In subtotally nephrectomized rats 22-oxacalcitriol suppresses parathyroid hormone with less risk of cardiovascular calcification or deterioration of residual renal function than 1,25(OH)₂D₃ vitamin D₃

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Abstract

Background. Although it effectively suppresses parathyroid hormone (PTH) secretion, vitamin D [1,25(OH)₂D₃] therapy often causes tissue calcification over the long term. In patients on chronic dialysis, cardiovascular calcification is clearly linked to an unfavourable prognosis. In pre-dialysis patients, renal calcification of the kidney leads to the deterioration of renal function.

Methods. We compared the propensities of 22-oxacalcitriol (OCT), with lesser calcaemic action, and 1,25(OH)₂D₃ for producing their potential side effects in rats: (i) metastatic calcification of heart and aorta, and (ii) renal dysfunction with nephrocalcinosis, using the same effective doses for hyperparathyroidism. OCT (1.25 and 6.25 mg/kg) or 1,25(OH)₂D₃ (0.125 and 0.625 mg/kg) solutions were administered intravenously to subtotally nephrectomized (SNX) rats three times weekly for 2 weeks.

Results. Despite the suppression of PTH to comparable levels, the calcification of the hearts, aortas and kidneys in the 1,25(OH)₂D₃-treated group was significantly greater than in the OCT-treated group. Of interest was that, in the OCT (6.25 mg/kg) group, the degree of calcification in hearts, aortas and kidneys were distinctly lower than those in the 1,25(OH)₂D₃ (0.125 mg/kg) group despite the comparable serum Ca × Pi products. Therefore, there may be different mechanisms behind the calcifications resulting from OCT and 1,25(OH)₂D₃. Deterioration of renal function, tubular changes, and atypical hyperplasia of proximal tubules associated with calcification were more severe in the 1,25(OH)₂D₃-treated group than in the OCT-treated group.

Conclusions. These results indicate that OCT may be an effective agent for the suppression of PTH with a lesser risk of cardiovascular calcification or deterioration of residual renal function.

Keywords: Ca × Pi product; metastatic calcification; 22-oxacalcitriol; 1,25(OH)₂D₃

Introduction

Vitamin D [1,25(OH)₂D₃] pulse therapy has been routinely used to treat secondary hyperparathyroidism (2HPT) [1]. Despite its effectiveness, this long-term therapy often causes metastatic calcification in several tissues, such as the heart, lung, aorta and kidney, because of hypercalcaemia [2]. Cardiovascular events represent the leading cause of death in dialysis patients, accounting for nearly half of all deaths among patients with end-stage renal disease (ESRD) [3,4]. These patients frequently have hyperphosphataemia as well as an excess calcium load, which elevate the Ca × Pi product, thereby contributing to the development of calcific complications [5]. Mismanagement of calcium and phosphorus metabolism during the treatment of 2HPT has been implicated as a major factor in the development of soft tissue calcification and cardiovascular disease. Nephrocalcinosis, which contributes to tubular damage and interstitial fibrosis, also causes renal dysfunction [6,7]. Thus, long-term 1,25(OH)₂D₃ therapy carries a high risk of accelerating cardiovascular disease and renal dysfunction associated with calcification.

In preclinical studies, 22-oxacalcitriol (OCT), a unique 1,25(OH)₂D₃ analogue, has been shown to
have less calcaemic [8] and less antiphosphaturic effects [9] than 1,25(OH)2D3. This treatment prevents 2HPT and renal osteodystrophy without causing hypercalcemia and hyperphosphataemia in rats [10] and dogs [11]. Therefore, we believe that OCT has an advantage over 1,25(OH)2D3 due to the lower risk of side effects.

In this study, we compared OCT and 1,25(OH)2D3 with respect to their risk of producing the following side effects: (i) metastatic calcification of heart and aorta, and (ii) renal dysfunction associated with nephrocalcinosis, using the same effective dose for 2HPT in subtotally nephrectomized (SNX) rats.

Materials and methods

Experimental protocol

Male, 7-week-old Sprague–Dawley rats were used as subjects. The nephrectomy was performed with a standard two-step operation. Briefly, after an acclimation period of 1 week, rats were anaesthetized with diethyl ether and two-thirds of their left kidneys were surgically removed. One week later, a right nephrectomy was performed. In sham-operated rats, both kidneys were only 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 Inc.). Food and water were provided ad libitum. At 10 weeks after the second step of nephrectomy, the rats were divided into five groups with comparable serum creatinines (Cr) and body weights (BW).

The treatment groups were as follows: Group 1, sham-operated vehicle-treated control group (sham-control group: n = 6); Group 2, SNX vehicle-treated control (SNX-control group: n = 9); Group 3, SNX-OCT 1.25 μg/kg BW (low-dose OCT-treated group: n = 8); Group 4, SNX-OCT 6.25 μg/kg BW (high-dose OCT-treated group: n = 8); Group 5, SNX-1,25(OH)2D3 0.125 μg/kg BW (low-dose 1,25(OH)2D3-treated group: n = 8); Group 6, SNX-1,25(OH)2D3 0.625 μg/kg BW (high-dose 1,25(OH)2D3-treated group: n = 8) as a positive control of calcification.

Results

Tissue preparation

The kidneys were dissected in a plane perpendicular to their interpolar axes and divided into three parts, of which two were fixed in 20% neutral-buffered formalin, and embedded in paraffin. The other was frozen at –20°C until Ca and Pi could be measured. One of the paraffin sections was stained by the von Kossa method and then counterstained with haematoxylin. The other was stained with HE stain. The aortas and hearts were removed and Ca and Pi concentrations in a part of each of them were determined. Their remnants were stained by the von Kossa method.

Measurements of Ca and Pi contents in aorta, heart and kidney

We performed the measurements of Ca and Pi contents in aortas, hearts and kidneys as described previously [12]. Briefly, after lyophilization, dried aortas, hearts and kidneys were delipidized with a mixed solution of chloroform and methanol (2:1) for 48 h, and then dehydrated with acetone for 3 h. Samples were incinerated to ash at 900°C for 12 h using an electric muffle furnace (KDF S90; Denken Co., Ltd, Kyoto, Japan), and then were dissolved in hydrochloric acid. These solutions were diluted with adequate volumes of distilled water, and Ca and Pi concentrations were measured using an autoanalyser (Hitachi 7170, Hitachi Co., Ltd, Tokyo, Japan).

Measurement of atypical hyperplasia of epithelial tubules (AHET)

To evaluate the relationship between renal dysfunction and morphological changes, we counted the foci of proliferative epithelial tubules in all areas of sections stained with HE. The foci consisted of bizarre epithelial cells projecting toward the lumen, which were designated as atypical hyperplasia of epithelial tubules (AHET). The histological evaluation and scoring of AHET were performed by two independent pathologists.

Statistical analysis

Statistical analysis was performed with statistical analysis software (SAS, version 6.12). A single ANOVA was used for comparisons between groups. The correlation was tested using Pearson’s product moment correlation coefficient. A P-value of < 0.05 was considered significant.

Results

Body weight and biochemical parameters

At sacrifice, the BW of SNX-control rats, OCT-treated groups and the 1,25(OH)2D3-treated group were slightly lower compared with sham-control rats. A significant elevation of serum N-PTH levels was
observed in SNX-control rats compared with sham-control rats (Table 1). In contrast, both OCT and 1,25(OH)₂D₃ inhibited the serum N-PTH levels significantly [OCT (1.25 μg/kg), \( P < 0.05 \); OCT (6.25 μg/kg), \( P < 0.01 \); 1,25(OH)₂D₃ (0.125 μg/kg), \( P < 0.05 \)] (Table 1).

To correlate the side effects of these compounds to their suppression of serum N-PTH levels at the same effective dose, we measured the concentrations of Ca and Pi in serum and urine at the end of treatment. Although both OCT and 1,25(OH)₂D₃ elevated serum Ca significantly, the increase associated with the OCT treatment was weaker compared with the 1,25(OH)₂D₃ treatment. OCT resulted in no significant dose-dependent elevation of Pi levels in serum or urine. Both OCT and 1,25(OH)₂D₃ elevated the serum Ca × Pi product significantly (Table 2). In addition, the serum Ca × Pi product in the 1,25(OH)₂D₃ group was comparable to that in the high-dose OCT group (Table 2). OCT and 1,25(OH)₂D₃ had no effect on creatinine clearance (CCr) (Table 1).

Metastatic calcification in heart and aorta

Figure 1 shows Ca and Pi concentrations in the heart and aorta at the end of treatment. There was no significant difference between the Ca and Pi concentrations in SNX-control rats and sham-control rats. Comparing the same effective doses for PTH inhibition, OCT treatment also produced levels of Ca and Pi comparable to those in the SNX-control groups, while 1,25(OH)₂D₃ treatment was associated with a significant elevation of both elements in the heart. Interestingly, despite the comparable effects on the Ca × Pi products, Ca and Pi concentrations in the tissues of the high-dose OCT group were significantly lower than their concentrations in the low-dose 1,25(OH)₂D₃ group.

Figure 2 shows representative sections of heart, coronary artery and aorta stained by von Kossa staining. Ca deposition in the high-dose 1,25(OH)₂D₃ group (Figure 2D) was observed in the valves (arrow) and muscle (asterisk) of the heart. Severe calcification and slight dilatation were also observed in the coronary arteries and the aortae in this group. On the other hand, there was no calcium deposition in the heart and the aorta in the high-dose OCT group.

Metastatic calcification in kidney

Figure 3 shows the Ca and Pi content of the kidney at the end of treatment. Both Ca and Pi in the kidneys of SNX-control rats were comparable with those in the sham-control rats. In contrast, Pi deposits in the low-dose 1,25(OH)₂D₃ group were elevated significantly compared with those in the low-dose OCT group. The deposits were not found even in the high-dose OCT group in spite of a significant elevation of uCa.

Figure 4 shows representative sections of a kidney stained by the von Kossa method. Prominent calcification was observed along the basement membranes of tubules in the high-dose 1,25(OH)₂D₃ group, but not in the high-dose OCT group.

Atypical hyperplasia in epithelial tubules

Figure 5 shows the effects of OCT and 1,25(OH)₂D₃ at the end of treatment on a number of AHET foci, which

### Table 1. Effects of OCT and 1,25(OH)₂D₃ on serum PTH, BW and CCr at the end of treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham control</th>
<th>SNX control</th>
<th>OCT (μg/kg)</th>
<th>1,25(OH)₂D₃ (μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.25</td>
<td>6.25</td>
</tr>
<tr>
<td>BW (g)</td>
<td>483.6 ± 16.5</td>
<td>446.4 ± 11.6</td>
<td>455.9 ± 19.9</td>
<td>441.6 ± 11.6</td>
</tr>
<tr>
<td>PTH (pg/ml)</td>
<td>18.2 ± 1.3</td>
<td>132.6 ± 44.2</td>
<td>32.1 ± 10.2</td>
<td>11.3 ± 1.7</td>
</tr>
<tr>
<td>CCr (ml/min)</td>
<td>2.0 ± 0.1</td>
<td>0.84 ± 0.08</td>
<td>0.78 ± 0.14</td>
<td>0.73 ± 0.1</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE. \( aP < 0.05 \) vs sham control; \( bP < 0.05 \), \( cP < 0.01 \) vs SNX control.

### Table 2. Effects of OCT and 1,25(OH)₂D₃ on calcium and phosphate in serum and urine at the end of treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham control</th>
<th>SNX control</th>
<th>OCT (μg/kg)</th>
<th>1,25(OH)₂D₃ (μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.25</td>
<td>6.25</td>
</tr>
<tr>
<td>Ca (mg/dl)</td>
<td>9.8 ± 0.1</td>
<td>9.9 ± 0.4</td>
<td>10.5 ± 0.2</td>
<td>11.7 ± 0.3</td>
</tr>
<tr>
<td>Pi (mg/ml)</td>
<td>6.9 ± 0.3</td>
<td>9.3 ± 1.0</td>
<td>10.4 ± 1.1</td>
<td>10.6 ± 0.7</td>
</tr>
<tr>
<td>Ca × Pi (mg/dl)²</td>
<td>67.3 ± 3.3</td>
<td>94.2 ± 12.2</td>
<td>110.7 ± 13.9</td>
<td>124.5 ± 10.1</td>
</tr>
<tr>
<td>uCa (mg/day)</td>
<td>0.46 ± 0.08</td>
<td>0.78 ± 0.14</td>
<td>1.75 ± 0.34</td>
<td>2.75 ± 0.36</td>
</tr>
<tr>
<td>uPi (mg/day)</td>
<td>20.1 ± 1.4</td>
<td>21.7 ± 1.2</td>
<td>22.2 ± 2.5</td>
<td>25.0 ± 2.1</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE. Ca, serum calcium; Pi, serum phosphorus. \( aP < 0.05 \) vs sham control; \( bP < 0.05 \), \( cP < 0.01 \) vs SNX control.
are represented by asterisks (Figure 6). The AHET and calcification (arrow) were observed along the basement membranes of the tubules. The mean AHET score (i.e. mean number of foci) in the SNX-control group was slightly, but not significantly, elevated. Although the low-dose 1,25(OH)₂D₃ group showed a significant elevation of the AHET score, the OCT group did not.

**Correlation analysis**

Table 3 shows the correlation between AHET and Ca and Pi concentrations in serum and kidney. A significant correlation with the AHET score was apparent for serum Pi, serum Ca × Pi, kidney Ca, and Pi content, and for the kidney Ca × Pi. In contrast,
therewasnosignificantcorrelationbetweenAHETand
serum Ca.

Discussion

We have previously demonstrated that 1,25(OH)2D3
was 10 times as potent as OCT in suppressing serum
PTH levels in the same setting [13,14]. In clinical
settings, OCT has usually been prescribed at 10 times
the dosage of 1,25(OH)2D3 (OCT, 10
\text{mg/patient/haemodialysis session}; 1,25(OH)2D3, 1
\text{mg/patient/haemodialysis session}). Therefore, we used OCT at 10
times the dosage of 1,25(OH)2D3 in this and previous
animal studies.

In this study, we demonstrated that using OCT
carried a significantly lower risk of inducing metastatic
calcification than did the use of 1,25(OH)2D3 when
these compounds were used in doses that exerted
comparable PTH suppression, and even when serum Ca × Pi products were similar. Although areas positive to von Kossa staining were not observed in the low dose 1,25(OH)₂D₃ group, the Ca and Pi concentrations in aorta, heart and kidney were appreciably higher than with OCT treatment. These findings suggest that different mechanisms might be operating in the induction of metastatic calcification by either of these compounds. The possible explanations for this effect are: First, it is well known that, in vitro, 1,25(OH)₂D₃ has a stimulatory effect on calcification in bovine vascular smooth muscle cells [15] and human aortic smooth muscle cells [16]. In keeping with this calcification process, it was revealed that 1,25(OH)₂D₃ promoted calcium deposits in cells [16]. Therefore, 1,25(OH)₂D₃ is considered to be one of the risk factors of vascular calcification. In spite of rapid clearance from blood circulation [17], OCT reaches the para-

Table 3. Correlation analysis between AHET and Ca and Pi

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Kidney</th>
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<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca Pi</td>
<td>Ca × Pi</td>
<td>Ca Pi</td>
<td>Ca × Pi</td>
<td></td>
</tr>
<tr>
<td>Correlation factor</td>
<td>0.15</td>
<td>0.581</td>
<td>0.536</td>
<td>0.66</td>
<td>0.795</td>
</tr>
<tr>
<td>P-value</td>
<td>NS</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

be valuable in treating them. This therapy, however, has a high risk of producing deterioration of renal function, and could be partly responsible for the nephrocalcinosis in tubules that is due to the hypercalcaemia associated with 1,25(OH)₂D₃ intake [19]. Nephrocalcinosis, which is observed in the early stages of renal disease in humans, is mainly promoted by HPT. Chronic PTH stimulation results in cellular changes and death, which induces the increase of ionized calcium in the cytosol in proximal tubular cells [8]. Following this, inflammatory and sclerotic reactions to the dead cells contribute to tubular damage, i.e. tubular degeneration and interstitial fibrosis [7]. In addition, it is known that calcium deposits are strongly correlated with the functional impairment of the kidney [7]. In our present study, supra-physiological doses of 1,25(OH)₂D₃ induced AHET related to nephrocalcinosis, but OCT affected neither AHET nor nephrocalcinosis. The AHET changes associated with calcification are partly similar to the micropolyp formation in medullary collecting tubules caused by high protein diets [20]. CCr in the high-dose 1,25(OH)₂D₃ group deteriorated with severe calcification in the tubules (data not shown). In addition, significant correlations were observed not only between AHET and the serum Ca × Pi products but also between it and the renal concentrations of Ca and Pi (Table 3). Therefore, we believe that it might be valuable in the management of the side effects of 1,25(OH)₂D₃ on tubular epithelium to assess the changes associated with calcification. Thus, the induction of AHET correlated with tubular calcification resulted in renal deterioration. We believe that the situation in which tubular epithelium shows high turnover due to subtotal nephrectomy to be at least partially paralleled in pre-dialysis patients. In all, OCT carries a lower risk of inducing AHET than does 1,25(OH)₂D₃ during PTH suppression, which suggests that OCT could be a more useful agent for controlling PTH levels of pre-dialysis patients without causing renal deterioration.

In conclusion, OCT might be a clinically useful and safe agent in the management of 2HPT, due to both its lower calcemic action and lower propensity to induce metastatic calcification. Further clinical studies are envisioned.

Conflict of interest statement. M.H., K.K., K.E., N.F. and H.O. are employees of Chugai Pharmaceutical Company Ltd. M.F. is a scientific advisor for this project performed by Chugai Pharmaceutical Company Ltd.

References


