Binding of highly concentrated maxacalcitol to the nuclear vitamin D receptors of parathyroid cells*

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Abstract

Background. Injection of maxacalcitol (OCT) directly into the parathyroid gland (PTG) is a clinically safe and effective treatment for advanced secondary hyperparathyroidism (A-SHPT) resistant to conventional medical treatment. In the present study, the degree of nuclear localization of directly injected OCT in parathyroid cells (PTC) was investigated by microautoradiography (mARG) in a model of A-SHPT.

Methods. The 5/6 nephrectomized Sprague–Dawley rats were fed a high-phosphate and low-calcium diet for 8 weeks and consequently the level of vitamin D receptor (VDR) in their PTC severely decreased. The bilateral PTG were surgically exposed and only the left gland were directly injected with 3H-OCT (DI-3H-OCT). The time course of the changes in both radioactivity and localization of 3H-OCT in the bilateral glands was analysed using a bioimaging analyser system and mARG, respectively. A very high dose of unlabelled calcitriol was administered intravenously (IV-1,25D3) prior to DI-3H-OCT, as a competitive study.

Results. Peak radioactivity levels in the directly injected and intact PTG occured immediately and 1 h, respectively, after DI-3H-OCT, and the difference was about 50-fold higher in the treated gland. The of mARG showed a marked concentration of silver grains in the nuclei of PTC in the gland treated with DI-3H-OCT and that concentration was significantly suppressed by IV-1,25D3.

Conclusions. Direct injection of OCT into the PTG enables the administration of the highly concentrated drug for specific binding to nuclear vitamin D binding sites, including VDR of PTC, which markedly suppresses the parathyroid hormone, improves the response to calcium and vitamin D and induces apoptosis in PTC.

Keywords: 22-oxacalcitriol; 5/6 nephrectomy; parathyroid gland; renal osteodistrophy; secondary hyperparathyroidism; vitamin D receptor

Introduction

Advanced secondary hyperparathyroidism (A-SHPT) unresponsive to medical treatment indicates the presence of severely hyperplastic parathyroid glands (PTG) in which the parathyroid cell (PTC) shows higher activities of synthesis and secretion of the parathyroid hormone (PTH), and of cell proliferation. Moreover, these PTC have very low levels of the vitamin D receptor (VDR) and calcium-sensing receptor (CaSR) [1,2]. When SHPT progresses to this advanced status (nodular hyperplasia), most patients are treated with parathyroidectomy-autotransplantation (PTx-AT), which is not without problems such as the need for general anaesthesia, hyper- or hypofunction of the autotransplanted PTG and psychological distress. In addition, the marked suppression of PTH levels after PTx-AT can induce not only adynamic bone disease, but also calcification of blood vessels [3].

The development of ultrasonography has enabled direct injection of drugs into the PTG and percutaneous ethanol injection therapy (PEIT), in particular, is as effective as PTx-AT for patients with A-SHPT [4]. However, it also may have complications, such as injury to the surrounding tissues caused by leakage of the highly concentrated ethanol from the PTG,
which can cause transient palsy of the unilateral recurrent and sympathetic nerve and severe pain. Thus there is a need for an effective and safe treatment for A-SHPT.

Vitamin D and its analogue are commonly used for the prevention and treatment of SHPT. They exert their effect through the interaction with the nuclear receptors that have an affinity for vitamin D, which includes the specific VDR, and the binding of their complexes to the vitamin D response element (VDRE) of the target gene in the PTC, which results in a reduction not only of the PTH level but also of cell proliferation [5]. Maxacalcitol (22-oxacalcitriol; OCT), an analogue of calcitriol (1,25-dihydroxy vitamin D$_3$; 1,25D$_3$), was developed to strongly suppress PTH, but have a low calcemic action, and in Japan its use has improved SHPT in chronic dialysis patients [6]. Direct injection of highly concentrated 1,25D$_3$ or OCT into the PTC was developed as a treatment that more safely suppresses PTH in patients with A-SHPT. Previous reports have shown that these new therapeutic techniques enable significant PTH reduction without significant problems and that the reduced PTH level is maintained for a long time by subsequent conventional medical SHPT treatment in these patients [7–9]. Moreover, we have developed a rat model of A-SHPT in which the VDR level in the PTC is severely decreased and thus allows us to investigate in detail the cellular effects of direct injection of OCT (DI-OCT) into the PTC. We have already reported that this treatment induces specific cellular effects, not only PTH suppression but also improvements in some of the important aetiological factors of the unresponsiveness to medical treatment; i.e. up-regulation of the VDR and CaSR in the PTC, and regression of the hyperplasia by induction of PTC apoptosis [10]. These specific effects of DI-OCT are maintained by subsequent intravenous OCT administration, and the control of the serum PTH level makes it possible to ameliorate osteitis fibrosa and the high rate of turnover of bone caused by A-SHPT [11].

In the present study to explain the mechanisms of the specific cellular changes in the PTG undergoing DI-OCT, particularly the difference in the degree of nuclear localization of OCT between local (i.e. direct) and systemic administration, using a bioimaging analyser system (BAS) and microautoradiography (mARG) with [26-$^3$H]-1,25-dihydroxy-22-oxacalcitriol ($^3$H-OCT), was investigated.

Subjects and methods

Animals

Seven-week-old male Sprague-Dawley rats underwent 5/6-nephrectomy under intraperitoneal pentobarbital anaesthesia (50 mg/kg body weight). The rats were fed with a normal diet (0.9% P, 1.12% Ca) until 1 week after this procedure and then switched to a high-P and low-Ca (HP-LC) diet (1.2% P, 0.4% Ca; Oriental Yeast, Inc., Chiba, Japan) for 8 weeks to progress to A-SHPT in which the VDR level of the PTC was severely decreased. Animal care, handling and euthanasia followed the ethical protocols of Wakayama Medical University and Chugai Pharmaceutical Co. Ltd, Tokyo, Japan.

Treatments and samplings

The bilateral PTG of the rats were surgically exposed under diethyl ether inhalation and [26-$^3$H]-1,25-dihydroxy-22-oxacalcitriol ($^3$H-OCT) (10 pg/ml) with a specific activity of 2.89 TBq/mmol (Amersham Biosciences Corp., Piscataway, NJ, USA) was directly injected into the left gland (DI-$^3$H-OCT) using a 30-gauge needle (specially made by Toyohata Co Ltd, Chiba, Japan). Immediately after the injection, any leakage was washed away with saline. The actual injected volume of solution was similar to the original PTG volume (2.47±0.65 µl/PTG; n=10), as previously reported [10]. Before, immediately, and at 0.25, 1, 4, 8 and 24 h after DI-$^3$H-OCT (n for each time point=1), blood was collected from the abdominal aorta, the animal euthanased by exsanguination, and all cervical organs, comprising the trachea, oesophagus, thyroid and parathyroid glands, were removed.

A very high dose of unlabelled 1,25D$_3$ (10 µg/kg) (IV-1,25D$_3$) was intravenously administered prior to DI-$^3$H-OCT (DI-$^3$H-OCT+IV-1,25D$_3$) as a competition study (n=1). Blood and cervical organs were harvested, as described earlier, 1 h later.

For a comparative examination of the results following intravenous administration of $^3$H-OCT (IV-$^3$H-OCT) at a dose sufficient to reach a plasma $^3$H-OCT concentration exceeding the peak level following DI-$^3$H-OCT (2 µg/kg: ≈800 pg/rat), blood and cervical organs were harvested 1 h after IV-$^3$H-OCT (n=1). The interval of 1 h was chosen because it has been reported as the time point at which there is a very high level of radioactivity in the PTG [12]. All samples of the excised cervical organs were mounted on tissue holders using liver paste derived from normal rats, and then frozen using liquid-nitrogen-cooled isopentane until the next procedure.

Laboratory measurements

Serum levels of intact-PTH, Ca$^{2+}$ and P, and other data were obtained 1 week before the experiments. The serum level of intact-PTH was measured by the two-antibody method using a rat intact-PTH ELISA kit (Immutopics Inc., San Clemente, CA, USA), which can measure between 1.6 and 800 pg/ml; therefore, the samples from all uraemic rats were diluted with the supplied 0 pg/ml standard at 1:10 and analysed together. All standards, controls and test samples were assayed in duplicate and averages were taken. Other data were determined using an automated analyser (7070, Hitachi, Tokyo, Japan).

Plasma $^3$H-OCT level

Immediately after being taken, each blood sample was centrifuged at 1600g for 10 min, and then 500 µl of plasma and 1 ml of Soluene® (PerkinElmer Inc., Wellesley, MA, USA) were mixed and dissolved by heating at 50°C using...
a Sand Bath, after which Ultima Gold® (PerkinElmer) was added to the solution. The radioactivity level of the resulting mixture was measured using a liquid scintillation counter and expressed in OCT equivalents estimated from the specific radioactivity of $^3$H-OCT.

Quantitative radioluminography of the PTG determined by BAS

Sections of 10µm thickness were prepared using a cryomicrotome, mounted on MAS-coated glass slides, and dried at $-35^\circ$C in a cryostat. The sections were exposed to the BAS imaging plates (TR2023, Fuji Photo Film, Tokyo, Japan) for 3 days at room temperature. Autoradiographs were processed using a computerized image analysis system (BAS5000, Fuji Photo Film). The distribution of radioactivity in each section was observed and photostimulated luminescence (PSL) was determined for each PTG as shown in the image using the BAS. The radioactivity of the PTG was quantified by dividing the PSL by the PTG area. The data from nine sections were averaged for the PSL level of each PTG.

Localization of directly injected OCT in the PTC determined by mARG

Sections of 4µm thickness were prepared using a cryomicrotome at $-35^\circ$C, thaw-mounted on nuclear emulsion-coated glass slides (NR-M2, Konica, Tokyo, Japan), and exposed in light-proof desiccator boxes at 4°C for 8 months. After exposure, the slides were developed, rinsed and stained with methylene blue-basic fuchsin [13]. As additional controls against artefacts, autoradiographs of PTG sections without radioactivity that had been exposed for the same time period were compared. There was no evidence of positive chemography.

For the quantitative analysis of radioactivity in the nuclei of the PTC, the ratios of silver grain area in the nucleus per total nuclear area of the PTC in mARG (nuclear mARG-index) following DI-$^3$H-OCT, DI-$^3$H-OCT+IV-$^1$25D$_3$ and IV-$^3$H-OCT were estimated using NIH images (National Institute of Health, USA). The cytoplasmic mARG-index (ratio of silver grain area in the cytoplasm per total cytoplasmic area of PTC) was similarly evaluated following DI-$^3$H-OCT+IV-$^1$25D$_3$. Data from 100 PTG were averaged for both mARG-indexes of each PTG.

Serum OCT level following DI-OCT

The relationship between the plasma level of $^3$H-OCT following DI-$^3$H-OCT and the actual serum OCT level following DI-OCT was analysed in 11 rats. Blood samples were taken before and at 0.25, 0.5, 1, 2, 4 and 6 h after DI-OCT of both PTG and centrifuged. The serum was frozen and stored at $-20^\circ$C until OCT level determination by high-performance liquid chromatography (HPLC) with tandem mass spectrometry (LC-MS-MS) [14].

Statistical analyses

Data are expressed as means ± SD. The differences in the peak PSL levels and nuclear mARG-indexes among the respective left and right PTG following DI-$^3$H-OCT and IV-$^3$H-OCT were analysed by analysis of variance (ANOVA) with post-hoc multiple comparisons using Tukey-Kramer’s tests. For determining the statistical significance of the difference between the left and the right PTG in terms of injection time and for the competition test (respective left and right PTG with and without IV-$^1$25D$_3$), Student’s paired and unpaired $t$ tests, respectively, were used. A $P$-value <0.05 was considered statistically significant.

Results

Laboratory data for the uraemic rats

The mean serum levels of intact-PTH, P, creatinine, and urea nitrogen were markedly high and that of Ca$^{2+}$ was low (3735 ± 1761 pg/ml, 9.96 ± 2.40 mg/dl, 1.20 ± 0.24 mg/dl, 59.2 ± 15.7 mg/dl and 1.20 ± 0.12 mmol/l, respectively). Thus, it is considered that the rats developed A-SHPT because of renal insufficiency and the HP–LC diet, as previously reported in the SHPT rat model in which the VDR level of the PTC was severely decreased [10].

Time course of changes in the plasma level of $^3$H-OCT

The serum level of OCT rapidly increased immediately after direct injection and then decreased to its baseline level 4 h later (Figure 1A). The plasma level of $^3$H-OCT also reached its peak immediately following direct injection and then rapidly decreased (Figure 1C). The time courses of the change in the serum level of OCT and of the plasma level of $^3$H-OCT following direct injection were almost the same.

The plasma level of $^3$H-OCT at 1 h after intravenous injection was higher than that following direct injection at all time points (Figure 1B and C). Thus, the intravenous dose was sufficient to reach a plasma level of $^3$H-OCT that exceeded the peak $^3$H-OCT level following direct injection.

Moreover, the plasma levels of $^3$H-OCT with or without IV-$^1$25D$_3$ at the same 1 h time point in the competition study were almost the same (1603 and 2356 pg eq/ml, respectively).

Radioactivity level in the PTG

Figure 2 shows representative photographs obtained using BAS. Immediately after DI-$^3$H-OCT, there was a very high level of radioactivity not only in the left PTG, but also in the space surrounding the cervical organs, which gradually decreased in almost all parts of the sample except the PTG. There was high radioactivity around the right PTG, although infiltration from the surface was not confirmed. One hour after DI-$^3$H-OCT, the radioactivity level in the right PTG peaked at almost the same level as in the PTG treated by IV-$^3$H-OCT.
Figure 3 shows the radioactivity level at 1 h after IV-3H-OCT and the time course of the changes in both PTG following DI-3H-OCT. The radioactivity in the left and right PTG peaked immediately and at 1 h, respectively, after DI-3H-OCT and the latter level was almost the same as at 1 h after IV-3H-OCT. A significant difference (≈50-fold) between the peak levels of the left and right PTG (including the level following IV-3H-OCT) was observed.
Localization of directly injected OCT in the PTC

Figure 4 shows representative photographs of the mARG of both PTG following individual treatment. Immediately after DI-\(^3\)H-OCT, the markedly high radioactivity level (silver grains) was confirmed in almost all parts of the left PTG (both inside and outside of the nuclei). More silver grains aggregated in the nuclei of PTC immediately and 0.25 h after DI-\(^3\)H-OCT, and these results were observed to a lesser degree in PTG not treated by DI-\(^3\)H-OCT (right PTG treated by IV-\(^3\)H-OCT). In the right PTG, there was infiltration of some silver grains from the surface but most were observed inside the gland, which suggested that almost all the \(^3\)H-OCT detected in the right PTG was delivered via the circulation.

Figure 5 shows the nuclear mARG-index 1 h after IV-\(^3\)H-OCT and the time course of the change in the nuclear mARG-indexes of both PTG following DI-\(^3\)H-OCT. The indexes of the left PTG (with DI-\(^3\)H-OCT) were significantly greater than those of the right PTG (without DI-\(^3\)H-OCT) and of the PTG treated by IV-\(^3\)H-OCT during the early phase following DI-\(^3\)H-OCT. However, the indexes at more than 4 h after DI-\(^3\)H-OCT were almost the same in both PTG. The nuclear mARG-index of the right PTG peaked 1 h after DI-\(^3\)H-OCT and was almost the same as that at 1 h after IV-\(^3\)H-OCT.

Discussion

One of the most important disorders of mineral metabolism in patients with end-stage renal disease is SHPT and control of the serum levels of PTH, Ca and P improves their prognosis [3]. Percutaneous maxacalcitol injection therapy (PMIT) was developed as a new technique for suppressing PTH levels in these patients with A-SHPT and it enables significant reduction of the PTH level without the complications of PTx-AT or PEIT and that reduced PTH level is maintained by subsequent medical treatment, including intravenous OCT administration (IV-OCT) [8]. We have previously reported that specific effects in the PTC are the basis of these preferable clinical effects [10] and in the present study, using our previously established rat model of A-SHPT and direct injection therapy, analyses by BAS and mARG techniques, respectively enabled visualization of the degree of OCT concentration in the PTG and its localization in PTC, revealing that DI-OCT delivers the highly concentrated drug to the nuclear vitamin D binding sites of PTC.

First, we should confirm that the plasma level of \(^3\)H-OCT following direct injection reflects the serum level of OCT following direct injection, and that the intravenous dose of \(^3\)H-OCT was sufficient to exceed the maximum plasma level of \(^3\)H-OCT following direct injection. Second, we had an appropriate control. A previous report showed that the level of \(^3\)H-OCT in the PTG at 1 h after intravenous administration was significantly high [12], so in the present study, the data obtained 1 h after IV-\(^3\)H-OCT were used as the reference. There was no significant difference in the

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Fig. 3. Radioactivity level determined by the BAS following (A) IV and (B) DI of \(^3\)H-OCT. Data are means ±SD. The number of sections from each PTG analysed for radioactivity level was 9; \(^*\)P < 0.01 compared with corresponding right PTG; \(^*\)Rt and \(^*\)IV/P < 0.01 analysed among the corresponding peak level of the left PTG (immediately after DI-\(^3\)H-OCT) and that of the right PTG (1 h after DI-\(^3\)H-OCT), and level 1 h after IV-\(^3\)H-OCT. Solid line: left PTG; dotted line: right PTG. OCT, maxacalcitol; PSL, photostimulated luminescence.
Fig. 4. The mARG of the PTG. High concentration of silver grains in the nuclei of the PTC can be seen in the left PTG (direct injection of $^{3}$H-OCT) (red arrows). Green bars = 20 μm. (Bottom panels) IV, 1 h after IV administration of $^{3}$H-OCT; DI-$^{3}$H-OCT+IV-1,25D$_{3}$, 1 h after DI-$^{3}$H-OCT and prior intravenous unlabelled calcitriol (1,25D$_{3}$) at an excessive dose. OCT, maxacalcitol.
levels of radioactivity (analysed by both BAS and mARG) between the right PTG and the PTG treated by IV-3H-OCT nor was there significant infiltration of 3H-OCT into the right PTG, although there was very slight surface penetration. Thus, we consider that the peak radioactivity level of right PTG following DI-3H-OCT and the level 1 h after IV-3H-OCT indicate the limitation of OCT uptake into PTG (and PTC) by the conventional administration in this A-SHPT model rats.

Next, we investigated whether DI-OCT was able to exceed this limitation (particularly in the nucleus of the PTC). A high level of radioactivity was observed in the left PTG (with DI-3H-OCT) and a significant difference between the peak levels of the left and right PTG (including PTG treated by IV-3H-OCT) was also confirmed. Very high concentrations of silver grains in the nuclei of PTC were observed immediately and 0.25 h after DI-3H-OCT, and a significant difference in the nuclear mARG-index between the left and right PTG was confirmed immediately, and at 0.25 and 1 h after DI-3H-OCT. Thus, we consider that a smaller dose of OCT by direct injection overcame the limitations of OCT uptake into the nuclei of PTC in which the VDR level is severely decreased (i.e. A-SHPT), with much less increase of the serum OCT level than with conventional administration of the drug (i.e. intravenous). Moreover, the marked decrease in both the radioactivity level determined by BAS and the mARG-indexes was confirmed in the competition study. In the left PTG, the decrease in the nuclear mARG-index was more significant than that of the cytoplasmic mARG-index. Thus, we consider that DI-OCT has immediate effects on the nuclear vitamin D binding sites particularly, as well on the cytoplasmic sites, in the PTC, because in the competition study 3H-OCT could not bind to these sites after high-dose 1,25D3 was administered, which occupied all the receptors. These results indicate that the specific cellular effects of PMIT (DI-OCT) in A-SHPT (i.e. marked suppression of PTH, up-regulations of the VDR and CaSR, and induction of apoptosis in PTC) were not expressed following conventional OCT administration (IV-OCT) [11], and are induced via effects based on the significantly high OCT concentration in the PTG.
In this model of A-SHPT, the VDR mRNA and immunohistochemical expression levels were very low in the PTG [10]; however, a high level of \(^{3}\)H-OCT was detected in the nuclei of PTC treated by DI-\(^{3}\)H-OCT in the early phase following injection. Previously, up-regulation of the VDR in PTC induced by 1,25D\(_3\) has been shown in both in vivo and in vitro studies [15,16]. Those reports showed that it took at least 6h to up-regulate VDR mRNA expression in the PTG following 1,25D\(_3\) administration, and we also previously reported a significant increase in the VDR mRNA expression level in the PTG at 24h after DI-OCT [10]. Small hydrophobic signal molecules, such as steroid hormones and vitamin D, diffuse directly across the plasma membrane of target cells and bind to intracellular receptor proteins. A recent study explained one of the mechanisms of the transport of these molecules. Megalin, a member of LDL receptor gene family, is expressed in the epithelial cells of the renal proximal tubule and delivers the precursor 25-OH vitamin D\(_3\) to tubular cells for conversion into 1,25-(OH)\(_2\) vitamin D\(_3\) [17]. Moreover, the co-expression of megalin and 25-hydroxyvitamin D\(_2\)-1\(\alpha\)-25-hydroxylase in PTC has been confirmed [18]. Thus, as the suggested mechanism of these findings, the simple diffusion of highly concentrated OCT into the PTC, the passive transport by the pressure of direct injection, the possibility of compensation for a defective megalin-mediated uptake of OCT by the PTC and the existence of the other vitamin D binding sites in the PTC are considered, after which the directly injected OCT will enter the cytosol and nuclei of PTC at a significantly high concentration and accumulate there because of its higher affinity with VDR (the original VDR and/or the other nuclear vitamin D binding sites, not as a result of up-regulation of VDR in the PTC). We obtained data from the same examination (only IV-\(^{3}\)H-OCT) using VDR knock-out mice to investigate the possibility of nuclear vitamin D binding sites in PTC other than the VDR. The results showed that \(^{3}\)H-OCT accumulated in PTG-specific in cervical organs and that some were localized in the nuclei of PTC (data not shown). Thus, we considered that \(^{3}\)H-OCT might bind not only VDR but also other vitamin D binding sites in the nucleus of PTC. Recent reports have shown various VDR-independent effects of vitamin D. The VDR-independent regulation of both the PTG and cartilaginous growth by Ca and vitamin D has been shown [19] and another report has shown that a higher dose of vitamin D compounds inhibits the proliferation of breast cancer cell ablated VDR [20]. In those reports, a higher dose of vitamin D was required for the expression of these effects in animals without VDR than in those with VDR, which indicates the existence of a functional binding site other than the VDR. We consider that there is a higher expression level of this site in A-SHPT than in the non-uraemic or non-SHPT condition. In any case, these previous and present findings indicate that the specific effects of DI-OCT in the PTC are caused by the local administration of this agent at high concentration, which enables specific interaction with nuclear vitamin D binding sites. However, more precise studies are required to elucidate the detailed mechanism of the original and/or alternative vitamin D signalling pathway in the PTC of A-SHPT and the induction of these cellular effects by this treatment.

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Conflict of interest statement. None declared.

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