are directly involved in RTC death and renal dysfunction, we compared cell resistance to cisplatin using a cell viability assay and FACS analysis with fluorescein isothiocyanate-conjugated annexin V and propidium iodide staining. The ratios of cell viability loss and cell death, both from apoptosis and necrosis, were higher in B6 PTCs than in other cells, while the ratios were comparable between *Fas*-mutant PTCs and *TNFR1*-deficient PTCs. Caspase-8 activity was increased in B6 PTCs, but not in *Fas*-mutant PTCs and *TNFR1*-deficient PTCs. Furthermore, the renal dysfunction and RTC death, both apoptosis and necrosis, induced by cisplatin were more severe in B6 mice in vivo.

Conclusion. Based on these data, we conclude that the Fas- and TNFR1-mediated apoptotic pathways are directly involved in the pathogenesis of cisplatin-induced RTC death process.

Key words: acute renal failure, Fas, TNF receptor, proximal tubule cells, kidney dysfunction, necrosis, cytoprotection, death receptor-mediated pathways.

Received for publication November 27, 2001
and in revised form July 3, 2002
Accepted for publication August 14, 2002

Experimental models of acute renal failure (ARF) induced by ischemia-reperfusion or nephrotoxic drugs exhibit morphological damage involving a variable degree of necrosis, especially of the S3 segment of the proximal tubules [1]. This tubular damage results in severe and acute reduction of the glomerular filtration rate, accompanied by increased serum concentrations of blood urea nitrogen (BUN) and creatinine. On the other hand, apoptosis has emerged as an important general mechanism by which renal tubular cells (RTCs) are injured, when subjected to a variety of stimuli, including ischemia [2–4], nephrotoxins [5], obstruction [6], polycystic disease [7], and transplant rejection [8], at an intensity below the threshold for necrotic cell death [9]. Although activation of endonucleases [10] has been implicated in the final postmortem phase [11], the proximal pathways involved in the stimulus recognition, signal transduction, and effector phases of RTC apoptosis are largely unknown.

A number of protein systems regulate the apoptotic events. One of these systems operates through the tumor necrosis factor (TNF) receptor family, including Fas antigen (Fas, CD95, APO-1) and TNF receptor 1 (TNFR1). Both Fas and TNFR1 contain conserved death domains in their cytoplasmic tails, which mediate defined protein-protein interactions [12, 13], 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 [14, 15]. Although a number of reports have documented Fas expression on RTCs and up-regulation of Fas expression during acute and chronic renal failure [4, 16–21], whether

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

Species	Gene	Primer 5′	Primer 3′	Anneal temp ℃	Cycle	Base pair
Rat	Fas	CTT TGA GGG TTT GGA GTT GA	ATT TGG TGT TGC TGG TTC GT	54	30	404
	FasL	GCA ACA GCC AGC CCC TAA GC	CCA CAC AGC AGC CCA AAA CT	55	30	472
	TNF-α	TAC TGA ACT TCG GGG TGA TTG GTC C	CAG CCT TGT CCC TTG AAG AGA ACC	59	32	295
	TNFR1	GAC TGG TTC CTT CTC TTG GT	GGT GTT CTG TTT CTC CTT AC	55	28	407
	GAPDH	TCC CTC AAG ATT GTC AGC AA	AGA TCC ACA ACG GAT ACA TT	55	23	308
Mouse	Fas	TCC TGC CTC TGG TGC TTG CT	TGT ATT GCT GGT TGC TGT GC	55	30	458
	FasL	GAG CAG TCA GCG TCA GAG TT	GAT TTG TGT TGT GGT CCT TC	55	30	323
	TNF-α	CCT CCC TCT CAT CAG TTC TA	AGC CAT AAT CCC CTT TCT AA	55	32	634
	TNFR1	TAC ATC CAT CAG GGG TCA CT	AGG CAC AAC TTC ATA CAC TC	55	28	525
	GAPDH	GCA GTG GCA AAG TGG AGA TT	GCA GAA GGG GCG GAG ATG AT	55	23	307

Abbreviations are: TNF-α, tumor necrosis factor-alpha; TNFR1, TNF receptor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; anneal temp, annealing temperature.

RTC apoptosis under those conditions depends on the Fas-Fas ligand (FasL) pathway remains controversial. Information regarding the involvement of TNFR1 in RTC apoptosis is limited and still preliminary [22–24]. Mice, in which TNFR1 expression was abolished, were reported to be more sensitive to certain types of infection and, in contrast, to exhibit increased resistance to endotoxin-induced lethality [25, 26]. However, to date there has been no report investigating the inhibitory effect of this receptor on RTC death.

In the present study, we examined the involvement of Fas and TNFR1 in RTC death using a cisplatin model of ARF in vivo, and cultured RTCs in vitro. Our results suggest that both Fas- and TNFR1-mediated apoptotic pathways contribute to RTC death in cisplatin-induced ARF, and lend support to the notion that inhibition of death receptor-mediated pathways may offer a novel approach to the cytoprotection of RTCs from a variety of stimuli.

METHODS

In vivo studies

Cisplatin-induced ARF in rats and mice. Male Sprague-Dawley rats weighing 200 to 250 g were obtained from Kyudo (Kumamoto, Japan). C57BL/6 (B6, wild-type) mice and C57BL/6-*lpr/lpr* (*Fas*-mutant) mice were obtained from Japan SLC (Hamamatsu, Japan). *TNFR1*-deficient (*TNFR1*$^{-/-}$) mice with a C57BL/6 background were a kind gift from Dr. Tak Wah Mak, Amgen Institute, University of Toronto. The animals were housed individually in standard laboratory cages and treated according to the principles outlined in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (Bethesda, MD, USA). Approval for the animal experiments was obtained in advance from the Animal Ethics Experimentation Committee of our institution. Cisplatin (Sigma, St. Louis, MO, USA) was administered to the rats by a single intravenous injection [8 mg/kg body weight (BW)] and to the mice by intraperitoneal injection (20 mg/kg BW). Rats and mice were sacrificed three or five days after injection. The kidneys were perfused in situ via the aorta or the heart with phosphate-buffered saline (PBS), pH 7.4, and then excised for further analyses. Control rats had saline injected instead of cisplatin.

Reverse transcription (RT)-PCR. Total RNA was extracted from the rat kidneys using the TRIzol method according to the protocol recommended by the manufacturer (Gibco BRL, Grand Island, NY, USA). Equal amounts (2.0 μg) of DNA-free total RNA from each sample were converted to cDNA using 200 U of Super-Script™ II reverse transcriptase (Gibco) with 500 ng of Oligo(dT)$_{12\text{-}18}$ primer (Gibco), 0.5 mmol/L of each dNTP (Promega, Madison, WI, USA), and 40 U of RNasin (Promega), in a 40-μL reaction volume. Reverse transcription was performed at 22°C for 10 minutes, at 42°C for 45 minutes, and at 95°C for 5 minutes. The reaction products (2.0 μL) were subjected to PCR amplification using 1.25 U of Taq DNA polymerase (Promega) in a 50-μL reaction volume with 0.4 μmol/L of each dNTP, 0.4 μmol/L of each specific primer, and 2 mmol/L $MgCl_2$. PCR was performed using the Perkin-Elmer (Foster City, CA, USA) thermal cycler according to the instructions provided by the manufacturer. The published sequences of rat and mouse Fas, FasL, TNF-α, TNFR1, and glyceraldehyde-3-phosphate dehydrogenase (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, USA). 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 were confirmed the identities of the fragments.

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.

Measurement of plasma BUN. Plasma BUN levels were measured using the urease-UV method by SRL Laboratories (Tokyo, Japan).

Morphological assessment. Three days after cisplatin injection, kidneys were excised from mice, immersed in Bouin's solution and fixed for 12 to 24 hours, and then embedded in paraffin and 3-μm sections were mounted on silane-coated glass slides. After deparaffinization and rehydration, the tissues were stained with periodic acid-Schiff (PAS), and the degree of morphological evidence of renal failure was determined using light microscopy, as described by Megyesi et al with some modifications [27], by an investigator blinded to the treatment protocol. Briefly, the following morphological parameters were selected to assess the severity of cisplatin-induced damage of the kidney: loss of proximal tubule brush border, tubule degeneration, tubule necrosis, and tubular cast formation. These parameters were evaluated on a scale of 0 to 4, which ranged from not present (0), mild (1), moderate (2), severe (3), to very severe (4). Each parameter was determined in at least five different animals.

Apoptosis assay. Histological aspects of apoptosis were studied by a standard terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) assay using ApopTag Plus Kit (Intergen, Inc., Gaithersburg, MD, USA) according to the instructions provided by the manufacturer. Briefly, 3-μm sections fixed in 10% formalin were pretreated for 30 minutes with PBS containing 3% H_2O_2, and then washed and incubated with proteinase K (20 μg/mL; Roche, Mannheim, Germany) at room temperature for 15 minutes. The sections were incubated with TdT and a mixture of digoxygenin-labeled nucleotides for 60 minutes. This was followed by incubation with anti-digoxygenin-peroxidase for 30 minutes and color development with H_2O_2-diaminobenzidine for three to six minutes. Then, the slides were counterstained with hematoxylin. For positive controls, specimens of thyroid tissue were provided by Intergen. Negative controls were performed by omission of TdT enzyme from the incubation buffers.

In vitro studies

Isolation of murine proximal tubular cells (PTCs). Murine PTCs were cultured from collagenase-digested fragments of the proximal tubules obtained from the cortices of C57BL/6 mice, C57BL/6-*lpr/lpr* mice and *TNFR1*$^{-/-}$ mice with a modification of methods described by Wuthrich et al [28]. Briefly, cortices were minced and incubated with 0.5 mg/mL collagenase (Sigma) in Hanks' solution (Gibco) for one hour and the cell suspension was sieved through steel meshes (250 μm, 150 μm, 75 μm, and 38 μm). After centrifugation at 200 × *g* for five minutes, cells were washed twice with PBS, and cultured in the growth medium.

Cell culture and cisplatin stimulation. Murine PTCs were cultured in 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F-12; Gibco) containing the following additives; 25 mmol/L HEPES (Gibco), 100 U/mL penicillin (Gibco), and 100 μg/mL streptomycin (Gibco), 10% fetal calf serum (FCS; Gibco), 5 μg/mL insulin (Sigma), 5 μg/mL transferrin (Sigma), 1 ng/mL prostaglandin E_1 (PGE_1; Sigma), 5×10^{-11} mol/L triiodothyronine (Sigma), 10^{-8} mol/L sodium selenite (Sigma), 5×10^{-8} mol/L hydrocortisone (Sigma), and 25 ng/mL epidermal growth factor (Sigma). Normal rat kidney (NRK52E) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 medium (Gibco) containing 10% FCS. The cultured cells were incubated with various concentrations of cisplatin or vehicle (PBS) for 24, 48, or 72 hours for the following analyses.

RT-PCR. Total RNA was extracted by the TRIzol method from NRK52E cells according to the protocol provided by the manufacturer, following the manipulations described earlier in the in vivo study.

Fas expression on the surface of PTCs. Cell surface Fas expression on murine PTCs was examined by fluorescence-activated cell sorter (FACS) analysis using a fluorescein isothiocyanate (FITC)-conjugated monoclonal hamster antibody to murine Fas (PharMingen, San Diego, CA, USA). After incubation with either 8 μmol/L cisplatin or vehicle for 72 hours, the murine PTCs were trypsinized and washed, and then incubated in the dark with 10 μL of either an anti-Fas antibody or a control antibody (FITC-conjugated hamster IgG; PharMingen) in Hanks' solution with 10% FCS for 30 minutes at 4°C. After incubation, the cells were washed twice, resuspended in 200 μL of PBS, and then analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) and the data were analyzed using CELLQUEST software (Becton Dickinson).

TNF-α protein excretion in cell culture medium. TNF-α protein excretion in cell culture medium of PTCs after incubation with cisplatin or vehicle for 24, 48, or 72 hours was measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the protocol recommended by the manufacturer (BioSource International, Camarillo, CA, USA).

Activity of caspase-8. Activity of caspase-8 in RTCs after incubation with cisplatin or vehicle for 72 hours was measured using ApoAlert Caspase-8 Colorimetric Assay Kit according to the protocol recommended by the manufacturer (Clontech, La Jolla, CA, USA).

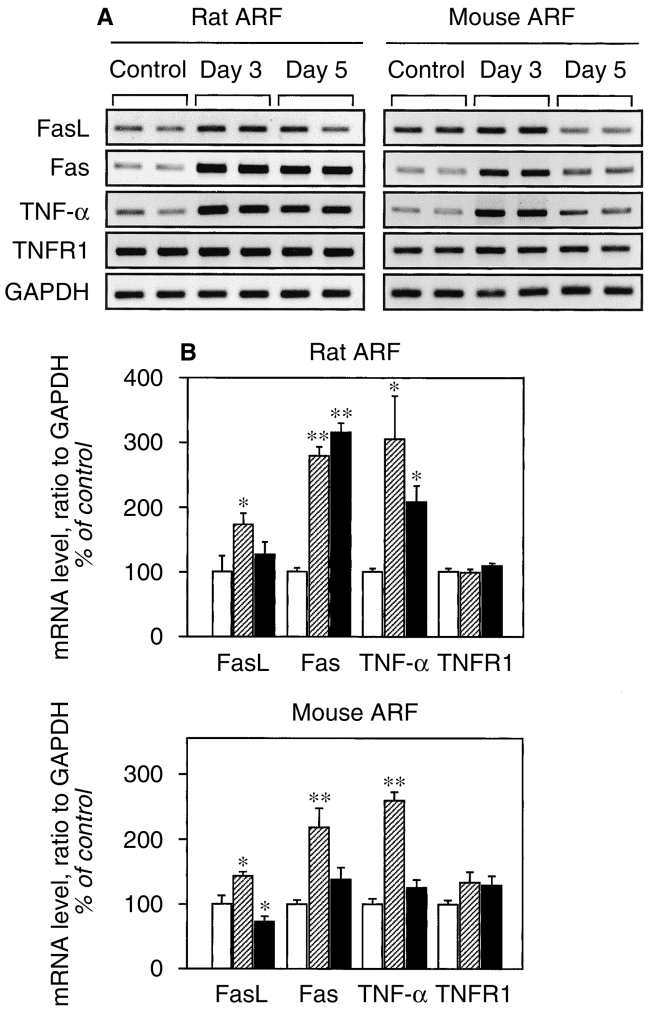

Fig. 1. mRNA expression of death receptors and their ligands in rat and murine kidneys determined by RT-PCR. (*A*) Representative expression of FasL, Fas, tumor necrosis factor-α (TNF-α), and TNF receptor 1 (TNFR1) mRNAs in control kidneys or in kidneys 3 days (Day 3) and 5 days (Day 5) after cisplatin injection. (*B*) Mean mRNA levels of these genes, quantified using NIH image software and normalized to the intensity of GAPDH bands, in control kidneys (□) and in kidneys 3 days (▨) and 5 days (■) after cisplatin injection are shown as a ratio of mean mRNA levels in control kidneys. The ratios represent means (± SEM). $*P < 0.05$, $**P < 0.01$ compared to control kidneys.

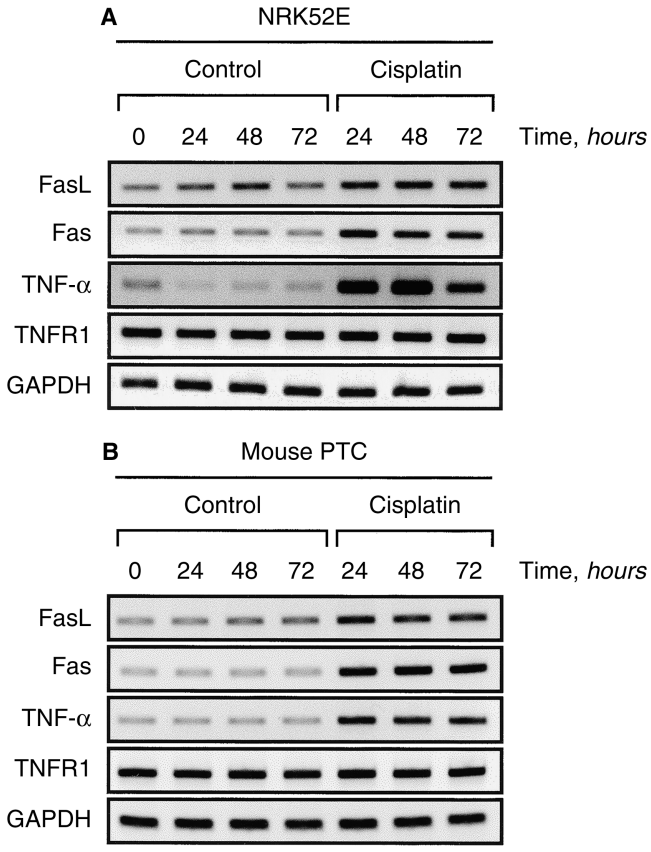

Fig. 2. mRNA expression of death receptors and their ligands in cultured renal tubular cells determined by RT-PCR. Representative expression of FasL, Fas, TNF-α and TNFR1 mRNAs in NRK52E cells (*A*) and in cultured PTCs of C57BL/6 mice (*B*) are shown after incubation with vehicle (Control) or cisplatin for the indicated times.

Cell viability assay. Cell viability was determined by the WST-1 assay, as described by Kawahara et al [29]. In brief, the murine PTCs (5×10^4 cells in 100 μL) were treated with various concentrations of cisplatin. 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 mmol/L, respectively, and incubated for one hour at 37°C. Using an ELISA autoreader, cell viability was determined by measuring the difference between the absorbance at 450 and 620 nm.

Cell death assay. Cell death was assayed by FACS analysis with FITC-conjugated annexin V (PharMingen) and propidium iodide (PI; PharMingen). Murine PTCs were incubated with either 8 μmol/L cisplatin or vehicle for 72 hours. The cells were trypsinized, washed, and incubated with FITC-conjugated annexin V and PI in binding buffer at concentrations suggested by the manufacturer on ice in the dark for 15 minutes. Stained cells were analyzed on a FACSCalibur flow cytometer. Data were analyzed using CELL-QUEST software.

Statistical analysis

Data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) with the Bonferroni modified *t* test was used for comparison with control values in the RT-PCR assay and with wild-type (C57BL/6) mice. Mann-Whitney's U-test (non-parametric analysis) was used for comparison with control values in ELISA of TNF-α and caspase-8 assay. Survival data were analyzed by Kaplan-Meier using log rank analysis to compare survival curves. Statistical significance was accepted at $P < 0.05$.

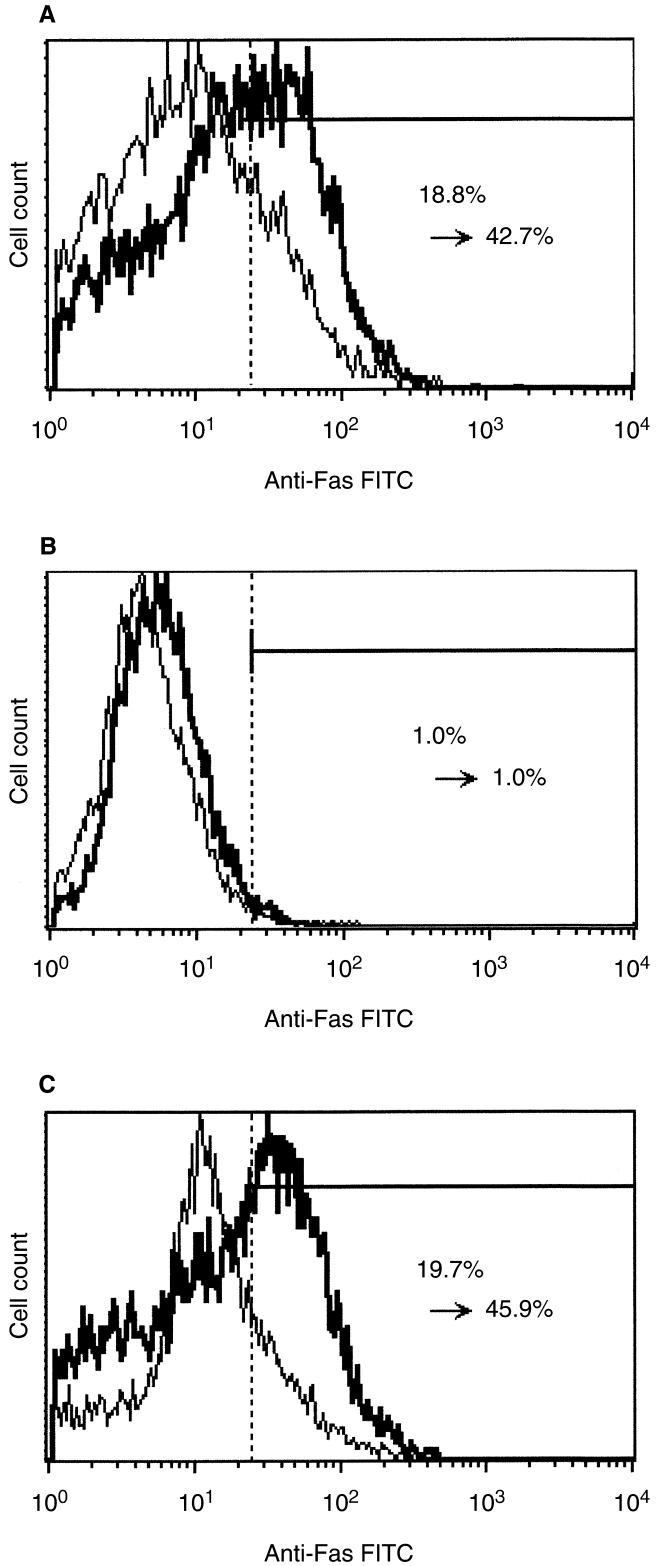

Fig. 3. Effects of cisplatin on Fas protein expression on the cell surface of murine proximal tubular cells (PTCs). Representative Fas protein expression on the surface of murine PTCs isolated from C57BL/6 mice (*A*), C57BL/6-*lpr/lpr* mice (*B*), and *TNFR1*$^{-/-}$ mice (*C*) was identified by FACS analysis using a monoclonal hamster antibody to murine Fas. The levels of fluorescence on the cisplatin-stimulated PTCs (bold lines) are higher than those on the vehicle-stimulated PTCs (fine lines) in mice C57BL/6 (A) and *TNFR1*$^{-/-}$ mice (C), whereas in C57BL/6-*lpr/lpr* mice, the levels are low and comparable between stimulated and unstimulated cells. The data represent three independent experiments. The upper values (fine lines) indicate percentages of positive staining for anti-Fas antibody in PTCs stimulated with vehicle and the lower values (bold lines) indicate those stimulated with cisplatin.

RESULTS

Cisplatin-induced up-regulation of Fas, FasL and TNF-α mRNA expression in the kidneys of rats and B6 mice in vivo, and NRK52E cells and B6 PTCs in vitro

In the in vivo study, low expression levels of FasL, Fas and TNF-α mRNAs were observed in the kidneys of control rats and B6 mice. The mRNA levels of these genes were up-regulated after cisplatin injection. In contrast, the level of TNFR1 mRNA was substantial in control kidneys, but was not altered by cisplatin injection in rats and was slightly increased in mice (Fig. 1A). The signal densities were quantified using the NIH image program and results were normalized to the intensity of GAPDH bands. Mean mRNA levels of these genes in control kidneys and in insulted kidneys three and five days after cisplatin injection are shown as a ratio to mean mRNA levels in control kidneys in Figure 1B.

In the in vitro study, low expression levels of FasL, Fas and TNF-α mRNAs were observed also in NRK52E cells and in B6 PTCs in the absence of cisplatin stimulation. The mRNA levels of these genes were increased by incubation with 8 μmol/L cisplatin for over 24 hours. Similar to the findings in the in vivo study, TNFR1 mRNA levels were substantial and constant, irrespective of stimulation by cisplatin (Fig. 2).

Up-regulation of Fas expression on the surface of murine PTCs by cisplatin stimulation

FACS analysis revealed Fas expression on the surface of PTCs of C57BL/6 and *TNFR1*$^{-/-}$ mice and the expression levels were increased by incubation with 8 μmol/L cisplatin for 72 hours. However, no positive staining was observed on the surface of PTCs of C57BL/6-*lpr/lpr* mice, irrespective of stimulation by cisplatin (Fig. 3).

Increased production of TNF-α protein in the medium of murine PTCs by cisplatin stimulation

ELISA analysis for TNF-α in the medium of B6 PTCs revealed that TNF-α production by PTCs increased following incubation with 8 μmol/L cisplatin and remained elevated over a period of more than 24 hours (Fig. 4).

Activation of caspase-8 in NRK52E cells and B6 PTCs by cisplatin stimulation

Caspase-8 activity assay revealed that the levels of caspase-8 activity in NRK52E cells and B6 PTCs incubated

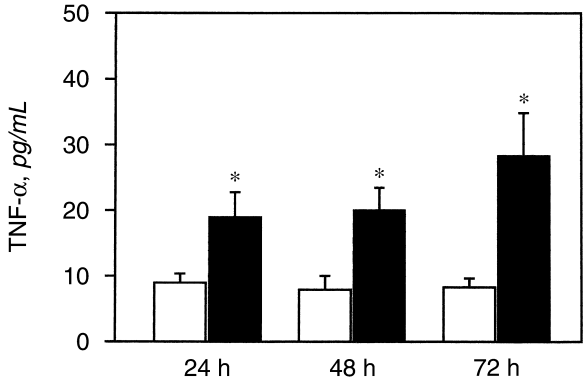

Fig. 4. Effects of cisplatin on TNF-α protein production by cultured renal tubular cells. The PTCs of C57BL/6 mice were incubated with 8 μmol/L cisplatin (■) or vehicle (□) for the indicated time. TNF-α concentrations in cell culture medium determined by enzyme-linked immunosorbent assay are shown. $*P < 0.05$ compared to Control.

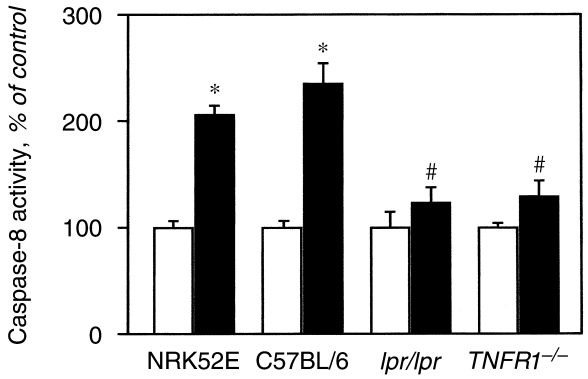

Fig. 5. Effects of cisplatin on caspase-8 activity in cultured renal tubular cells. NRK52E cells and PTCs of C57BL/6 mice, C57BL/6-*lpr/lpr* mice, and *TNFR1*$^{-/-}$ mice were incubated with 8 μmol/L cisplatin (■) or vehicle (Control; □) for 72 hours. Activities of caspase-8 are shown as percent change from Control. $*P < 0.05$ compared to Control. $\#P < 0.05$ compared to C57BL/6 mice.

with 8 μmol/L cisplatin for 72 hours were significantly higher than in those cells incubated with vehicle. However, only slight increases were observed in the PTCs of C57BL/6-*lpr/lpr* mice and *TNFR1*$^{-/-}$ mice after stimulation by cisplatin (Fig. 5).

Effects of *Fas* or *TNFR1* gene ablation on amelioration of RTC death and renal dysfunction induced by cisplatin

Serum levels of BUN three days and five days after cisplatin injection were significantly lower in C57BL/6-*lpr/lpr* mice and *TNFR1*$^{-/-}$ mice than in C57BL/6 mice (Fig. 6). Histological examination of kidneys excised from C57BL/6 mice three days after cisplatin injection revealed severe RTC injuries, such as proximal tubule brush border loss, tubule degeneration, tubule necrosis, and tubule cast formation in the corticomedullary region (Fig. 7A). Such RTC injury was less severe in kidneys of

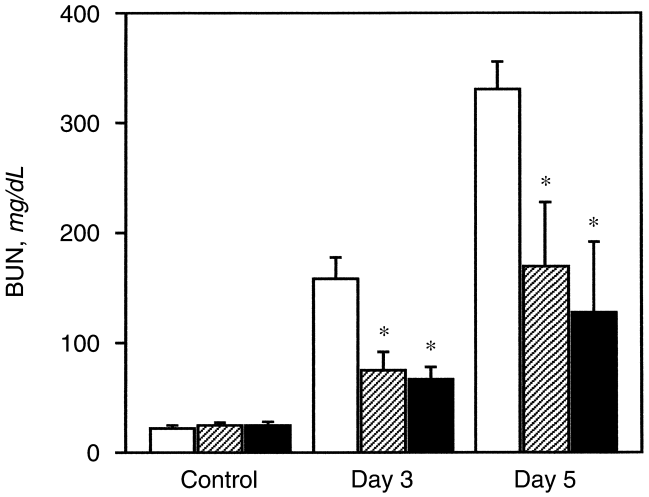

Fig. 6. Renal function of cisplatin-injected mice. Plasma levels of BUN 3 days (Day 3) and 5 days (Day 5) after cisplatin injection are shown in C57BL/6 mice (□), C57BL/6-*lpr/lpr* mice (▨), and *TNFR1*$^{-/-}$ mice (■). Values, in milligrams per deciliter, represent means (± SEM) of at least five mice. $*P < 0.05$ compared to C57BL/6 mice.

C57BL/6-*lpr/lpr* mice and *TNFR1*$^{-/-}$ mice than of C57BL/6 mice (Fig. 7 B, C). These histological parameters are quantified in Figure 7D. The numbers of TUNEL-positive apoptotic cells observed in outer medulla three days after cisplatin injection were significantly smaller in kidneys of C57BL/6-*lpr/lpr* mice and *TNFR1*$^{-/-}$ mice than of C57BL/6 mice (Fig. 8).

The cell viability assay revealed a significant dose-dependent loss of cell viability by incubation with various concentrations (2, 4, or 8 μmol/L) of cisplatin for 72 hours in all kinds of PTCs. However, PTCs of C57BL/6-*lpr/lpr* mice and *TNFR1*$^{-/-}$ mice were significantly more resistant to cisplatin stimulation than those of C57BL/6 mice (Fig. 9).

FACS analysis demonstrated increased ratios of annexin V-positive (apoptotic) cells and PI-positive (necrotic) cells, in PTCs of C57BL/6 mice, following incubation with 8 μmol/L cisplatin for 72 hours (Fig. 10A). However, no increases were seen in these ratios in PTCs of C57BL/6-*lpr/lpr* mice and *TNFR1*$^{-/-}$ mice (Fig. 10 B, C).

Effects of *Fas* or *TNFR1* gene ablation on decrease of mortality after cisplatin administration

All genotypes of mice had a high mortality after the 20 mg/kg cisplatin injection in this study. We compared the survival rate after cisplatin injection among all three genotypes of mice. As a result, the survival rate was significantly higher in C57BL/6-*lpr/lpr* mice and *TNFR1*$^{-/-}$ mice than in C57BL/6 mice (Fig. 11).

DISCUSSION

The present study demonstrates that cisplatin induces apoptosis of RTCs in association with increased levels

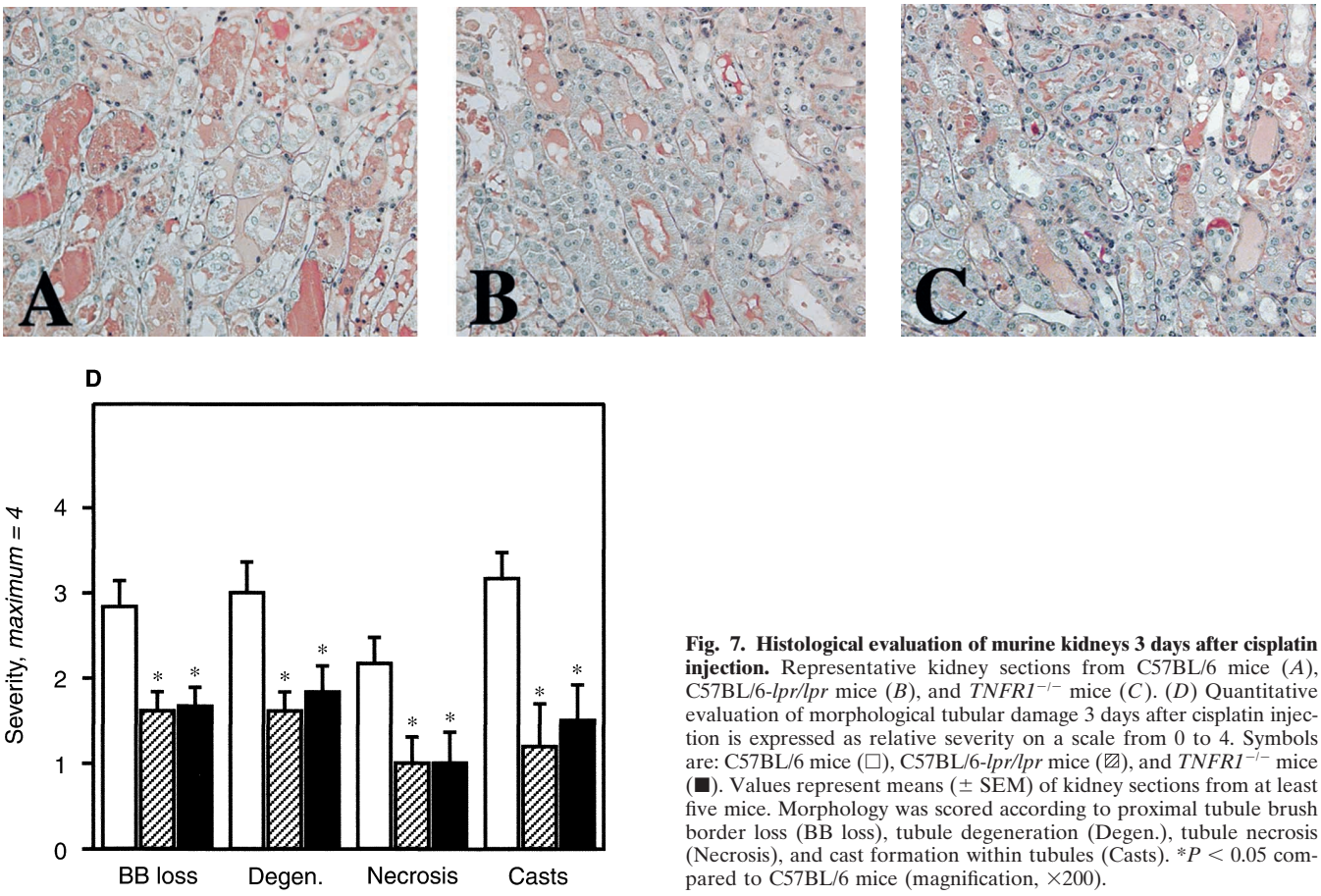

Fig. 7. Histological evaluation of murine kidneys 3 days after cisplatin injection. Representative kidney sections from C57BL/6 mice (*A*), C57BL/6-*lpr/lpr* mice (*B*), and *TNFR1*$^{-/-}$ mice (*C*). (*D*) Quantitative evaluation of morphological tubular damage 3 days after cisplatin injection is expressed as relative severity on a scale from 0 to 4. Symbols are: C57BL/6 mice (□), C57BL/6-*lpr/lpr* mice (▨), and *TNFR1*$^{-/-}$ mice (■). Values represent means (± SEM) of kidney sections from at least five mice. Morphology was scored according to proximal tubule brush border loss (BB loss), tubule degeneration (Degen.), tubule necrosis (Necrosis), and cast formation within tubules (Casts). *$P < 0.05$ compared to C57BL/6 mice (magnification, ×200).

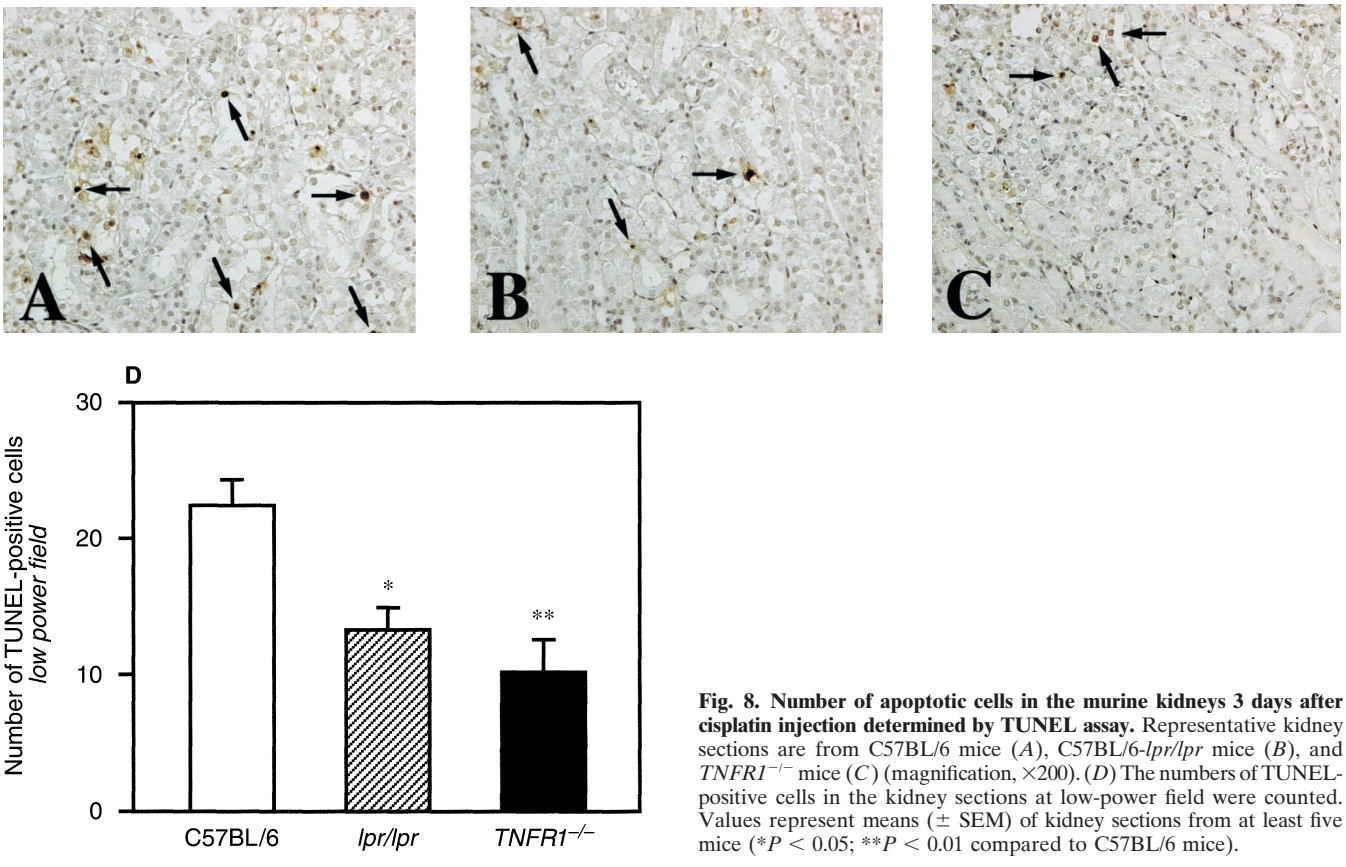

Fig. 8. Number of apoptotic cells in the murine kidneys 3 days after cisplatin injection determined by TUNEL assay. Representative kidney sections are from C57BL/6 mice (*A*), C57BL/6-*lpr/lpr* mice (*B*), and *TNFR1*$^{-/-}$ mice (*C*) (magnification, ×200). (*D*) The numbers of TUNEL-positive cells in the kidney sections at low-power field were counted. Values represent means (± SEM) of kidney sections from at least five mice (*$P < 0.05$; **$P < 0.01$ compared to C57BL/6 mice).

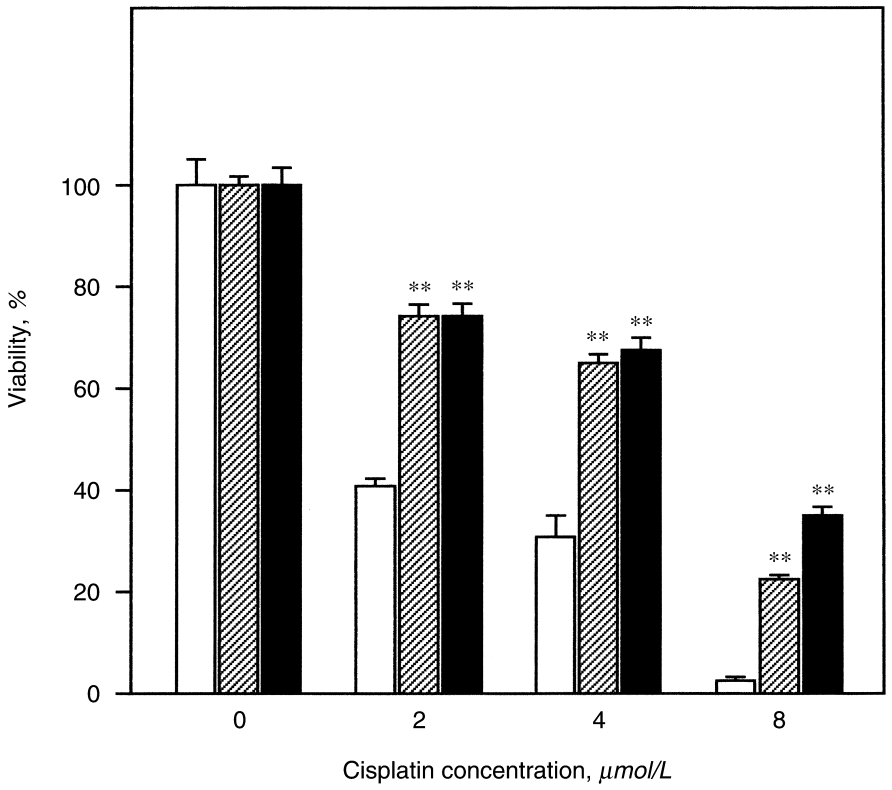

Fig. 9. Cell viability of murine PTCs determined by WST-1 assay. Symbols are: C57BL/6 mice (□), C57BL/6-*lpr/lpr* mice (▨), and *TNFR1*$^{-/-}$ mice (■). Cell viability was determined by WST-1 assay as described in the **Methods** section. Results are expressed as the percentage of the value obtained without cisplatin. The experiments were performed in triplicate, and the mean values (± SEM) are plotted. A dose-dependent killing of PTCs by the 72-hour exposure to cisplatin is shown. The PTCs of C57BL/6-*lpr/lpr* mice and *TNFR1*$^{-/-}$ mice were significantly more resistant to cisplatin stimulation than those of C57BL/6 mice. $^{**}P < 0.01$ compared to C57BL/6 mice.

of gene expression of death receptors and ligands, with the exception of *TNFR1*. To our knowledge, this shows for the first time that ablation of these death receptor genes ameliorates RTC damage and renal dysfunction induced by cisplatin in vivo and in vitro.

A previous study by Lieberthal, Triaca and Levine demonstrated that high concentrations of cisplatin (800 μmol/L) led to necrotic cell death over a few hours, whereas much lower concentrations of cisplatin (8 μmol/L) led to apoptosis, which resulted in loss of the cell monolayer over several days [9]. Our results in murine PTCs, demonstrating that annexin V-positive cells are increased more than PI-positive cells by 72 hours of incubation with lower concentrations (8 μmol/L) of cisplatin, are in agreement with that study, even taking into account the different methods of detecting apoptosis and necrosis (FACS analysis with annexin V and PI versus DNA gel electrophoresis and morphological analysis with trypan blue and fluorescent dyes such as ethidium homodimer-1 and H-33342). These findings are in concordance with recent perspectives on the difference between necrosis and apoptosis, that is, that a mild insult can lead to apoptosis while a severe form of the same insult can induce necrosis [30, 31].

Although there are only a few reports on the involvement of the TNF-mediated pathway in RTC apoptosis [4, 32, 33], those reports have not considered this pathway to be important in the pathogenesis of RTC death for the following reasons: First, in vitro studies have reported TNF-α alone did not induce RTC apoptosis [34, 35]. However, it is considered that TNF-α is unlikely to act alone in vivo, but in association with other cytokines such as interleukin-1 and interferon-γ, and with interacting with many apoptosis regulatory proteins such as c-myc, Bax and Bcl2. Therefore, elucidation of the contribution of TNFR1 requires stimulation of RTCs by such cytokines, in addition to the stimulation by TNF-α. Indeed, they demonstrated that TNF-α promoted marked RTC apoptosis under conditions of serum depletion, with increased Bax expression and reduced Bcl2 expression [35]. Secondly, unlike Fas, the cytotoxicity mediated by TNFR1 is regulated by two kinds of opposing activities: induction of apoptosis and activation of the transcriptional factor, nuclear factor-kappa B (NF-κB). Indeed, a protective effect of TNF-α against TNF-mediated apoptosis in the liver mediated by stimulation of NF-κB has been reported [36]. However, in the kidney, many studies have demonstrated deleterious roles of NF-κB in RTC damage [37, 38]. Therefore, we believe that it is possible that the TNF-mediated pathway plays a crucial role in RTC death.

Recently, Choi et al reported increased expression of Fas, FasL, TNFR1, and signal transduction adaptor molecules such as Fas-associated death domain (FADD), TNFR1-associated death domain (TRADD), and caspase-8, in chronic obstructive nephropathy [24]. Our

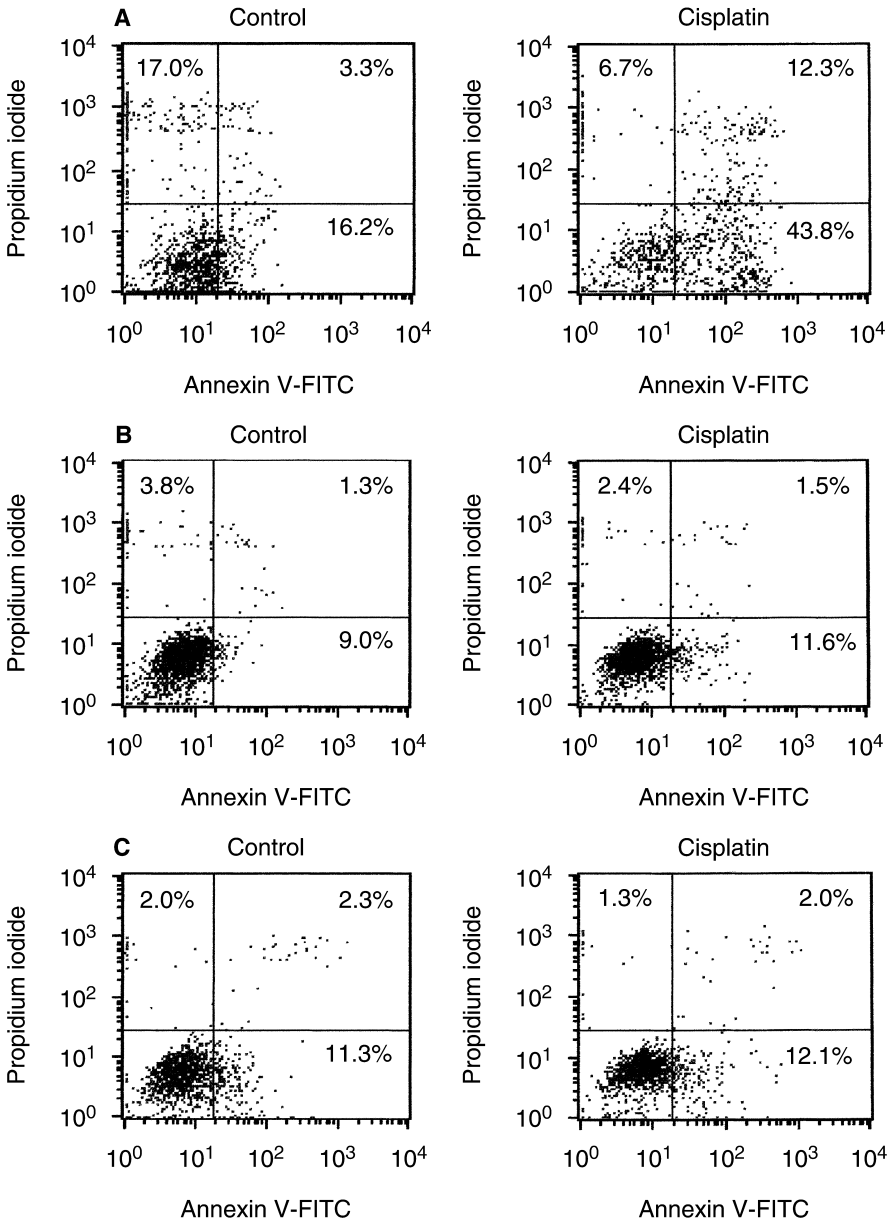

Fig. 10. Percentages of apoptotic cells among murine PTCs determined by FACS analysis using annexin V-FITC and PI. Murine PTCs of C57BL/6 mice (*A*), C57BL/6-*lpr/lpr* mice (*B*), or *TNFR1*$^{-/-}$ mice (*C*) were incubated without cisplatin (Control), or with 8 μmol/L cisplatin for 72 hours and were stained with FITC-conjugated annexin V and PI, and analyzed using a FACSCalibur flow cytometer. Increased ratios of annexin V-positive cells and PI-positive cells induced by cisplatin stimulation were seen in PTCs of C57BL/6 mice (A), whereas no increases were observed in PTCs of C57BL/6-*lpr/lpr* mice (B), and *TNFR1*$^{-/-}$ mice (C). The data represent three independent experiments. Values on the upper left, upper right, and lower right indicate the percentages of necrotic, late apoptotic, and apoptotic cells, respectively.

present study used *TNFR1*-deficient mice to examine the effect of *TNFR1* gene ablation on the susceptibility of PTCs to cisplatin, and demonstrated that the TNF-mediated apoptotic pathway was involved in cisplatin-induced RTC death. The roles of TNFR1 and TNFR2 receptors in tubulointerstitial fibrosis of obstructive nephropathy were reported by Guo et al using the respective knockout mice [22]. However, the relationship between these receptors and RTC death was not examined, although these investigators discussed the possible involvement of TNF-mediated apoptosis in tubular damage. Therefore, to the best of our knowledge, our study is the first report demonstrating the direct involvement of TNFR1 in RTC death.

Tumor necrosis factor-α is a pleiotropic, pro-inflammatory peptide cytokine that is produced by macrophages, T cells, and some non-hematopoietic cells, and is cytotoxic in picogram quantities. Recent reports have documented the ability of RTCs to express TNF-α protein in response to ischemia, lipopolysaccharide and interleukin-1α, and membrane-associated TNF-α is not limited to hematopoietic cells [39, 40]. Similar to TNF-α, FasL expression has been demonstrated in RTCs [17, 41]. Our results demonstrated a low level expression of both TNF-α and FasL mRNA in RTCs and their increased expression in vitro in response to cisplatin, indicating that RTCs may have undergone apoptosis by lymphocyte-independent activation of the Fas-FasL and TNF-TNFR1

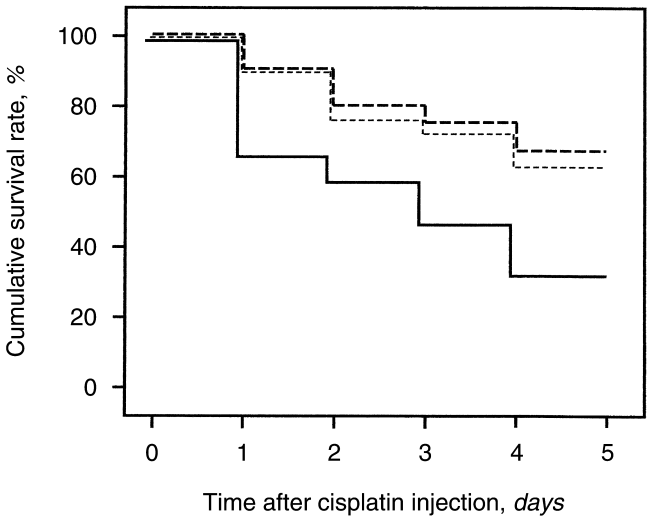

Fig. 11. Effects of ablation of *fas* and *tnfr1* on survival after cisplatin injection. Cumulative survival rate of C57BL/6 (solid line) vs. C57BL/6-*lpr/lpr* (dotted line) ($P = 0.02$) and *TNFR1*$^{-/-}$ mice (dashed line) ($P = 0.04$), 1 to 5 days after injection with cisplatin is shown as Kaplan-Meier survival curve.

systems between adjacent RTCs. This finding is consistent with previous reports demonstrating that RTCs have antigen-presenting properties reminiscent of those of "professional" antigen-presenting cells such as macrophages [28, 42, 43].

The association of Fas and TNFR1 on RTCs remains unclear. We demonstrated Fas protein expression on the surface of PTCs of *TNFR1*-deficient mice at a level similar to that in wild-type mice, indicating that Fas expression in RTCs is independent of TNFR1. This finding suggests that, in the present study, the effect of ablation of each gene on inhibition of RTC damage was independent, and that concomitant ablation of both genes could be more effective for inhibition of apoptotic change in RTCs. However, Zhou et al reported that *Fas*/*TNFR1* double knockout mice exhibited accelerated lymphoproliferation and increased rates of autoimmune disease, associated with high mortality, indicating that the Fas and TNFR1 signaling and apoptosis pathways may compensate for each other [44]. Further studies are required to elucidate the association between these death receptors.

Cisplatin is a well-known DNA-damaging agent and current thinking is that DNA platination is an essential first step in the cytotoxic activity of the drug. Cisplatin-induced DNA damage may lead to expression of p53 protein, which subsequently induces both expression of downstream p21$^{WAF1/CIP1/SDI1}$ protein and G1 phase cell cycle arrest, and in the event of irreparable DNA damage, p53 protein induces apoptosis [45]. The degree to which p53 is activated may be central to the cellular decision of whether to arrest the cell cycle or to initiate cell death after exposure to DNA damage or other stresses [46]. Megyesi et al showed increased levels of p53 and p21 protein in the kidney during cisplatin-induced ARF, and demonstrated a renoprotective role of p21 in this insult using *p21* gene knockout mice [1, 27]. We also observed increased levels of p53 and p21 protein in the nuclei of PTCs in the outer medulla of the rat kidneys three days after cisplatin injection (unpublished data). This finding is consistent with a recent study reported by Miyaji et al [47]. However, it is unclear whether the main role of p53 induction is as an effector of G1 phase cell cycle arrest or as an effector of apoptosis in cisplatin-induced RTC death. The physiological significance of cross talk between Fas and p53 protein in apoptosis is controversial [48–50]. Studies using *p53* knockout mice are required to elucidate its role in RTC death induced by cisplatin, and will provide information about mechanisms of such insults. In this respect, a study using *TNFR* knockout mice and *p53* knockout mice, which underwent lung injury by treatment with bleomycin, demonstrated that TNFR rather than p53 contributed to apoptosis of alveolar macrophages and affected the fibroproliferative response in the lungs of bleomycin-treated mice [51].

In summary, the present study demonstrates that cisplatin-induced RTC death is mediated by the Fas-FasL and TNF-TNFR1 pathways. Further studies are necessary to investigate the role of mitochondrial pathways in RTC death. Elucidation of such pathways may lead to a better understanding of the mechanisms of RTC atrophy and tubulointerstitial fibrosis observed in chronic renal failure, in addition to RTC death in ARF, and may provide clues for the rational design of novel therapeutic interventions.

ACKNOWLEDGMENTS

We are grateful to Ms. H. Noguchi for her excellent technical assistance and Dr. F. G. Issa for editing of the manuscript. Part of this study was conducted at the Morphology Core, Graduate School of Medical Sciences, Kyushu University.

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