# L-Arginine treatment may prevent tubulointerstitial nephropathy caused by germanium dioxide

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*Background.* Long-term oral ingestion of germanium dioxide (GeO$_2$) causes progressive renal failure derived from tubulointerstitial nephropathy in humans and animals. The characteristic of GeO$_2$-induced nephropathy is the renal tissue injury persisting for a long time, even after cessation of GeO$_2$ ingestion. However, a treatment that can suppress the long-lasting renal tissue injury has not yet been established.

*Methods.* Using the methods of immunohistochemistry and reverse transcription-polymerase chain reaction, we examined the expression of ED1-positive cells (macrophages/monocytes), transforming growth factor (TGF)-$\beta_1$ mRNA and protein and collagen type IV mRNA and protein in the kidneys of rats with GeO$_2$-induced nephropathy. Concomitantly, the effects of L-arginine treatment on their expression was explored in the kidneys of rats with GeO$_2$-induced nephropathy.

*Results.* Chronic administration of GeO$_2$ caused tubulointerstitial nephropathy characterized by leukocyte invasion into the enlarged tubulointerstitial space in rats. The expression of ED1-positive cells, TGF-$\beta_1$ protein and collagen type IV protein was markedly increased in the tubulointerstitium of the renal cortex from rats with GeO$_2$-induced nephropathy. Similarly, TGF-$\beta_1$ and collagen type IV mRNA were significantly enhanced in the renal cortex of rats with GeO$_2$-induced nephropathy. A small number of tubulointerstitial cells expressing TGF-$\beta_1$ protein were also observed in the renal cortex of rats with GeO$_2$-induced nephropathy. However, L-arginine treatment led to a parallel decrease in the expression of ED1-positive cells, TGF-$\beta_1$ mRNA and collagen type IV mRNA and protein in rats with GeO$_2$-induced nephropathy.

*Conclusions.* In general, collagen synthesis is driven by TGF-$\beta_1$ in the fibrotic process associated with a variety of renal disorders. TGF-$\beta_1$ is secreted by TGF-$\beta_1$ producing cells such as macrophages, fibroblasts and myofibroblasts. Thus, the present study indicates that the expression of collagen type IV may be mediated by TGF-$\beta_1$ released from invading macrophages and, to a lesser extent, released from tubulointerstitial cells, presumably fibroblasts and/or myofibroblasts in GeO$_2$-induced ne-

phropathy. L-Arginine treatment inhibits collagen type IV synthesis possibly by suppressing macrophage invasion and the resultant TGF-$\beta_1$ expression in this nephropathy. L-Arginine treatment may be beneficial in the prevention of tubulointerstitial fibrosis, which is considered to be the terminal stage of GeO$_2$-induced nephropathy.

Germanium (Ge), carbon (C), tin (Sn), and lead (Pb), all belong to group IV of the periodic system. Ge has been utilized in the industrial field as a semiconductor and in the production of lenses [1]. Ge compounds, particularly organogermanium derivatives, are known to have some biological activities including anti-tumor effects and immunomodulative actions [2, 3]. These compounds were previously used to maintain good health and as a panacea for patients with cancer or AIDS in Japan, the USA and some European countries [4]. Consequently, it was noted that long-term oral ingestion of Ge compounds led to progressive renal failure in humans. The causative substances are now considered to be either germanium dioxide (GeO$_2$) [5–7] or germanium lactate citrate [4, 8, 9].

The histopathological findings concerning GeO$_2$-induced nephropathy are characterized by tubulointerstitial nephropathy with no remarkable changes in glomeruli [7, 10]. The sites of the tubular injury caused by GeO$_2$ are the distal tubules and the collecting ducts [10]. A clinical feature of this nephropathy is subacute renal failure, although there have been no abnormal findings such as proteinuria or hematuria in the urine [7, 10]. However, the most important characteristic is the long-lasting renal dysfunction, based on the renal tissue damage, that persists even after cessation of germanium-containing compounds [7, 8, 10]. Studies by Sanai et al have demonstrated that long-term administration of GeO$_2$ causes tubulointerstitial nephropathy in rats as well as in humans [10]. The same has been described in European patients after long-term ingestion of germanium lactate citrate [4, 8].

The transforming growth factor (TGF)-$\beta$ family, par-

ticularly TGF-$\beta_1$, plays a central role in the regulation of the collagen accumulation that contributes to the formation of tubulointerstitial fibrosis [11, 12]. In the kidneys, TGF-$\beta_1$ is mainly expressed in tubules, glomeruli, invading macrophages and interstitial cells [11, 13, 14]. It was recently reported that L-arginine treatment blocked macrophage infiltration and the resultant interstitial cell involvement and collagen type IV synthesis in tubulointerstitial fibrosis associated with ureteral obstruction and puromycin-induced nephrosis [15, 16]. These findings suggest the importance of early macrophage infiltration in the TGF-$\beta_1$-induced collagen deposition seen in tubulointerstitial fibrosis.

As shown by Sanai et al [10] and also demonstrated in the present study, the long-term oral ingestion of GeO2 leads to tubulointerstitial nephropathy in rats, and it is of enormous value to find a means to block the activity of this progressive renal disorder. Using the kidneys of rats with GeO2-induced nephropathy, we therefore examined the expression of ED1-positive cells (macrophages/monocytes), TGF-$\beta_1$ mRNA and collagen type IV mRNA and protein, which are considered to play key roles in the development of the tubulointerstitial fibrosis associated with GeO2-induced nephropathy. Concomitantly, we explored whether L-arginine had an inhibitory effect on their expression in the kidneys of rats with GeO2-induced nephropathy.

## METHODS

### Chemicals and reagents

L-Arginine was purchased from Life Technologies (Gibco BRL, Baltimore, MD, USA). Monoclonal mouse antibody (Ab) recognizing the cytoplasmic antigen ED1 of macrophages/monocytes (monoclonal Ab ED1) was supplied by BMA Biomedicals Ltd. (Augst, Switzerland). Polyclonal rabbit Ab against TGF-$\beta_1$ was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Polyclonal rabbit Ab against collagen type IV was obtained from LSL C. Ltd. (Tokyo, Japan). Immunohistochemical staining kits were supplied by Dako Corp. (San Diego, CA, USA). Block Ace, a blocking reagent was purchased from Dainippon Pharmaceutical Corp., Ltd. (Osaka, Japan).

### Experimental protocol

Germanium dioxide treatment was performed according to the protocol reported by Sanai et al [10] with slight modification. Female Wistar rats (approximately 175 g, Japan Biochemical Supplement Center, Tokyo, Japan) were pair-fed either a diet not containing GeO2 (standard diet; $N = 15$) or a diet containing 0.15% GeO2 (GeO2 diet; $N = 24$) for 20 weeks. The basic compositions of both diets such as protein, carbohydrates, fat, minerals and vitamin mixtures were identical except for the addi-

tion of GeO2. The amount of GeO2 ingested by the rats in the GeO2 diet group ($N = 18$) amounted to $5.16 \pm 0.48$ (means $\pm$ SD) g over the 20 weeks. The induction of tubulointerstitial nephropathy was examined in a histopathological study using kidney sections, which were stained with hematoxylin and eosin (H&E), from another group of rats at 16 weeks after the start of the GeO2 diet (standard diet, $N = 6$ and GeO2 diet, $N = 10$). All rats ($N = 39$) involved in the final study were allowed free access to tap water until the start of the L-arginine treatment.

Following the histopathological study, rats from both the standard diet group and the GeO2 diet group were further divided into two groups each and given tap water alone (standard diet, $N = 9$ and GeO2 diet, $N = 18$) or 1% L-arginine dissolved in tap water ($N = 6$ in each group) [14, 15] for the last three weeks (weeks 17 to 20) of the feeding period. The body weight of rats was monitored at four week intervals throughout the study.

### Kidney preparation

Under light ether anesthesia, both kidneys were thoroughly perfused with ice-cold phosphate-buffered saline (PBS) through the bifurcation of the aorta [17, 18]. The kidneys were immediately taken out and decapsulated. One kidney was longitudinally sliced at a thickness of approximately 3 mm. The sliced preparations were then fixed overnight at 4°C and 4% paraformaldehyde dissolved in PBS in preparation for the subsequent immunohistochemical study. The other kidney was dissected on ice into the cortex and the medulla. The cortex was used for the extraction of total RNA.

### Immunohistochemical study

The immunohistochemical analysis was performed as reported previously [17, 18]. To remove paraffin, kidney sections (4 μm) were incubated serially in xylene ($\times 3$), 99.5% ethanol ($\times 2$) and 70% ethanol ($\times 1$). H&E staining was then carried out on the sections from each group of rats. For the immunohistochemical study, the sections were treated with 0.125% tripsin diluted with a TBS buffer solution (50 mmol/L Tris-HCl, pH 7.8, 0.05% Tween 20, 0.3 mol/L NaCl and 0.02% NaN$_3$). The endogenous peroxidase activity of the sections was inactivated using a 0.3% hydrogen peroxide treatment. The sections were then blocked in a blocking solution (20% Block Ace), and incubated for two hours with either monoclonal Ab ED1 (1:50 dilution) or antiserum against collagen type IV (1:100 dilution) in a TBS buffer solution containing 0.1% bovine serum albumin (BSA). Each dilution of preimmune serum was used as a negative control. The sections were further incubated with a biotin-conjugated second Ab and horseradish peroxidase-labeled streptavidin, and stained with diaminobenzidine. Nucleus staining was carried out with hematoxylin. In

addition, double immunostaining for ED1-positive cells and TGF-$\beta_1$ was done using monoclonal Ab ED1 and antiserum against TGF-$\beta_1$ (1:200 dilution) according to the protocol described above. These antibodies had alkaline phosphatase and horseradish peroxidase, respectively, through the binding of biotin and streptavidin. Color development was performed with fast red/naphtol and diaminobenzidine, respectively. Monoclonal Ab ED1 was specific for the cytoplasmic antigen ED1 of macrophages/monocytes [18, 19]. The antiserum against collagen type IV or TGF-$\beta_1$ showed no cross reactivity with laminin, fibronectin and collagen types I, II, III, V and VI, or with TGF-$\beta_2$ and TGF-$\beta_3$ when examined by Western blot analysis.

### Determination of the ED1-positive cell number

The ED1-positive cell number was determined by counting the number of cells within the renal cortex that reacted with monoclonal Ab ED1 [16]. Ten randomly chosen fields from the same kidney section were photographed under 200× magnification. The ED1-positive cells were then counted and averaged (cells/200× field). The average number of ED1-positive cells in each group was determined by examining five rats.

### Semiquantitative analysis of collagen type IV

To evaluate the severity of GeO₂-induced tubulointerstitial fibrosis, the deposition score of collagen type IV was determined in the cortical tubulointerstitium of kidney sections that were immunostained with antiserum against collagen type IV as reported previously [20]. Fifty nonoverlapping fields from the same kidney section were assigned a score of 0 to 3 under a microscopic examination (magnification ×200). A score of 0 was assigned when the immunohistochemical appearance was equivalent to that of the control kidney. An increasing score of 1 to 3 was assigned depending on the intensity and distribution of collagen type IV accumulation in the tubulointerstitium (1 = mild deposition; 2 = moderate deposition; and 3 = severe deposition). The rate of the fields assigned each score (X) was calculated as follows.

$$X(\%) = \frac{\text{the number of the fields assigned each score}}{50 \text{ fields}} \times 100$$

Also, the average score exhibiting the severity of tubulointerstitial fibrosis (Y) was determined as follows.

$$Y = \frac{Z_0 + Z_1 + Z_2 + Z_3}{50 \text{ fields}}$$

$$Zn = (N: \text{each score}) \times \left(\begin{array}{c}\text{the number of fields} \\ \text{assigned the score}\end{array}\right)$$

The mean values of X and Y in each group were obtained from six rats.

### Extraction of RNA

The total RNA was extracted from the renal cortex using the guanidinium thiocyanate method [17, 18]. Briefly, the renal cortex was homogenized in ice-cold ISOGEN (Nippon Gene, Toyama, Japan) and the total RNA was precipitated using isopropanol. The RNA pellets were then washed in 75% ethanol, dried and dissolved in RNase-free, distilled water. The quantitative analysis of the total RNA was carried out at 260 nm and 280 nm using a Beckman UV 640 spectrophotometer. The $OD_{260}/OD_{280}$ ratio of all RNA samples used was over 1.9.

### cDNA synthesis and amplification

The polymerase chain reaction (PCR) coupled with the reverse transcription (RT) of RNA was performed for collagen type IV and TGF-$\beta_1$ mRNA expression using an RNA PCR kit (Takara Biochemicals, Shiga, Japan) [17, 18]. Total RNA from the renal cortex was reverse transcribed to first-stand cDNA in an RT buffer solution consisting of 10 mmol/L Tris-HCl, 50 mmol/L KCl, 5 mmol/L MgCl₂, 1 mmol/L deoxynucleotide triphosphate (dNTP) mixture, 1 U/μl RNase inhibitor, 0.25 U/μl avian myeloblastosis virus (AMV) reverse transcriptase, and 2.5 μmol/L random primers. The incubation conditions were 30°C for 10 minutes and 42°C for 30 minutes. Following the incubation period, the reaction mixture was heated at 99°C for five minutes and immediately chilled to 4°C.

Polymerase chain reaction amplification was carried out for collagen type IV, TGF-$\beta_1$, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA obtained from the renal cortex. In brief, collagen type IV cDNA (363 bp) was amplified with 37 cycles in the reaction mixture consisting of the PCR amplification buffer (1 mmol/L Tris-HCl, 50 mmol/L KCl, 4 mmol/L MgCl₂, 200 μmol/L dNTP mixture, and 0.025 U/μl Takara Tag DNA polymerase), 1 μmol/L of collagen type IV cDNA amplification primers (sense: 5′-GTGCGGTTTGTGAAGCACCG-3′ and antisense: 5′-GTTCTTCTCATGCACACTT-3′ [20]) and reverse transcribed cDNA obtained from 1 μg of total RNA under the following conditions: denaturation at 94°C for one minute, annealing at 60°C for one minute and extension at 72°C for two minutes. Similarly, TGF-$\beta_1$ cDNA (498 bp) and the housekeeping gene, GAPDH cDNA (515 bp), were amplified with 37 cycles under the same amplification protocol in the reaction mixture composed of the PCR amplification buffer, 1 μmol/L of primers for each cDNA amplification (TGF-$\beta_1$, sense, 5′-AATACGTCAGACATTCGGGAAGCA-3′, and antisense, 5′-GTCAATGTACAGCTGCCGTACACA-3′ [21]; GAPDH, sense, 5′-AATGCATCCTGCACCACCAA-3′, and antisense, 5′-GTAGCCATATTCATTGTCATA-3′ [17, 18, 20, 21]) and reverse transcribed cDNA obtained from 0.5 μg of total RNA. A pair of primers for collagen type
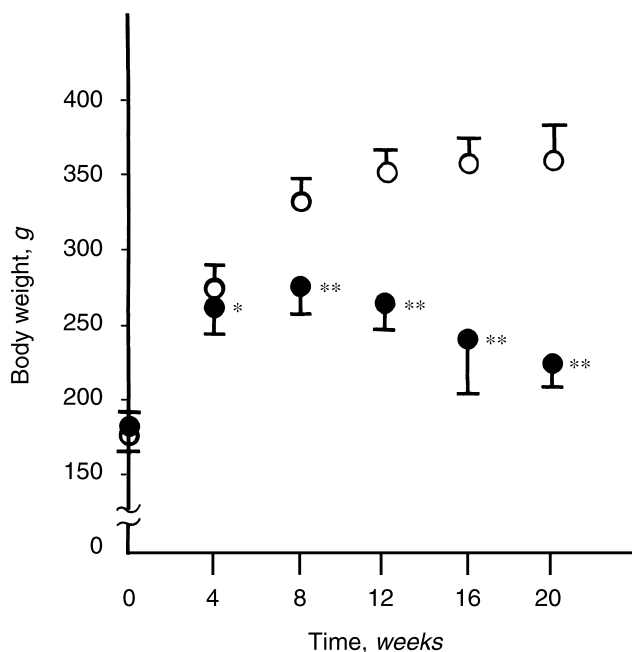
**Fig. 1. Changes in rat body weight after administration of a standard diet (○) or a GeO₂ diet (●).**

IV, TGF-$\beta_1$ or GAPDH cDNA amplification has been confirmed to specifically amplify the objective cDNA through sequencing [20, 21]. In addition, there was a linear increase in each PCR product at least up to 40 cycles when examined using total RNA obtained from control rats and rats with GeO₂-induced nephropathy.

## Semiquantitative analysis of PCR products

Polymerase chain reaction amplification products were separated utilizing 2.0% agarose gel electrophoresis [17, 18]. The gel, containing ethidium bromide (0.5 μg/ml), was visualized with UV light and photographed with Polaroid Type 667 positive film. The intensity of bands was measured by densitometry for semiquantification. Relative levels of collagen type IV or TGF-$\beta_1$ mRNA were determined by the ratio of collagen type IV cDNA/GAPDH cDNA or TGF-$\beta_1$ cDNA/GAPDH cDNA.

## Statistical analysis

All data reported represent means ± SD. Comparisons were evaluated using the unpaired Student's $t$-test or two-way analysis of variance (ANOVA) with Student's $t$-test. Differences were considered significant when $P < 0.05$.

## RESULTS

Figure 1 shows the changes in body weight of rats fed a standard ($N = 9$) or a GeO₂ ($N = 18$) diet for 20 weeks. Rats fed a standard diet showed a progressive increase in body weight for 12 weeks after the start of the diet. Thereafter, the rats showed no substantial changes in body weight. Rats fed a GeO₂ diet had a sluggish increase in body weight for the initial eight weeks of the diet and subsequently exhibited a progressive reduction in body weight. L-Arginine treatment did not affect the body weight of rats fed a standard ($N = 6$) or a GeO₂ diet ($N = 6$; data not shown). The changes in body weight observed in rats fed a GeO₂ diet was probably the result of GeO₂ toxicity rather than calorie restriction, as the two groups of rats were pair-fed isocaloric diets.

To assess the effect of GeO₂ on the induction of tubulointerstitial nephropathy, H&E staining was carried out on kidney sections from rats at 16 weeks after the start of a standard diet ($N = 6$) or a GeO₂ diet ($N = 10$). Figure 2 shows representative photomicrographs of the renal cortex obtained from rats fed either a standard or a GeO₂ diet for 16 weeks. There were no significant histopathological findings in rats fed a standard diet for 16 weeks (Fig. 2A). The H&E staining, however, indicated tubulointerstitial nephropathy characterized by leukocyte infiltration into the enlarged tubulointerstitial space in rats fed a GeO₂ diet (Fig. 2B). There was a loss of proximal tubules, distal tubules and collecting ducts in the enlarged tubulointerstitium. This may have been the result of tubular epithelial cell injury caused by GeO₂ exposure [10]. In addition, some dilated collecting ducts were observed. Glomeruli were mostly intact. These observations were noted in all of the rats examined, thus indicating that with the dietary protocol used in this study, tubulointerstitial nephropathy develops at latest by 16 weeks after exposure to GeO₂.

Table 1 shows data on the number of ED1-positive cells found in the tubulointerstitium of the renal cortex from control rats and rats with GeO₂-induced nephropathy that either did or did not receive L-arginine ($N = 5$ in each group). Figure 3 shows representative photomicrographs of the immunohistochemical staining of ED1-positive cells in the renal cortex from each group of rats ($N = 5$). Few ED1-positive cells were detected in control rats (Fig. 3A). There was a marked increase in ED1-positive cells found in rats with GeO₂-induced nephropathy (Fig. 3B) compared to those found in control rats. Administration of L-arginine did not influence the number of ED1-positive cells found in control rats (Fig. 3C), but it significantly decreased the number of these cells found in rats with GeO₂-induced nephropathy (Fig. 3D). However, the number of ED1-positive cells was still significantly greater in rats with GeO₂-induced nephropathy than in control rats. In addition, no significant expression of ED1-positive cells was observed in glomeruli from each group of rats.

Figure 4 shows representative photomicrographs of the double immunostaining for ED1-positive cells (pink) and TGF-$\beta_1$ (brown) in the renal cortex of control rats

**Table 1.** The number of ED1-positive cells found in the renal cortices of control rats and rats with GeO$_2$-induced nephropathy that did or did not receive L-arginine

| Tap Water | | L-Arginine | |
|---|---|---|---|
| Control | GeO$_2$ | Control | GeO$_2$ |
| 0.9 ± 0.4 | 55.2 ± 19.0[a] | 0.7 ± 0.4 | 11.8 ± 3.8[a,b] |

Data reported are mean ± SD of values obtained from five rats. The number of ED1-positive cells was determined as described in the **Methods** section. Comparisons were based on two-way ANOVA using Student's *t*-test.
[a] $P < 0.005$ compared to each control value
[b] $P < 0.005$ compared to each tap water treatment value

and rats with GeO$_2$-induced nephropathy ($N = 5$ in each group). Nucleus staining was not performed to make the contrast of double staining for ED1-positive cells and TGF-$\beta_1$ clear. There was no significant expression of ED1-positive cells and TGF-$\beta_1$ in control rats (Fig. 4A). However, the expression of ED1-positive cells and TGF-$\beta_1$ was markedly increased in GeO$_2$-induced nephropathy (Fig. 4B). The major cells responsible for the expression of TGF-$\beta_1$ were ED1-positive cells (Fig. 4C, D). However, there were a small number of TGF-$\beta_1$ expressing tubulointerstitial cells that did not react with monoclonal Ab ED1.

Figure 5 shows representative photomicrographs of the immunohistochemical staining of collagen type IV in the renal cortex of control rats and rats with GeO$_2$-induced nephropathy that either did or did not receive L-arginine ($N = 6$ in each group). In control rats, slight expression of collagen type IV was detected in the tubular basement membrane and the peritubular capillary (Fig. 5A). This observation was similar to that reported previously [20]. However, the expression of collagen type IV was substantially potentiated in the tubulointerstium of rats with GeO$_2$-induced nephropathy (Fig. 5B). In particular, increased deposition of collagen type IV was observed in the peritubular capillary and the enlarged tubulointerstitial space. L-Arginine treatment did not affect the expression of collagen type IV in control rats (Fig. 5C). However, L-arginine treatment markedly reduced the expression of collagen type IV in the tubulointerstium of rats with GeO$_2$-induced nephropathy (Fig. 5D). In addition, no significant deposition of collagen type IV was seen in glomeruli from each group of rats.

Table 2 shows the deposition score of collagen type IV in the renal cortex of rats with GeO$_2$-induced nephropathy that either did or did not receive L-arginine ($N = 6$ in each group). A score of 0 to 3 was determined on the basis of the representative photomicrographs presented in Figure 4 (0 = no deposition or faint deposition as shown in Fig. 4A or 4C; 1 = mild deposition as shown in Fig. 4D; 2 = moderate deposition showing the intermediate staining of Fig. 4B and 4D; and 3 = severe deposition as shown in Fig. 4B). The distribution of colla-

gen type IV deposition was the greatest at score 3 in rats with GeO$_2$-induced nephropathy. L-arginine treatment decreased the greatest distribution of collagen type IV deposition to score 1. Consequently, L-arginine treatment significantly reduced the average score of the severity of tubulointerstitial fibrosis from 2.28 ± 0.19 to 1.26 ± 0.15. However, the average score was still higher in rats with GeO$_2$-induced nephropathy than in control rats.

Table 3 shows the relative levels of TGF-$\beta_1$ and collagen type IV mRNA (Fig. 6) found in the renal cortex of control rats and rats with GeO$_2$-induced nephropathy that either did or did not receive L-arginine ($N = 6$ in each group). TGF-$\beta_1$ and collagen type IV mRNA were clearly detected in control rats. Both renal tubules and glomeruli may be the major contributors of TGF-$\beta_1$ mRNA in control rats [11]. Similarly, collagen type IV mRNA may be derived from renal tubules and peritubular capillaries [20]. Relative to that found in control rats, the expression of TGF-$\beta_1$ and collagen type IV mRNA was significantly augmented in rats with GeO$_2$-induced nephropathy. L-Arginine treatment had no effect on the expression of TGF-$\beta_1$ and collagen type IV mRNA in control rats. In contrast, L-arginine treatment substantially decreased the expression of TGF-$\beta_1$ and collagen type IV mRNA in rats with GeO$_2$-induced nephropathy, although the expression of these substances was still significantly increased in rats with GeO$_2$-induced nephropathy relative to that observed in control rats. The expression of TGF-$\beta_1$ and collagen type IV mRNA paralleled the number of ED1-positive cells (Table 1) found in both control rats and rats with GeO$_2$-induced nephropathy that either did or did not receive L-arginine. In addition, there was a parallel relationship between the expression of mRNA and protein in TGF-$\beta_1$ and collagen type IV.

## DISCUSSION

Long-term oral ingestion of GeO$_2$ or germanium lactate citrate, but probably not of carboxyethyl-germanium sesquioxide (Ge-132), leads to chronic tubulointerstitial nephropathy through its cytotoxic effect in humans and animals [4, 7, 8, 10]. There is a marked accumulation of Ge in the renal tissue (particularly in the tubular epithelial cells) of GeO$_2$-induced nephropathy [10]. The striking feature of this nephropathy is the progressive and long-lasting renal damage that is not ameliorated by cessation of germanium administration, although urinalysis is almost normal [7, 8, 10]. In the present study, we showed that long-term administration of GeO$_2$ causes tubulointerstitial nephropathy characterized by leukocyte infiltration into the enlarged tubulointerstitium as a consequence of tubular epithelial cell injury in rats. The tubular damage occurred in the proximal tubules, distal tubules and collecting ducts. However, Sanai et al reported previously that GeO$_2$ was primarily cytotoxic
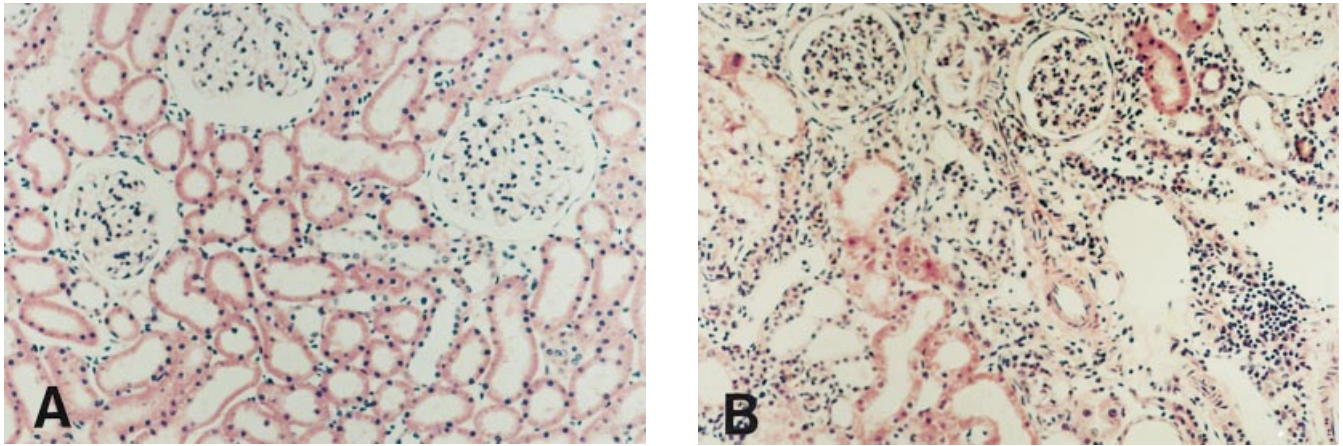
**Fig. 2. Photomicrographs of the kidney sections stained with hematoxylin and eosin.** (*A*) Renal cortex from rats fed a standard diet for 16 weeks. (*B*) Renal cortex from rats fed a GeO₂ diet for 16 weeks. Invading leukocytes are observed in the enlarged tubulointerstitial space of the renal cortex from rats fed a GeO₂ diet for 16 weeks. Original magnification, ×100.
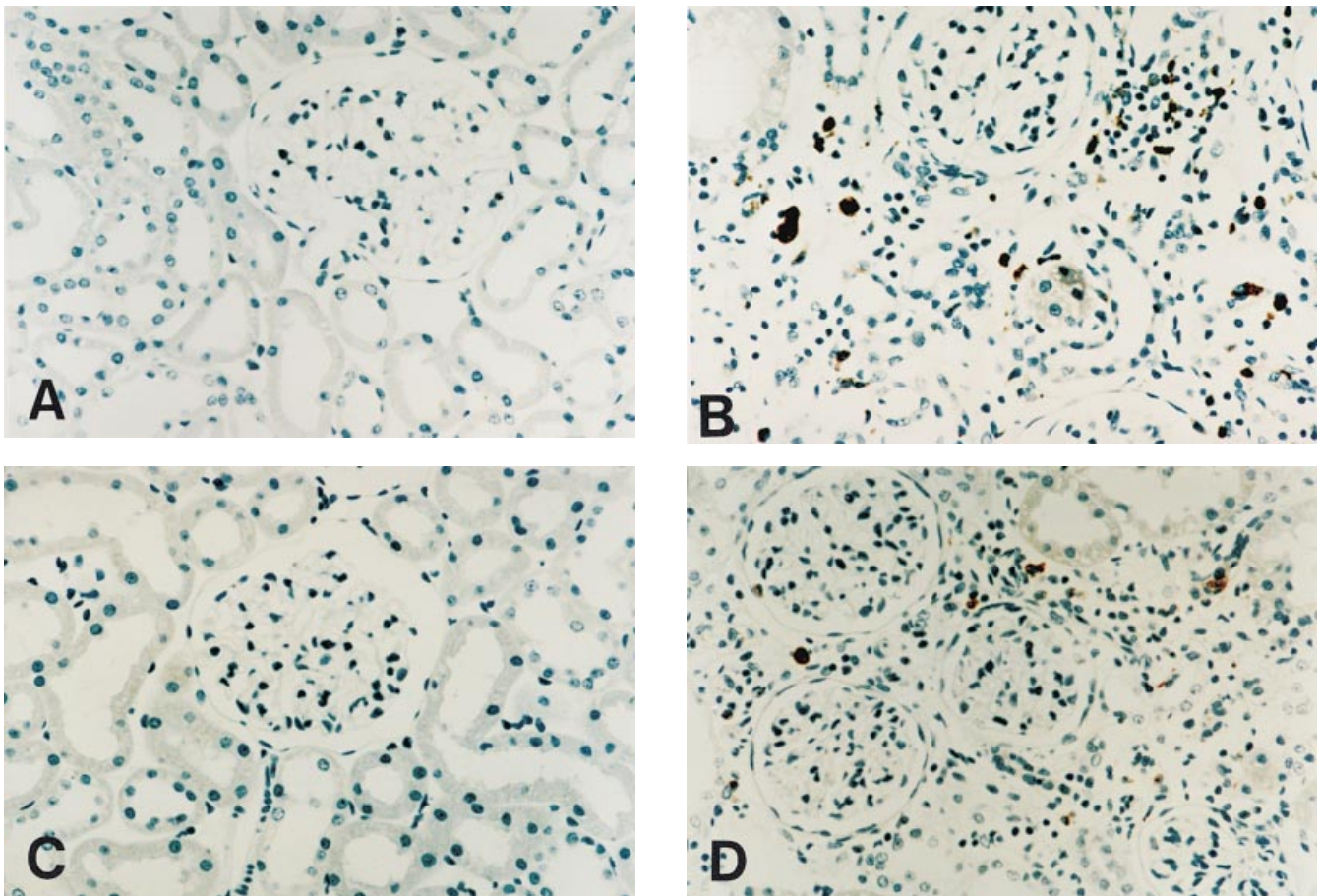


**Fig. 3. Photomicrographs of the immunohistochemical study of ED1-positive cells.** (*A*) Renal cortex from control rats. (*B*) Renal cortex from rats with GeO₂-induced nephropathy. (*C*) Renal cortex from control rats treated with L-arginine for 3 weeks. (*D*) Renal cortex from rats with GeO₂-induced nephropathy treated with L-arginine for 3 weeks. Details of the protocol are described in the **Methods** section. ED1-positive cell infiltration was increased in the renal cortex of rats with GeO₂-induced nephropathy. L-Arginine treatment, however, reduced ED1-positive cell infiltration in the renal cortex of these rats. Original magnification, ×200.

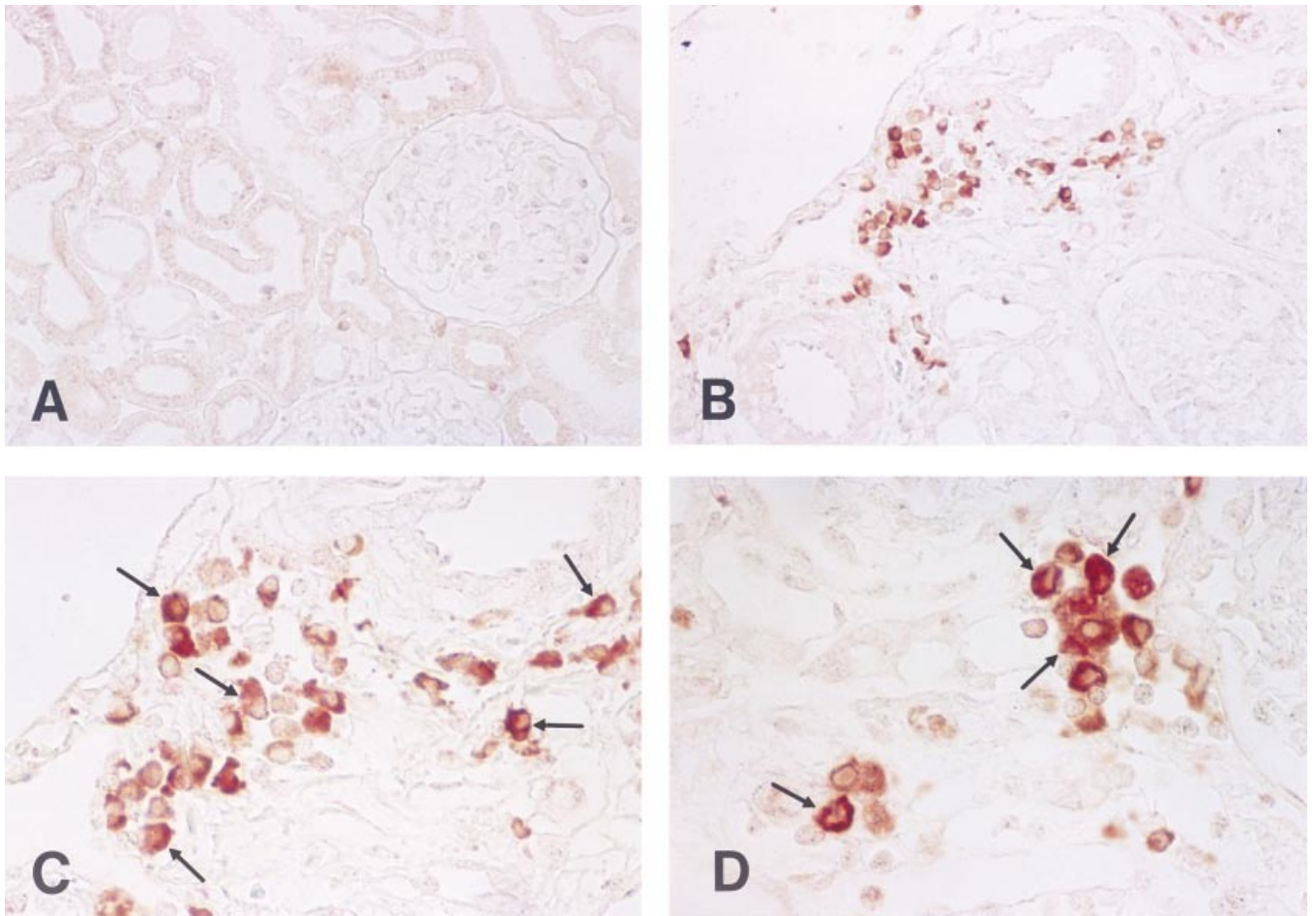**Fig. 4. Photomicrographs of the double immunostaining of ED1-positive cells and transforming growth factor (TGF)-$\beta_1$.** (*A*) Renal cortex from control rats (original magnification, $\times$ 200). (*B*) Renal cortex from GeO₂-induced nephropathy (original magnification, $\times$200). (*C* and *D*) Renal cortex from GeO₂-induced nephropathy (original magnification, $\times$400). Arrows (C and D) show ED1-positive cells (pink) expressing TGF-$\beta_1$ (brown). Details of the protocol are described in the **Methods** section. Nucleus staining was not carried out to make the contrast of double staining clear. Most of the cells responsible for TGF-$\beta_1$ expression were ED1-positive cells.

in the distal tubules and the collecting ducts [10]. This result is somewhat different from ours, and suggests that our study presents a more progressive model of GeO₂-induced nephropathy.

The immunohistochemical/RT-PCR analysis revealed a marked increase in the expression of ED1-positive cells, TGF-$\beta_1$ mRNA and protein, and collagen type IV mRNA and protein in the renal cortex of rats with GeO₂-induced nephropathy relative to that observed in control rats. L-Arginine treatment, although exhibiting no influence in control rats, significantly reduced the expression of these substances in rats with GeO₂-induced nephropathy. There was a parallel relationship between the expression of ED1-positive cells, TGF-$\beta_1$ mRNA and protein, and collagen type IV mRNA and protein in control rats and rats with GeO₂-induced nephropathy that either did or did not receive L-arginine. However, double immunostaining exhibited a small number of TGF-$\beta_1$ expressing tubulointerstitial cells that did not react with

monoclonal Ab ED1. In general, macrophages, TGF-$\beta_1$ and collagens, particularly early infiltrating macrophages, play a key role in the pathophysiology of tubulointerstitial nephropathy. After the onset of tubulointerstitial nephropathy, macrophages infiltrate the tubulointerstitial lesion and act as chemoattractants, thereby stimulating the proliferation of migrated fibroblasts [22]. Macrophages [23], fibroblasts [14, 24] and myofibroblasts [24, 25] are the major TGF-$\beta_1$ producing cells in fibrosis. TGF-$\beta_1$ produced the differentiation of fibroblasts into myofibroblasts [26] and the transdifferentiation of renal tubule epithelial cells into myofibroblasts [27]. The resultant increase in the profibrotic factor TGF-$\beta_1$ increases the synthesis of collagen mRNA and protein in fibroblasts [11], myofibroblasts [28] and renal tubule epithelial cells [29]. Thus, the present study indicates that the synthesis of collagen type IV may be driven by TGF-$\beta_1$ that is derived from invading macrophages and, to a lesser extent, derived from tubulointerstitial cells, presumably
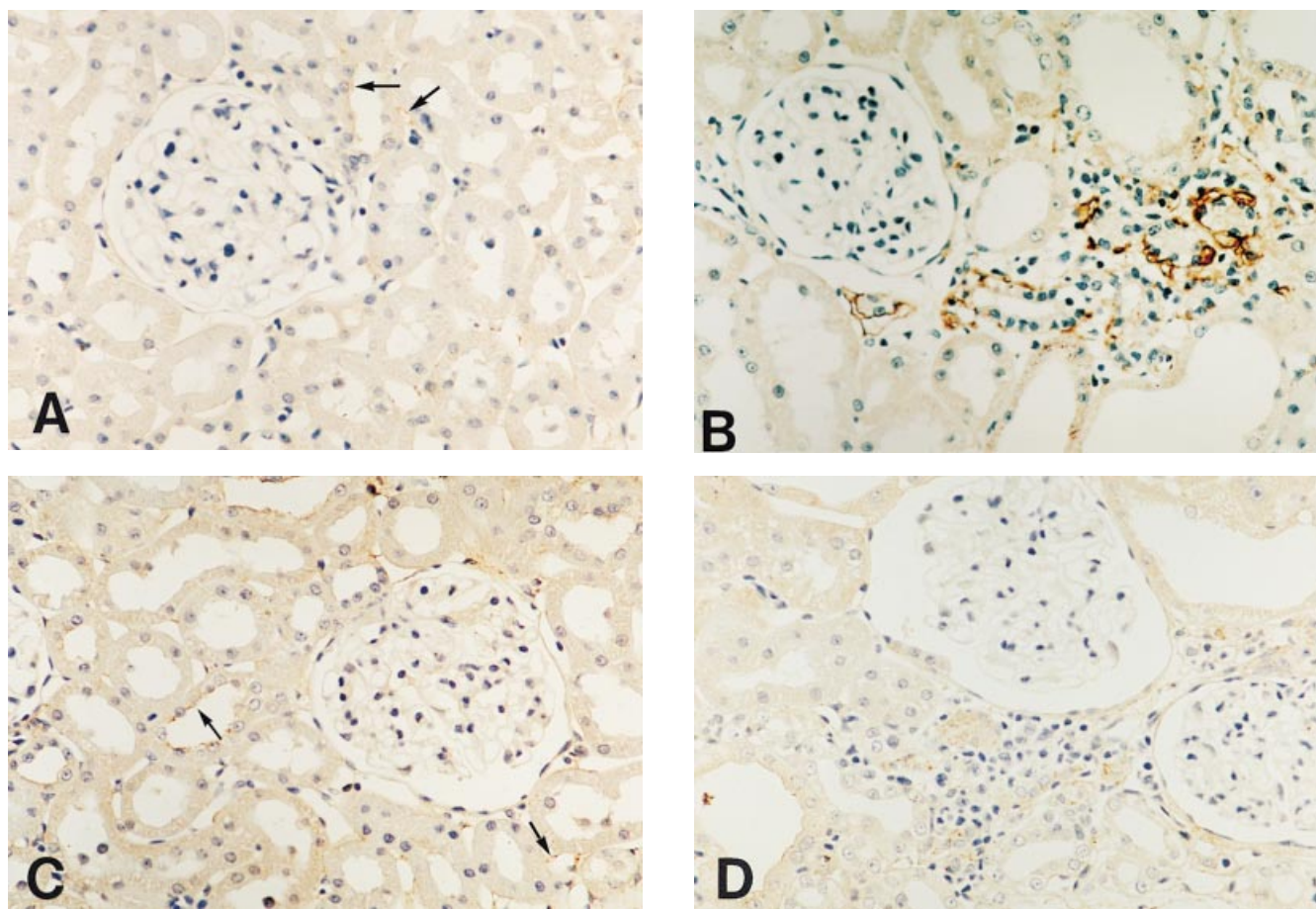
**Fig. 5. Photomicrographs of the immunohistochemical study of collagen type IV.** (*A*) Renal cortex from control rats. (*B*) Renal cortex from rats with GeO₂-induced nephropathy. (*C*) Renal cortex from control rats treated with ʟ-arginine for 3 weeks. (*D*) Renal cortex from rats and GeO₂-induced nephropathy treated with ʟ-arginine for 3 weeks. Arrows (A and C) show collagen type IV expression in the tubular basement membrane and the peritubular capillary. Details of the protocol are described in the **Methods** section. The expression of collagen type IV was increased in the renal cortex of rats with GeO₂-induced nephropathy. ʟ-Arginine treatment, however, reduced the expression of collagen type IV in the renal cortex of these rats. Original magnification, ×200.

**Table 2.** Collagen type IV deposition score in the renal cortices of rats with GeO₂-induced nephropathy that did or did not receive ʟ-arginine

| | Deposition score % | | | | Average score |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | |
| Tap water | $10.0 \pm 3.8$ | $9.3 \pm 3.9$ | $23.7 \pm 9.4$ | $57.0 \pm 12.9$ | $2.28 \pm 0.19$ |
| ʟ-Arginine | $13.3 \pm 4.3$ | $54.0 \pm 7.0^a$ | $25.7 \pm 7.3$ | $7.0 \pm 3.9^a$ | $1.26 \pm 0.15^a$ |

Data reported are means ± SD of values obtained from six rats. Criteria for the deposition score of collagen type IV and the average score of collagen type IV deposition were determined as described in the **Methods** section. The deposition score of collagen type IV was zero in the renal cortices of control rats (not shown). Comparisons were based on unpaired Student's *t*-test.

$^a P < 0.005$ compared to each tap water treatment value

fibroblasts and/or myofibroblasts in GeO₂-induced nephropathy. Subsequently, collagen type IV deposition may contribute to the remodeling of the enlarged tubulo-interstitium that is seen as a consequence of this nephropathy.

It has been recently reported that ʟ-arginine treatment reduces the ED1-positive cell infiltration and the resultant α-smooth muscle actin (a marker of myofibroblasts)

involvement, and collagen type IV mRNA and protein expression that are up-regulated in some renal diseases such as obstructive nephropathy and puromycin-induced nephrosis [15, 16]. This effect of ʟ-arginine may be related to increased synthesis of nitric oxide (NO) by the endothelial NO synthase [16, 30]. Similarly, the administration of NO-generating compounds decreases the expression of ED1-positive cells, TGF-β₁ mRNA, and col-

**Table 3.** Relative levels of transforming growth factor-$\beta_1$ and collagen type IV mRNA found in the renal cortices of control rats and rats with GeO₂-induced nephropathy that did or did not receive L-arginine

| | Tap water | | L-arginine | |
|---|---|---|---|---|
| | Control | GeO₂ | Control | GeO₂ |
| Transforming growth factor-$\beta_1$ | $0.57 \pm 0.11$ | $1.66 \pm 0.15$[a] | $0.49 \pm 0.13$ | $0.94 \pm 0.26$[b,d] |
| Collagen type IV | $0.53 \pm 0.06$ | $1.83 \pm 0.31$[a] | $0.60 \pm 0.16$ | $1.05 \pm 0.25$[c,d] |

Data reported are means (arbitrary units) $\pm$ SD of values obtained from six rats. Relative levels of transforming growth factor-$\beta_1$ and collagen type IV and mRNA were determined as described in the **Methods** section. Comparisons were based on two-way ANOVA using Student's $t$-test.

[a] $P < 0.005$, [b] $P < 0.01$ and [c] $P < 0.025$ compared to each control value

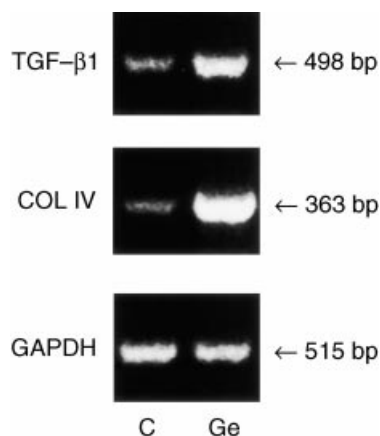[d] $P < 0.005$ compared to each tap water treatment value



**Fig. 6. Photographs of transforming growth factor (TGF)-$\beta_1$, collagen type IV (COL IV) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in the renal cortex of control rats (C) and rats with GeO₂-induced nephropathy (Ge).** Each mRNA expression was determined using the method of reverse transcription-polymerase chain reaction (see **Methods** section).

lagen type IV mRNA and protein that is enhanced in obstructive nephropathy [20], and suppresses TGF-$\beta_1$ mRNA expression and collagen synthesis in cultured vascular smooth muscle cells and mesangial cells [31, 32]. These observations suggest that the inhibitory effect of L-arginine treatment on the fibrotic events associated with some renal diseases results from the suppression of the macrophage/TGF-$\beta_1$/collagen pathway linked to the fibrotic formation by NO produced through this treatment. Indeed, we have shown in the present study that L-arginine treatment prevents collagen type IV mRNA and protein synthesis, as well as suppresses macrophage infiltration and TGF-$\beta_1$ mRNA expression in the renal cortex of rats with GeO₂-induced nephropathy.

It is known that L-arginine supplementation ameliorates systemic and glomerular hypertension [12]. Hypertension usually plays a role in the progression of a variety of renal disorders. Again, hypertension appears with the development of renal injury in many renal disorders. To the best of our knowledge, it is not apparent that GeO₂-induced nephropathy leads to hypertension. It is suggested that L-arginine treatment also contributes in part

to preventing the progression of GeO₂-induced nephropathy by ameliorating hypertension if GeO₂-induced nephropathy develops hypertension.

Nitric oxide also directly inhibits the synthesis of matrix proteins such as collagens [33]. However, there was a parallel reduction in ED1-positive cell invasion, TGF-$\beta_1$ mRNA expression, and collagen type IV mRNA and protein expression in rats with GeO₂-induced nephropathy when the animals were treated with L-arginine. These results suggest that L-arginine treatment causes a decrease in collagen type IV accumulation by inhibiting TGF-$\beta_1$ mRNA expression through the blocking of macrophage infiltration rather than by directly suppressing collagen type IV synthesis.

Of greater interest in the present study is the fact that L-arginine treatment was still effective in suppressing the fibrotic formation associated with GeO₂-induced nephropathy, even when the administration of L-arginine was initiated after the onset of the nephropathy. This implies a therapeutic effect of L-arginine. As noted previously, GeO₂-induced nephropathy is characterized by progressive renal injury that is not alleviated by cessation of GeO₂ ingestion. Thus, L-arginine treatment appears to be useful for preventing tubulointerstitial fibrosis associated with this type of renal disorder. Accordingly, it may be worth examining whether L-arginine is also beneficial in blocking the fibrotic information following renal damage caused by heavy metal compounds other than GeO₂.

In summary, the present study demonstrates that long-term administration of GeO₂ leads to chronic tubulointerstitial nephropathy. This nephropathy is accompanied by macrophage infiltration, a rise in TGF-$\beta_1$ mRNA and protein expression, and an increase in collagen type IV mRNA and protein expression. The increased expression of collagen type IV may play a central role in the formation of tubulointerstitial fibrosis associated with GeO₂-induced nephropathy. L-Arginine treatment produces an inhibitory effect on collagen type IV mRNA and protein synthesis, possibly through suppression of macrophage infiltration and the resultant TGF-$\beta_1$ mRNA expression in GeO₂-induced nephropathy. Thus, L-arginine treatment may be a means of preventing the tubulointerstitial

fibrosis that is considered to be the terminal feature of GeO$_2$-induced nephropathy.

## REFERENCES

1. FURST A: Biological testing of germanium. *Toxicol Industr Health* 3:167–204, 1987
2. ASO H, SUZUKI F, YAMAGUCHI T, HAYASHI Y, EBINA T, ISHIDA N: Induction of interferon and activation of NK cells and macrophages in mice by oral administration of Ge-132, an organic germanium compound. *Microbiol Immunol* 29:65–74, 1985
3. SUZUKI F, BRUTKIEWICZ RR, POLLARD RB: Importance of T-cells and macrophages in the antitumor activity of carboxyethylgermanium sesquioxide (Ge-132). *Anticancer Res* 5:479–484, 1985
4. VAN DER SPOEL JI, STRICKER BH, ESSEVELD MR, SCHIPPER ME: Dangers of dietary germanium supplements. *Lancet* 336:117, 1990
5. NAGATA N, YONEYAMA T, YANAGIDA Y, USHIO K, YANAGIHARA S, MATSUBARA O, EISHI Y: Accumulation of germanium in the tissues of a long-term user of germanium preparation died of acute renal failure. *J Toxicol Sci* 10:333–341, 1985
6. MATSUSAKA T, FUJII M, NAKANO T, TERAI T, KURATA A, IMAIZUMI M, ABE H: Germanium-induced nephropathy: Report of two cases and review of the literature. *Clin Nephrol* 30:341–345, 1988
7. SANAI T, OKUDA S, ONOYAMA K, OOCHI N, OH Y, KOBAYASHI K, SHIMAMATSU K, FUJIMI S, FUJISHIMA M: Germanium dioxide-induced nephropathy: A new type of renal disease. *Nephron* 54:53–60, 1990
8. HESS B, RAISIN J, ZIMMERMANN A, HORBER F, BAJO S, WYTTENBACH A, JAEGER P: Tubulointerstitial nephropathy persisting 20 months after discontinuation of chronic intake of germanium lactate citrate. *Am J Kidney Dis* 21:548–552, 1993
9. KRAPF R, SCHAFFNER T, ITEN P: Abuse of germanium associated with fatal lactic acidosis. *Nephron* 62:351–356, 1992
10. SANAI T, OKUDA S, ONOYAMA K, OOCHI N, TAKAICHI S, MISUHIRA V, FUJISHIMA M: Chronic tubulointerstitial changes induced by germanium dioxide in comparison with carboxyethylgermanium sesquioxide. *Kidney Int* 40:882–890, 1991
11. SHARMA K, ZIYADEH FN: The emerging role of transforming growth factor-β in kidney diseases. *Am J Physiol* 226:F829–F842, 1994
12. PETERS H, NOBLE AN: Dietary L-arginine in renal disease. *Semin Nephrol* 16:567–575, 1996
13. DIAMOND JR, KEES-FOLTS D, DING G, FRYE JE, RESTREPO NC: Macrophages, monocyte chemoattractant peptide-1 and TGF-β$_1$ in experimental hydronephrosis. *Am J Physiol* 266:F926–F933, 1994
14. JOHNSON RJ, HUGO C, HASELEY L, PICHLER R, BASSUK J, THOMAS S, SUGA S, COUSER WG, SHANKLAND SJ: Mechanisms of progressive glomerulosclerosis and tubulointerstitial fibrosis. *Clin Exp Nephrol* 2:307–312, 1998
15. REYES AA, PORRAS BH, CHASALOW FI, KLAHR S: L-Arginine decreases the infiltration of the kidney by macrophages in obstructive nephropathy and puromycin-induced nephrosis. *Kidney Int* 45:1346–1354, 1994
16. MORRISSEY JJ, ISHIDOYA S, MCCRACKEN R, KLAHR S: Nitric oxide generation ameliorates the tubulointerstitial fibrosis of obstructive nephropathy. *J Am Soc Nephrol* 7:2202–2212, 1996
17. YANAGISAWA H, NODERA M, KURIHARA N, WADA O: Altered expression of endothelin-1 and endothelial nitric oxide synthase in the juxtaglomerular apparatus of rats with HgCl$_2$-induced acute renal failure. *Toxicol Let* 98:181–188, 1998
18. YANAGISAWA H, NODERA M, UMEMORI Y, SHIMOGUCHI Y, WADA O: Role of angiotensin II, endothelin-1, and nitric oxide in HgCl$_2$-induced acute renal failure. *Toxicol Appl Pharmacol* 152:315–326, 1998
19. DIJKSTRA CD, DOPP EA, JOLING P, KRAAL G: The heterogeneity of mononuclear phagocytes in lymphoid organs: Distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED$_1$, ED$_2$, and ED$_3$. *Immunology* 54:587–599, 1985
20. KANETO H, MORRISSEY J, MCCRACKEN R, REYES A, KLAHR S: Enalapril reduces collagen type IV synthesis and expansion of the interstitium in the obstructed rat kidney. *Kidney Int* 45:1637–1647, 1994
21. KANETO H, MORRISSEY J, KLAHR S: Increased expression of TGF-β mRNA in the obstructed kidney of rats with unilateral ureteral ligation. *Kidney Int* 44:313–321, 1993
22. KUNCIO GS, NEILSON EG, HAVERTY T: Mechanisms of tubulointerstitial fibrosis. *Kidney Int* 39:550–556, 1991
23. DING G, DIAMOND IP, DIAMOND JR: Cholesterol, macrophage, and gene expression of TGF-β$_1$ and fibronectin during nephrosis. *Am J Physiol* 264:F577–F584, 1993
24. CAMPBELL SE, KATWA LC: Angiotensin II stimulated expression of transforming growth factor-beta 1 in cardiac fibroblasts and myofibroblasts. *J Mol Cell Cardiol* 29:1947–1958, 1997
25. MCKAIG BC, MAKH SS, HAWKEY CJ, PODOLSKY DK, MAHIDA YR: Normal human colonic subepithelial myofibroblasts enhance epithelial migration (restitution) via TGF-beta 3. *Am J Physiol* 276:G1087–G1093, 1999
26. DIAMOND JR, VAN GOOR H, DING G, ENGELMYER E: Myofibroblasts in experimental hydronephrosis. *Am J Pathol* 146:121–129, 1995
27. FAN J-M, NG Y-Y, HILL PA, NIKOLIC-PATERSON DJ, MU W, ATKINS RC, LAN HY: Transforming growth factor-β regulates tubular epithelial-myofibroblast transdifferentiation in vitro. *Kidney Int* 56:1455–1467, 1999
28. TANG WW, VAN GY, QI M: Myofibroblast and α1 (III) collagen expression in experimental tubulointerstitial nephritis. *Kidney Int* 51:926–931, 1997
29. CREELY JJ, DIMARI SJ, HOWE AM, HARALSON MA: Effects of transforming growth factor-beta on collagen synthesis by normal rat kidney epithelial cells. *Am J Pathol* 140:45–55, 1992
30. TSAO PS, MCEVOY LM, DREXLER H, BUTCHER EC, COOKE JP: Enhanced endothelial adhesiveness in hypercholesterolemia is attenuated by L-arginine. *Circulation* 89:2176–2182, 1994
31. KOLPAKOV V, GORDON D, KULIK TJ: Nitric oxide-generating compounds inhibit total protein and collagen synthesis in cultured vascular smooth muscle cells. *Circ Res* 76:305–309, 1995
32. CRAVEN AP, STUDER KR, FELDER J, PHILIPS S, DERUBERTIS RF: Nitric oxide inhibition of transforming growth factor-β and collagen synthesis in mesangial cells. *Diabetes* 46:671–681, 1997
33. TRACHTMAN H, FUTTERWEIT S, SINGHAL P: Nitric oxide modulates the synthesis of extracellular matrix proteins in cultured rat mesangial cells. *Biochem Biophys Res Commun* 207:120–125, 1995