Studies on the Renoprotective Effect of Bovine Lactoferrin on Cisplatin-Induced Nephrotoxicity

シスプラチン誘発性腎毒性に対する ウシ由来ラクトフェリンの腎保護効果に関する研究

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CONTENTS

GENERAL INTRODUCTION

Cisplatin (*cis*-diamminedichloroplatinum II) is one of the most effective chemotherapeutic agents used to treat a variety of solid tumors, but it has limited clinical application because it leads to the development of nephrotoxicity [13, 47]. It is well known that cisplatin administered to rats damages the proximal tubule [2, 6, 30, 32, 34, 37] and that the kidney injury induced by cisplatin is similar to ischemic damage [11, 46]. The acute renal failure caused by cisplatin is typically characterized by signs such as a severe reduction in the glomerular filtration rate (GFR) [24, 40], a variable fall in the renal blood flow [11, 46], a decrease in urinary concentrating ability, and changes in urine volume and creatinine clearance [36]. It is essential to develop practical ways to counteract or minimize the side effects of cisplatin.

Increases of various reactive oxygen species (ROS) occur during cisplatin treatment of cultured renal tubular cells, kidney slices, and *in vivo* in whole animals [37]. It is known that increased expression of lipid peroxidation markers, such as malondialdehyde and 4-hydroxy-2-nonenal (4-HNE), is closely associated with the severity of cisplatin-induced nephrotoxicity [7, 15, 16, 52]. However, it remains unclear whether protein oxidation markers such as dityrosine and advanced oxidation protein products (AOPP), are associated with cisplatin-induced nephrotoxicity. Dityrosine

recently has been developed as a biomarker of protein oxidation [4, 14, 28]. The oxidation of tyrosine generates a tyrosyl radical, and dityrosine is then formed by the reaction of two tyrosyl radicals [4, 14, 28]. Dityrosine is formed by ROS, enzymatic reactions, ultraviolet irradiation and lipid peroxidation [21, 22, 27, 29]. AOPP are defined as dityrosine-containing cross-linked protein products and are considered to be reliable markers for estimating the degree of protein oxidation [5, 10, 48]. Renal injury by cisplatin mainly occurs in the urinary tubule [32], and oxidative damage is known to trigger the renal injury. Several anti-oxidants such as $_{L}$-carnitine, crocin, and lycopene have been investigated for their protective effects on cisplatin-induced nephrotoxicity in experimental models [6, 2, 34].

Lactoferrin (Lf), an iron-binding glycoprotein with a molecular weight of approximately 80 kDa, is found in milk, tears, saliva, other external secretions, and in the secondary granules of neutrophils. Some physiological effects of Lf have been reported, including anti-inflammatory [31, 55], antinociception [18, 19], antistress [25, 42], anti-oxidant [44], cell proliferative [9], and immunoregulatory properties [1, 54]. Åbrink *et al.* [1], investigating the expression of Lf in the human kidney, showed that Lf may act as an anti-oxidant defense systems protecting the kidney against nonmicrobial oxidative injury. However, to our knowledge, there have been no published studies to

date on the protective effect of Lf against nephrotoxicity.

To clarify the physiological effects of Lf, the characteristic transporting system for Lf has been investigated. Takeuchi *et al.* [43] demonstrated that intraduodenally administered bovine Lf (bLf) is transported into the blood circulation via thoracic duct lymph fluid in adult rats. Their findings indicate that the bLf transported to epithelial cells from the lumen reaches the blood circulation via the lymphatic pathway, suggesting that it could be distributed to the whole body if an effective dose was administered.

The present studies were carried out to clarify the novel function of Lf as a potent therapeutic agent in acute renal failure. Cisplatin has been used to produce acute renal failure model in rats. First, we investigated the expressions of protein oxidation markers in cisplatin-induced nephrotoxicity in rats. Secondly, we investigated the protective effect of bLf on cisplatin-induced nephrotoxicity in rats. We analyzed the one of the mechanisms of renoprotective effect of bLf by assessing urine output and accumulated platinum (Pt) contents in the kidney.

CHAPTER 1

Expressions of Protein Oxidation Markers, Dityrosine and Advanced Oxidation Protein Products in Cisplatin-Induced Nephrotoxicity in Rats

1.1 Introduction

The mechanism of the anticancer activity of cisplatin is not completely understood, but a widely held view is that cisplatin binds to DNA, leading to the formation of inter- and intrastrand cross-links [8]. Cross-linking results in defective DNA templates and the arrest of DNA synthesis and replication [37]. In rapidly dividing cells, such as neoplastic cells, cross-linking can further induce DNA damage [37].

Oxidative stress has been recognized as an important factor that contributes to cisplatin-induced nephrotoxicity [37, 51]. It is known that increased expression of lipid peroxidation markers is closely associated with the severity of cisplatin-induced nephrotoxicity [7, 15, 52]. However, it remains unclear whether protein oxidation markers such as dityrosine and advanced oxidation protein products (AOPP), are associated with cisplatin-induced nephrotoxicity.

In the present study, to examine whether the expression of dityrosine and AOPP reflect the severity of cisplatin-induced nephrotoxicity, we investigated the immunohistochemical expression of dityrosine in kidney tissue and the concentration of AOPP in plasma in cisplatin-induced nephrotoxicity in rats until Day 4 following a single injection of cisplatin.

1.2 Materials and Methods

1.2.1 Animals and Sampling

All experiments were performed on 7-week-old, male Wistar strain rats weighing 223.15 ± 5.18 g (mean ± SE) obtained from the Institute of Animal Reproduction (Ibaraki, Japan). The animals were maintained at a controlled temperature of 22 ± 2^{o}C with a 12:12-h 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), (2) cisplatin-treated rats (n=24). Cisplatin-treated rats received cisplatin (5 mg/kg body weight; Nippon Kayaku, Tokyo, Japan) by intraperitoneal injection. Blood and kidney samples were collected under pentobarbital anesthesia (100 mg/kg, intraperitoneal injection) at Days 1, 2, 3 and 4 after cisplatin injection. Serum blood urea nitrogen (BUN) and creatinine levels were determined by means of a biochemical autoanalyzer (Dri-Chem 3000; Fuji Film Company, Tokyo, Japan).

1.2.2 Histological examination by hematoxylin and eosin stain

Kidney tissues were fixed in 10% buffered formaldehyde, 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*. [52]. Tubules (n=100) were selected randomly in 25 samples at ×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 ≧25% but <50% of the tubular profile; 3 = similar changes involving ≧50% but <75% of the tubular profile; 4 = similar changes involving ≧75% of the tubular profile. Those conducting the histopathological examinations were blinded to the study treatments.

1.2.3 Analysis of apoptosis with TUNEL

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, USA). A semiquantitative analysis was performed by counting the number of TUNEL-positive cells per field at ×400 magnification as described by Zhou *et al*. [53].

1.2.4 Immunohistochemical examination and the assessment of AOPP

For immunohistochemistry, the following primary antibody was used: anti-dityrosine mouse monoclonal antibody (Nikken Seil Co., Ltd., Shizuoka, 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. Slides were incubated with primary antibody (1:100 dilution) at 4^{o}C overnight and, after rinsing with TBS, were 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. Plasma AOPP concentration was assayed by a colorimetric method with a commercial kit (Cell Biolabs, Inc., San Diego, U.S.A.).

1.2.5 Statistical analysis

All data are expressed as means ± 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.

1.3 Results

1.3.1 Blood Chemistry

Figure 1 shows the time course of the serum concentrations of BUN (Fig. 1A) and creatinine (Fig. 1B) in the acute renal failure rats induced by cisplatin administration. In the initial 2-day period after exposure to cisplatin, serum BUN levels remained below 20 mg/dl (Fig. 1A). At Day 3, serum BUN increased significantly and reached a maximum level at Day 4. At and after Day 3, serum creatinine levels increased and reached a maximum at Day 4 (Fig. 1B).

1.3.2 Histological examination by hematoxylin and eosin stain

Figure 3 shows the time course of renal tubular morphology in cisplatin-induced nephrotoxicity. No histopathological changes were observed at Day 1. At Day 2, degenerative changes were seen in the straight portion of the proximal tubule, and they consisted of hydropic degeneration, cytoplasmic vacuolization and tubular dilation. These histopathological changes were severely time-dependent. 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 third segment (S3). The tubular damage score

increased time-dependently at and after Day 2 (Fig. 2A).

1.3.3 Analysis of apoptosis with TUNEL

There were no changes between control and Day 1, whereas there was a significant increase in the number of TUNEL-positive cells at Days 3 and 4 (Fig. 2B).

1.3.4 Immunohistochemical examination and the assessment of AOPP

Figure 4 shows the time course of immunohistochemical expressions of dityrosine. Tubular epithelial cells in saline-treated control rats and rats at Day 1 were negative for dityrosine. However, at and after Day 2, they were positive for dityrosine and their immunostaining intensity increased time-dependently. At Day 2, dityrosine was faintly immunostained in the cytoplasm of degenerative proximal tubular epithelial cells in the straight portion. At Day 3, the cytoplasms of damaged tubular epithelial cells in the corticomedullary area were moderately positive for dityrosine and they were strongly positive for dityrosine at Day 4. In the initial 2-day period after cisplatin injection, plasma AOPP levels remained as low as those of the control group (Fig. 5). There was a significant increase in plasma AOPP levels at Days 3 and 4.

1.4 Discussion

Recently, dityrosine has been developed as a biomarker of protein oxidation and there are some reports of dityrosine expression in humans [26] and experimental animals [4, 14, 28, 45, 50]. Dityrosine has been detected immunohistochemically in lipofuscin of pyramidal neurons of aged human brains [26], atherosclerotic lesions of apolipoprotein E-deficient mice [28] and cholesterol-fed rabbits [14], and renal proximal tubules in diabetic mice [45]. Dityrosine concentration significantly increased in the liver of rats chronically intoxicated with ethanol [4] and cooking-oil fumes-induced acute lung injury in rats [50]. To our knowledge, there have been no reports about expression of dityrosine in cisplatin-induced nephrotoxicity, and this is the first one.

In the present study, rats were injected 5 mg/kg of cisplatin at the first day of experiment and followed the occurrence of acute renal injury for four days. The dose of cisplatin was the same dose as previously reported and enough to produce nephrotoxicity [6]. Serum BUN and creatinine concentrations started increasing at Day 3, while tubular damage score showed significant increase at Day 2. It is suggested that histological changes in kidney occur earlier period of time than blood chemical changes are obvious. Also, the expression of dityrosine was detected from Day 2, and it

suggested that the expression of dityrosine was correlated with early changes in the kidney. Although dityrosine was proved as a protein oxidative marker especially in chronic disease process [4, 14, 26, 28 45], the present study showed that the expression of dityrosine occurs in the acute phase of renal changes.

To assess the oxidative stress in the kidney, many parameters have been measured. It is reported that levels of renal glutathione peroxidase activity, glutathione concentration, total thiol concentration, and catalase activity were decreased, while malondialdehyde was increased in cisplatin-induced nephrotoxicity [2, 34]. Witko-Sarsat *et al.* demonstrated that plasma levels of AOPP starts increasing at an early stage of chronic renal failure and has a significant inverse relationship between the plasma concentrations of AOPP and the glomerular filtration rate [49]. They showed that *in vivo* levels of AOPP strongly correlate with creatitine clearance, indicating that AOPP are excellent biomarkers of the progression of chronic renal failure and uremia [48], and that AOPP accumulation coexists with decreased glutathione peroxidase level, while the plasma concentration of malondialdehyde remains stable [49]. They concluded that AOPP are more accurate markers of oxidative stress than lipid peroxidation products [49]. In the present study, the plasma AOPP concentration showed significant increase at Day 3 and 4 in according with the progression of the

acute renal injury. The present study described that plasma AOPP concentration was useful as a biomarker of oxidative protein damage in acute renal failure induced by cisplatin.

In vivo, plasma levels of AOPP closely correlate with plasma concentration of dityrosine [48]. In the present study, the changes of plasma AOPP concentration were also similar to those of the immunostaining intensity of dityrosine. The immunostaining intensity of dityrosine in the kidney tissue and plasma AOPP concentration increased significantly at Days 3 and 4 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.

In conclusion, the present study clearly showed that immunohistochemical expression of dityrosine in the kidney tissue and plasma AOPP concentration associate closely with development of cisplatin-induced nephrotoxicity and that they might be useful biomarkers for estimating the degree of oxidant-mediated protein damage in cisplatin-induced nephrotoxicity.

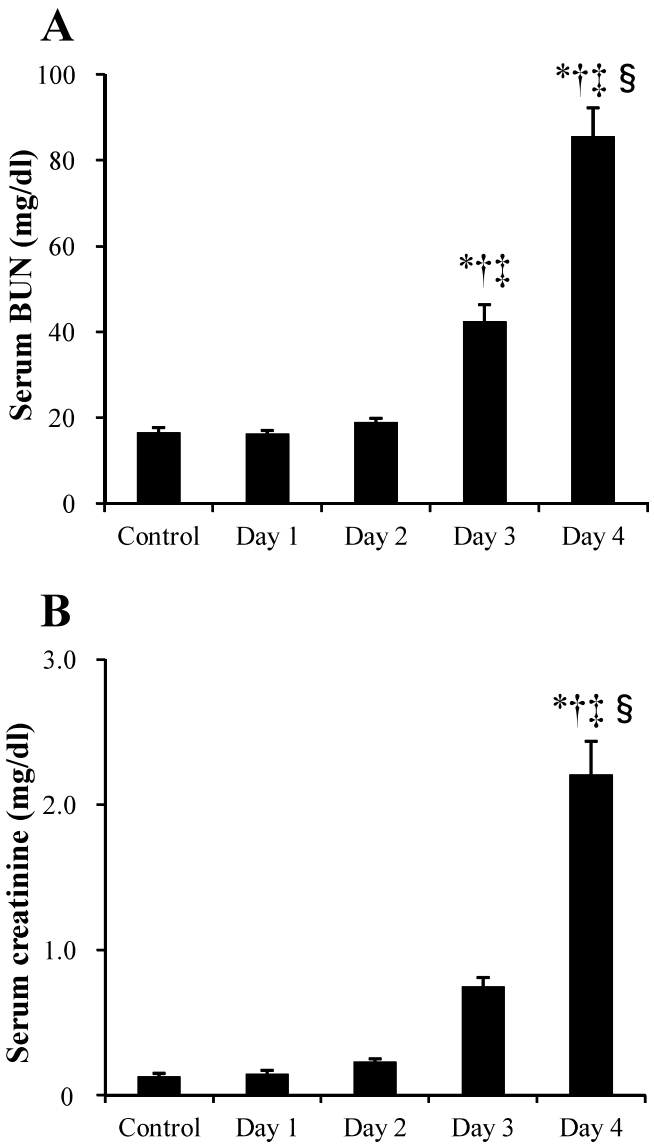

Fig. 1. Time course of renal function in cisplatin-induced nephrotoxicity. Each graph shows the serum concentrations of BUN (A) and creatinine (B). Values are expressed means ± SE (n=6).
*: $p<0.05$, compared with control group, †: $p<0.05$, compared with Day 1 Group, ‡: $p<0.05$, compared with Day 2 group, § : $p<0.05$, compared with Day 3 group.

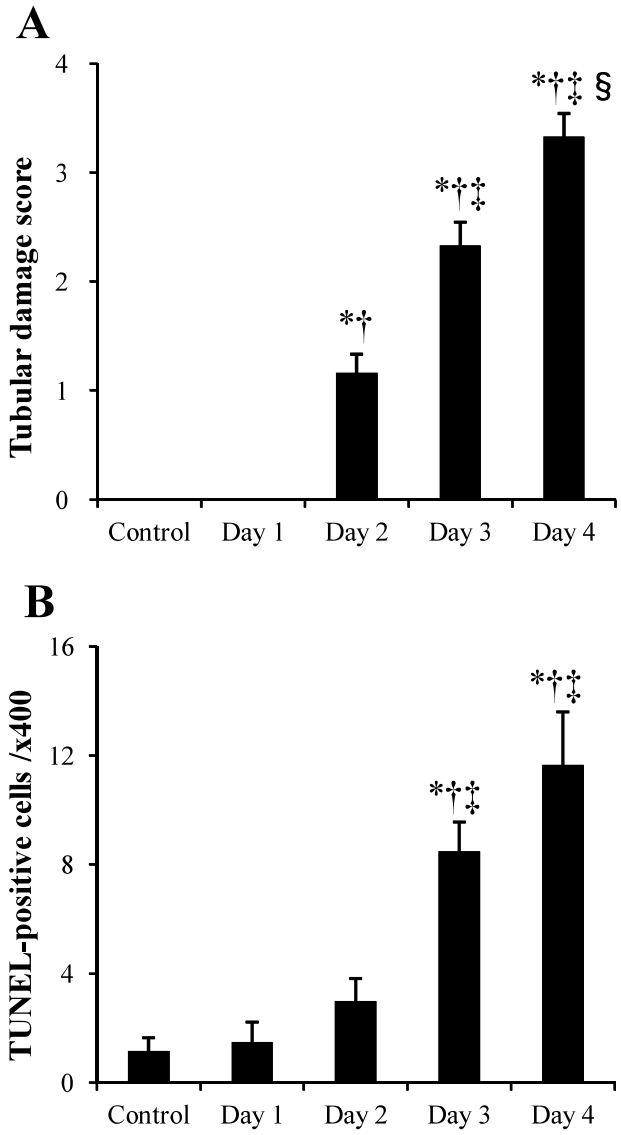

Fig. 2. Time course of tubular morphology in cisplatin-induced nephrotoxicity. Each graph shows the tubular damage score (A) and the number of TUNEL-positive cells per field at ×400 magnification (B). Values are expressed means ± SE (n=6). *: $p<0.05$, compared with control group, †: $p<0.05$, compared with Day 1 Group, ‡: $p<0.05$, compared with Day 2 group, §: $p<0.05$, compared with Day 3 group.

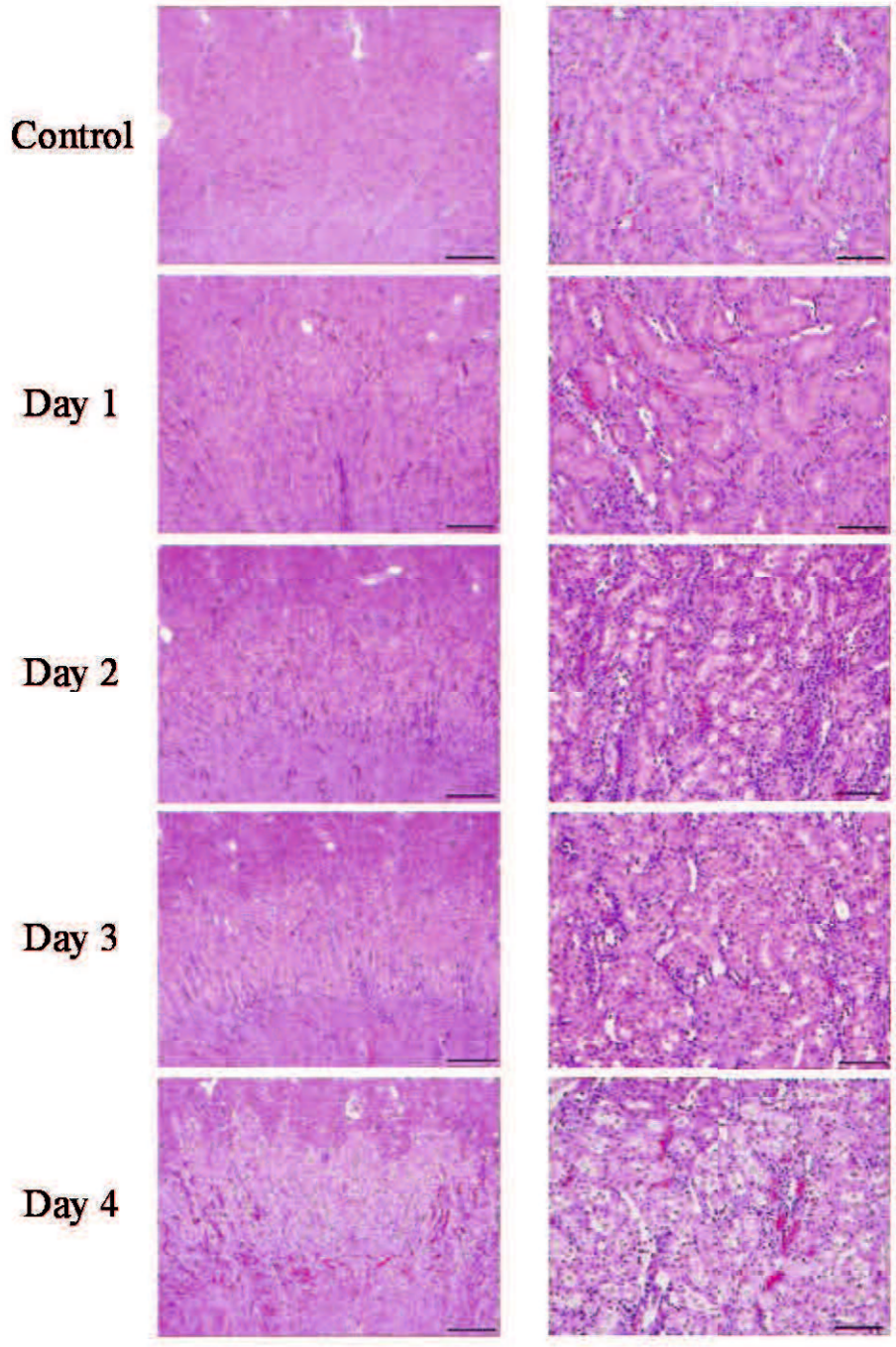

Fig. 3. Changes of histochemical expression in time course of renal tubular morphology in cisplatin-induced nephrotoxicity. Images from the top to the bottom; Control, Day 1, 2, 3, and 4. Images at the left; HE stain, Bar=500μm. Images at the right; HE stain, Bar=200 μm. No changes were observed at Day 1. Degenerative changes were seen in the straight portion of proximal tubule at Day 2, 3 , and 4.

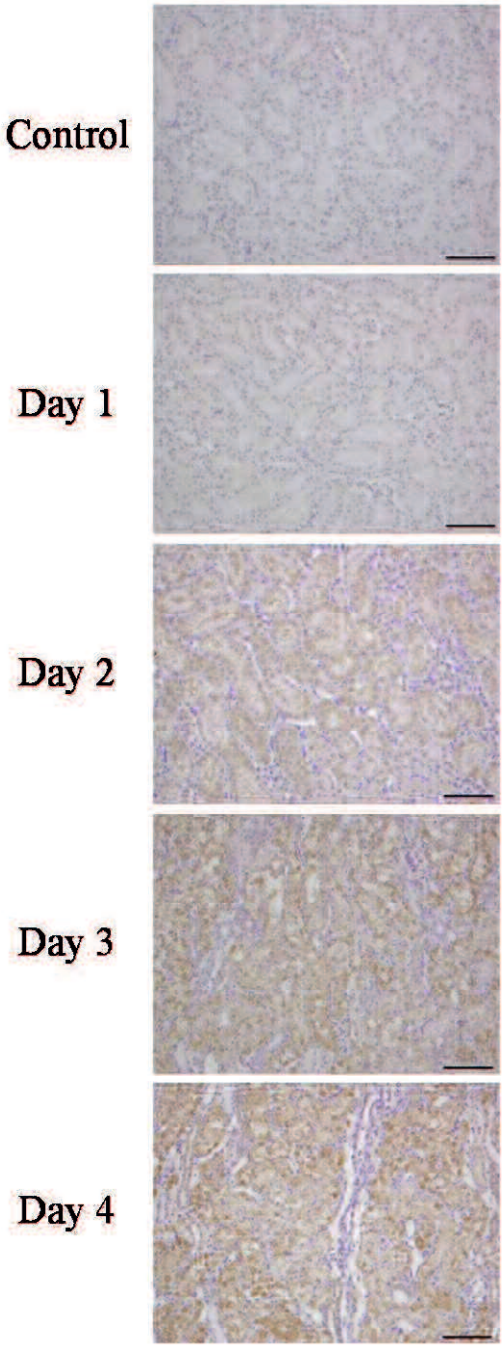

Fig. 4. Changes of imunohistochemical expression in time course of renal tubular morphology in cisplatin-induced nephrotoxicity. Images from the top to the bottom; Control, Day 1, 2,3 and 4; Immunohistochemistry for dityrosine, Bar=200 μm. In control and Day 1 group rats after cisplatin injection, tubular epithelial cells were negative for dityrosine. At Day 2 after injection, damaged tubular cells were positive for dityrosine, but the intensity was still weak. The cytoplasm of damaged tubular epithelial cells at Day 3 were moderately positive for dityrosine and strongly positive for dityrosine at Day 4.

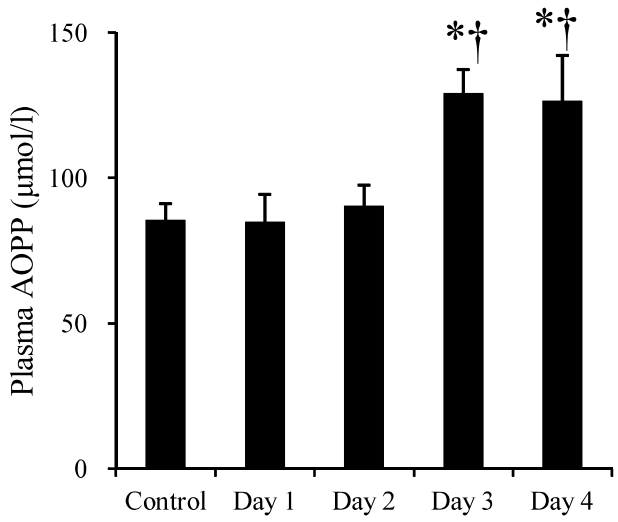

Fig. 5. Plasma AOPP concentration in cisplatin-induced nephrotoxicity.
Values are expressed means ± SE (n=6).
*: $p<0.05$, compared with control group,
†: $p<0.05$, compared with Day 1 group.

CHAPTER 2

Protective effect of Lactoferrin

on Cisplatin-Induced Nephrotoxicity in Rats

2.1 Introduction

Cisplatin is one of the most effective chemotherapeutic agents, but it has severe side effects, such as nephrotoxicity [47]. Lf is well known that it has a wide variety of physiological effect throughout the body. We have performed many researches about Lf from various aspects including its transporting system [17, 43] and its function as a milk protein [25, 42]. Åbrink *et al.* [1], investigating the expression of Lf in the human kidney, showed that Lf may act as an anti-oxidant defense systems protecting the kidney against nonmicrobial oxidative injury.

In the present study, we assessed the protective effect of bLf on cisplatin-induced nephrotoxicity in rats. We also investigated whether bLf affects the accumulation of platinum (Pt) in the kidney.

2.2 Materials and Methods

2.2.1 Animals and blood chemistry and histological analysis of the kidney

Seven-week-old male Wistar rats were obtained from the Institute of Animal Reproduction (Ibaraki, Japan). The animals were maintained at a controlled temperature of 24 ± 1 °C with a 12h-12h light-dark cycle (light cycle, 07:00-19:00), and were given standard chow (CE-2; Nihon Clea, Tokyo, Japan). The use of these animals and the

procedures performed were approved by the Animal Research Committee of Tottori University.

Cisplatin was purchased from Nippon Kayaku (Tokyo, Japan). BLf was purchased from Wako Chemical Co.Ltd. (Osaka, Japan).

Twenty-four rats were randomly divided into four groups of six rats each. The rats in the control group were orally administered saline (3 ml/kg) daily from the day before the study began (Day 0) to the fifth day (Day 5) of the study. Saline (14 ml/kg, i.p.) was injected to the rat on Day 1. The rats in the bLf alone group were orally administered bLf (300 mg/kg) daily from Day 0 to Day 5, and injected with saline (14 ml/kg, i.p.) on Day 1. The rats in the cisplatin alone group were orally administered saline (3 ml/kg) daily from Day 0 to Day 5, and injected with cisplatin (7 mg/kg, i.p.) 2 hrs after the saline administration on Day 1. This dose of cisplatin has been well documented to induce nephrotoxicity in rats [2]. The rats in the cisplatin+bLf group were orally administered bLf (300 mg/kg) daily from Day 0 to Day 5 and injected with cisplatin (7 mg/kg, i.p.) 2 hrs after the bLf administration on Day 1. On Day 5, the rats were anesthetized with sodium pentobarbital (Somnopentyl, 50 mg/kg, i.p.; Kyoritsu Pharmaceutical Co., Ltd., Tokyo, Japan), and blood samples were collected from the caudal venae cavae. These samples were transferred to heparinized tubes, then

centrifuged at 4 °C for 15 min. Plasma was separated and stored at -20 °C until biochemical analyses were performed. Plasma BUN and creatinine were determined by means of a biochemical autoanalyzer (Dri-Chem 3000 V3.1-P01; Fuji Film Company, Tokyo, Japan). Both kidneys were rapidly removed, and the blood was washed away with saline. Each kidney was sliced longitudinally and one piece from each side of the kidney was fixed in 10% neutral buffered formalin. The kidney tissues were processed for histological examination by the conventional method [41], and stained with hematoxylin and eosin (HE).

2.2.2 Effect of bLf on the accumulation of cisplatin in the kidney

Forty rats were divided into four groups (control, bLf alone, cisplatin alone, and cisplatin+bLf) of 10 rats each. Oral administration (saline or bLf) and intraperitoneal injection (saline or cisplatin) were the same as described above. The collection of blood and kidneys was carried out on Day 2, because it is well known that the onset of oxidation damage in the kidney is at around 48 hrs after the cisplatin injection. After 48 hrs of saline or cisplatin treatment, the rats were anesthetized with sodium pentobarbital (Somnopentyl, 50 mg/kg, i.p.), and blood samples were collected from the caudal venae cavae. A kidney was removed from each rat and weighed, and

then stored at -20 °C until the analysis of the accumulated Pt content. The kidney tissue was decomposed to mimic pyrolysis by adding nitric acid and hydrogen peroxide. Pt was measured using an inductively coupled plasma-optical emission spectrometry (ICP-OES) system (Optima 4300DV; PerkinElmer Japan, Yokohama, Japan). The range of Pt measurement was 0-500 ppb. The accumulated Pt contents in the kidney were represented as μg/g tissue.

2.2.3 Diuretic effect of bLf in normal rats

Seven healthy rats were used for this experiment. Under general anesthesia with 25% urethane (1 g/kg, s.c.) and sodium pentobarbital (Somnopentyl, 25 mg/kg, i.p.), a cannula was inserted into the external jugular vein to control the depth of anesthesia by an additional injection of sodium pentobarbital. Saline (4.5 ml/kg/hr) was infused constantly by syringe pump (TOP-5300, TOP Co., Ltd, Tokyo, Japan) to maintain blood circulation and blood pressure. Another cannula was inserted into the external carotid artery to monitor blood pressure via an amplifier (PowerLab; ADInstruments, Sydney, Australia). The heart rates of all rats were also monitored by electrocardiography (PowerLab; ADInstruments). Body temperature was monitored by a rectangular sensor (SK-1250MC III α; Sato Keiryoki Mfg. Co., Ltd., Tokyo, Japan).

During the experiment, the blood pressure of the rats was maintained within the range of 80-120 mmHg, the heart rate within 270-330 BPM, and the body temperature within 36.5-37.0 °C. An abdominal midline incision was made in each rat, and the bladder and both ureters were exposed. Polyethylene tubes (SP-10; Natsume Co., Ltd., Tokyo, Japan) were placed into both ureters. BLf was administered to the rats through the external jugular vein at three doses (3, 10, and 30 mg/kg) at 40, 100 and 160 min after the start of urine sampling. During the procedures, urine samples were collected into plastic tubes every 10 min and urine volume was measured.

2.2.4 Statistical analysis

All data are expressed as means ± SE. Statistical comparisons were performed by analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. A probability level of $P<0.05$ was considered statistically significant.

2.3 Results

2.3.1 Effect of bLf on blood chemistry and histological findings in the kidney

Figure 6 shows the effects of bLf on the plasma concentrations of BUN (Fig. 6A) and creatinine (Fig. 6B) in normal rats and rats with acute renal failure induced by

cisplatin administration. The administration of bLf alone did not change BUN or creatinine. Cisplatin caused a significant increase in BUN (14.47±0.38 mg/dl in the control group and 124.32±11.39 mg/dl in the cisplatin alone group) and creatinine (0.17±0.02 mg/dl and 2.56±0.17 mg/dl, respectively). These renal abnormalities induced by cisplatin were significantly improved by pretreatment with oral bLf (the cisplatin+bLf group).

Histopathological examination of the kidney revealed that cisplatin strongly impaired proximal tubule. In the cisplatin alone group (Figs. 7C and 7E), marked tubular necrosis was observed especially in the straight portion of the proximal tubule in the outer stripe of the medulla, while bLf alone and saline groups (Figs. 7A and 7B, respectively) did not cause any pathological changes. The lesions in the cisplatin+bLf group were smaller and milder than those of cisplatin alone group (Figs. 7D and 7F). BLf administration was found to reduce the epithelial damage induced by cisplatin.

2.3.2 Effect of bLf on the accumulation of cisplatin in the kidney

The accumulated Pt contents in the kidney were measured at 48 hrs after saline or cisplatin (7 mg/kg, i.p.) treatment (Table 1). As reported in the previous study [14], the tubular damages occurred at 48 hrs after cisplatin treatment. The Pt contents in the

cisplatin alone and the cisplatin+bLf groups were 16.70±1.96 μg/g tissue and 9.8±2.1 μg/g tissue, respectively (Table 1). The Pt content in the kidney was decreased significantly by bLf treatment. In this experiment, the BUN and creatinine concentrations in plasma were also measured at 48 hrs after saline or cisplatin (7 mg/kg, i.p.) injection. The administration of bLf (300 mg/kg, p.o.) alone did not affect BUN or creatinine, while cisplatin caused a mild increase in both (cisplatin alone group). These changes induced by cisplatin were significantly improved by pretreatment with oral bLf (cisplatin+bLf group).

2.3.3 Diuretic effect of bLf in normal rats

Intravenous bLf administration caused a significant increase in urine volume in a dose-dependent manner (Fig. 8A). Compared with the average urine volume during the first 30 min, those values after 10 and 30 mg/kg bLf administrations resulted in increments of 157% and 250%, respectively. There were no significant effects of bLf on an arterial blood pressure and a heart rate (Figs. 8B and 8C).

2.4 Discussion

The present study indicated that orally administered bLf has a protective effect

against the acute renal failure induced by cisplatin in rats. This protective effect of bLf was suggested by biochemical and morphological evidences, including lower BUN and creatinine levels and lower damage in the proximal tubles compared with the cisplatin alone group. It is reported that oxidative damage triggers the renal injury induced by cisplatin [32, 37, 51], and several anti-oxidants such as L-carnitine, crocin, and lycopene have the protective effects on cisplatin-induced nephrotoxicity in experimental models [6, 2, 34]. Tsubota *et al.* [44] showed that oral administration of bLf inhibits oxidative liver damage in Long-Evans Cinnamon rats. They described one possible mechanism for this antioxidative action might be the ability of Lf to bind transition metal irons. Koike *et al.* reported that intraperitoneal injection of human Lf had protective effects on pancreatitis in rats [31]. They also concluded that Lf acts an antioxidant by binding iron ions in the pancreas and prevent tissue damage. It is suggested that one of mechanism for renoprotective effect of Lf may correlate with antioxidative effect and that Lf has potent therapeutic property. However, further researches are required.

We also demonstrated that the Pt content in the kidneys in the cisplatin+bLf group was significantly lower than that in the cisplatin alone group. Esteban-Fernandez *et al.* [12] reported that the maximum concentration of Pt in the kidney was achieved within the third day after the cisplatin treatment in rats. In our previous study,

proximal tubular injury developed from the second day to the fourth day after the cisplatin injection [30]. Therefore, we measured the Pt content in the kidney at 48 hrs after the cisplatin injection. Muenyi *et al.* [33] reported that the systemic Pt accumulation occurred in the following order: kidney > liver = spleen > heart > brain. It is well known the accumulated Pt contributes to the occurrence of damage to the proximal tubules. However, Esteban-Fernandez *et al.* [12] mentioned that the toxic effects produced by cisplatin in the kidney are probably not only related to the Pt accumulation and distribution, but also to the drug-biomolecule interaction, because they found that two fractions ranging below 50 kDa represented approximately 50% of the total Pt in the cytosol of the kidney. In any case, the reduction of accumulated Pt in the kidney might be related with the prevention of tubular damage induced by cisplatin.

In the present study, intravenous injection of bLf caused diuresis in a dose-dependent manner. Cisplatin causes decreased renal blood flow by renal vasoconstriction as well as ischemic damage in the proximal tubule [11, 36, 40, 46]. It is well known that several diuretic drugs such as furosemide and mannitol have a renoprotective effect in cisplatin-treated animals [35]. The increment of urine volume leads to suppression of the reabsorption of cisplatin at the proximal tubules. In our study, urine volume increased up to 2.5 times higher than the basal volume when the rat

received bLf at a dose of 30 mg/kg. Hayashida *et al.* [20] reported that bLf causes a nitric oxide (NO)-dependent relaxation of smooth muscle in the rat's aorta, suggesting that bLf can affect the blood circulation in individual organs. The involvement of NO in the renoprotective effect in cisplatin-injected rats was supported by a treatment with L-arginine resulting in NO modulation [38]. Hydration with saline also has a renoprotective effect on cisplatin-injected rats [39]. In our experiment regarding the diuretic effect of bLf, the rats were infused with saline throughout the experimental period to maintain the blood circulation. Saline infusion had no significant effects on the urine volume, heart rate or blood pressure by itself. In contrast, bLf resulted in a significant increase in the urine volume without affecting the heart rate or blood pressure. Further experiments are required to clarify the precise mechanisms of the diuretic effect of bLf.

For the clinical application of bLf, the oral route may be safest, because bLf has a risk of antigenicity. Atkinson and Meredith [3] reported that bLf given through i.p. exhibited antigenicity in the Brown Norway rat model using carrageenan as an adjuvant. Ishikado *et al.* [23] also compared the oral administration and subcutaneous administration of bLf in guinea pigs in terms of antigenicity. Their report indicated that the oral administration caused less passive cutaneous anaphylaxis than the

subcutaneous administration. In the present study, orally administered bLf actually indicated the protective effect on the cisplatin-induced renal failure. In our previous study, orally administered Lf was detected in plasma as an intact protein [17]. Moreover, we demonstrated that intraduodenally administered bLf is transported into the blood circulation via thoracic duct lymph fluid in adult rats [43]. These characteristic transportation systems of Lf contribute to its variety of clinical effects throughout the body.

In conclusion, the present study indicates that pretreatment with bLf produces a protective effect against cisplatin-induced nephrotoxicity. It is also suggested that bLf evokes diuresis and reduces the accumulation of cisplatin in the kidney.

Table 1. Effect of bLf administration on the plasma concentrations of BUN and creatinine and the Pt content in the kidney tissue in cisplatin-induced renal injury.

Group	BUN (mg/dl)	Creatinine (mg/dl)	Pt content (μg/g tissue)
Saline	14.87 ± 0.49	0.13 ±0.02	N.D.
BLf alone	16.24 ± 0.69	0.11 ± 0.01	N.D.
Cisplatin alone	24.32 ± 1.86**	0.31 ± 0.03**	16.7 ± 1.96
Cisplatin+bLf	18.26 ± 1.66	0.15 ± 0.02##	9.8 ± 2.1#

All rats were evaluated at 48 hr after the administration of saline or cisplatin (7 mg/kg, ip). Data represent means ± SE from 10 rats. Significant differences are shown as **; $p<0.01$ vs. saline group, ##; $p<0.01$ vs. cisplatin alone group, and #; $p<0.05$ vs. cisplatin alone group. N.D.: not detected.

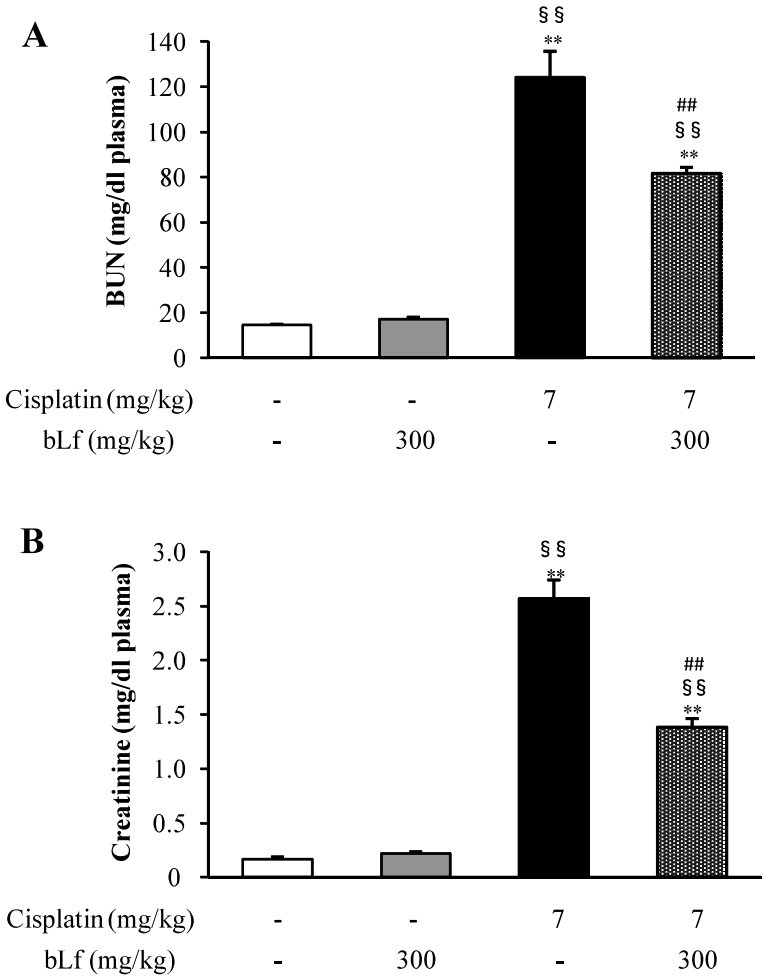

Fig. 6. Effect of bLf administration on the plasma concentrations of BUN and creatinine in cisplatin-induced renal injury. Each graph shows the plasma concentrations of BUN (A) and creatinine (B) in the cisplatin-induced rats on the 5th day after the cisplatin (7 mg/kg) injection. BLf (300 mg/kg) was orally administered in the bLf alone and cisplatin+bLf groups from Day 0 to Day 5. Data represent means ± SE from 6 rats. Significant differences are shown as **; $p<0.01$ vs. control group, § §; $p<0.01$ vs. bLf alone group, and ##; $p<0.01$ vs. cisplatin alone group, respectively.

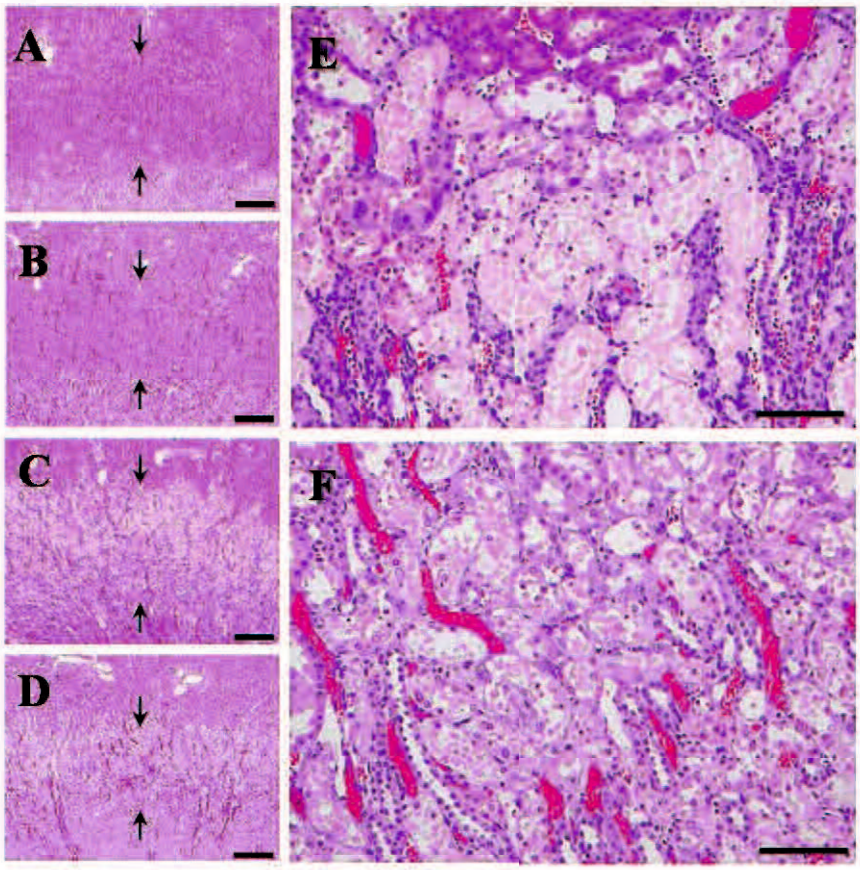

Fig. 7. Histopathological findings of cisplatin–induced renal tubular injury. Bar=500μm (A-D). Bar =50μm (E and F). Photographs A-D show low magnification. Photographs E and F show high magnification. A: saline, B: bLf alone, C and E: cisplatin alone, D and F: cisplatin+bLf. Cisplatin treatment caused severe renal tubular injury in the outer stripe of the medulla (arrows) in the cisplatin alone group (C). The injured area was smaller and milder in the cisplatin+bLf group (D and F) than the cisplatin alone group (C and E).

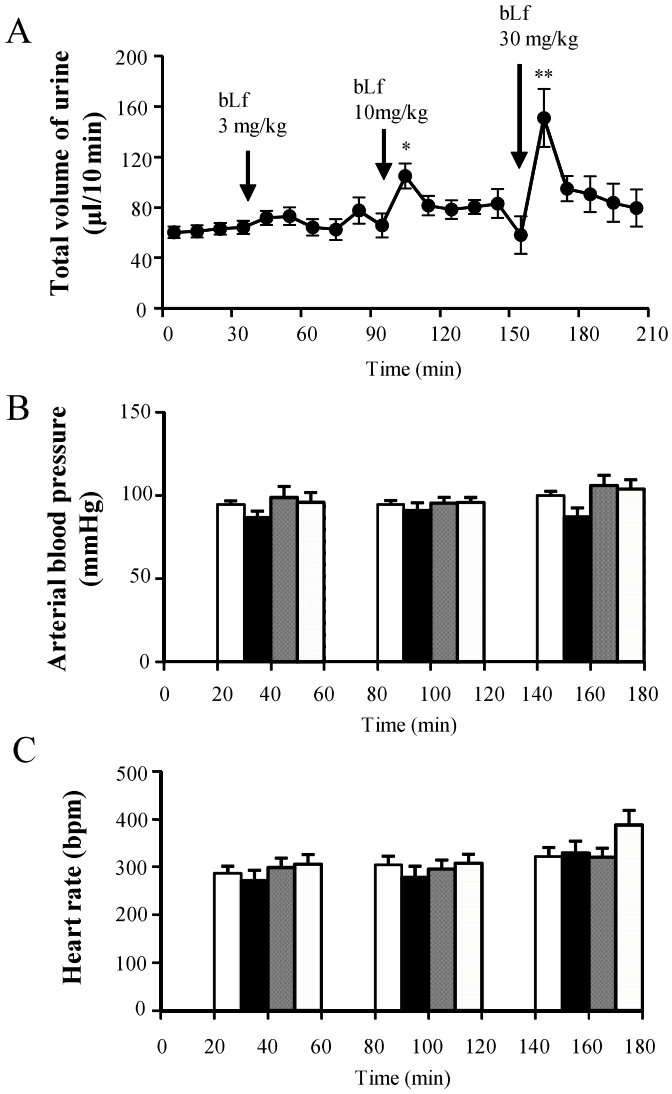

Fig. 8. Time course of urine output and the diuretic effect of bLf in normal rats. Each graph shows the total value of the urine (A), arterial blood pressure (B) and heart rate (C), respectively. Three doses of bLf (3, 10 and 30 mg/kg) were intravenously administered at 40, 100 and 160 min after the start of urine sampling. With respect to the arterial blood pressure (B) and heart rate (C), the effect of the drug was compared with the value measured 10 min before each drug injection. Each column shows the mean value in each 10-min interval. Data represent means ± SE from 7 rats. Significant differences are shown as *, **, corresponding to $p< 0.05$, $P<0.01$ vs. the average volume of urine during the first 30 min, respectively.

GENERAL CONCLUSION

First, we examined cisplatin-induced nephrotoxicity morphologically and immunohistochemically using novel protein oxidation markers, dityrosine and advanced oxidation protein products (AOPP). The results of our experiments demonstrate that cisplatin has a site-specific nephrotoxic effect on the straight portion of the proximal tubule in the rat kidney and that immunohistochemical expression of dityrosine in the kidney tissue and plasma AOPP concentration correlate with development of cisplatin induced nephrotoxicity. Therefore, it is suggested that dityrosine and AOPP might be useful biomarkers for estimating the degree of oxidant-mediated protein damage in cisplatin-induced nephrotoxicity.

Secondly, we investigated the protective effect of bLf on cisplatin-induced nephrotoxicity in rats. We analyzed the mechanisms of renoprotective effect of bLf by assessing urine output and accumulated platinum (Pt) contents in the kidney. Cisplatin caused a significant increase of the plasma concentrations of BUN and creatinine. These renal deteriorations were well related to the histological changes, that is tubular injury. We demonstrated that pre-treatment with bLf to cisplatin-induced acute renal failure rats ameliorates renal tubular injury histologically and suppresses the deterioration of renal function. The renal abnormalities induced by cisplatin were significantly recovered by

the oral administration of bLf. We showed that intravenous administration of bLf has a potent diuretic effect on the normal rats. We assumed that bLf suppresses reduction of renal blood flow by means of improving vascular resistance induced by cisplatin.

To clarify one of the mechanisms of the renoprotective effect of bLf, we evaluated the Pt content in the kidney tissue at 48 hrs after cisplatin treatment. In the present study, the Pt content in the kidney of cisplatin treated rats tended to be larger than that of bLf pretreated rats. It is suggested that the less amount of Pt content in bLf treated acute renal failure rats was responsible for reduction of the tubular injury.

The present study clearly showed that bLf treatment provides significant amelioration against cisplatin-induced nephrotoxicity. It was suggested that when cisplatin-induced renal injury occurs, bLf causes the increment of urine output and prevents the accumulation of cisplatin in the renal tubule, consequently ameliorating renal injury.

SUMMARY

The purposes of these studies were to clarify one of the mechanisms of cisplatin-induced nephrotoxicity and the renoprotective effect of bLf. We have had special interest in its variety of properties of Lf and investigated the novel functions of Lf based on its characteristic transportation systems.

The results obtained are as follows:

1. The immunostaining intensity of dityrosine in the kidney tissue and plasma AOPP concentration increased significantly after cisplatin injection, and the present changes reflect those of morphological tubular injury and blood biochemical findings of renal function.
2. Morphological and biochemical deteriorations induced by cisplatin were significantly recovered by pretreatment with oral administration of bLf.
3. Intravenous bLf administration caused a significant increase in urine volume in normal rats.
4. Pretreatment of bLf in cisplatin-induced nephrotoxicity rats decreased the accumulation of the Pt content in the kidney. It is suggested that the less amount of Pt content in bLf treated acute renal failure rats was responsible for reduction of the tubular injury.

Consequently, it was suggested that when cisplatin-induced renal injury occurs, bLf causes the increment of urine output and prevents the accumulation of cisplatin in the renal tubule. Thus bLf treatment provides significant amelioration against cisplatin-induced nephrotoxicity.

In this study, we found a new property of bLf which has a protective effect on cisplatin-induced nephrotoxicity in rats. However, the further mechanisms such as the carrier mediated cisplatin transportation in the tubular cells and the site bLf actually working are still unclear. Further studies are needed, but our findings will inspire to develop a new approach to prevent and treat acute renal failure and moreover chronic renal failure in our patients.

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