Expressions of Lipid Oxidation Markers, N^ε-Hexanoyl Lysine and Acrolein in Cisplatin-Induced Nephrotoxicity in Rats

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ABSTRACT. The purpose of this study was to evaluate whether N^ε-hexanoyl lysine (N^ε-HEL) and acrolein reflect the severity of cisplatin-induced nephrotoxicity. Immunoreaction of N^ε-HEL and acrolein in kidneys and their urinary concentration were examined up to day 4 post-cisplatin injection in rats. Cisplatin-induced tubular injury was observed histopathologically on days 2–4 after injection and became more severe time-dependently. On days 2–4, N^ε-HEL and acrolein were immunostained in the cytoplasm of damaged tubular cells. Their immunostaining intensity and urinary levels increased as tubular injury became more severe. These results suggest that expressions of N^ε-HEL and acrolein were associated with the pathogenesis of cisplatin-induced nephrotoxicity.

KEY WORDS: acrolein, cisplatin, N^ε-hexanoyl lysine, nephrotoxicity, oxidative damage.

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Cisplatin [*cis*-diamminedichloroplatinum (II)] is a major antineoplastic drug used for the treatment of solid tumors in the testis, ovary, head and neck, and elsewhere in human and veterinary medicine [19, 35]. The most common side effect limiting its clinical use is nephrotoxicity [6, 19, 35, 37]. Cisplatin-induced nephrotoxicity is characterized by proximal tubular injury and decreased glomerular filtration [9, 19]. Cisplatin causes tubular injury through multiple mechanisms, including oxidative stress, DNA damage, apoptosis and inflammation [19, 35].

Cisplatin generates active oxygen species such as superoxide anion and hydroxyl radical [13, 16] and stimulates renal lipid peroxidation [24, 25]. It is known that increased expression of lipid peroxidation biomarkers, malondialdehyde and 4-hydroxy-2-nonenal (4-HNE), is closely associated with the development of cisplatin-induced nephrotoxicity [3, 8, 36]. However, it remains unclear whether N^ε-hexanoyl lysine (N^ε-HEL) and acrolein, are associated with cisplatin-induced nephrotoxicity.

N^ε-HEL and acrolein have recently been developed as novel biomarkers of oxidative stress and have been detected in the development of oxidative stress-related disease in humans [2, 4, 10–12, 23, 26–28, 31, 32] and experimental animal models [7, 17, 18, 20, 30, 34]. N^ε-HEL, which is formed from the reaction between lipid hydroperoxide and a lysine residue, is considered to be one of the stable markers for lipid-peroxidation-derived protein modification [12]. Acrolein is one of the major lipid peroxidation products with cytotoxic and mutagenic activity [33]. Among all α , β -unsaturated aldehydes including acrolein and 4-HNE are by

far the strongest electrophiles and react with nucleophiles, such as the sulfhydryl group of cysteine, imidazole group of histidine and amino group of lysine [5]. Of these, lysine generated the most stable product [33]. Acrolein-lysine adducts can be used as a reliable index of lipid peroxidation *in vivo* [21].

Recently, useful urinary biomarkers for estimating the severity of cisplatin induced nephrotoxicity have been identified, such as kidney injury molecule-1 (KIM-1), alpha-glutathione S-transferase (GST- α), GST- μ [29] and malondialdehyde [36]. However, there has been little study as to whether oxidative stress markers, which are known to be related to cisplatin-induced nephrotoxicity, increase in urine and can predict the development of cisplatin-induced nephrotoxicity.

In the present study, to examine whether the expression of lipid oxidation markers N^ε-HEL and acrolein reflect the severity of cisplatin-induced nephrotoxicity, we investigated the immunohistochemical expression of N^ε-HEL and acrolein in kidney tissue and their urinary concentrations in cisplatin-induced nephrotoxicity in rats until Day 4 following a single injection of cisplatin.

All experiments were performed on 7-week-old, male Wistar strain rats weighing 223.15 ± 5.18 g (mean $\pm$ SE) obtained from the Institute of Animal Reproduction (Ibaraki, Japan). The animals were maintained at a controlled temperature of $22 \pm 2^\circ\text{C}$ with a 12:12-hr light/dark cycle (light cycle, 7:00–19:00), and were given standard chow (CE-2; Nihon Clea, Tokyo, Japan). The use of these animals and the procedures performed on them were approved by the Animal Research Committee at Tottori University.

A total of 30 animals were divided into two different groups as follows: (1) saline-treated control rats (n=6), and (2) cisplatin-treated rats (n=24). Cisplatin-treated rats received cisplatin (5 mg/kg body weight; Nippon Kayaku,

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Tokyo, Japan) by intraperitoneal injection. Blood, urine and kidney samples were collected under pentobarbital anesthesia (100 mg/kg, intraperitoneal injection) at Days 1, 2, 3 and 4 after cisplatin injection (each group; $n=6$). Blood urea nitrogen (BUN) and serum creatinine levels in serum were determined by means of a biochemical autoanalyzer (Dri-Chem 3000; Fuji Film Company, Tokyo, Japan). Urinary N^e-HEL and acrolein concentrations were assayed by ELISA method with commercial kits (Nikken Seil Co., Ltd., Shizuoka, Japan; NOF Corporation, Tokyo, Japan).

Kidney tissues were fixed in 10% neutral buffered formalin, processed for histological examination by conventional methods, and stained with hematoxylin and eosin (HE). The kidney pathology was scored as described by Zhou *et al.* [37]. Tubules ($n=100$) were selected randomly in 25 samples at $\times 400$ magnification and scored for each animal according to the following criteria: 0=normal; 1=areas of tubular epithelial cell, hydropic degeneration, vacuolar degeneration, tubular dilation, necrosis and desquamation involving $<25\%$ of the tubular profile; 2=similar changes involving $\geq 25\%$ but $<50\%$ of the tubular profile; 3=similar changes involving $\geq 50\%$ but $<75\%$ of the tubular profile; 4=similar changes involving $\geq 75\%$ of the tubular profile. Those conducting the histopathological examinations were blinded to the study treatments. This examination was conducted in the corticomedullary area of kidney tissue.

Apoptotic bodies in kidney tissue were detected by terminal deoxynucleotidyl-transferase (TdT)-mediated deoxyuridine triphosphate-digoxigenin (dUTP) nick-end labeling (TUNEL), which was performed using an *in situ* apoptosis detection kit (Trevigen, Inc., Gaithersburg, MD, U.S.A.). A semiquantitative analysis was performed by counting the number of TUNEL-positive cells per field at $\times 400$ magnification as described by Zhou *et al.* [37]. This examination was conducted in the corticomedullary area of kidney tissue.

For immunohistochemistry, the following primary antibodies were used: anti- N^e-HEL mouse monoclonal antibody (Nikken Seil Co., Ltd., Shizuoka, Japan) and anti-acrolein mouse monoclonal antibody (NOF Corporation, Tokyo, Japan). All sections were dewaxed, rehydrated, rinsed with 0.05 M tris-buffered saline (TBS; pH 7.6), treated with 3% hydrogen peroxide, and then rinsed again with TBS. Tissue sections used for the detection of acrolein were immersed in 0.01 M citrate buffer (pH 6.0) and autoclaved for 15 min at 121°C to retrieve antigen. Slides were

incubated with primary antibody (N^e-HEL, 1:75 dilution; acrolein, 1:100 dilution) at 4°C overnight and, after rinsing with TBS, then treated with Simple Stain MAX-PO (M) (Nichirei, Tokyo, Japan) for 30 min at room temperature. They were then rinsed with TBS before being treated with a 3,3'-diaminobenzidine solution containing 0.01% hydrogen peroxide to facilitate a peroxidase color reaction. After a further wash with TBS, the slides were counterstained with Mayer's haematoxylin.

All data are expressed as means $\pm$ SE of all rats in each group. The results in each group were compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test with statistical software (SSRI Co., Ltd., Tokyo, Japan). $P<0.05$ was considered to be statistically significant.

In the initial 1-day period after exposure to cisplatin, BUN and serum creatinine levels remained as low as those of the saline-treated control group (Table 1). BUN and serum creatinine increased at and after Day 2, and reached a maximum level at Day 4 after the dose (Table 1). No histopathological changes were observed in saline-treated control rats and at Day 1 (Figs. 1a, 1b, 2a and 2e). At Days 2 and 3, degenerative changes seen in the straight portion of the proximal tubule consisted of hydropic degeneration, cytoplasmic vacuolization and tubular dilation. These histopathological changes were more severe at Day 3 than Day 2 (Figs. 2b, 2c, 2f and 2g). At Day 4, many pyknotic nuclei were observed in the straight portion of the proximal tubule, and widespread desquamation and necrosis of tubular epithelial cells of the corticomedullary area were noted, predominantly in the straight portion of the proximal tubule (Figs. 1d and 1h). The tubular damage score increased time-dependently at and after Day 2 (Table 1). There was a significant increase in the number of TUNEL-positive cells at Days 3 and 4 (Table 1).

Tubular epithelial cells in saline-treated control rats and cisplatin-treated rats at Day 1 were negative for N^e-HEL or acrolein (Figs. 1c, 1d, 2i and 2m). However, at and after Day 2, cisplatin-treated rats were positive for N^e-HEL and acrolein and their immunostaining intensity increased time-dependently. At Day 2, N^e-HEL and acrolein were faintly immunostained in the cytoplasm of degenerative proximal tubular epithelial cells in the straight portion (Figs. 2j and 2n). At Day 3, the cytoplasm of damaged tubular epithelial cells in the corticomedullary area were moderately positive

Table 1. Time course of renal function and tubular morphology in cisplatin-induced nephrotoxicity

	Control	Day 1	Day 2	Day 3	Day 4
BUN	16.7 $\pm$ 1.02	16.3 $\pm$ 0.74	19.0 $\pm$ 0.86	42.6 $\pm$ 3.73*†‡	85.6 $\pm$ 6.61*†‡§
Serum creatinin	0.13 $\pm$ 0.02	0.15 $\pm$ 0.02	0.23 $\pm$ 0.02	0.55 $\pm$ 0.06	2.21 $\pm$ 0.23*†‡
Tubular damage score	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	1.17 $\pm$ 0.17*†	2.33 $\pm$ 0.21*†‡	3.33 $\pm$ 0.21*†‡§
Number of TUNEL-positive cells / $\times 400$	1.17 $\pm$ 0.48	1.50 $\pm$ 0.72	3.00 $\pm$ 0.82	8.50 $\pm$ 1.06*†‡	11.7 $\pm$ 1.92*†‡§

Values are expressed means $\pm$ SE ($n=6$). *: $P<0.05$, compared with control group, †: $P<0.05$, compared with Day 1 group, ‡: $P<0.05$, compared with Day 2 group, §: $P<0.05$, compared with Day 3 group.

Fig. 1. Histopathological and immunohistochemical findings in kidney of saline-treated control rats. (a) and (b) HE stain. (c) Immunohistochemistry for N^ε-HEL. (d) Immunohistochemistry for acrolein. (a) Bar=500 μ m. (b)–(d) Bar=100 μ m. No histopathological and immunohistochemical changes were observed in kidney of saline-treated control rats.

Fig. 2. Changes of immunohistochemical expression in time course of renal tubular morphology in cisplatin-induced nephrotoxicity. (a)–(d); HE stain, low magnification view, Bar=500 μ m. Tubular injury areas are indicated by arrows. (e)–(h); HE stain, high magnification view, Bar=100 μ m. (i)–(l); Immunohistochemistry for N^ε-HEL, Bar=100 μ m. (m)–(p); Immunohistochemistry for acrolein, Bar=100 μ m. Cisplatin-induced tubular injury was observed histopathologically on Days 2–4 after injection and became more severe time-dependently. On Days 2–4, N^ε-HEL and acrolein were immunostained in the cytoplasm of damaged tubular cells. Their immunostaining intensity showed a tendency to increase as tubular injury became more severe.

Fig. 3. Urinary N^ε-HEL and acrolein concentration in cisplatin-induced nephrotoxicity. Values are expressed means $\pm$ SE (n=6). *: $P < 0.05$, compared with control group, †: $P < 0.05$, compared with Day 1 group.

for N^ε-HEL and acrolein (Figs. 2k and 2o) and they were strongly positive for N^ε-HEL and acrolein at Day 4 (Figs. 2l and 2p). In the initial 2-day period after cisplatin injection, urine levels of N^ε-HEL and acrolein remained as low as those of the control group (Fig. 3). Urinary N^ε-HEL and acrolein levels showed a tendency to increase time-dependently and there was significant increase at Day 4 (Fig. 3).

The major findings of the present study were the following: (1) N^ε-HEL and acrolein immunostained in damaged tubular cells induced cisplatin, and (2) immunostaining intensity of N^ε-HEL and acrolein and their urinary levels increased as renal tubular injury became more severe.

Renal tubular cell death is common histopathological feature of cisplatin nephrotoxicity [19]. In the present study, number of TUNEL-positive cells in the corticomedullary junction area showed a tendency to increase time-dependently as well as immunostaining intensity of N^ε-HEL and acrolein. This result suggested that apoptotic cell death caused by cisplatin related to oxidative damage of lipid. Cisplatin induce the generation of various reactive oxygen species (ROS) through inactivation of cellular antioxidant system, disruption of mitochondrial respiratory chain, or interaction with microsomal cytochrome P450 [19]. By their broad reactive nature, ROS target and modify multiple

molecules in the cells such as lipids, proteins and DNA [19]. ROS induce apoptosis by regulating the expression of various apoptosis regulatory proteins such as bcl-2 and p53 protein, too [1, 14].

N^ε-HEL has been found in the reaction between linoleic hydroperoxide and lysine moiety [11]. The formation of N^ε-HEL has been shown to be a good marker for oxidative modification by oxidized ω -6 fatty acids such as linoleic acid and arachidonic acid [11]. The immunohistochemical evidence of N^ε-HEL presence in atherosclerotic lesions of humans [11] and cholesterol-fed rabbits [7], lipopolysaccharide-induced liver injury in D-galactosamine-sensitized mice [18] and renal proximal tubules in diabetic mice [34] has already been reported. N^ε-HEL concentration significantly increased in hepatic ischemia-reperfusion injury in rats [30] and exercised rat tissues [17]. The urinary N^ε-HEL levels were higher in human patients with diabetes [12] and rheumatoid arthritis [10]. Serum N^ε-HEL level increased in human patients with systemic sclerosis [23] and metabolic syndrome [28] and high-cholesterol diet-fed rats [20].

Acrolein protein adducts have been immunohistochemically detected in traumatic spinal cord injury [15], neurofibrillary tangles in Alzheimer's disease [2] and diabetic glomerular lesions [26] of humans. Urinary levels of acrolein-lysine adduct are elevated in human patients with type 1 and type 2 diabetes [4, 31], atopic dermatitis [32] and severe motor and intellectual disabilities of humans [27].

Cisplatin causes the generation of oxygen free radicals, such as hydrogen peroxide, superoxide anions and hydroxyl radicals [13, 16]. In particular, the hydroxyl radical is highly reactive among oxygen radicals. Once excessive hydroxyl radicals are released, lipid peroxidation, which causes changes in the fluidity and permeability of membranes, is induced [22]. Cisplatin induced nephrotoxicity is closely associated with increased lipid oxidation markers such as malondialdehyde and 4-HNE in kidney tissue [3, 8, 36]. However, it remains unclear whether N^ε-HEL and acrolein, are associated with cisplatin-induced nephrotoxicity. To our knowledge, this is the first report showing an association of N^ε-HEL and acrolein with cisplatin-induced nephrotoxicity.

In conclusion, the immunostaining intensity of N^ε-HEL and acrolein in kidney tissue and their urinary concentration showed a tendency to increase time-dependently after cisplatin injection, and the present changes reflect those of morphological tubular injury and blood biochemical findings of renal function, such as BUN and creatinine. These findings suggest that expression of N^ε-HEL and acrolein in kidney tissue associate closely with pathogenesis of cisplatin-induced nephrotoxicity.

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