22-Oxacalcitriol prevents progressive glomerulosclerosis without adversely affecting calcium and phosphorus metabolism in subtotally nephrectomized rats

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Abstract

Background. 22-Oxacalcitriol (OCT), an analogue of vitamin D, has been shown to inhibit cell proliferation in cultured mesangial cells. OCT also prevented albuminuria and glomerular injury in an acute model of anti-Thy1 glomerulonephritis. However, potential side effects, including calcemic actions and tubular dysfunction, of chronic OCT treatment remain unclear. In the present study, we evaluated the effect of OCT in a chronic model of progressive glomerulosclerosis in subtotally nephrectomized (SNX) rats.

Methods. At one week after subtotal nephrectomy, SNX rats were divided into 3 groups having equivalent serum creatinine levels and body weight. OCT (0.08 or 0.4 µg/kg body weight) was administered intravenously three times per week for 8 weeks to SNX rats. We evaluated effects of OCT on renal function during treatment and on morphologic parameters in glomeruli at 8 weeks. We additionally measured calcium and phosphate levels in serum and urine, and tubular dysfunction markers, including β₂-microglobulin (β₂m) and N-acetyl-β-D-glycosaminidase (NAG) levels in urine.

Results. OCT treatment significantly suppressed urinary albumin excretion, prevented increases in serum creatinine and serum urea nitrogen, and inhibited glomerular cell number, glomerulosclerosis ratio and glomerular volume in SNX rats at 8 weeks. At that time, OCT-treated groups did not show hypercalcemia, hypercalciuria or hyperphosphaturia. Furthermore, OCT treatment did not affect β₂m or NAG levels in urine, and did not induce histological changes in tubular or interstitial regions.

Conclusions. These findings suggest that OCT may provide a clinically useful agent for preventing the progression of glomerulosclerosis without adversely affecting calcium and phosphorus metabolism or causing subsequent tubular dysfunction.

Keywords: glomerulosclerosis; hypercalcemia; hyperphosphataemia; 22-oxacalcitriol (OCT); tubular dysfunction

Introduction

Mesangial cell proliferation is one of the hallmarks of glomerular diseases, such as lupus nephritis, mesangial proliferative glomerulonephritis, IgA nephritis and even diabetic nephropathy [1]. Anticoagulant treatment with heparin prevents glomerular matrix expansion due to a reduction in mesangial cell proliferation in experimental models [2].

1,25(OH)₂D₃ (1,25D₃) exerted anti-proliferative effects in mesangial cells in vitro [3], reduced proteinuria in active Thy1-nephritis rats [4] and inhibited progressive glomerulosclerosis in subtotally nephrectomized (SNX) rats [5]. However, this compound causes hypercalcemia and hyperphosphataemia, which are risk factors for the progression of end-stage kidney disease. Therefore, these properties may limit the clinical usefulness of 1,25D₃.

22-Oxacalcitriol (OCT) is an analogue of vitamin D that has reduced calcemic effects [6] but demonstrates a strong action on cell differentiation [7]. The weaker calcemic effect of OCT has been mainly attributed to its short half-life in the blood stream. In kidney cultured mesangial cells, anti-proliferation effects of OCT have been demonstrated that act in a TGF-β mediated manner [8]. We have recently shown that OCT
effectively prevents urinary albumin excretion, the development of glomerulosclerosis and mesangial cell proliferation without inducing hypercalcaemia in an acute model of anti-Thy1 glomerulonephritis [4]. However, it remains unclear whether long-term OCT treatment prevents progressive glomerulosclerosis in a chronic renal failure model without causing side effects such as hypercalcaemia and hyperphosphataemia.

In the present study, we examined the effects of OCT on glomerulosclerosis in SNX rats using histomorphometric analysis of the glomerulus as well as biochemical measurements of serum and urine. We employed a morphological analysis of glomerular cell number (cell count), glomerular volume (GV) and the glomerulosclerosis ratio (GS) by means of an image analyzer with a microscope (IPAP). For evaluation of side effects, we examined calcium and phosphorus levels in serum and urine, as well as tubular degeneration, dilatation and calcification by histopathology. Furthermore, we measured $\beta_2$-microglobulin ($\beta_2$-m) and N-acetyl-$\beta$-d-galactosaminidase (NAG) levels in urine, which are thought to be specific markers for tubular dysfunction secondary to chronic interstitial renal disease [9].

Subjects and methods

Experimental design

Seven-week-old male Sprague–Dawley (SD) rats were used as a model of SNX. Renal nephrectomy was performed with a standard two-step operation. In brief, after one week acclimation, rats were anaesthetized with diethyl ether and two-thirds of the left kidney was surgically removed. One week later, right nephrectomy was performed. In sham-operated rats, both kidneys were decapsulated. All rats were then maintained in sterilized cages and fed standard rodent chow containing 1.18% calcium and 1.03% phosphate (CE-2, CLEA Japan, Tokyo, Japan). Food and water were provided ad libitum. At 1 week after nephrectomy, SNX rats were divided into 3 groups having equal levels of serum creatinine (Cr) and body weight. OCT was synthesized at Chugai Pharmaceutical Co., Ltd (Gotemba, Japan). The compound was dissolved in phosphate-buffered saline (pH 8.0) containing 0.2% ethanol and 0.01% Tween 20. The treatment groups were as follows: group 1, sham-operated vehicle-treated controls (sham-control: n = 7); group 2, SNX vehicle-treated controls (SNX-control: n = 14); group 3, SNX-OCT, given 0.08 μg/kg body weight OCT (n = 15); and group 4, SNX-OCT, given 0.4 μg/kg body weight OCT (n = 12). OCT or vehicle solutions (volume 100 ml/100 g body weight) were administered intravenously, three times per week for 8 weeks. Blood samples were collected at 4 and 8 weeks, and urine samples were collected at 8 weeks. Histological studies and histomorphometrical analysis were performed in remnant kidneys at sacrifice. The present study was carried out in accordance with Chugai Pharmaceutical’s ethical guidelines for animal care and the research protocols were approved by the animal care committee of the institution.

Biochemical measurements

Measurements of blood ionized calcium (iCa) were performed with an automatic Ca$^{2+}$/pH analyzer (THE 634 Ca$^{2+}$/pH Analyzer, CIBA-Corning, Tokyo, Japan). Serum phosphorus (P), serum urea nitrogen (SUN), serum creatinine (Cr), urinary calcium (uCa), urinary phosphorus (uP), urinary creatinine (uCr) and urinary NAG concentrations were determined using an autoanalyzer (Hitachi 7170, Hitachi, Tokyo, Japan). Urinary albumin (uALB) was measured using a rat albumin ELISA kit (NEPHRAT II, Philadelphia, USA). Urinary $\beta_2$-m was measured using a rat $\beta_2$-m ELISA kit (Panastet, Panafirm Laboratories Co., Ltd, Kumamoto, Japan).

Tissue preparations

At sacrifice, the kidneys were dissected in a plane perpendicular to the interlobar axis. These samples were fixed in Methyl Carnoy’s solution and embedded in paraffin. Coronal sections were cut in 2 μm thicknesses and were stained with haematoxylin/eosin (HE), periodic acid–Schiff reagent (PAS) and periodic acid–methylene silver (PAM).

Morphometric analysis

Glomerular lesions. Glomerulosclerosis and glomerular hypertrophy were evaluated in PAM-stained tissues. The PAM-positive areas and glomerular areas were measured using an image analyzer with a microscope (IPAP, Sumitomo Chemical Co., Ltd, Osaka, Japan). In brief, 50 glomeruli, randomly selected from both superficial cortical and juxta-medullary areas, were evaluated per section. Glomerulosclerosis was expressed as GS (%), which was defined as the ratio of PAM-positive area to glomerular area. Glomerular hypertrophy was expressed as GV (μm$^3$/glomerulus). Glomerular volume was derived from the mean equatorial area of glomeruli from each specimen, as described in the method by Dehoff and Phines [10] for analyzing mean sizes of particles having similar shapes. The mean area of each glomerulus ($A_1, A_2 \ldots A_n$) was calculated by using an image analyzer. Glomerular volume was calculated from the following equations:

$$\text{Mean equatorial area} = \frac{1}{n} \times \frac{3.14}{2} B$$

$$Z = \frac{1}{n} (\frac{1}{A_1} + \frac{1}{A_2} + \ldots + \frac{1}{A_n})$$

$$\text{GV} = 4/3 \times 3.14 \times B \times (1/B \times 1/3.14)^{1/2}$$

Glomerular cell proliferation was estimated by the measurement of total glomerular cell number per glomerulus in HE-stained sections.

Tubular and interstitial lesions. The severity of tubular degeneration, dilatation, calcification and interstitial fibrosis were individually evaluated. A semiquantitative analysis (using a scale of 0 to 3+) was applied for the following features: tubular epithelial degenerative and regenerative changes, tubular atrophy, interstitial oedema, and inflammation fibrosis. These were scored 0 for absent, 1 for <10%, 2 for 10–25%, 3 for 25–50% and 4 for >50% of the section.
Statistical analysis

All values were expressed as means ± SEM. Statistical analysis was performed with a statistical analysis system (SAS, version 6.12). Student’s t-tests were used for comparisons between sham-control and SNX-control. Dunnett’s tests were used for comparisons of SNX-control with OCT-treated groups. A P-value of <0.05 was considered significantly different.

Results

Urinary protein excretion

The uALB excretion in SNX-control rats was markedly elevated at 8 weeks after treatment. In contrast, OCT treatment at both doses significantly prevented the elevation in uALB excretion (Figure 1).

Serum creatinine and urea nitrogen

Serum creatinine (A) and SUN (B) concentrations were significantly increased in SNX-control rats compared with sham-controls throughout the treatment. Treatment with OCT significantly lowered these parameters at 8 weeks but not at 4 weeks (Figure 2).

Calcium and phosphorus

Table 1 shows measurements of calcium and phosphorus metabolism in SNX rats at the end of treatment. Ionized Ca concentrations in OCT-treated rats were within the normal range. Serum phosphorus in OCT-treated rats was slightly but non-significantly elevated. The uCa/uCr and uP/i/uCr values in SNX-control rats were significantly higher than in sham-controls. OCT treatment did not affect these parameters.

Histologic studies

Figure 3 shows representative histological sections of glomeruli and interstitial areas. Prominent glomerular cell growth, glomerulosclerosis and glomerular hypertrophy were observed in SNX-control rats (B). In contrast, OCT-treated rats showed apparent amelioration of glomerulosclerosis and glomerular hypertrophy.

Figure 4 shows the number of glomerular cells (cell count, A), the mean GV (B) and the GS (C) at the end of treatment. Significant increases in cell numbers, GV and GS, which represented the increase in the mesangial matrix, were observed in SNX rats compared with sham-controls. Treatment with OCT significantly ameliorated each of these parameters.

Table 2 shows histological changes in both tubular and interstitial areas. Although SNX-controls showed a significant degeneration and dilatation of tubules and fibrosis in the interstitial area, these changes were not observed in OCT-treated rats. OCT did not induce calcification in interstitial areas and also showed a tendency towards prevention of tubular dilatation.

Tubular dysfunction parameters

Urinary excretion of the \( \beta_2 \)m and the NAG to uCr ratios are shown in Table 3. A significant elevation

![Fig. 1. Effect of OCT on the urinary ALB excretion at the end of treatment in SNX rats. Values show means ± SE. *P < 0.05 vs sham-control group, **P < 0.01 vs SNX-control group.

![Fig. 2. Sequential changes in serum creatinine concentration (A) and SUN concentration (B) in sham-control group (closed diamonds), SNX-control group (closed squares) and OCT-treated groups (closed triangles: 0.08 μg/kg; open triangles: 0.4 μg/kg) after the start of treatment. Values show means ± SE. *P < 0.05 vs sham-control group, **P < 0.01 vs SNX-control group.](https://academic.oup.com/ndt/article-abstract/17/12/2132/1821323)
2m uCr was observed in SNX-control rats. OCT-treated rats were not significantly different from SNX-controls. Despite the loss of tubular number by nephrectomy, NAG/uCr in SNX-control rats was similar to that in sham-control rats. Therefore, urinary NAG excretion per tubular cell in SNX-control rats might be higher than in sham-control rats. OCT slightly lowered this parameter.

Discussion

Schwarz et al. [5] demonstrated that 1,25D3 could attenuate the progression of glomerulosclerosis in SNX rats. However, the effective dose caused a significant elevation in urinary Ca excretion. In cultured mesangial cells, the anti-proliferative action of 1,25D3 was comparable to that of OCT. In addition, we recently reported that OCT reduces glomerular injury without inducing hypercalcaemia in an anti-Thyl nephritis model [4]. We therefore evaluated whether long-term OCT prevents progressive glomerulosclerosis without causing hypercalciuria in SNX rats.

OCT treatment at a dose of 0.08 µg/kg body weight significantly reduced Cr, uALB secretion and morphometrical parameters, indicating reductions in the number of glomerular cells, the mean GV and the GS in glomeruli. In an SNX model, using immunostaining to mark the proliferating cell nuclear antigen, Floege et al. [11] reported that mesangial cells proliferated more markedly than endothelial cells after subtotal nephrectomy. These findings suggest that reductions in cell number by OCT may mainly reflect inhibition of mesangial cells. A possible mechanism underlying

Table 1. Effect of OCT on calcium and phosphorus in SNX rats at the end of treatment

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<th>Parameters</th>
<th>Sham-control</th>
<th>SNX-control</th>
<th>OCT (µg/kg)</th>
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<tr>
<td></td>
<td></td>
<td>0.08</td>
<td>0.4</td>
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<td>Blood or serum</td>
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<tr>
<td>iCa (mmol/l)</td>
<td>1.46 ± 0.02</td>
<td>1.37 ± 0.02$^*$</td>
<td>1.45 ± 0.01</td>
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<td>Pi (mg/dl)</td>
<td>5.7 ± 0.2</td>
<td>6.3 ± 0.3$^*$</td>
<td>7.0 ± 0.2</td>
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<td>Urinary excretion ratio</td>
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<tr>
<td>uCa (mg/mg uCr)</td>
<td>54.1 ± 5.9</td>
<td>95.3 ± 11.1$^*$</td>
<td>110.2 ± 9.9</td>
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<tr>
<td>uPi (mg/mg uCr)</td>
<td>0.8 ± 0.1</td>
<td>1.5 ± 0.1$^*$</td>
<td>1.4 ± 0.1</td>
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Values are presented as means ± SE. $^*P<0.05$ vs sham-control.

Fig. 3. Light micrographs of a glomerulus from a SNX-control rat (A, B) and OCT-treated SNX rats at the dose of 0.08 µg/kg (C, D). PAS stain. Magnification: left, ×25; right, ×100.
the anti-proliferative action of 1,25D3 may be the activation of the Cdk inhibitor p21 [12] through the vitamin D receptor (VDR), which exists in mesangial cells [3]. In addition, we reported that OCT also exerted anti-proliferative effects by causing TGF-β type-II receptor up-regulation without an elevation in the TGF-β ligand [8]. Therefore, OCT may inhibit mesangial cell proliferation not only through p21 dependent cell-cycle regulation but also by a TGF-β signal. It has been recently reported that the antiproteinuric effect of 1,25D3 is associated with a reduction in the glomerular inflammatory process. Since anti-inflammatory effects of OCT have also been shown in rats with carrageenin-induced inflammation [13], the attenuated glomerulosclerosis in this study may be partly due to anti-inflammatory actions of the analogue.

There is strong correlation between glomerular size and the degree of glomerulosclerosis. Indeed, subtotal nephrectomy, which imposes a hypertrophic stimulus on the remnant kidney, has been found to accelerate glomerulosclerosis. In the present study, SNX rats had significant elevations in GV and GS as well as increases in type I and IV collagens in the mesangial area (data not shown). Therefore, the efficacy of OCT to suppress glomerular hypertrophy and sclerosis was mainly due to the prevention of mesangial matrix synthesis and mesangial cell growth via the VDR, which is found in mesangial cells [4].

Although the data are not shown in the present study, we evaluated the effects of both OCT and angiotensin converting enzyme inhibition with enalapril on arterial blood pressure. We found that enalapril significantly prevented increases in serum creatinine by lowering blood pressure, whereas OCT prevented creatinine increases without causing haemodynamic changes. These data suggest that OCT and enalapril reduce glomerulosclerosis through different mechanisms.

Chronic active vitamin D treatment and the resulting hypercalcaemia and hyperphosphataemia induce a high risk of calcification in soft tissues, including arteries and kidney [14]. In the present study, OCT did not induce hypercalcaemia, hypercalcuria or hyperphosphaturia, and did not produce tubular calcification. OCT produces weaker calcemic and phosphataemic effects than 1,25D3. The reduced calcaemic actions of OCT have been partly explained by its rapid metabolic clearance due to both its low affinity for vitamin D-binding proteins and selective tissue distribution [15]. For instance, a single intravenous administration in normal rats produces a blood half-life of OCT (about 20 min) that is much shorter than that of 1,25D3 (about 60 h) [16]. In addition, an attenuated effect of OCT on intestinal calcium absorption acting

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Table 2. Histochemical analysis of tubules and interstitium in SNX rats at the end of treatment

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Values are presented as means ± SE.

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Table 3. Effects of OCT on tubular dysfunction markers in SNX rats at the end of treatment

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Values are presented as means ± SE. *P < 0.005 vs sham-control. **P < 0.05 vs SNX-control.
on calcium-binding proteins, especially on CaBP–D9K in the intestine, may be responsible for the lower calcemic effect of OCT [17]. Furthermore, the reduced influence of OCT on phosphorus metabolism has been explained in part by weaker stimulating effects on both intestinal absorption and urinary excretion of calcium and phosphorus in normal rats, and by a decreased action on bone resorption in parathyroidectomized rats [18]. These findings therefore indicate that OCT produces reduced calcification in soft tissues compared with 1,25D3.

Abundant VDR is also found in tubular epithelial cells and 1,25D3–DBP complexes are endocytosed into the proximal tubular epithelium via megalin from the basolateral site of the tubule [19]. To assess whether long-term treatment with OCT induces tubular damage, we histologically evaluated the renal tubules and measured NAG and β2M, which are useful markers of tubular dysfunction. We found that OCT did not induce significant morphological changes in tubules and did not affect tubular dysfunction markers. OCT is transported by low-density lipoprotein (LDL), which is thought to be an additional carrier protein of the vitamin D analogue, and enters cells via an LDL-receptor mediated pathway [20]. Importantly, these receptors are expressed in the mesangial cells. Therefore, it was suggested that OCT, which binds to LDL, is incorporated more effectively into mesangial cells. These characteristics may explain the efficiency of OCT on reducing glomerulosclerosis despite its rapid clearance from the circulation.

In conclusion, the present findings demonstrated that OCT prevented the progression of glomerulosclerosis in SNX rats without affecting calcium and phosphorus homeostasis or tubular morphology and function. Further studies are warranted to evaluate the mechanisms through which OCT prevents the progression of glomerulosclerosis.

Acknowledgements. Part of this study was presented at the Thirty-First Annual Meeting of the American Society of Nephrology at Miami, 1999; and appeared as an abstract in J Am Soc Nephrol 1999: 10: A3345. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture, Japan (Nos. 11877175 and 12470210) and ‘Research for the Future (RFTF)’ of Japan Society for the Promotion of Science (JSPS-RFTE97L00805).

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Received for publication: 23.8.01
Accepted in revised form: 28.6.02