The Effect of Antioxidant on Development of Fibrosis by Cisplatin in Rats

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Abstract. Cisplatin causes chronic interstitial disease with fibrosis, but the development mechanism of interstitial fibrosis is not yet understood. We examined the effect of an antioxidant, N,N’-diphenyl-1,4-phenylenediamine (DPPD), on development of interstitial fibrosis induced by cisplatin. Cisplatin increased blood urea nitrogen (BUN), plasma creatinine, and elicited glucosuria and enzymuria at 3 days after administration, but these changes were restored to the normal level after 14 days. Type III collagen increased from 7 days after administration of cisplatin and the expansion of the interstitial fibrosis area became evident at 14 days. Sustained renal fibrosis worsened renal function again at 56 days. Administration of DPPD, which was started at 3 days after cisplatin treatment, significantly inhibited the increase in renal type III collagen contents and the expansion of the interstitial fibrosis area without affecting enzymuria and increased BUN. These results indicate that anti-fibrotic action of DPPD is not secondary due to the inhibition of acute renal injury but is rather a direct effect on renal fibrogenesis. DPPD did not prevent the infiltration of macrophages by cisplatin, suggesting that anti-fibrotic action of DPPD was not mediated by the inhibition of inflammatory cellular influx. It is suggested that reactive oxygen species are involved in cisplatin-induced renal interstitial fibrosis.

Keywords: cisplatin, renal injury, macrophage, interstitial fibrosis, oxidative stress

Introduction

cis-Diaminedichloroplatinum (II) (cisplatin) is a potent and an effective antitumor agent for testis, bladder, head and neck, lung, and ovarian cancers. Cisplatin causes severe side effects such as acute renal failure, gastrointestinal toxicity, and ototoxicity. In particular, its clinical use is limited due to the induced acute renal failure with tubular necrosis in the kidney (1, 2). The acute renal failure induced by cisplatin has been well described in animals and humans. Antioxidants or radical scavengers prevented an acute renal failure induced by cisplatin administration to rats (3, 4). In addition, cisplatin increased the lipid peroxidation level in rat renal slices (5) and the synthesis of hydrogen peroxide in cultured renal cells (6). These findings suggest that reactive oxygen species (ROS) play an important role in the pathogenesis of cisplatin-related acute renal failure.

It is known that cisplatin causes chronic interstitial nephritis with interstitial fibrosis in humans (7). In experimental animals, cisplatin causes renal interstitial fibrosis in the long term (8). Interstitial fibrosis is a common lesion in most chronic kidney diseases (9). The development of interstitial fibrosis is thought to cause irreversible renal dysfunction (9). However, a few reports have described the relationship between a renal function and development of fibrosis by cisplatin. Furthermore,
the pathogenic mechanism of interstitial fibrosis and the effect of fibrosis on renal function induced by cisplatin remain to be clarified.

Since ROS played an important role in acute renal failure by cisplatin, we investigated the role of ROS in the development of fibrosis induced by cisplatin in the kidney. We have reported that an antioxidant, \(N,N'-\)diphenyl-1,4-phenylenediamine (DPPD), prevented the increases in content of lipid peroxides and nephrotoxicity induced by cisplatin (10). So, we determined the effect of DPPD on the development of fibrosis induced by cisplatin in rats. In this case, interstitial fibrosis may be suppressed merely due to the inhibition of cisplatin-induced acute renal injury by the antioxidant. To eliminate an effect of antioxidant on acute renal injury in the progression of fibrosis by cisplatin, we administered an antioxidant to the rats at a time that does not influence the acute renal injury induced by cisplatin.

**Materials and Methods**

*Animals and drugs*

All experimental procedures were performed according to the Guide to the Care and Use of Laboratory Animals of Osaka University of Pharmaceutical Sciences. Male Sprague-Dawley rats weighing 170 – 220 g were purchased from Japan SLC (Shizuoka). These rats were kept in a room maintained at 24 ± 1°C and humidity of 55 ± 10%, with a 12-h light/dark cycle and had free access to rat chow and tap water. Cisplatin (Sigma, St. Louis, MO, USA) was dissolved in saline (2.5 mg/mL). The antioxidant \(N,N'-\)diphenyl-1,4-phenylene-diammine (DPPD; Tokyo Kasei, Tokyo) was suspended in corn oil (0.125 mg/mL).

*Experimental protocols*

**Time course of cisplatin nephrotoxicity:** In order to examine the time course effects of cisplatin on renal function and renal histology, rats received an intravenous bolus injection of either cisplatin (7.5 mg/kg) or saline. At 3, 7, 14, 28, and 56 days after cisplatin or saline administration, rats were housed in metabolic cages and 18-h urine collection was made. Thereafter, blood samples were obtained and kidneys were removed under anesthesia (pentobarbital, 50 mg/kg, i.p.).

**Effects of DPPD treatment on cisplatin nephrotoxicity:** We have shown that the antioxidative effect of single injection of DPPD (0.5 g/kg, i.p.) on the increases in lipid peroxides continued for 4 days after treatment with cisplatin (10). Therefore, we administered the same dose of DPPD every two days to rats. The rats were divided into four groups: 1) Cisplatin: Rats received cisplatin (7.5 mg/kg, i.v.) at the start of experiments; 2) Cisplatin + DPPD: Three days after cisplatin administration (7.5 mg/kg, i.v.), rats were given DPPD (0.5 g/kg, i.p.) every two days; 3) Saline: Rats received intravenous saline instead of cisplatin and served as the control; and 4) Saline + DPPD: Three days after intravenous saline, rats were given DPPD (0.5 g/kg, i.p.) every two days. Rats in groups 1 and 3 received corn oil instead of DPPD. At 14 days after cisplatin (or saline) administration, urine and blood samples were obtained and kidneys were removed.

**Kidney functions**

Plasma creatinine, blood urea nitrogen (BUN), and urinary glucose and N-acetyl-β-d-glucosaminidase (NAG) concentrations were measured by standard laboratory methods.

**Determination of type III collagen, α-SMA, and 4-hydroxy-2-nonenal (4-HNE) by Western-blotting analysis**

Protein levels were determined by Western-blotting analysis as previously described (11). The protein samples were separated on a 10% SDS-polyacrylamide gel and then transferred to a PVDF membrane. Immunoblots were performed with primary antibody; mouse monoclonal anti α-SMA (Sigma), mouse monoclonal anti-type III collagen (Sigma), mouse monoclonal anti-4-HNE (Japan Institute for the Control of Aging, NIKKEN SEIL, Shizuoka), and mouse monoclonal anti-β-actin (Sigma). After washing, the membranes were reacted with a secondary antibody, horseradish peroxidase–conjugated goat anti-mouse IgG and detected by the chemiluminescence of ECL (GE Healthcare UK, Ltd., Buckinghamshire, UK). Chemiluminescence was quantified by densitometric scanning (LAS-3000; Fuji Film, Tokyo).

**Histological examination**

The kidney was fixed in 10% neutral-buffered formalin solution and embedded in paraffin. The paraffin-embedded kidney tissues were sectioned at a thickness of 4 μm. The sections were stained with Masson’s trichrome to determine interstitial fibrosis. We determined the degree of the interstitial fibrosis area in the outer medulla of the kidney using image analysis software Scion Image (NIH) (12). Immunohistochemical examination was performed as described before (13). Briefly, myofibroblasts were immunostained with anti-α-SMA antibody (Sigma). Color development was performed with Fast Red (Sigma). Macrophage infiltration was identified by staining ED-1–positive cells (AbD Serotec, Oxford, UK) using Histofine Simple Stain Rat MAX PO (Nichirei Biosciences, Tokyo). We counted ED-1–positive cells and evaluated the cell count per
observation field area.

Statistical analyses
We used Sheffe’s F-test for multiple comparison calibration after one-way layout analysis of variance (one-way ANOVA) when comparing more than three groups.

Results
We investigated the time course effects of cisplatin on renal function. Renal function was evaluated using BUN, plasma creatinine, and urinary excretions of NAG and glucose as parameters of renal injury after cisplatin treatment (Fig. 1). BUN and plasma creatinine increased from 3 days after treatment with cisplatin and peaked at the 7 days administration. Increases in BUN and plasma creatinine induced by cisplatin returned to normal levels at the 14 days administration. BUN and plasma creatinine rose again at 56 days after cisplatin (Fig. 1: A and B). Urinary excretion of glucose and NAG as indices of injury to proximal tubules increased 3 days after treatment with cisplatin and returned to the normal level after 14 days (Fig. 1: C and D).

We examined the influence of tubulointerstitial fibrosis by changes in contents of type III collagen and α-SMA using Western-blottting analysis (Figs. 2: A and B) and by Masson’s trichrome staining (Fig. 3). Cisplatin increased the renal content of type III collagen and α-SMA from 7 days, and they remained elevated even at 56 days after single injection of cisplatin (Fig. 2: A and B). In addition, cisplatin significantly enlarged the fibrosis area in the tubular interstitium from 14 days after single injection of cisplatin. The fibrosis area was not reduced even at 56 days after treatment, similar to type III collagen contents (Fig. 3). Histological examination revealed that renal tubules were remarkably dilated in the corticomedullary junction of the kidney.

Next, we examined the expression site of α-SMA in the kidney caused by cisplatin using immunohistochemistry. In a normal kidney, α-SMA was confirmed in vascular smooth muscle cells. The administration of cisplatin induced the expression of α-SMA in the tubular interstitium (Fig. 2: C and D). This expression site of α-SMA was consistent with the fibrosis area induced by cisplatin.

To determine the involvement of ROS in the development of cisplatin-induced fibrosis, we measured the change in the content of 4-HNE modified protein, an index of oxidative stress, in the kidney tissues (Fig. 4).

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![Fig. 1](image1.png)

**Fig. 1.** Time course of changes in plasma creatinine (A), BUN (B), and urinary excretion of glucose (C) and NAG (D) after treatment with cisplatin (CDDP, 7.5 mg/kg, i.v.) in rats. Data are presented as the mean ± S.E.M., *P<0.05, **P<0.01 vs. control. N = 3 – 16 for each time point.
Renal tissue content of 4-HNE modified protein at 65 kDa was elevated by cisplatin treatment. DPPD administration decreased the cisplatin-induced increases in renal 4-HNE modified protein content. DPPD alone did not affect the baseline of 4-HNE modified protein level.

We previously reported that acute renal failure by cisplatin was relieved by an antioxidant (3); therefore, we examined whether ROS were implicated in the development of interstitial fibrosis by cisplatin. We
used the antioxidant DPPD to examine the role of ROS in tubulointerstitial fibrosis induced by cisplatin. Antioxidant administration was started from 3 days after administration of cisplatin when acute renal disease by cisplatin became evident. DPPD administration did not influence BUN increases or NAG excretion in urine induced by cisplatin at 7 days and 14 days after treatment (Fig. 5: A and B). Although DPPD did not affect acute renal injury, it significantly blocked the expansion of interstitial fibrosis area and increase of type III collagen induced by cisplatin (Fig. 6). Furthermore, DPPD significantly inhibited α-SMA overexpression by cisplatin (Fig. 7A).

In contrast, although cisplatin caused the infiltration of macrophages into the tubular interstitium and tubular lumen 14 days after treatment (Fig. 7B), the infiltration of macrophages into the tubular interstitium by cisplatin was unaffected by the antioxidant DPPD.

**Discussion**

Cisplatin caused tubulointerstitial fibrosis at 14 days after single injection of cisplatin at a time when acute renal disease had recovered. The antioxidant inhibited the increases in 4-HNE modified protein level and interstitial fibrosis induced by cisplatin without influencing acute renal disease. The elevated level of 4-HNE modified protein resulted from increased formation of ROS in the kidney (14). Thus, these results indicated that ROS are directly involved in the development of interstitial fibrosis induced by cisplatin.

There are reports that antioxidants such as taurine or extracts from adzuki beans inhibit interstitial fibrosis induced by cisplatin (15, 16). However, in these studies, antioxidants were administered at the time of cisplatin. In our preliminary experiment, an antioxidant administered prior to cisplatin administration prevented both acute renal failure and interstitial fibrosis by cisplatin (data not shown). Thus, it is possible that interstitial fibrosis may be a process of resolution from cisplatin-induced acute renal injury. Accordingly, the anti-fibrotic action of these antioxidants could merely result from the inhibition of acute renal disease. In order to eliminate this possibility, we administered an antioxidant after the onset of acute renal injury in this study. Consequently, the antioxidant inhibited interstitial fibrosis without affecting acute renal injury induced by cisplatin. The treatment with DPPD did not prevent a renal dysfunction.
at 14 days after administration of cisplatin. These data indicated that histological changes, such as interstitial fibrosis, did not bring about influences for renal dysfunction. Our results indicate that ROS is involved in not only acute renal injury but also in the progression of interstitial fibrosis induced by cisplatin.

Although our studies revealed that ROS was involved in the development of renal interstitial fibrosis induced by cisplatin, downstream mechanisms remain to be elucidated. There are a number of reports demonstrating that antioxidants inhibit the kidney disease and fibrosis in experimental models of hypertension, cyclosporine nephrotoxicity, and diabetic nephropathy (17–19). In addition, the participation of ROS in the development of interstitial fibrosis has been reported (20).

Fibrosis occurs at least in part as a result of a chronic inflammatory reaction that is involved in the infiltration of macrophages (21). Activation of NF-κB stimulates transcription of various pro-inflammatory chemokines and elicits inflammatory cell influx. (22). ROS stimulates the infiltration of macrophages into interstitium and the production of profibrotic mediators that are released from macrophages (23, 24). In the unilateral ureteral obstruction, a model of renal interstitial fibrosis, inhibition of macrophage infiltration prevented the increase of renal interstitial fibrosis (25). Our present results showed that the administration of antioxidant from 3 days after single injection of cisplatin did not prevent the infiltration of macrophages. Thus, anti-fibrotic action of the antioxidant, DPPD, is not related to macrophage infiltration. Nevertheless, macrophages that infiltrated into the interstitium can release ROS (26). It is reported that ROS generated by the infiltrated macrophages contribute in the development of renal disease (27). Thus, DPPD may have scavenged macrophage-derived ROS and attenuated ROS-mediated fibrogenetic process, a possibility that must be tested in future studies.

Generally, interstitial fibrosis is a common pathological feature in most chronic kidney disease (9, 28). In the present study, the development of fibrosis persisted even at 56 days and renal dysfunction reappeared again, although it once recovered 14 days after single injection of cisplatin. Similar results have been reported by Yang et al. (29). Sustained fibrosis induced by cisplatin may elicit progressive decline in renal function. Thus, seeking therapeutic options of chronic cisplatin nephrotoxicity may be of value in preventing delayed renal dysfunction.

In summary, the development of interstitial fibrosis induced by cisplatin can lead to the chronic dysfunction
of the kidney. Therapeutic administration of antioxidant inhibited the increment of interstitial fibrosis induced by cisplatin without any influence on acute renal failure. Thus, our present results indicate that an antioxidant may be useful for retarding the progression of renal interstitial fibrosis occurring after acute tubular injury. Further studies are needed to determine the role of ROS in pathogenic and progressive mechanisms leading to the fibrosis induced by cisplatin.

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References