Cisplatin Nephrotoxicity Is Mediated by Deoxyribonuclease I

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Cisplatin is commonly used for chemotherapy in a wide variety of tumors; however, its use is limited by kidney toxicity. Although the exact mechanism of cisplatin-induced nephrotoxicity is not understood, several studies showed that it is associated with DNA fragmentation induced by an unknown endonuclease. It was demonstrated previously that deoxyribonuclease I (DNase I) is a highly active renal endonuclease, and its silencing by antisense is cytoprotective against the in situ hypoxia injury of kidney tubular epithelial cells. This study used recently developed DNase I knockout (KO) mice to determine the role of this endonuclease in cisplatin-induced nephrotoxicity. The data showed that DNase I represents approximately 80% of the total endonuclease activity in the kidney and cultured primary renal tubular epithelial cells. In vitro, primary renal tubular epithelial cells isolated from KO animals were resistant to cisplatin (8 µM) injury. DNase I KO mice were also markedly protected against the toxic injury induced by a single injection of cisplatin (20 mg/kg), by both functional (blood urea nitrogen and serum creatinine) and histologic criteria (tubular necrosis and in situ DNA fragmentation assessed by the terminal deoxynucleotidyl transferase nick end-labeling). These data provide direct evidence that DNase I is essential for kidney injury induced by cisplatin.

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Cisplatin (cis-diaminedichloroplatinum II) is commonly used for chemotherapy in a wide variety of tumors; however, it has nephrotoxicity as a major side effect (1–3). After a single injection of cisplatin, 28 to 36% of patients develop dose-dependent nephrotoxicity (4). Cisplatin has been shown to accumulate in the kidney to a greater degree than in other organs (4).

Although a number of cellular targets of cisplatin have been identified (5), the mechanism of cisplatin nephrotoxicity is unclear. Multiple studies showed that cisplatin nephrotoxicity is associated with DNA fragmentation. It is visualized either by a 200-bp DNA ladder in agarose gel (6,7) or by using the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate (TdT) nick-end labeling (TUNEL) assay (8–10). The appearance of a 200-bp ladder indicates that the enzyme is an endonuclease, capable of internucleosomal fragmentation of nuclear DNA. This type of DNA fragmentation is usually attributed to apoptosis (11). Positive TUNEL staining, which is also characteristic for apoptotic DNA fragmentation, suggests that the endonuclease produces 3′OH DNA termini, a substrate for TdT. Earlier studies suggested that the apoptotic DNA fragmentation is catalyzed by a Ca/Mg-dependent endonuclease (12). Despite several attempts to isolate the enzyme (13,14), this hypothetical endonuclease had never been cloned. So far, deoxyribonuclease I (DNase I) is the most well known Ca/Mg-dependent endonuclease that produces 3′OH DNA ends and is involved in DNA fragmentation during cell death (15,16).

We showed previously that DNase I is highly expressed in the kidney and is upregulated during kidney injury induced by ischemia-reperfusion in vivo (17,18). The suppression of DNase I by antisense was protective against the hypoxia/reoxygenation injury of renal tubular epithelial cells in vitro (17). In the present study, we chose to investigate the effect of DNase I inactivation in vivo using recently produced DNase I–deficient mice (19) to determine whether DNase I is important for kidney injury induced by cisplatin. Unlike previously described DNase I knockouts (KO) in 129×C57BL/6 background, the DNase I−/− CD-1 mice used in the current study do not develop lupus. Our experience showed that DNase I−/− mice are healthy, suggesting that DNase I is dispensable in normal tissues. That distinguishes DNase I from other cell death endonucleases, for example DNase II or endonuclease G (EndoG), inactivation of which was lethal (20–22). The role of DNase I during tissue injury has not been previously studied in any organ, including the kidney.

Materials and Methods

Animals

DNase I−/− KO mice (CD-1 background) were obtained from T. Moroy (University of Essen, Essen, Germany). The mice were bred as heterozygotes and genotyped by PCR as suggested by Napirei et al. (19). Female 8- to 12-wk-old (20 to 30 g) mice were used in all of the experiments. We administered 20 mg/kg cisplatin (cis-diaminedichloroplatinum II; Bedford Laboratories, Bedford, OH) in a single...
intrapеритонеal injection in DNase1−/− and DNase1+/+ (wild-type [WT]) mice. The control mice received an injection of saline. All experiments were approved by the Animal Care and Use Committee of the Central Arkansas Veterans Healthcare System.

**Primary Tubular Epithelial Cells**

Mouse tubular epithelial cells were freshly isolated from DNase 1 KO and WT mice as described by Nowak et al. (23) and cultured up to 10 d (passages 1 and 2) before the experiment. The cells were treated in vitro with 8 μM cisplatin in serum-free media for 4 d. We assessed cell death by lactate dehydrogenase (LDH) using the LDH release assay kit (Promega, Madison, WI). Cytotoxicity was expressed as the ratio of the LDH release in the treated cell medium to that of the maximal LDH release. Activation of caspase-3 was measured using the cell ELISA procedure described by Frahm et al. (24). The cells were grown in 96-well plates; permeabilized; and fixed with 4% paraformaldehyde, 0.012% saponin, and buffered phosphate saline for 10 min at room temperature. Polyclonal rabbit anti-active caspase-3 (titer 1:1000; Chemicon, Temecula, CA) was used as the primary antibody, which was detected with secondary anti-rabbit antibody conjugated with HRP (titer 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA). The HRP activity was measured with TMB substrate (Sigma, St. Louis, MO) at 450 to 540 nm using the Synergy HT-I microplate reader (Bio-Tek, Winooski, VT). After measurement and triple washing, the wells were reprobed with anti–actin-FITC antibody (titer 1:100; Santa Cruz), washed again, and measured at 485/528 nm. All measurements were done in quadruplicate and were repeated at least three times in different plates.

**Histology and Histochemistry**

Kidney samples were fixed with 10% neutral formalin (Sigma) for 24 h, dehydrated, and embedded in paraffin. Sections of 3-μm thickness were cut and stained with hematoxylin and eosin for routine histologic analysis. Tubular necrosis was assessed by measurement of the decreased number of tubular epithelial cells attached to the basement membrane as described by Soley et al. (25). The data were presented as the number of cell nuclei per mm² as suggested by Ishikawa and Kitamura (26). We performed TUNEL staining using the In Vivo Cell Death Detection Kit (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s manual, followed by counterstaining with 10 μM 4',6-diamidino-2-phenylindol (DAPI; Sigma) for 5 min. Slides were mounted under the ProLong Antifade Kit (Molecular Probes, Eugene, OR) and analyzed with a Carl Zeiss microscope with the green (fluorescein, TUNEL) or blue spectrum (DAPI).

**Endonuclease Activity**

We prepared total kidney extracts or cell extracts and measured the endonuclease activity using the plasmid incision assay with pBR322 plasmid (New England Biolabs, Beverly, MA) as substrate as described previously (17). The total endonuclease activity was measured in 2 mM CaCl₂, 5 mM MgCl₂, 10 mM Tris-HCl (pH 7.4), and 0.5 mM dithiothreitol. We accepted 1 unit as the amount of endonuclease capable of converting 1 μg of covalently closed supercoiled plasmid DNA to open circular or linear isoforms in 1 h at 37°C. The protein was measured using the BCA protein assay (Pierce, Rockford, IL). BSA was used as a standard. Zymogram gel electrophoresis of total protein extracts was performed as described by us previously (17).

**Statistical Analyses**

Statistical analysis was performed with ANOVA and t test. Results were expressed as mean ± SEM. P < 0.05 was considered significant.

**Results**

**Characterization of the Models**

To determine whether DNase I becomes important during kidney tissue injury, we started with an in vitro approach, considering that a significant effect of cisplatin can be observed at the cellular level (1). In the kidney, the preferential damage by cisplatin is located in tubular epithelial cells (2,3). To have an in vitro model for studying the role of DNase I in cisplatin renal injury, we used primary tubular epithelial (PTE) cells prepared as described by Nowak et al. (23). We did not observe any difference in morphology or viability between nontreated DNase1−/− and DNase1+/+ PTE cells.

Zymogram gel electrophoresis of total protein extracts obtained from mouse kidneys or PTE cells showed that the 34-kD activity band corresponding to DNase I is absent in KO mice (Figure 1A). We measured the endonuclease activities of total protein extracts from PTE cells and mouse kidneys using the plasmid incision assay under conditions that, according to our previous studies (17,18), are sufficient for all known cell death endonucleases. The activity in PTE cell extracts was 25 ± 3 units/μg protein in WT cells and 5 ± 2 units/μg protein in KO cells (Figure 1B). The endonuclease activity of the total kidney was 105 ± 10 units/μg protein in WT mice and 15 ± 2 units/μg protein in KO mice (Figure 1B). We did not observe an activation of any known endonuclease in kidneys or PTE cells from KO mice or an overexpression of any known endonuclease mRNA measured using reverse transcriptase-PCR (data not shown). Taken together, these data suggested that DNase I represents the majority (approximately 80%) of the total Ca/Mg-dependent endonuclease activity both in the kidney

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**Figure 1.** Endonuclease deficiency in deoxyribonuclease I-deficient (DNase I−/−) primary tubular epithelial (PTE) cells and animals. (A) Zymogram gel electrophoresis of DNase I in total mouse kidney or PTE cell protein extracts performed as described previously (17). (B) Although specific endonuclease activity measured by plasmid incision assay is lower in PTE cells, the total endonuclease activity is decreased approximately 5 times in kidney extracts and PTE cells from knockout (KO) versus wild-type (WT) mice (the number of independent cell culture preparations, n = 6 in each cohort). *P < 0.01; **P < 0.001.
and in the PTE cells, and, thus, PTE cells can be used as a cellular model to study the role of DNase I in vitro.

Protection of DNase I–Deficient PTE Cells against Cisplatin Injury In Vitro

To determine the role of DNase I in cisplatin injury in vitro, we treated PTE cells with 8 μM cisplatin for 96 h. The treatment with this concentration of cisplatin was shown previously to induce apoptosis in renal tubular epithelial cells in vitro (27). Our experiments demonstrated that cisplatin induces irreversible PTE cell death measured by LDH release, whereas PTE cells isolated from KO mice showed a significant resistance to cisplatin injury. Cisplatin treatment induced LDH release from 9.5 ± 1.1% in nontreated controls to 19.4 ± 1.0% in WT cells (P < 0.05) and from 10.1 ± 0.3% in controls to 11.2 ± 0.9% of total LDH in KO cells (in each group, n = 6; Figure 2A). These data suggest that DNase I is essential for the direct cisplatin cytotoxicity to the kidney tubular epithelium cells in vitro. We measured caspase-3 activation in DNase I KO and WT cells after 8 μM cisplatin injury. These experiments showed that active caspase-3 amount normalized by β-actin was not significantly different between DNase I WT and KO cells before impact (WT, 0.82 ± 0.04; KO, 0.78 ± 0.02) or after cisplatin treatment (WT, 1.11 ± 0.05; KO 1.05 ± 0.03; mean ± SEM, n = 4 in each group). Thus, after cisplatin exposure, caspase-3 was almost equally and significantly activated in both types of cells (P < 0.001 in each group). These data suggest that even after caspase-3 activation, DNase I is required for cell death in this model and the contribution of DNase I as an upstream event to caspase-3 is negligible.

Decreased Mortality of DNase I–Deficient Mice after Cisplatin Ingestion

A dose-dependent nephrotoxicity can be observed after a single injection of cisplatin (4). In mice, cisplatin injury is usually associated with tubular necrosis and kidney failure starting from day 2 and reaching maximum at days 3 to 4, depending on the dose of the cisplatin (28,29). To determine the role of DNase I in kidney injury by cisplatin in vivo, we injected a single dose of cisplatin (20 mg/kg) into KO and WT mice intraperitoneally and monitored mortality, kidney failure, and tubular necrosis for 4 d. In our experiments, the animal deaths started at day 3. Our data showed that the mortality of KO mice at days 3 to 4 after cisplatin injection was markedly reduced compared with WT mice. It was 11.1% in the KO mice (n = 18) versus 38.1% in the WT mice (n = 21; Figure 2B). We did not observe animal deaths in the control groups of WT and KO mice that received an injection of saline (n = 10 in each group). There was no mortality observed after day 4.

Protection of Kidney Function in DNase I–Deficient Mice during Cisplatin Injury

We assessed kidney function by blood urea nitrogen (BUN) and creatinine measured in blood serum at days 0, 2, 4, and 8 after the cisplatin injection. Neither functional parameters was different between the two genotypes before the experiment or significantly changed 2 d after cisplatin injection. However at day 4, surviving WT mice showed marked kidney failure both by serum BUN and creatinine, which increased up to 250 ± 28 and 2.7 ± 0.5 mg/dl, respectively (Figure 3). DNase I KO mice showed significant protection of kidney function: The BUN was 125 ± 29 mg/dl and the creatinine decreased to 0.9 ± 0.2 mg/dl (n = 10 in each group, P < 0.05 versus WT mice for both BUN and creatinine). Kidney function normalized at day 8 in both genotypes.

Protection of Kidney Structure in DNase I–Deficient Mice during Cisplatin Injury

To determine the morphologic changes of the kidney after cisplatin treatment, we performed histologic analyses of kidneys that were isolated from animals that survived to determine tubular necrosis using established histologic criteria (25). This analysis showed the profound renal tubular necrosis in WT mice at day 4 (Figure 4). The necrosis was associated with

Figure 2. Correlation of animal mortality and PTE cell death induced by cisplatin with the inactivation of DNase I. (A) WT and KO PTE cell death after 96 h of exposure with 8 μM cisplatin measured by lactate dehydrogenase (LDH) release into media. (B) Animal mortality at days 3 to 4 after 20 mg/kg cisplatin injection. *P < 0.05.

Figure 3. Protection of kidney function in DNase I KO mice after cisplatin injection. We measured blood urea nitrogen and creatinine in blood serum collected from mice that survived after a single-dose intraperitoneal injection of 20 mg/kg cisplatin. *P < 0.05 versus WT.
tubular dilation, accumulation of casts, loss of tubular epithelium, and interstitial inflammation visualized by leukocyte infiltration. Often, tubular basement membranes were ruptured and necrotic epithelial cells were present in the tubular lumen. Histologic analysis of kidneys that were isolated from KO survivors showed that these mice had almost no tubular necrosis. To quantify the kidney injury, we measured the density of tubular cells by a manual count of the tubular cell nuclei. It was shown to be significantly decreased in KO mice compared with the WT mice (Figure 5).

**Attenuation of DNA Fragmentation in DNase I–Deficient Mice during Cisplatin Injury**

We measured DNA fragmentation using the TUNEL assay, which allows detection of 3’OH DNA ends commonly associated with endonuclease-mediated DNA fragmentation. The degree of DNA destruction in WT mice 4 d after cisplatin injection was high and very heterogeneous throughout the tissue. Importantly, TUNEL staining was strongly suppressed in KO mice compared with WT mice (Figure 6). In WT mice, nuclei counterstaining with DAPI showed a decreased number of nuclei after cisplatin injury. Some nuclei looked partially deformed. It is interesting that we observed a small number of apoptotic bodies in WT mice that were treated with cisplatin. No apoptotic bodies were found in the cisplatin-treated KO mice.

**Discussion**

The degradation of cellular DNA by endonucleases is an important component of renal tubular epithelial cell death induced by ischemia or nephrotoxins. Several studies showed activation of endonuclease(s) after cisplatin injury in kidney cells, which was visualized by an increased 200-bp ladder DNA fragmentation (6,7,30,31) or TUNEL (8–10). Our previous studies indicated that endonucleases are important executioners of renal cell death mechanisms induced by a variety of stimuli, including hypoxia/reoxygenation in vitro (32), toxic injury in vitro (33), and renal ischemia (34–36).
vivo (33), and ischemia/reperfusion of the kidney in vivo (17). The inhibition of renal endonucleases by aurintricarboxylic acid, a pan-endonuclease inhibitor (34), or suppression of DNase I using antisense was protective against DNA fragmentation and cell death in vitro (17). Takeda et al. (7) also showed that the cisplatin-induced fragmentation of DNA in renal S3 cells can be inhibited by aurintricarboxylic acid. These data demonstrated a cause–effect relationship among endonuclease activation, DNA fragmentation, and renal cell death. However, the endonuclease responsible for DNA fragmentation in cisplatin-induced renal injury in vitro or in vivo has not been identified.

The mechanism of the cisplatin-induced cytotoxicity is not fully understood. It was shown that cisplatin is capable of binding to several cellular components, including membrane phospholipids, thiols, cytoskeletal microfilaments, proteins, RNA and DNA (5). Apparently, this may involve more than one mechanism of cell death. Indeed, both apoptosis and necrosis were observed in cells treated with cisplatin (6,27). These studies revealed that low doses of cisplatin induce apoptosis, while high doses lead to necrosis (6,27). It was demonstrated recently that apoptosis induced by cisplatin in cultured renal tubular epithelial cells and epithelial cells of other origins is to a significant degree independent from p53 and caspases 3, 8, and 9 (10,35). Because DNA fragmentation is observed in all of these models, it can be suggested that apoptosis and necrosis pathways, as well as caspase-dependent and caspase-independent pathways, are likely to be shared at the level of cell death endonucleases (2). Our measurements of caspase-3 activation indicated that during low-dose cisplatin injury in vitro, DNase I does not use the caspase-3-mediated apoptosis pathway. However, it is still possible that DNase I may be regulated by caspase-3.

Cell death endonucleases are a recently recognized group of enzymes that include cytoplasmic DNase I and caspase-activated DNase (16,36), mitochondrial EndoG (37), lysosomal DNase II (38), and perinuclear DNase γ (39). In the kidney, DNase I is highly expressed (18,40). We showed recently that among other endonucleases, EndoG and DNase II are also highly expressed in the kidney, whereas the expression of caspase-activated DNase and DNase γ is very low (17).

Our data are the direct evidence that DNase I is important for both in vitro and in vivo cisplatin-induced renal cell death, and it mediates cisplatin-induced DNA fragmentation, tubular necrosis, and possibly tubular apoptosis. Unlike in our study of kidney ischemia-reperfusion injury (32), cisplatin nephrotoxicity was not associated with an activation of DNase I (data not shown). This can be explained by the fact that DNase I is a bivalent cation-dependent endonuclease that is inhibited directly by heavy metals such as platinum (15). The comparison of these two models may also suggest that the activation of DNase I after impact is not as important as its presence at the moment of injury and the availability of nuclear DNA for fragmentation. DNase I does not have known nuclear localization signals; however, the nuclear translocation of DNase I had been previously demonstrated (16). This translocation may be promoted by nuclear membrane damage, which was shown to be induced by cisplatin (41).

The use of DNase I KO mice allowed the determination of the endonuclease activity produced by DNase I in the kidney and the identification of its role in cisplatin injury using the cause–effect approach. The DNase I–mediated kidney cell death was evident by both functional and morphologic criteria. Although our data did not determine whether all DNA fragmentation was produced by DNase I alone or was only guided by DNase I, it demonstrated that inactivation of DNase I is protective against cisplatin-induced DNA fragmentation, whereas other endonucleases participated in DNA destruction. DNase I was previously described as an endonuclease that participates in apoptosis and is capable of cooperating with EndoG (16,37). Further studies will be necessary to determine whether DNase I induces renal tubular necrosis directly or through apoptosis and whether it functions in cooperation with other endonucleases.

Cell death endonucleases act both premortem, leading to cell death, and postmortem, providing a “clean-up” after cell death (11). Importantly, our study showed that DNase I acts at the premortem level because its inactivation caused protection of the kidney. This is an encouraging result for potential applications of DNase I inactivation in future therapeutic strategies to protect the kidney against toxic acute renal failure.

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