Stimulatory Effect of Cisplatin on Production of Lipid Peroxidation in Renal Tissues

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Abstract—Cisplatin (cis-diamminedichloroplatinum II), an anticancer chemotherapeutic agent with the dose-limiting side effect of nephrotoxicity, caused a statistically significant increase in lipid peroxidation, monitored by measuring the production of malondialdehyde, in rat kidney 72 hr after injection. Treatment of rats beforehand with the antioxidant α-tocopherol or N-N'-diphenyl-p-phenylenediamine (DPPD) effectively decreased such peroxidation. DPPD was a more effective inhibitor than α-tocopherol, since it is known for its ability to scavenge free radicals more powerfully. The ability of renal cortical slices to accumulate p-aminohippurate (PAH) was examined as a biochemical parameter that would change in nephrotoxicity. The ability to accumulate PAH by the incubated slices decreased 72 hr after administration of cisplatin. The pretreatment with DPPD prevented the decrease in PAH accumulation in the slices from rats treated with cisplatin. Structural changes of the renal proximal tubule caused by cisplatin, analyzed in a transmission electron microscope, were also prevented by the pretreatment with DPPD. The results suggest that cisplatin affects renal tissues in which free radicals generated by cisplatin may interact with membrane lipids to cause the production of lipid peroxidation, which affects both cellular structure and function.

cis-Diamminedichloroplatinum II (cisplatin) is a potent anticancer chemotherapeutic agent effective in the treatment of cancers of the testis, ovary, head and neck, and elsewhere. Unfortunately, it has dose-limiting toxicity, the main effect being renal dysfunction caused by an unknown mechanism. McGinness et al. (1) found that Orgotein (having superoxide dismutase activity) ameliorates the nephrotoxicity of cisplatin. They concluded that the production of oxygen radicals is responsible for this nephrotoxicity. We have tried to evaluate further the importance of reactive free radicals in the nephrotoxicity of cisplatin (2). The increase in blood urea nitrogen and decrease in body weight caused by injection of cisplatin into rats are modified when an antioxidant, α-tocopherol or N-N'-diphenyl-p-phenylenediamine (DPPD), is given before the cisplatin injection. These results suggest that the toxic effect of cisplatin may be related to damage caused by free radicals. Abnormal levels of reactive oxygen radicals react with membrane lipids and cause an increase in lipid peroxidation. Uncontrolled lipid peroxidation can profoundly affect both membrane structure and function, and this may cause cell death (3, 4). The relationship of lipid peroxidation to the nephrotoxicity of cisplatin is not understood.

Here, experiments were designed to detect the production of lipid peroxidation in the kidney of rats treated with cisplatin. We also wished to see if an antioxidant such as DPPD or α-tocopherol, which are nonspecific free radical scavengers, would affect the production of lipid peroxidation and biochemical parameters.
is caused by cisplatin.

Materials and Methods

Animals: The animals used were male Sprague-Dawley rats. They were fed standard chow and given free access to water until they were killed. Their weight at the start of the experiment was 200 g on an average.

Treatment: The rats were injected i.p. with 5.0 or 7.5 mg of cisplatin per kilogram of body weight. A dose of 0.5 g/kg DPPD in corn oil, or else a dose of 1667 IU/kg α-tocopherol in 0.1% Tween 80, was given i.p. 24 hr before the injection of cisplatin in the experiment with antioxidant. Control rats received saline injections. At 24, 48, 72, 96 or 120 hr after the injection of cisplatin, the animals were anesthetized with sodium pentobarbital (30 mg/kg, i.p.). Both kidneys were rapidly removed and decapsulated in ice-cold saline (0.9% NaCl). The kidneys were homogenized in a solution containing 0.15 M KCl and 20 mM Tris-HCl (pH 7.4) for measurement of lipid peroxidation. Renal protein was measured by the method of Lowry et al. (5) using bovine serum albumin as the standard. On day 3 after cisplatin treatment, urine was collected over a three-hour period for the determination of platinum (Pt) content by atomic absorption spectrophotometry using a Shimadzu AA-670 spectrophotometer. Thereafter, plasma and renal tissue samples were collected from the anesthetized animals. Pt content in the plasma and kidney, which was digested in 61% HNO₃ at room temperature overnight, was also analyzed.

Measurement of lipid peroxidation in kidney: Lipid peroxidation in homogenates of renal tissues was monitored by measuring the production of malondialdehyde using the thiobarbituric acid assay. The determination procedure was essentially the same as the method of Buege and Aust (6).

Determination of p-aminohippurate (PAH) accumulation in renal cortical slices: The ability of slices to accumulate PAH was determined as a biochemical parameter that would change in nephrotoxicity. At 72 hr after cisplatin treatment, renal cortical slices were prepared free-hand with a razorblade on an ice-cold Petri-dish. The slices, weighing about 150 mg, were incubated at 37°C for 30 min in 10 ml of incubation medium containing 134 mM NaCl, 5.9 mM KCl, 1.5 mM CaCl₂, 1.2 mM MgCl₂, 11.5 mM glucose, 5.8 mM N,N′-hydroxyethylylperazine-N′-2-ethanesulfonic acid buffer (pH 7.4), 0.074 mM PAH and 1% inulin which was added to estimate the extracellular space of the slices. After incubation, the PAH and inulin levels in the slices and medium were analyzed by the methods of Bratton and Marshall (7) and Roe et al. (8), respectively. PAH accumulation was calculated as the ratio of concentration of PAH in the intracellular fluid (S) to that in the medium (M), the S/M ratio. The experimental methods of the slice-study were essentially the same as those already described (9).

Electron microscopy: Renal cortical tissue was fixed immediately in 2.5% glutaraldehyde in phosphate buffer, then postfixed with osmium tetroxide. The tissue was serially dehydrated in ethanol, infiltrated with propylene oxide, and mounted in Epon. Thin sections were stained with uranylacetate and lead nitrate, and analyzed in a Hitachi H-600 transmission electron microscope.

Chemicals and statistics: Cisplatin and α-tocopherol were purchased from the Sigma Chemical Co. (St. Louis, MO). DPPD was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Other chemicals were of the highest purity available from commercial sources.

Data are expressed as means±S.E. Statistical analysis was done by analysis of variance. Significant differences at the level of P<0.05 were determined among multiple comparisons with Dunnett’s test (10).

Results

Kidneys of rats injected with cisplatin (5 mg/kg) had a time-dependent increase in lipid peroxidation (Fig. 1). Up to 48 hr after the injection of cisplatin, the malondialdehyde level did not increase, but it was progressively increasing at 72 hr. Since vitamin E is an antioxidant and radical scavenger (11–13), our experiment was to determine if α-tocopherol, one of the four tocopherols which constitute vitamin E, could prevent cisplatin induced lipid peroxidation. Treatment
of rats with α-tocopherol before the cisplatin injection delayed the increase in lipid peroxidation until almost 120 hr after the injection (Fig. 1).

The α-tocopherol did not completely prevent lipid peroxidation, but merely delayed it, so we tried the effect of DPPD, a more powerful free-radical scavenger (14, 15). Rats given DPPD before the injection of cisplatin (5 mg/kg) produced no significant change in the level of malondialdehyde in the kidney compared to the level at time zero (Fig. 1). Neither α-tocopherol or DPPD alone affected the malondialdehyde level in the kidney (data not shown).

Our previous study showed that administration of cisplatin developed marked nephrotoxicity on day 3, with an increase in plasma urea nitrogen, and the nephrotoxicity was attenuated by DPPD pretreatment (2). Renal high Pt content, which was accumulated 6 hr after i.p. injection of cisplatin, decreased thereafter at a relatively rapid rate, but the rate of decrease in the Pt content in the kidney was relatively slow from 48 hr over several days in rats and mice (16–18). Then, we examined whether the antioxidant, given 24 hr before the injection of cisplatin, could have the effect on Pt distribution 3 days after cisplatin injection. The Pt content in renal cortical tissue and plasma and urinary Pt

![Fig. 1. Changes in renal malondialdehyde level caused by cisplatin in rats treated with α-tocopherol (●), N-N'-diphenyl-p-phenylenediamine (DPPD, △) or not (○). α-Tocopherol (1867 IU/kg) or DPPD (0.5 g/kg) was given i.p. 24 hr before the injection of cisplatin (5 mg/kg, i.p.). Data are the means±S.E. of six experiments. *P<0.05 vs. the control value at time zero. **P<0.01 vs. the control value at time zero. $P<0.05 vs. the intoxicated group (cisplatin alone, ○) at the respective time. 8P<0.01 vs. the intoxicated group (cisplatin alone, ○).

Table 1. Effect of N-N'-diphenyl-p-phenylenediamine (DPPD) on platinum (Pt) distribution

| Treatment       | Urinary Pt excretion (μg/100 g/3 hr) | Kidney cortical Pt (μg/g kidney) | Plasma Pt (μg/ml) |
|-----------------|-------------------------------------|---------------------------------|------------------|
| Cisplatin       | 0.285±0.11                          | 5.35±0.33                       | 0.09±0.02        |
| DPPD+Cisplatin  | 0.286±0.06                          | 4.26±0.59                       | 0.08±0.03        |

N-N'-Diphenyl-p-phenylenediamine (0.5 g/kg) was given i.p. 24 hr before the injection of cisplatin (7.5 mg/kg). Data are expressed as the mean±S.E. of eight experiments. There was no significant effect of DPPD on platinum distribution.

Table 2. Effect of DPPD on cisplatin-induced decrease of PAH accumulation in incubated slices

| Treatment                  | PAH accumulation (S/M) |
|----------------------------|------------------------|
| None                       | 12.59±0.94             |
| Cisplatin alone            | 8.74±0.40*             |
| DPPD+Cisplatin after 24 hr | 10.81±0.59             |
| DPPD alone                 | 12.92±0.65             |

N-N'-Diphenyl-p-phenylenediamine (DPPD, 0.5 g/kg) was given 24 hr before the injection of cisplatin (7.5 mg/kg). Kidney cortical slices were prepared from rats treated 72 hr after the injection of cisplatin and incubated at 37°C for 30 min. Data are expressed as the mean±S.E. of four to six experiments. *P<0.01 vs. "None". #P<0.05 vs. "Cisplatin alone".
excretion were not affected by DPPD pre-treatment.

Table 2 shows that injection of cisplatin (7.5 mg/kg) significantly decreased the capacity of the incubated slices to accumulate PAH. Pretreatment with DPPD prevented the decrease. DPPD alone had no effect on the capacity to accumulate PAH by the slices.

Transmission electron microscopy was used to examine the effect of DPPD pre-treatment on structural changes induced by the administration of cisplatin. After 3 days of cisplatin treatment (7.5 mg/kg), proximal tubular cells of kidney showed numerous cytoplasmic vacuoles, rounded mitochondria with disordered cristae, disarrangement of basal infoldings and dissociation of mitochondria from basal infoldings (Fig. 2B) as compared to the electron micrograph of proximal tubular cells from a control rat (Fig. 2A). These observations induced by the drug were almost consistent with those reported by Dobyan et al. (19). These structural changes caused by cisplatin were prevented by the pretreatment with DPPD (Fig. 2C). DPPD alone had no effect on the structure of the cells (data not shown).

Discussion

The nephrotoxicity of cisplatin is alleviated by Orgotein, which contains superoxide dismutase (1) and by antioxidants such as α-tocopherol and DPPD (2), which suggests that pathologic levels of oxygen radicals initiate lipid peroxidation. Because of its simplicity, lipid peroxidation was monitored using the level of malondialdehyde. Results of this study showed that the renal level of malondialdehyde had started to increase 72 hr after cisplatin was injected into the rats. This indicates that renal lipid peroxide and blood urea nitrogen (2) increased at the same time, 72 hr after cisplatin administration. We investigated whether the renal malondialdehyde level could be reduced by antioxidants and found that α-tocopherol given beforehand delayed the onset of the increase in renal lipid peroxides caused by cisplatin. DPPD, a powerful free radical scavenger, completely prevented the increase in the lipid peroxidation caused by the drug without affecting Pt distribution, i.e., Pt content in the plasma, kidney cortex and urine 3 days after cisplatin injection. These results provide.
indirect evidence that cisplatin generates free radicals that interact with membrane lipids and consequently cause production of lipid peroxides. There is no direct evidence that lipid peroxidation plays a causative role in the development of cisplatin-induced nephrotoxicity. However, if the increase in lipid peroxidation were a result of cisplatin-induced nephrotoxicity rather than a cause of the nephrotoxicity, antioxidants such as α-tocopherol and DPPD would not inhibit both increases in lipid peroxidation and urea nitrogen, but only the former. In fact, antioxidants inhibited both.

Biochemical evidence was presented in the present study to demonstrate that administration of DPPD actually modified a parameter that changes in nephrotoxicity, that is, DPPD pretreatment prevented the decrease in PAH accumulation in renal cortical slices from rats treated with cisplatin. Goldstein et al. (20) described that PAH accumulation in renal cortical slices from F-344 rats given cisplatin i.v. in the dose of 5 mg/kg did not significantly decrease in comparison with that in the slices from control rats. We do not know whether the difference in our results came from the difference in the strain of rats used or from the different dose. The in vitro technique utilizing renal cortical slices for measurement of PAH accumulation is one of the methods used to study the secretory mechanism for PAH as the prototype for organic acids. Therefore, it appears that the decrease in the capacity to accumulate PAH by the slices from rats treated with cisplatin in the present study probably reflects the decrease in renal clearance and excretion of PAH induced by administration of cisplatin to experimental animals as reported already (20–22). In addition, histological evidence was also presented to demonstrate that DPPD pretreatment actually prevented the ultrastructural changes in renal proximal tubular cells of rats treated with cisplatin.

Fujita (23) demonstrated that cisplatin was found to elevate urinary activities of enzymes, N-acetyl-beta-glucosaminidase which is rich in lysosomes and alanine aminopeptidase in brush border membranes of renal cells, in patients. The possibility that membrane lipid peroxidation has a role as a primary event in the toxicity of chemicals has been suggested in recent years. The peroxidation of membrane lipids in lysosomes and brush borders probably results in enzyme leakage in tubules. Urinary enzyme activity was not monitored in the present study. Biochemical and histological evidence in the present study showed that DPPD prevented renal proximal tubular damage induced by cisplatin. So, we think that the antioxidant DPPD may have a preventive effect on the alterations in urinary enzyme activity by coping with the drug-induced membrane lipid peroxidation.

Kuo et al. suggested that lipid peroxidation in cephaloridine-induced nephrotoxicity is initiated by superoxide radicals produced through redox cycling of the antibiotic in renal microsomes (24, 25). Increased formation of lipid peroxides in kidneys of rats poisoned with mercuric chloride may be due at least in part to decreased activity of glutathione peroxidase (26). It is still unknown whether the mechanism involved in cisplatin nephrotoxicity is in accord with either of the above concepts or not. Glutathione protects proteins and cell membranes against free radicals and peroxides; hydrogen peroxides and organic peroxides are metabolized by glutathione peroxidase. Studies on the effect of cisplatin on glutathione metabolism are in progress in our laboratory.

The present study shows that the antioxidants, which attenuated cisplatin nephrotoxicity (2), protected against the increase in lipid peroxidation and the biochemical and histological changes induced by cisplatin. We suggest that the nephrotoxic effect of cisplatin is associated at least in part with lipid peroxidation mediated by free radicals.

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