Inhibitory effects of L-arginine treatment on the progression of GeO_2-induced tubulointerstitial nephropathy

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Summary

Long-term oral ingestion of germanium dioxide (GeO_2) causes progressive renal failure derived from tubulointerstitial nephropathy in humans. The characteristic of GeO_2-induced nephropathy is the renal tissue injury persisting for a long time even after cessation of GeO_2 ingestion. To elucidate the mechanisms involved, we examined the expression of ED_1-positive cells (macrophages/monocytes), transforming growth factor (TGF)-β_1 and collagen type Ⅳ in the kidneys of rats with GeO_2-induced nephropathy. Concomitantly, we explored the effect of L-arginine treatment on their expression in the kidneys of rats with GeO_2-induced nephropathy. Chronic administration of GeO_2 caused tubulointerstitial nephropathy characterized by leukocyte invasion into the enlarged tubulointerstitial space in rats. The expression of ED_1-positive cells, TGF-β_1 and collagen type Ⅳ was markedly increased in the tubulointerstitium of the renal cortex from rats with GeO_2-induced nephropathy. However, L-arginine treatment led to a parallel decrease in the expression of ED_1-positive cells, TGF-β_1 and collagen type Ⅳ in rats with GeO_2-induced nephropathy. In general, collagen synthesis is driven by TGF-β_1 in the fibrotic process associated with a variety of renal disorders. TGF-β_1 is secreted by TGF-β_1 producing cells such as macrophages. Thus, the present study indicates that the expression of collagen type Ⅳ may be mediated by TGF-β_1 released from invading macrophages. L-Arginine treatment inhibits collagen type Ⅳ synthesis possibly by suppressing macrophage invasion and the resultant TGF-β_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.

Introduction

Germanium (Ge) compounds, particularly organogermanium derivatives, are known to have some biological activities including anti-tumor effects and immunomodulative actions [1,2]. These compounds

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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 [3]. 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) or germanium lactate citrate [3].

The histopathological findings concerning GeO_2-induced nephropathy are characterized by tubulointerstitial nephropathy with no remarkable changes in glomeruli [4,5]. The sites of the tubular injury caused by GeO_2 are the distal tubules and the collecting ducts [4,5]. 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 [4]. 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 [4,5].

As demonstrated in the present study, the long-term oral ingestion of GeO_2 also leads to tubulointerstitial nephropathy in rats. It is of enormous value to find a means to block the activity of this progressive renal disorder. Using the kidneys of rats with GeO_2-induced nephropathy, we therefore examined the expression of ED_1-positive cells (macrophages/monocytes), transforming growth factor (TGF)-β_1 and collagen type Ⅳ which is considered to play a key role in the development of the tubulointerstitial fibrosis associated with GeO_2-induced nephropathy. Concomitantly, we explored whether L-arginine had an inhibitory effect on their expression in the kidneys of rats with GeO_2-induced nephropathy.

Methods

1. Female Wistar rats (approximately 175g) were pair-fed either a diet not containing GeO_2 (standard diet) or a diet containing 0.15% GeO_2 (GeO_2 diet) for 20 weeks. The basic compositions of both diets such as protein, carbohydrates, fat, minerals and vitamin mixtures were identical except for the addition of GeO_2. The amount of GeO_2 ingested by the rats in the GeO_2 diet group (n=18) amounted to 5.16 (means)± 0.48 (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-eosin (H-E), from another group of rats at 16 weeks after the start of the GeO_2 diet. All rats involved in the final study were allowed free access to tap water until the start of the L-arginine treatment. Following the histopathological study, the rats from both the standard diet group and the GeO_2 diet group were further divided into 2 groups each and given tap water alone or 1% L-arginine dissolved in tap water for the last 3 weeks (weeks 17 to 20) of the feeding period.
2. Kidneys from each group of rats were harvested and decapsulated following dietary treatment. The immunohistochemical study for the expression of ED_1-positive cells (macrophages/monocytes), transforming growth factor (TGF)-β_1 and collagen type Ⅳ in the renal cortex was performed using

monoclonal antibody (Ab) ED_1, polyclonal TGF-β_1 Ab and polyclonal collagen type Ⅳ Ab, respectively. Concomitantly, relative levels of TGF-β_1 and collagen type Ⅳ mRNA were examined in the renal cortex by means of reverse transcription-polymerase chain reaction.

3. The ED_1-positive cell number was determined by counting the number of cells within the renal cortex that reacted with monoclonal Ab ED_1. Ten randomly chosen fields from the same kidney section were photographed under 200x magnification. The ED_1-positive cells were then counted and averaged (cells/200x field). The average number of ED_1-positive cells in each group was determined by examining five rats.
4. To evaluate the severity of GeO_2-induced tubulointerstitial fibrosis, the deposition score of collagen type Ⅳ was determined in the cortical tubulointerstitium of kidney sections that were immunostained with polyclonal collagen type Ⅳ Ab. Fifty non-overlapping fields from the same kidney section were assigned a score of 0 to 3 under a microscopic examination (magnification: x 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(%)=the number of the fields assigned each score/50 fields x 100

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

Y= Z0 + Z1 +Z2 + Z3 / 50 fields

Zn = (n: each score) x (the number of fields assigned the score)

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

Results

1. There were no significant histopathological findings in rats fed a standard diet for 16 weeks. H-E staining, however, indicated tubulointerstitial nephropathy characterized by leukocyte infiltration into the enlarged tubulointerstitial space in rats fed a GeO_2 diet. There was a loss of proximal tubules, distal tubules and collecting ducts in the enlarged tubulointerstitium. Glomeruli were mostly intact (not shown).
2. Few ED_1-positive cells were detected in control rats. There was a marked increase in ED_1-positive cells found in rats with GeO_2-induced nephropathy compared to those found in control rats. Administration of L-arginine did not influence the number of ED_1-positive cells found in control rats, but it significantly decreased the number of these cells found in rats with GeO_2-induced nephropathy. However, the number of ED_1-positive cells was still significantly greater in rats with

GeO_2-induced nephropathy than in control rats (Table 1).

Table 1. The number of ED_1-positive cells found in the renal cortex 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 means ± SD of values obtained from five rats. The number of ED_1-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.

3. Double immunostaining exhibited no significant expression of ED_1-positive cells and TGF-β_1 in control rats. However, the expression of ED_1-positive cells and TGF-β_1 was markedly increased in GeO_2-induced nephropathy. The major cells responsible for the expression of TGF-β_1 were ED_1-positive cells (not shown).
4. Immunostaining exhibited slight expression of collagen type Ⅳ in the tubular basement membrane and the peritubular capillary in control rats. However, the expression of collagen type Ⅳ was substantially potentiated in the tubulointerstium of rats with GeO_2-induced nephropathy. 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. However, L-arginine treatment markedly reduced the expression of collagen type Ⅳ in the tubulointerstium of rats with GeO_2-induced nephropathy (not shown).
5. The distribution of collagen 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 providing 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 2).
6. The expression of TGF-β_1 and collagen type Ⅳ mRNA was clearly detected in control rats. Relative to that found in control rats, the expression of TGF-β_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-β_1 and collagen type Ⅳ mRNA in control rats. In contrast, L-arginine treatment substantially decreased the expression of TGF-β_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.

Table 2. Collagen type IV deposition score in the renal cortex of rats with GeO_2-induced nephropathy that did or did not receive L-arginine

	Deposition Score (%)				Average Score
	0	1	2	3	
Tap Water	10.0 ± 3.8	9.3 ± 3.9	23.7 ± 9.4	57.0 ± 12.9	2.28 ± 0.19
L-Arginine	13.3 ± 4.3	54.0 ± 7.0[a]	25.7 ± 7.3	7.0 ± 3.9[a]	1.26 ± 0.15[a]

Data reported are means ± SD of values obtained from six rats. Criteria for the deposition sore 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 cortex 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.

Table 3. Relative levels of transforming growth factor-β_1 and collagen type Ⅳ mRNA found in the renal cortex 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
Transforming Growth Factor-β_1	0.57 ± 0.11	1.66 ± 0.15[a]	0.49 ± 0.13	0.94 ± 0.26[b,d]
Collagen Type Ⅳ	0.53 ± 0.06	1.83 ± 0.31[a]	0.60 ± 0.16	1.05 ± 0.25[c,d]

Data reported are means (arbitrary units) ± SD of values obtained from six rats. Relative levels of transforming growth factor-β_1 and collagen type Ⅳ 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.

Discussion

1. The present study demonstrated 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 as well as in humans.
2. In general, macrophages, TGF-β_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 [6]. Macrophages are the major TGF-β_1 producing cells in fibrosis [7]. The profibrotic factor TGF-β_1 increases the synthesis of collagen mRNA and protein in fibroblasts. Thus, the present study indicates that the synthesis of collagen type Ⅳ may be driven by TGF-β_1 that is primarily derived from invading macrophages in GeO_2-induced nephropathy. Subsequently, collagen type Ⅳ deposition may contribute to the remodeling

of the enlarged tubulointerstitium that is seen as a consequence of this nephropathy.

3. It has been recently reported that L-arginine treatment reduces the infiltration of ED_1-positive cells and the expression of TGF-β_1 and collagen type Ⅳ that are upregulated in a variety of renal diseases[8]. This effect of L-arginine may be related to increased synthesis of NO by the endothelial NO synthase[8]. Thus, it is suggested that the inhibitory effect of L-arginine treatment on the fibrotic events associated with GeO_2-induced nephropathy results from the suppression of the macrophage/TGF-β_1/collagen pathway linked to the fibrotic formation by NO produced through this treatment.
4. GeO_2-induced nephropathy is characterized by the progressive renal injury that is not alleviated by cessation of GeO_2 ingestion[4,5]. 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 formation following renal damage caused by heavy metal compounds other than GeO_2.

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