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Effects of Green Tea Tannin on Cisplatin-induced Nephropathy in LLC-PK$_1$ Cells and Rats

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Abstract

A study was conducted to clarify whether green tea tannin ameliorated cisplatin-induced renal injury in terms of lactate dehydrogenase and malondialdehyde leakage from a renal epithelial cell line, swine-derived LLC-PK$_1$ cells in culture. Green tea tannin was shown to suppress the cytotoxicity of cisplatin, the suppressive effect increasing with the dose of green tea tannin. The effect of cisplatin was then investigated in rats given green tea tannin for 40 days before cisplatin administration and in control rats given no green tea tannin.

In control rats, blood, urinary and renal parameters and the activities of antioxidative enzymes in renal tissue deviated from the normal range, indicating dysfunction of the kidneys. In contrast, rats given green tea tannin showed decreased blood levels of urea nitrogen and creatinine, and decreased urinary levels of protein and glucose, reflecting less damage to the kidney. In this group, the activity of catalase in the renal tissue was increased, while the level of malondialdehyde was decreased, suggesting the involvement of radicals in the normalizing of kidney function.

Based on the evidence available it appeared that green tea tannin eliminated oxidative stress and was beneficial to renal function.

The number of therapeutic agents used clinically has been increasing year by year. Although these agents are essential for the treatment of patients, they are also responsible for an increasing number of various adverse reactions and organ disorders. Among these, drug-induced nephropathy is particularly frequent. This is probably because the kidney is supplied with a large volume of blood accounting for 20% of total cardiac output, and is therefore likely to be affected by secondary effects of drugs and their metabolites that are concentrated through the urine concentration mechanism (Guyton 1986). In our ageing society, the number of elderly patients with age-related renal hypofunction who are receiving drug treatment has been increasing, raising the problem of potentially total renal disorders due to such drugs.

Cisplatin is a platinum chelate that exerts a potent antitumour action on cancers of the testis, ovary, urinary bladder, prostate, and head and neck. Unfortunately, the drug has adverse effects on the kidney, bone marrow and digestive organs; the risk of nephropathy, in particular, is so large as to limit the indications for use of this agent (Blachley & Hill 1981; Goldstein & Mayor 1983; Weiner & Jacobs 1983; Litterst 1984). To reduce the risk of cisplatin-induced nephropathy while enhancing the antitumour effect of the drug, combined use of other drugs such as diuretics and development of a non-nephrotoxic cisplatin derivative have been attempted (Gemba 1991). Suppression of nephrotoxicity by antioxidants and the radical scavenger superoxide dismutase has been demonstrated in a rat model of cisplatin-induced acute renal failure and in an in-vitro system of cultured renal epithelial cells (Gemba 1991; Gemba & Fukuishi 1991), suggesting the involvement of free radicals in cisplatin-induced nephropathy.

We have searched for agents among various Oriental medicines that might be useful for the treatment of renal failure, and carried out serial studies on the beneficial effects, mechanisms of

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action, and active components of some candidate agents (Yokozawa et al 1991a, b, 1992, 1994, 1995a, 1996a; Chung et al 1995). Since renal failure is a condition of oxidative stress, we focused on the mechanism of creatinine oxidation, exploiting the discovery of a creatinine oxide, creatol (Ienaga et al 1991; Nakamura et al 1991; Yokozawa et al 1991c, 1993a, 1995b, 1997; Fujitsuka et al 1993, 1994), and demonstrated that tannin-containing crude drugs have antioxidant activity (Yokozawa et al 1993b, 1995c, d).

Since large amounts of tannin are also present in tea leaves, we examined the effects of green tea tannin in nephrectomized rats in which the free radical scavenging system had been destroyed, and found that the tannin normalized the kidney under increased oxidative stress (Yokozawa et al 1996b). It was also suggested that green tea tannin might have a direct effect on mesangial cells, exerting some influence on free radicals (Yokozawa et al 1993c). In this study, we have investigated whether green tea tannin can improve cisplatin-induced nephropathy, using cultured renal epithelial cells and rats.

Materials and Methods

Green tea tannin

The tea tannin used was Sunphenon (Taiyo Kagaku Co., Yokkaichi, Japan), prepared from a hot-water extract of green tea, as reported previously (Sakanaka et al 1989). It was composed mainly of (−)-epigallocatechin 3-O-gallate, (−)-gallocatechin 3-O-gallate, (−)-epicatechin 3-O-gallate, (−)-epigallocatechin, (+)-gallocatechin, (−)-epicatechin and (+)-catechin.

Medium and reagents

Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12) and foetal calf serum were purchased from Life Technologies, Inc. (Grand Island, NY) and Cell Culture Laboratories (Cleveland, OH), respectively. Cisplatin was obtained from Sigma Chemical Co. (St Louis, MO). A commercial kit (lactate dehydrogenase CII-Test Wako) for assaying lactate dehydrogenase was obtained from Wako Pure Chemical Industries, Ltd, Osaka, Japan.

Cultured cell experiment

Commercially available renal epithelial cell lines, swine-derived LLC-PK_1 cells, were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air on culture plates with 5% foetal calf serum-supplemented DMEM/F-12 medium. After confluence had been reached, the cells were seeded on fresh 96-well culture plates at 10^4 per well. Cisplatin (0·25 μM) and/or green tea tannin were added to the culture 2-h later, and the plates were incubated for 48 h. Leakage of lactate dehydrogenase into the culture medium was assayed as an index of cytotoxicity using a commercial kit. The level of lipid peroxide released from the cultured cells was estimated by measuring the amount of malondialdehyde, as described by Yagi (1976). Five determinations were performed for all assays.

Animal preparation

Male LWH-Wistar rats (150–160 g) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). They were kept in a wire-bottomed cage under a conventional lighting regimen with a dark night. The room temperature (approx. 25°C) and humidity (approx. 60%) were controlled automatically. Laboratory pellet chow (CLEA Japan Inc., Tokyo, Japan; comprising 24·0% protein, 3·5% lipid and 60·5% carbohydrate) and water were given freely. Following several days of adaptation, the animals were divided into four groups, avoiding any intergroup difference in body weight gain. Two groups were given water, while the other groups were given green tea tannin orally at a dose of 10 or 20 mg kg^{-1}/day for 40 consecutive days.

A single dose of cisplatin (6 mg kg^{-1}) was administered intravenously to rats which had been given green tea tannin, or an equivalent volume of water orally for the preceding 40 days, and urine specimens were collected for 1–2 days after cisplatin administration. Blood samples were obtained by cardiac puncture without anaesthesia, and the serum was separated immediately by centrifugation. The kidneys were subsequently extirpated from each rat following renal perfusion through the renal artery with ice-cold physiological saline. Six rats were used for each experimental group.

Determination of serum, kidney and urine samples

Urea nitrogen and creatinine were determined using the commercial reagents BUN Kainos and CRE-EN Kainos (Kainos Laboratories, Tokyo, Japan). Malondialdehyde was determined using the method of Naito & Yamanaka (1978). Protein and glucose were assayed by the sulphosalicylic acid method (Sakagishi 1968) and the Momose method (Momose et al 1963), respectively.

Enzyme assays. The kidney was homogenized with a 4-fold volume of iced physiological saline, and the activities of enzymes in the homogenate were determined. Superoxide dismutase activity was assayed by the nitrous acid method (Oyanagui 1984), and catalase activity was determined in terms of the decrease in the amount of hydrogen peroxide (Aebi 1974). Glutathione peroxidase activity was determined by colorimetry of 2-nitro-5-thiobenzoic acid, a compound produced through the reaction of glutathione and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman 1959). Protein was determined by the method of Itzhaki & Gill (1964), with bovine serum albumin as a standard.

Statistics

Data are presented as mean ± s.e. Differences among groups were analysed by Dunnett's test. Significance was accepted at $P < 0{\cdot}05$.

Results

Lactate dehydrogenase leakage from cultured cells

During normal conditions, approximately 110 m int. units mL^{-1} lactate dehydrogenase was released from the cells into the culture medium. This leakage increased to 280·4 m int. units mL^{-1} after cisplatin had been added to the medium (Table 1). Although there was no suppression of the cytotoxic effect of cisplatin in the presence of 0·25 μg mL^{-1} green tea tannin, 0·5 μg suppressed the leakage significantly (237·7 m int. units mL^{-1}). This suppression became more marked as the concentration of green tea tannin increased. When green tea tannin was added to the medium to give a final concentration of 10 μg mL^{-1}, leakage of the enzyme due to cisplatin was significantly reduced to 170·6 m int. units mL^{-1}.

Table 1. The effect of green tea tannin on lactate dehydrogenase leakage from LLC-PK_1 cells exposed to cisplatin.

Material	Concentration (μg mL^{-1})	Lactate dehydrogenase activity (m int. units mL^{-1})
Tannin	–	280·4 ± 12·0
	0·25	265·8 ± 16·6
	0·5	237·7 ± 15·9[a]
	2·5	212·2 ± 15·1[b]
	5	187·9 ± 18·0[b]
	10	170·6 ± 14·2[b]
Normal	–	110·2 ± 6·9

[a] $P < 0{\cdot}01$, [b] $P < 0{\cdot}001$ compared with non-addition values.

Amount of malondialdehyde released into culture medium

Malondialdehyde (0·104 nmol/well) was detected in the culture medium of control cells incubated under normal conditions, and exposure to cisplatin increased sharply the malondialdehyde level in the control cell medium, approximately 1·6-fold (0·170 nmol/well; Table 2). The inhibition of this event in the green tea tannin-treated group resembled that for lactate dehydrogenase, i.e. cells pretreated with a concentration ranging from 5 to 10 μg mL^{-1} were significantly affected.

Blood components

Table 3 shows the effect of orally administered green tea tannin on parameters of blood constituents. The urea nitrogen level in control rats with induced renal failure increased significantly to reach 145·5 mg dL^{-1} (13·4 mg dL^{-1} in normal rats). The blood level of creatinine was also increased significantly in these rats. In contrast, the level of blood urea nitrogen in rats given 20 mg kg^{-1} green tea tannin for 40 days decreased from 145·5 to 87·9 mg dL^{-1} (a 40% change, $P < 0{\cdot}01$). Similarly, the creatinine level in rats given 20 mg kg^{-1} green tea tannin orally for 40 days showed a significant decrease compared with the control rats (Table 3).

Table 2. The effect of green tea tannin on malondialdehyde released from LLC-PK_1 cells exposed to cisplatin.

Material	Concentration (μg mL^{-1})	Malondialdehyde level (nmol/well)
Tannin	–	0·170 ± 0·014
	0·25	0·166 ± 0·013
	0·5	0·156 ± 0·012
	2·5	0·149 ± 0·010
	5	0·140 ± 0·012[a]
	10	0·130 ± 0·013[b]
Normal	–	0·104 ± 0·005

[a] $P < 0{\cdot}01$, [b] $P < 0{\cdot}001$ compared with non-addition values.

Table 3. The effect of green tea tannin on urea nitrogen and creatinine in serum.

Group	Dose (mg kg^{-1}/day)	Urea nitrogen (mg dL^{-1})	Creatinine (mg dL^{-1})
Normal	–	13·4 ± 0·3	0·41 ± 0·03
Rats with induced renal failure			
Control	–	145·5 ± 36·9[a]	6·11 ± 0·89[a]
Tannin	10	165·1 ± 25·4[a]	6·35 ± 0·69[a]
Tannin	20	87·9 ± 4·9[ab]	3·81 ± 0·25[ac]

[a] $P < 0{\cdot}001$ compared with normal rats; [b] $P < 0{\cdot}01$, [c] $P < 0{\cdot}001$ compared with control rats with renal failure.

Urine components

Although there was no significant difference in urine output between the normal and control rats (Table 4), the urinary excretion of protein and glucose in control rats was increased by 3·4- and 3·5-fold, respectively, compared with the normal rats. Administration of green tea tannin at both the 10 and 20-mg kg^{-1} dosage levels reduced the urinary excretion of protein. However, there were no significant differences between the control and green tea tannin-treated groups. A reduction of glucose excretion was also observed in green tea tannin-treated rats with induced renal failure. The urinary excretion of glucose (98·7 mg/day in control rats) decreased to 71·7 mg/day in rats given 20 mg kg^{-1}/day green tea tannin, although this decrease was not significant.

Table 4. The effect of green tea tannin on urinalysis.

Group	Dose (mg kg^{-1}/day)	Urine volume (mL/day)	Protein (mg/day)	Glucose (mg/day)
Normal	–	10·1 ± 2·0	8·8 ± 1·0	28·2 ± 7·9
Rats with induced renal failure				
Control	–	10·9 ± 2·9	29·9 ± 4·6[a]	98·7 ± 18·4[a]
Tannin	10	11·9 ± 1·4	26·1 ± 1·9[a]	74·9 ± 23·5[a]
Tannin	20	12·0 ± 2·0	27·1 ± 1·5[a]	71·7 ± 23·2[a]

[a] $P < 0·001$ compared with normal rats.

Malondialdehyde in serum and renal tissue

In the cisplatin-treated rats, the serum malondialdehyde level increased to 8·35 nmol mL^{-1} (2·05 nmol mL^{-1} in normal rats), reflecting lipid peroxidation (Table 5). Administration of 10 mg kg^{-1} green tea tannin for 40 days reduced the serum malondialdehyde level significantly from 8·35 to 3·59 nmol mL^{-1}. An increase in the dose to 20 mg kg^{-1} produced a further decrease in the malondialdehyde level. Oral administration of 20 mg kg^{-1} green tea tannin for 40 days caused a 76% decrease in the serum level of malondialdehyde compared with control rats. The renal malondialdehyde level in rats given 20 mg kg^{-1} green tea tannin orally for 40 days showed a significant decrease compared with control rats (Table 5).

Enzyme activities

Table 6 shows the activity of reactive oxygen species-scavenging enzymes. Compared with normal rats, rats with induced renal failure given no green tea tannin showed significantly decreased enzyme activities, the values being 46% lower for superoxide dismutase activity, 83% lower for catalase activity and 19% lower for glutathione peroxidase activity. In rats given 20 mg kg^{-1}/day green tea tannin, the superoxide dismutase activity decreased significantly to 8·94 units (mg protein)$^{-1}$, compared with control rats (10·72 units

Table 5. The effects of green tea tannin on malondialdehyde.

Group	Dose (mg kg^{-1}/day)	Serum malondialdehyde (nmol mL^{-1})	Kidney malondialdehyde (nmol g^{-1})
Normal rats	–	2·05 ± 0·12	3·05 ± 0·18
Rats with induced renal failure			
Control	–	8·35 ± 1·68[a]	14·84 ± 1·90[a]
Tannin	10	3·59 ± 0·72[b]	12·50 ± 1·28[a]
Tannin	20	2·01 ± 0·17[b]	9·38 ± 1·50[ab]

[a] $P < 0·001$ compared with normal rats, [b] $P < 0·001$ compared with control rats with renal failure.

Table 6. The effect of green tea tannin on oxygen species-scavenging enzymes in kidney.

Group	Dose (mg kg^{-1}/day)	Superoxide dismutase (units (mg protein)$^{-1}$)	Catalase (units (mg protein)$^{-1}$)	Glutathione peroxidase (units (mg protein)$^{-1}$)
Normal	–	19·85 ± 0·57	247·5 ± 5·0	153·7 ± 4·5
Rats with induced renal failure				
Control	–	10·72 ± 0·73[b]	42·1 ± 8·9[b]	124·5 ± 13·5[a]
Tannin	10	10·24 ± 0·44[b]	56·3 ± 2·7[bc]	127·4 ± 8·0[a]
Tannin	20	8·94 ± 0·26[bd]	62·7 ± 2·6[bd]	111·0 ± 12·5[b]

[a] $P < 0·01$, [b] $P < 0·001$ compared with normal rats; [c] $P < 0·01$, [d] $P < 0·001$ compared with control rats with renal failure.

(mg protein)$^{-1}$). However, green tea tannin significantly increased the activity of catalase from 42·1 (control rats) to 56·3 units (mg protein)$^{-1}$ at 10 mg kg^{-1} (a 34% change, $P < 0{\cdot}01$) and to 62·7 units (mg protein)$^{-1}$ at 20 mg kg^{-1} (a 49% change, $P < 0{\cdot}001$). There was no obvious variation in glutathione peroxidase activity.

Discussion

The nephrotoxicity of drugs and their metabolites is often manifested as proximal tubule disorders, which result in the release of enzymes held in the proximal tubules. Weiner & Jacobs (1983) reported that the major site of the renal disorder induced by cisplatin is the S3 segment in the straight part of the proximal tubule. Therefore, using the swine kidney-derived culture epithelial cell line LLC-PK_1, which has proximal tubule-like features, we examined the effects of cisplatin. As a result, we observed that cisplatin caused leakage of the lysosomal enzyme lactate dehydrogenase into the culture medium, increased the production of malondialdehyde, and damaged the cell membrane. However, green tea tannin suppressed these effects of cisplatin in a dose-dependent manner.

Gemba (1991) found that the leakage of lactate dehydrogenase increased when cisplatin was added to subcultures of cells, or to mature cells which had completed proliferation, and that cisplatin in the culture medium exerted a cytotoxic effect on renal cells—particularly an increase in cell permeability—at a concentration similar to that in blood. These findings suggested the usefulness of cultured cells as an in-vitro model system reflecting conditions in-vivo. However, the kidney consists of various cells, and we believe that an in-vivo experimental system is necessary for investigating the kidney as a whole. Therefore, we used rats in this study.

Rats given cisplatin intravenously showed markedly increased urinary excretion of glucose and deteriorated renal function (blood levels of urea nitrogen and creatinine), indicating dysfunction of proximal tubules. In contrast, in rats administered green tea tannin orally before intravenous cisplatin, there was a tendency for decreased glucose excretion, while levels of urea nitrogen and creatinine in blood were significantly decreased, confirming the effect of green tea tannin against nephropathy in culture cells. With regard to the mechanism of cisplatin nephrotoxicity, Gemba et al (1991) reported that cisplatin-induced nephropathy in rats was suppressed by the antioxidant *N*,*N*′-diphenyl-p-phenylenediamine and the hydroxyl radical ($^{\bullet}OH$) scavenger *N*,*N*′-dimethyl thiourea (DMTU). McGinness et al (1978) also reported that superoxide dismutase suppressed such nephropathy. In experiments using rat renal cortical sections, cisplatin was found to induce increased production of lipid peroxides and renal cell dysfunction (Sugihara et al 1987; Hannemann & Baumann 1988), suggesting the involvement of free radicals in the nephropathy due to cisplatin. Taking into account the results obtained with DMTU and superoxide dismutase, involvement of the superoxide (O_2^-) → hydrogen peroxide (H_2O_2) → H_2O system seemed likely. When the antioxidant enzymes involved in this system were measured, the activities of superoxide dismutase, catalase, and glutathione peroxidase were all decreased significantly, suggesting that the free radical-scavenging system was destroyed in rats given cisplatin. However, when green tea tannin was administered before intravenous cisplatin, there was a decrease in the activity of superoxide dismutase, an enzyme catalysing the disproportionation of O_2^- into H_2O_2 (Marklund 1980). There was also an increase in the activity of catalase, which specifically eliminates H_2O_2 and suppresses the production of $^{\bullet}OH$ and hypochlorite (OCl^-) (Agar et al 1986; Winterbourn & Stern 1987), suggesting an increase of O_2^- and a decrease of H_2O_2. Since H_2O_2 is stable and passes quickly through the cell membrane, unlike O_2^-, the possibility that it exerts a toxic effect even at areas distant from the site of its production has been suggested. It is also known that H_2O_2 produces more active $^{\bullet}OH$ through the Fenton reaction in the presence of transition metals, and is used as the substrate of myeloperoxidase to produce highly active OCl^- in the presence of chloride ions. Therefore, as Yoshikawa et al (1991) explained, catalase, which specifically eliminates H_2O_2 and suppresses the production of $^{\bullet}OH$ and OCl^-, is a more important antioxidant enzyme than superoxide dismutase. It seems that such scavenging enzymes are also involved in the improvement of malondialdehyde levels in blood and renal tissue by green tea tannin as observed in this study.

Active oxygen species are produced in mesangial cells, vascular endothelial cells, epithelial cells, neutrophils, and phagocytes, and not only elicit disorders of glomeruli and renal tubules but also affect renal haemodynamics as vasoconstrictors (Katusic & Vanhoutte 1989). Brady & Brenner (1994) reported that direct injection of H_2O_2 into the renal artery causes a decrease in Kf. Enzymes that can eliminate these active oxygen species are present in the kidney, and nephropathy is considered to be the result of an imbalance between

these enzymes and active oxygen species (Inoue 1990). Thus, destruction of the active oxygen-scavenging system has been attracting attention in recent years. Based on the evidence available it appears that green tea tannin eliminates oxidative stress and is beneficial to renal function. We anticipate that it may open a new avenue to the development of a new medicament for treatment of nephropathy that is more effective than the drugs currently available.

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