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Acute nephrotoxicity of aristolochic acids in mice

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Abstract

Aristolochic acids (AA), present in *Aristolochia* plants, are the toxin responsible for Chinese herbs nephropathy (CHN), a rapidly progressive tubulointerstitial nephritis (TIN). To clarify the mechanisms of the development of CHN, we tried to induce TIN in mice using AA. Three strains of inbred mice, BALB/c, C3H/He and C57BL/6, received 2.5 mg kg^{-1} of AA or AA sodium salt (AANa) daily by intraperitoneal or oral administration, 5 days a week for 2 weeks. Serum and renal tissue were obtained at sacrifice. Twelve-hour urine samples were individually collected in a metabolic cage at one-week intervals. In the AA-injected groups, severe tubular injury, with the appearance of acute tubular necrosis, and rare cell infiltration into the interstitium, were seen in BALB/c mice. C3H/He mice also developed TIN with prominent cell infiltration into the interstitium and interstitial fibrosis. In C57BL/6 mice, only mild and focal tubulointerstitial changes were seen. Serum creatinine and blood urea nitrogen increased in BALB/c and C3H/He mice. Immunofluorescent study revealed no deposition of immune components in kidneys. In the AANa-treated groups, TIN was also seen in all groups, but even more severe tubulointerstitial changes were induced by intraperitoneal injection. Further examination using purified AAI, AAII, AAIVa and aristolactam I (ALI) revealed that AAI induced strong nephrotoxicity in mice, and that AAII resulted in mild nephrotoxicity. However, AAIVa and ALI caused no nephrotoxicity in this experimental system. There are strain differences in mice in their susceptibility to AA nephropathy. AAI exerted the strongest nephrotoxic effect in mice.

Introduction

Chinese herbs nephropathy (CHN) is a rapidly progressive interstitial renal fibrosis reported in about 100 women who had been on a slimming regimen using Chinese herbs in Belgium (Vanherweghem et al 1993; Vanherweghem 1998). These tubulointerstitial changes have been traced to the ingestion of *Aristolochia fangchi*, containing aristolochic acids (AA), inadvertently included in slimming pills (Vanhaelen et al 1994). Exposure to AA was confirmed by the detection of AA-DNA adducts in the kidney tissue samples from CHN patients (Schmeiser et al 1996; Nortier et al 2000), and then the main features of human CHN (renal interstitial fibrosis and urothelial malignancy) were reproduced in rodents (Zheng et al 2001; Debelle et al 2002). These results removed any doubt concerning the aetiological role of AA in the genesis of CHN, and a better term for the interstitial nephropathies in which the unequivocal role of AA has fully documented would be AA nephropathy (Arlt et al 2002; Cosyns 2003). Other sporadic cases of CHN-like disorders have been reported in Spain, the UK and Japan (Pena et al 1996; Yokoi 1998; Lord et al 1999). Furthermore, a similarity in the morphological and clinical patterns of CHN and Balkan endemic nephropathy was pointed out, as well as the possibility that the two nephropathies have a common aetiological agent, AA (Cosyns et al 1994).

AA is a mixture of structurally related nitrophenantherene carboxylic acids from *Aristolochia* plants (Chen & Zhu 1987). There are two major components of AA – aristolochic acid I (AAI) and aristolochic acid II (AAII) – and aristolochic acid IVa (AAIVa) is also detected in Chinese herbs, such as *Asiasarum* root.

Experimental studies with rats (Mengs 1983; Mengs & Stotzem 1993) and mice (Mengs 1987) revealed that mixtures of chiefly AAI and AAII induced nephrotoxic

effects, especially in the renal tubular epithelium, and were carcinogenic. Experimental attempts to reproduce the clinical features of CHN have been performed. Intraperitoneal injections of 0.1 mg kg^{-1} AA (5 days per week, for 17–21 months) into New Zealand white rabbits (NZW) led to hypocellular interstitial fibrosis and urothelial atypia (Cosyns et al 2001), but the length of AA exposure required in rabbits makes the experimental model less useful for the study of renal interstitial fibrosis. Salt-depleted Wistar rats that received daily subcutaneous injections of AA 10 mg kg^{-1} developed similar interstitial lesions and urothelial dysplasia within 35 days (Debelle et al 2002). Salt-depletion conditioning and a high dose of AA seem to be essential for the development of AA-induced fibrosis in rats. There seem to be some interspecies differences in susceptibility to AA nephrotoxicity. Indeed, interspecies differences in the metabolism of AA have been reported in mammals (Krumbiegel et al 1987).

Strain differences are also possible. In this study, acute nephrotoxicity of AA was examined in three strains of inbred mice, BALB/c, C3H/He and C57BL/6, which are representative inbred mice that have been used in various experiments. We found that these three strains showed different susceptibility to experimental autoimmune interstitial nephritis (Ueda et al 1988). The investigation of the strain differences might contribute to clarification of the mechanism of AA-induced nephrotoxic effects. A high susceptibility to tubulointerstital changes by AA was revealed in BALB/c and C3H/He, and then the toxicities of AAI, II, IVa, and of a derivative of AAI, aristolactam I (ALI), were compared using these two highly susceptible strains of mice. We report these results in comparison with the other experimental models of AA-induced interstitial renal fibrosis.

Materials and Methods

Animals and aristolochic acids

Six-week-old inbred male mice of three strains, BALB/c, C3H/He and C57BL/6, were purchased from Japan SLC, Inc. (Shizuoka, Japan).

Aristolochic acids (Sigma-Aldrich Japan, Tokyo, Japan) used in this study contained 55% aristolochic acid I (AAI) and 45% AAII. Aristolochic acid sodium salt (AANa; Sigma, St Louis, MO) contained 70% AAI and 25% AAII. AAI and AAII were purified from aristolochic acids (1:1 mixture of AAI and II; Biomol Research Laboratories Inc., Plymouth Meeting, PA). The aristolochic acids were subjected to preparative HPLC to afford AAI and AAII. This was performed with 50% acetonitrile–acetic acid (600:1) on an APCELL PAK C18 AG120 (5 μm, 20 × 250 mm; Shiseido, Tokyo, Japan) column employing a UV monitoring flow system (400 nm) at a flow rate of 10.0 mL min^{-1}.

AAIVa and ALI were purified from *Asiasarum* root (*A. heterotropoides* var. *mandshuricum*; Tochimoto Tenkaido Co., Osaka, Japan) imported from North Korea. Dry roots of *Asiasarum* root (9 kg) were extracted twice with methanol under reflux. The methanol solvent was evaporated under reducing pressure, isolating a residue (953 g), which was then suspended in water and successively extracted with n-hexane, diethyl ether and n-butanol. After evaporation of the diethyl ether soluble layer, the residue was poured into a silica gel column, eluted with n-hexane and then with a gradient of acetic acid (0–100%) in n-hexane to produce 10 fractions. Fraction 8 was chromatographed on Toyopearl HW-40 (fine) using methanol and recrystallized from methanol to isolate ALI (90 mg). Fraction 9 was applied to a silica gel column eluting with chloroform–methanol–H_2O (90:10:1) and recrystallized from methanol to isolate AAIVa (100 mg). They were identified by a comparison of ^{1}H NMR and MS spectral data with the reported data (Che et al 1984; Priestap 1987, 1989).

Experimental designs

As shown in Table 1, 160 male mice of BALB/c and C3H/He strain were divided into 9 groups according to the materials administered and the vehicles used. One hundred and twenty male mice of C57BL/6 strain were divided into 5 groups. The mice were kept in individual

Table 1 Experimental groups of mice administered aristolochic acids (AA) and aristolactam I.

Vehicle Route	AA Oil i.p.	AANa Saline i.p.	AANa Distilled water p.o.	AAI Oil i.p.	AAII Oil i.p.	AAIVa Saline i.p.	ALI Oil i.p.	Control Oil i.p.	Control Saline i.p.
BALB/c	40	20	20	10	10	10	10	20	20
C3H/He	40	20	20	10	10	10	10	20	20
C57BL/6	40	20	20	n.d.	n.d.	n.d.	n.d.	20	20

Numbers denote no. of mice in each group. Each mouse received 2.5 mg kg^{-1} of material in corn oil or saline for 2 weeks (5 times per week, total 25 mg kg^{-1}). Half the mice from each group were sacrificed one day after the final injection and the other half were sacrificed 14 days after. In the AA-injected groups, to examine the time course of renal functional and histological changes, 5 mice were sacrificed one day after 1, 3, 6 and 9 injections. AA, aristolochic acids; AANa, aristolochic acids sodium salt; AAI aristolochic acid I; AAII, aristolochic acid II; AAIVa, aristolochic acid IVa; ALI, aristolactam I; Oil, corn oil; i.p., intraperitoneal; p.o., per os; n.d., not done.

plastic cages in fully air-conditioned rooms with a 12-h light–dark cycle and a temperature of $22 \pm 1\,^{\circ}C$. Standardized food pellets, CRF-1 (Oriental Yeast Co. Ltd, Tokyo, Japan) and tap water were freely available. The experimental protocol, animal care and treatment were approved by the committee for animal studies at Chiba University.

AA, AAI, AAII and ALI dissolved poorly in saline, so they were dissolved in corn oil (Wako Pure Chemical Industries Ltd, Osaka, Japan) at a concentration of $0.5\,mg\,mL^{-1}$, and AANa and AAIVa were dissolved in saline at the same concentration.

Each mouse received $2.5\,mg\,kg^{-1}$ of material in corn oil or in saline daily for two weeks (5 days/week, total $25\,mg\,kg^{-1}$). The oral administrations were performed by gavage. In the AA-injected groups, ten mice each were sacrificed 14 days after the first injection of AA, and another ten mice each 28 days after. In these groups, to examine the time course of renal functional and histological changes, 5 mice were sacrificed one day after 1, 3, 6 and 9 injections. AAI-treated BALB/c and C3H/He mice were anaesthetized just before death, when they became sick and showed emaciation, muscle weakness and low mobility.

Serum and renal tissue were obtained at sacrifice. Twelve-hour urine samples were individually collected in a metabolic cage at one-week intervals. The body weight of each mouse was measured from the day before the initial administration to the day of sacrifice, at 3-day intervals.

Histological examination

Blocks of renal tissue were fixed in 10% buffered formalin for routine histological examination. Because of the minimal change in the glomerulus, the severity of renal changes was graded by the degree of tubulointerstitial changes. We assessed on periodic acid–Schiff (PAS)-stained sections at a magnification of $\times 200$, and findings for the cortex were semi-quantatively scored. The scores used were defined as follows: 0, degeneration of the tubular epithelium absent; 1, one group or a single degenerated tubule; 2, several clusters of degenerated tubules; 3, moderate degeneration of the tubular epithelium; 4, more severe degeneration of the tubular epithelium; 5, the severest degeneration of the tubular epithelium, with massive necrosis and atrophy. Mononuclear cell infiltration into the interstitium: 0, absent; 1, few scattered cells; 2, groups of mononuclear cells; 3, dense widespread infiltrate. Existence of hyaline cylinders in distal tubules: 0, absent; 1, existent. Interstitial fibrosis: 0, absent; 1, mild diffuse fibrosis; 2, moderate fibrosis; 3, severe fibrosis. The histological score of each mouse was expressed as the sum of these four aforementioned scores.

Immunofluorescent study

Small blocks of kidney obtained at autopsy were quickly frozen in acetone dry ice and cut into 4-μm sections. The cryostat sections were stained with FITC-conjugated goat anti-mouse IgG and goat anti-mouse complement C3 (Cappel, ICN Pharmaceuticals Inc., Aurora, OH).

Haematological examination and urinalysis

The serum levels of creatinine and blood urea nitrogen (BUN) were determined by standard enzymatic methods for chemistry using an autoanalyser; creatininase–sarcosine oxidase–peroxidase method for creatinine and urease–glutamate dehydrogenase for BUN. Urinary protein concentration was measured by the Folin–Lowry method (Lowry et al 1951). Urinary protein was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). SDS-PAGE was performed on laboratory-made discontinuous (Laemmli 1970) polyacrylamide gels consisted of separating gels and stacking gels (10% and 3% polyacrylamide gels, respectively), using Mini PROTEAN II cell (Bio-Rad Laboratories Inc., Hercules, CA) and slab gel electrophoretic apparatus. Gels were stained for 3 min with 0.1% Coomassie Brilliant Blue R-250 (Wako Pure Chemical Industries Ltd, Osaka, Japan) in fixative (50% methanol and 5% acetic acid), and then destained with 5% methanol–7% acetic acid to remove background.

Statistics

Results were expressed as mean $\pm$ s.d. Comparisons were made by one-way analysis of variance. Significant analysis of variance was followed by the Post-hoc test (Dunn's procedure as a multiple comparison procedure). Nonparametric variables were analysed with the Kruskal–Wallis test and the Mann–Whitney U-test. Statistically significant differences between groups were defined as $P < 0.05$.

Results

Body weight and total condition

The body weight of AA-injected mice was significantly lower than that of control mice in all three strains. The difference became statistically significant after the fifth injection (day 7). BALB/c and C3H/He mice were already lighter in weight at that time compared with before the first injection (day 0 vs day 7, 18.6 ± 0.84 g vs 16.5 ± 0.86 g, 20.3 ± 0.82 g vs 17.4 ± 0.58 g, BALB/c and C3H/He mice, respectively). In the AA-injected groups, 20% of BALB/c mice died of debility with renal insufficiency before day 28. The body weight of C57BL/6 mice increased gradually after the first injection (day 0 vs day 7, 18.1 ± 0.88 g vs 18.4 ± 0.77 g, not significant). After the fifth injection, the body weight of C3H/He and C57BL/6 mice had slightly increased (18.0 ± 0.86 g and 19.0 ± 0.88 g, respectively on day 14), whereas that of BALB/c mice continued to decrease during the remainder of the administration period (16.3 ± 0.75 g). After the final administration, the body weight of BALB/c mice increased to nearly day 0 level. Similar changes in body weight were observed

in the AANa-treated groups (i.p. and p.o.; data not shown).

Morphological findings

Light microscopic study

Control mice receiving intraperitoneal injections of the vehicles (corn oil, saline or distilled water) did not exhibit renal morphologic alterations (Figure 1A). Light microscopic study revealed severe tubular damage in all BALB/c mice 14 days after the first administration of AA in oil. The main change was diffuse degeneration of the proximal tubular epithelium (PTE), like acute tubular necrosis (Figure 1B). This change was accompanied by prominent hyaline cylinders in distal tubules (Figure 1C), but not by interstitial cell infiltration. Despite these tubular changes, there was no remarkable glomerular change. In C3H/He mice, there was also degeneration of PTE with hyaline cylinders on the 14th day, as well as in BALB/c mice. In addition to these changes, C3H/He mice developed moderate infiltration of mononuclear cells into the interstitium (Figure 1D). In C57BL/6 mice, there were no tubular changes such as seen in the two other strains (Figure 1E).

On day 28, proliferation of PTE was seen in a part of the tubules in C3H/He mice, whereas most proximal tubules had no PTE in BALB/c mice (Figure 1F). The tubulointerstitial change of BALB/c at that point differed from day 14 in terms of the moderate interstitial mononuclear cell infiltration. In C3H/He, infiltration of mononuclear cells became more prominent, and interstitial fibrosis had clearly progressed (Figure 1G). In C57BL/6 mice, although there was no prominent degeneration of PTE, moderate interstitial mononuclear cell infiltration was apparent on day 28 (data not shown).

The changes were reflected in the histological scores. The median scores for degeneration of the tubular epithelium, mononuclear cell infiltration into the interstitium, existence of hyaline cylinders in distal tubules and interstitial fibrosis were calculated from individual data collected for each treatment group (Figure 2). In BALB/c mice, the total histological score was lower than in C3H/He on day 14 ($P < 0.01$); there was no other change except for degeneration of the tubular epithelium and hyaline cylinders. On day 28, the histological score had increased in BALB/c, as the scores of mononuclear cell infiltration and interstitial fibrosis were added ($P < 0.01$). However, cell infiltration into the interstitium and interstitial fibrosis were prominent at an early stage (day 14) in C3H/He mice because of the recovery of the tubular epithelium. In C57BL/6 mice, the histological score was the lowest among the three strains, and the main histological change was cell infiltration.

In the AANa-injected (i.p.) groups, nephrotoxicity was similar to that of the AA group of each strain, and HS was rather higher than in the AA groups in C3H/He and C57BL/6 mice on day 28. Tubulointerstitial changes were also induced by the p.o. administration of AANa in BALB/c and C3H/He mice, but the changes were milder than those of the AA- or AANa-injected groups, as indicated by the histological scores.

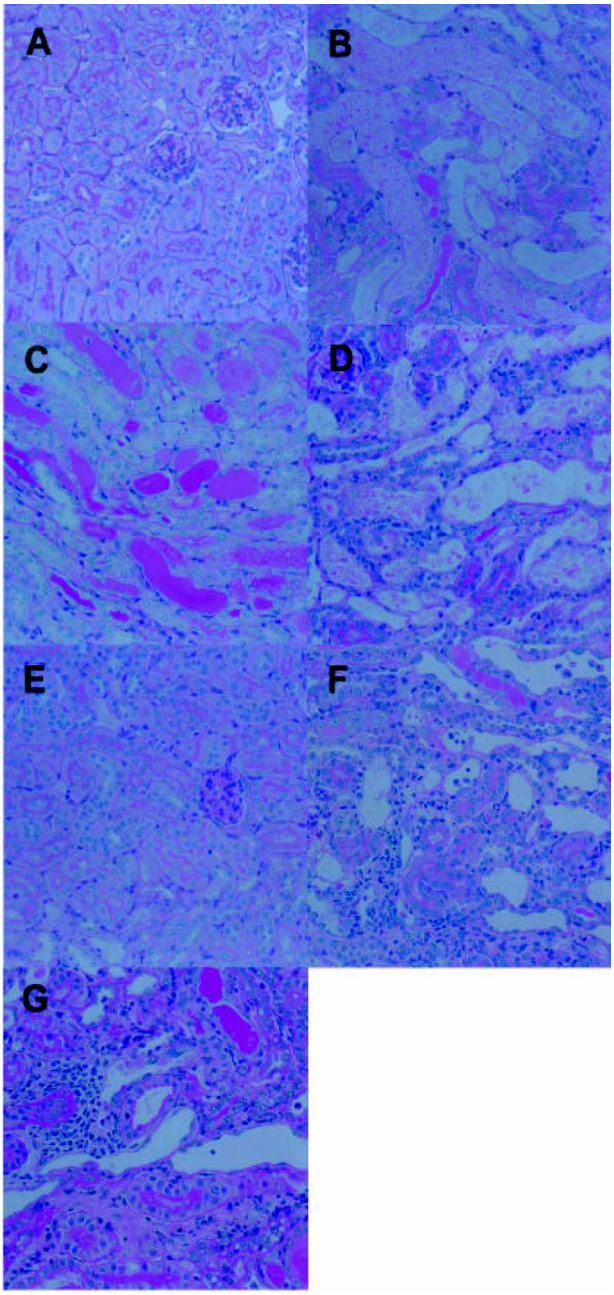

Figure 1 Photomicrographs of periodic acid–Schiff (PAS) staining of kidney sections from mice treated with AA. A. Control (BALB/c mouse). Severe tubular damage was seen in BALB/c and C3H/He mice 14 days after the first administration of AA. In BALB/c mice, the main change was diffuse degeneration of the proximal tubular epithelium (PTE), such as acute tubular necrosis (B), accompanied by prominent hyaline cylinders in distal tubules (C). In addition to these changes, C3H/He mice developed moderate infiltration of mononuclear cells into the interstitium (D). In C57BL/6 mice, there were no tubular changes such as seen in the two other strains (E). Proliferation of PTE was seen in a part of tubules with moderate interstitial mononuclear cell infiltration in BALB/c mice 28 days after the first administration (F). In C3H/He, infiltration of mononuclear cells became more prominent, and interstitial fibrosis had clearly progressed (G). Magnification × 200.

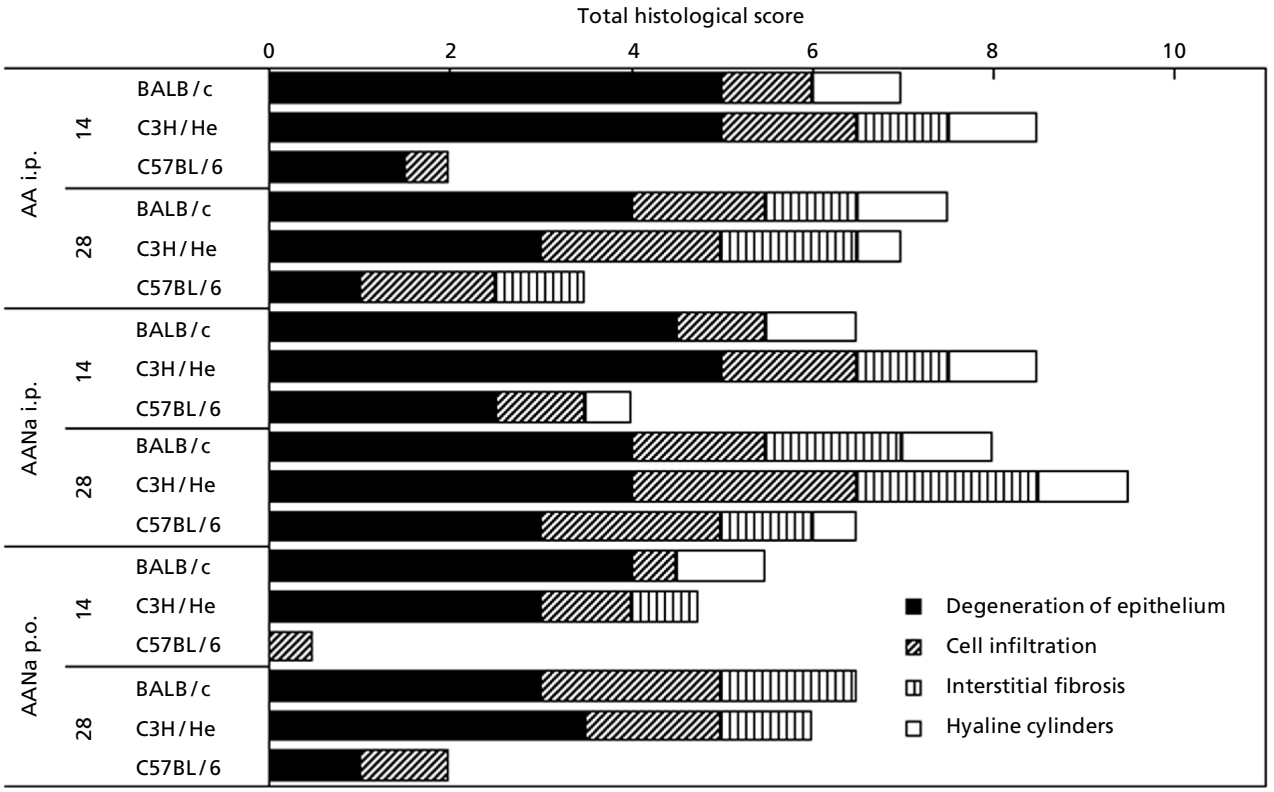

Figure 2 Histological score of mice treated with AA or AANa. The grading system used for classifying tubulointerstitial lesions is described in Methods. Data express total histological score, as the sum of each histological score: degeneration of tubular epithelium (0–5) (filled columns), mononuclear cell infiltration into interstitum (0–3) (hatched columns), interstitial fibrosis (0–3) (striped columns), and existence of hyaline cylinders in distal tubules (0 or 1) (open columns). AA, aristolochic acid; AANa, aristolochic acid sodium salt; C, control group; i.p., intraperitoneal; p.o., per os; Day 14, the data of day 14, just after the final administration of AA or AANa; Day 28, the data of day 28, 2 weeks after the cessation of AA or AANa.

In all animals, immunofluorescent study revealed no deposition of IgG or complement C3 on the tubular basement membrane or glomerulus.

Electron microscopy study showed prominent mononuclear cell infiltration into the interstitium and detachment of PTE from tubular basement membrane (Figure 3A), but no remarkable glomerular changes (Figure 3B) in C3H/He mice on the 28th day after the first administration of AA. Electron microscopy study of kidneys from the other mice also showed only interstitial changes.

Blood chemistry and urinalysis

Following AA administration, serum creatinine and BUN levels increased with the number of injections in BALB/c and C3H/He mice, reaching statistically significant values on day 7, compared with control mice ($P < 0.05$). However, in C57BL/6 mice, the levels remained similar to those of control. After the cessation of AA treatment on day 14, serum creatinine kept increasing in BALB/c mice and decreased slowly in C3H/He mice, whereas the BUN level decreased immediately in both strains. There were remarkable increases in serum creatinine and BUN levels following intraperitoneal injection of AANa in BALB/c and C3H/He mice on day 14, compared with AA-injected mice ($P < 0.05$). There was no significant increase in the two strains following oral administration, except for a slight increase of BUN in BALB/c mice (data not shown).

Urinary protein significantly increased in BALB/c mice 14 days after the first treatment with AANa compared with control mice. In C3H/He mice, a significant increase was seen only on day 14 following oral administration of AANa. No such increase was seen in C57BL/6 mice. In AA-treated BALB/c and C3H/He mice, electrophoresis of urinary protein showed different patterns from control. In these strains, an albumin broad band (67 kDa) was observed, with another one around 20 kDa, which was observed in the control groups. However, there was no difference between treated and control C57BL/6 mice (data not shown).

Further examination using purified AAI, AAII, AAIVa and ALI revealed that AAI possessed strong nephrotoxicity in BALB/c and C3H/He mice (Figure 4A), and that administration of AAII induced focal mild interstitial change (Figure 4B). However, AAIVa and ALI caused no significant renal morphologic alteration in this experimental system, as well as control groups (Figure 4C,

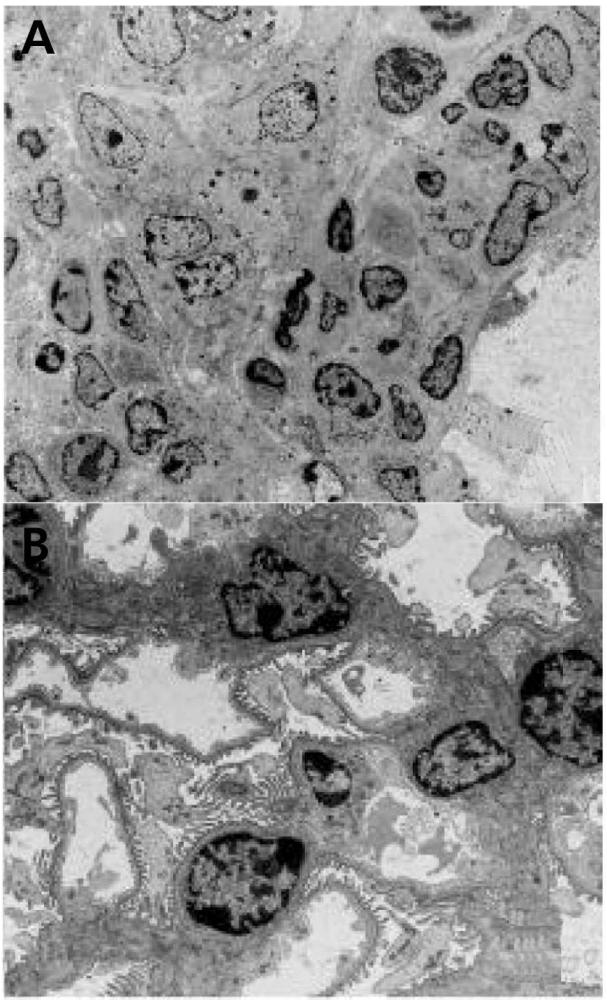

Figure 3 Electron microscopy of kidneys from mice treated with AA (day 14). There were prominent mononuclear cell infiltration into interstitium and detachment of PTE from the tubular basement membrane (A), but no remarkable glomerular changes (B) in C3H/He mice on day 28. Electron microscopy study of kidneys from the other mice also showed only interstitial changes. Magnification × 2280.

D). These changes were reflected in the histological scores (Table 2).

AAI-treated mice became progressively weak. Half of the BALB/c mice were dead due to debility before the final injection, and the administration was discontinued after 7 injections because the remaining mice also were moribund. C3H/He mice were sacrificed one week after the final injection, when their condition had become worse. In these groups, the serum levels of creatinine and BUN were significantly higher than control and the other treatment groups (Table 3).

Discussion

Close association of Chinese herbs containing aristolochic acids (AA) with rapidly progressive uraemia and unique interstitial renal fibrosis in man has been reported in several countries during the last decade. There have been several laboratory studies of AA toxicity in animals. Mengs reported acute toxicity induced by a single administration of a high dose (10–300 mg kg^{-1}) of AA in rats and mice (Mengs 1987; Mengs & Stotzem 1993). The main renal histological change was severe tubular necrosis, and almost all animals died within 15 days. In these animals, renal fibrosis, a characteristic of human AA nephropathy, was not prominent. Cosyns et al (2001) reported chronic nephrotoxicity of AA in rabbits, which was induced by long-term intraperitoneal administration (17 or 21 months) of 0.1 mg kg^{-1} of AA. All rabbits treated with AA developed renal interstitial fibrosis without mononuclear cell infiltration into the interstitium, but the length of AA exposure required makes the experimental model less useful for the study of renal interstitial fibrosis. Salt-depleted Wistar rats that received daily subcutaneous injections of AA 10 mg kg^{-1} developed similar interstitial lesion and urothelial dysplasia within 35 days (Debelle et al 2002). Salt-depletion conditioning and a high dose of AA seem to be essential for AA to induce fibrosis in rats. There seem to be some interspecies differences in susceptibility to AA nephrotoxicity.

In this study, a moderate dose of AA was selected to clarify which strains of mice are susceptible to AA nephropathy. Each mouse received 10 intraperitoneal or oral administrations of 2.5 mg kg^{-1} of AA over 2 weeks. Just after the final AA treatment (day 14), the characteristic histological changes in BALB/c mice were severe tubular necrosis and remarkable hyaline cylinders. In addition to these changes, in C3H/He mice there were infiltration of mononuclear cells into the interstitium and mild interstitial fibrosis. However, in C57BL/6 mice, only mild and focal tubular cell damage was observed on day 14. On day 28, mononuclear cell infiltration became marked among the damaged tubular cells in BALB/c mice. In C3H/He mice, prominent cell infiltration into the interstitium and moderate interstitial fibrosis were observed, and in C57BL/6 mice, moderate cell infiltration into the interstitium was seen. None of the groups showed any glomerular changes. With regard to tubular necrosis, severity was greatest in BALB/c mice, followed by C3H/He mice. In C57BL/6 mice, the tubular necrosis was mild and focal. Interstitial fibrosis, however, was the most severe in C3H/He mice. Thus, there were distinct strain differences in the histological changes induced by AA, but the common characteristic was chiefly tubulointerstitial damage without glomerular injury.

In this experimental system, the degeneration of tubular cells, like acute tubular necrosis, was similar to the result of the aforementioned single-dose toxicity study by Mengs (Mengs 1987; Mengs & Stotzem 1993). The dose of AA used in this study was lower than that of Mengs' study, but similar severe tubular necrosis was also induced by repeated smaller doses. There was extensive tubular necrosis throughout the renal cortex in these different studies. As for interstitial fibrosis, however, this characteristic differed from the findings of the chronic toxicity study by Cosyns et al (2001). In our study, severe degen-

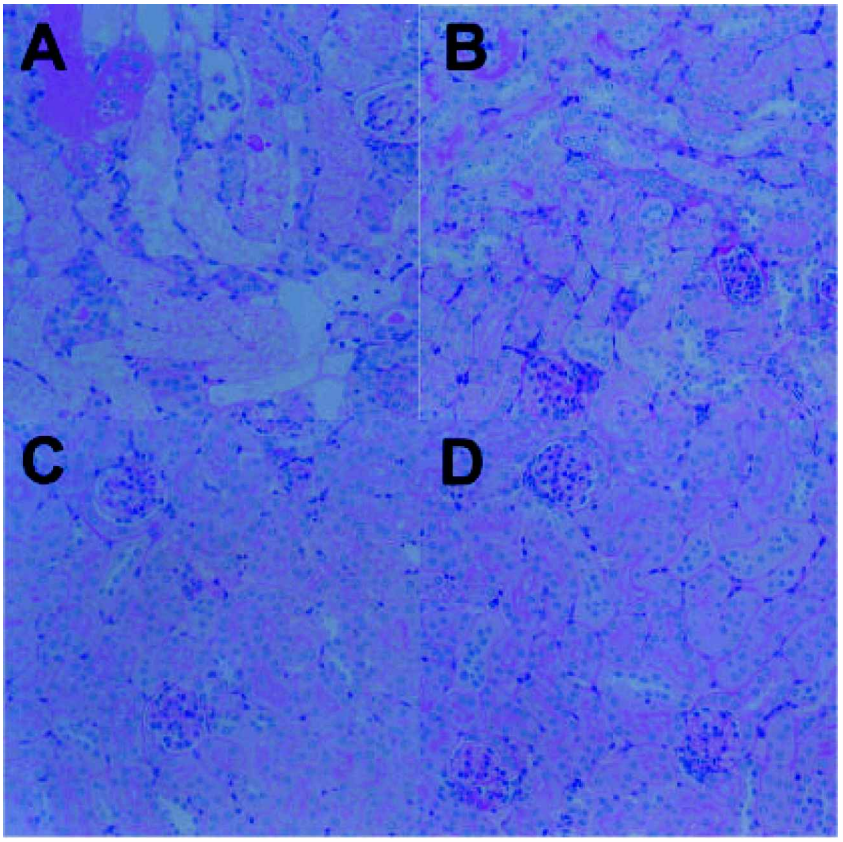

Figure 4 Photomicrographs of kidneys from BALB/c mice treated with AAI, AAII, AAIVa or ALI on day 14. Sections were stained with PAS, magnification × 200. A. In the AAI-treated group, severe degeneration of the tubular epithelium was observed. B. In the AAII-treated group, focal mild interstitial change was seen. AAIVa (C) and ALI (D) caused no nephrotoxicity in this experimental system.

Table 2 Total histological score of mice treated with AAI, AAII, AAIVa or ALI.

	Day	AAI	AAII	AAIVa	ALI
BALB/c	14	7 (6.5–7)	1 (1–1)	0	0
	28	n.d.[a]	2 (1–4)	0	0
C3H/He	14	7.5 (7–8.5)	1.5 (1–2.5)	0	0
	28	8 (7–9.5)[b]	0 (0–1)	0	0

Data are median values (minimum–maximum). Day 14, experimental day 14, just after the final injection of each material; day 28, experimental day 28, 2 weeks after the cessation of each material; n.d., not done. [a]All of the BALB/c mice treated with AAI died of renal insufficiency before day 28. [b]AAI-treated C3H/He mice were sacrificed 3 weeks after the first injection (day 21), when their condition became worse.

erative tubular damage was followed by interstitial fibrosis with mononuclear cell infiltration. Early accumulation of immnunocompetent cells in areas of active tubulointerstitial injury was also observed in salt-depleted rats (Debelle et al 2002). So, it was thought that this interstitial fibrosis in rodents differed in nature from that in the long-term rabbit model used by Cosyns et al (2001), which was induced slowly by long-term administration of a small amount of AA.

Serum creatinine and BUN reflected the severity of tubular necrosis. They revealed remarkable increases in BALB/c and C3H/He, but not in C57BL/6 mice. Cessation of AA administration immediately improved the serum BUN level, while the serum creatinine level decreased slowly. Part of the increase in BUN may also have resulted from non-renal events, such as dehydration or fasting. The BUN level is linked to the total body condition of mice, and its decrease would accompany

Table 3 Blood chemistry of mice treated with aristolochic acids (AA) or ALI.

		Day	AAI	AAII	AAIVa	ALI	Control
BALB/c	BUN (mg dL^{-1})	14	409.35 ± 26.80^{a}	15.87 ± 2.20	19.01 ± 0.92	20.46 ± 1.19	24.85 ± 2.19
		28	n.d.[b]	17.88 ± 1.45	17.60 ± 1.19	20.15 ± 2.19	30.25 ± 2.76
	Cre (mg dL^{-1})	14	0.62 ± 0.02^{a}	0.03 ± 0.02	0.05 ± 0.03	0.07 ± 0.04	0.11 ± 0.04
		28	n.d.[b]	0.08 ± 0.01	0.07 ± 0.01	0.11 ± 0.01	0.12 ± 0.00
C3H/He	BUN (mg dL^{-1})	14	328.60 ± 24.19^{a}	12.30 ± 1.50	24.10 ± 0.35	21.14 ± 1.78	29.65 ± 1.20
		28	317.40 ± 33.02^{ac}	21.10 ± 1.01	28.50 ± 1.24	27.80 ± 1.84	33.30 ± 2.55
	Cre (mg dL^{-1})	14	0.83 ± 0.14^{a}	0.03 ± 0.03	0.07 ± 0.03	0.07 ± 0.03	0.09 ± 0.04
		28	0.58 ± 0.09^{ac}	0.09 ± 0.03	0.17 ± 0.03	0.09 ± 0.02	0.11 ± 0.35

Data are mean ± s.d. BUN, blood urea nitrogen; Cre, serum creatinine; Day 14, experimental day 14, just after the final injection of each material; Day 28, experimental day 28, 2 weeks after the cessation of each material; n.d., not done. [a]$P < 0.001$ vs control. [b]All BALB/c mice treated with AAI died of renal insufficiency before day 28. [c]AAI-treated C3H/He mice were sacrificed 3 weeks after the first injection (day 21), when their condition became worse.

recovery of the body condition due to cessation of the drug. The serum creatinine level might reflect more closely the tubular necrosis and renal function conditions.

An increase in urinary protein was observed in some AANa-treated BALB/c and C3H/He mice, but proteinuria then decreased after the cessation of AANa administration. The proteinuria in CHN patients was mild (not albuminuria) but was of a tubular type, low-molecular-weight proteinuria (Kabanda et al 1995). However, in this study, albuminuria was observed in AA-treated BALB/c and C3H/He mice. Since there was no histological glomerular injury, it was believed that this albuminuria reflected the decrease in tubular reabsorption. Recent data (Lebeau et al 2001) could explain this hypothesis – AA impedes receptor-mediated endocytosis of low-molecular-weight proteins and albumin in the opossum kidney cell line, a model for proximal tubular epithelial cells, suggesting that formation of AA-DNA adducts and decreased megalin expression might be responsible for the inhibition of luminal protein reabsorption by proximal tubule cells.

The major components of AA, AAI and AAII, are known to be genotoxic mutagens forming DNA adducts after metabolic activation (Schmeiser et al 1988; Pfau et al 1990, 1991). In addition to their potential role in mutagenesis and carcinogenesis, AA-DNA adduct formation seems to be responsible for the destructive fibrotic process in kidney. Further quantification studies of DNA-adducts in kidney tissues by 32P post-labelling studies could provide new information about the mechanism of AA toxicity. Interspecies differences in the metabolism of AA have been reported in mammals (Krumbiegel et al 1987). From our histopathological results, it was expected that the metabolism of AA, including DNA-adduct formation, might differ among three strains of mice. Nitroreduction seems to be the crucial step in the pathway of metabolic activation of AA to their ultimate DNA binding species (Arlt et al 2002). Additional experiments to estimate the activity of hepatic microsomal drug metabolizing enzymes in different strains could also contribute to clarification of the AA-induced renal fibrotic process.

Aristolochic acids, representatives of the substituted 10-nitrophenanthrene-1-acids, have been found among *Aristolochia* plants. AAI and AAII are exponents of AA, and their DNA-adducts have both been discovered in the kidneys of CHN patients (Schmeiser et al 1996; Bieler et al 1997; Nortier et al 2000). But, to our knowledge, it has not been evident whether they have similar nephrotoxicity. In this experimental system, it was demonstrated that AAI had the strongest nephrotoxicity, which induced severe tubulointerstitial changes like acute tubular necrosis. In the AAII-treated groups, relatively milder tubular atrophy was seen in both strains. On the other hand, AAIVa and ALI, included in several herbs such as *Asiasarum* root (*Asiasarum sieboldi*), caused no renal abnormality indicated by blood chemistry or histological change.

Conclusion

In conclusion, this study demonstrated that AA induced tubulointerstitial changes in inbred mice, and that the susceptibility to AA differed in the three strains tested. Strain differences might reflect differences in metabolism or detoxification of AA. Further investigations would contribute to the clarification of mechanisms involved in the development of AA nephropathy.

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