EXPERIMENTAL STUDY

Relationship between parathyroid calcium-sensing receptor expression and potency of the calcimimetic, cinacalcet, in suppressing parathyroid hormone secretion in an in vivo murine model of primary hyperparathyroidism

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Abstract
Cinacalcet HCl, an allosteric modulator of the calcium-sensing receptor (CaR), has recently been approved for the treatment of secondary hyperparathyroidism in patients with chronic kidney disease on dialysis, due to its suppressive effect on parathyroid hormone (PTH) secretion. Although cinacalcet’s effects in patients with primary and secondary hyperparathyroidism have been reported, the crucial relationship between the effect of calcimimetics and CaR expression on the parathyroid glands requires better understanding. To investigate its suppressive effect on PTH secretion in primary hyperparathyroidism, in which hypercalcemia may already have stimulated considerable CaR activity, we investigated the effect of cinacalcet HCl on PTH-cyclin D1 transgenic mice (PC2 mice), a model of primary hyperparathyroidism with hypo-expression of CaR on their parathyroid glands. A single administration of 30 mg/kg body weight (BW) of cinacalcet HCl significantly suppressed serum calcium (Ca) levels 2 h after administration in 65- to 85-week-old PC2 mice with chronic biochemical hyperparathyroidism. The percentage reduction in serum PTH was significantly correlated with CaR hypo-expression in the parathyroid glands. In older PC2 mice (93 – 99 weeks old) with advanced hyperparathyroidism, serum Ca and PTH levels were not suppressed by 30 mg cinacalcet HCl/kg. However, serum Ca and PTH levels were significantly suppressed by 100 mg/kg of cinacalcet HCl, suggesting that higher doses of this compound could overcome severe hyperparathyroidism. To conclude, cinacalcet HCl demonstrated potency in a murine model of primary hyperparathyroidism in spite of any presumed endogenous CaR activation by hypercalcemia and hypo-expression of CaR in the parathyroid glands.

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Introduction
Primary hyperparathyroidism is characterized by hyperfunctioning parathyroid tissue that responds with decreased sensitivity to extracellular calcium (Ca) concentration in suppressing parathyroid hormone (PTH) secretion, resulting in the development of hypercalcemia. Sporadic nonfamilial parathyroid adenomas are the most frequent cause of primary hyperparathyroidism. Two specific genes have been identified as participating in their tumorigenesis: the cyclin D1/PRAD1 oncogene (1) and the multiple endocrine neoplasia type 1 (MEN1) tumor-suppressor gene (2, 3). Other oncogenes and/or tumor-suppressor genes, still unidentified, also contribute to the tumorigenesis of parathyroid adenomas (3, 4). Even though no mutations of the calcium-sensing receptor (CaR) gene were detected in parathyroid tumors (5, 6), reduced CaR expression in parathyroid glands has been observed in primary and secondary hyperparathyroidism (7, 8). CaR on the parathyroid chief cells is the primary PTH regulator, detecting extracellular Ca levels, working quickly when activated by serum Ca elevation through intracellular signaling pathways to suppress PTH secretion (9, 10). An abnormal secretory set-point of the PTH–Ca sigmoidal curve was also associated with reduced CaR expression in parathyroid glands in vivo in CaR-knockout mice (11) and a murine model of primary hyperparathyroidism (12).
Parathyroidectomy is currently the only definitive treatment for moderate to severe primary hyperparathyroidism (13). Some patients with asymptomatic primary hyperparathyroidism who do not meet the guidelines for parathyroidectomy, and are monitored without surgical therapy, develop bone and kidney complications (13, 14). Although bisphosphonates, such as alendronate (15), or estrogen therapies (16) have met with some success in preventing bone loss, other complications such as nephrocalcinosis may not be prevented. Other patients clearly meet the criteria for definitive surgical therapy but either refuse or are not candidates for surgery for other reasons. Calcimimetic compounds are low molecular weight phenylalkylamine derivatives that act as allosteric modulators of the CaR (17). These compounds enhance the sensitivity of the CaR to extracellular Ca, thus exerting a suppressive effect on PTH secretion. Cinacalcet HCl has recently been approved for the treatment of secondary hyperparathyroidism in patients on maintenance hemodialysis, and has been shown to reduce PTH secretion both in vitro (bovine parathyroid cell assay) and in vivo (normal rats) (18). Although cinacalcet HCl has met with success as a novel therapeutic agent for the treatment of secondary (19–23) and primary (24, 25) hyperparathyroidism in clinical studies, the relationship between the effect of this compound and CaR expression on the abnormal parathyroid glands is unknown.

The aim of this study was to evaluate the effects of cinacalcet HCl in the suppression of serum PTH and Ca levels in vivo in a murine model of primary hyperparathyroidism and also to investigate its molecular mechanism. We aimed to learn more about the effect of cinacalcet on parathyroid glands with reduced CaR expression.

Materials and methods

Materials

Cinacalcet HCl (αR)-(−)-α-methyl-N-[3-[3-trifluoromethylphenyl]propyl]-1-naphthalenemethanamine hydrochloride was generated by Kirin Brewery Co. Ltd (Takasaki, Japan). Methylcellulose was obtained from Sigma (St Louis, MO, USA). NPS Pharmaceutical, Inc. (Salt Lake City, UT, USA) kindly provided anti-CaR antibody (4638 polyclonal antibody). Anti-PTH antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Animals

FVB/N background PTH-cyclin D1 transgenic mice (PC2), which exhibit parathyroid-targeted overexpression of the human cyclin D1 oncogene, were used in this study as a model of primary hyperparathyroidism (12). Offspring were genotyped by Southern blotting using human(h)-cyclin D1 cDNA as a probe (12), to select the mice bearing the transgene. All mice were provided with the commercially available rodent diet, CE-2 (Crea Japan, Inc., Tokyo, Japan), containing 1.03% Ca and 0.97% phosphate. Food and water were available ad libitum. Studies were approved by the appropriate institutional animal care committees at Osaka City University Medical School.

Experimental protocols

Wild-type (WT) mice, 65–85 weeks old, were used for the time-course changes of serum Ca levels after the oral administration of various doses of cinacalcet HCl. Mice were given 10, 30 or 100 mg cinacalcet HCl/kg body weight in 0.5% methylcellulose or vehicle, and blood samples were collected at 0, 2, 8, and 24 h after the oral administration. Serum Ca levels were measured to determine the proper dose of cinacalcet HCl for mice. To examine the effects of cinacalcet HCl, 65- to 85-week-old WT and PC2 mice, and 93- to 99-week-old PC2 mice were used for the study. The 65- to 85-week-old WT and PC2 mice were given 30 mg cinacalcet/kg orally, and blood samples were collected at 0 and 2 h thereafter. The inhibitory rate of PTH secretion (%) was defined as (PTH 2 h – PTH 0 h) / PTH 0 h × 100. The 93- to 99-week-old PC2 mice were also given cinacalcet HCl twice, at the initial dose of 30 mg/kg followed by 100 mg/kg. The interval between the first treatment (30 mg/kg) and the second treatment (100 mg/kg) was 1 week so as to wash out the effect of the first administration.

Measurement of biochemical parameters

Blood samples were collected from orbital cavities. Serum PTH was measured by a rat PTH IRMA kit (Immutopics, Inc., San Clemente, CA, USA). Serum 1,25(OH)2D concentrations were measured by a radio-immunoassay kit (Immunodiagnostics Systems Ltd, Boldon, Tyne and Wear, UK). Serum biochemistries, including total Ca, phosphate, urea nitrogen (SUN) and alkaline phosphatase (ALP) were determined by a series of TESTWAKO kits (Wako Pure Chemical Industries Ltd, Osaka, Japan.).

Parathyroid histology

Resected parathyroid glands were immediately fixed in 10% formalin for 2 days, and embedded in paraffin. Total parathyroid glands were sliced into 3 μm-thick sections, and the number of slides that contained parathyroid tissue was counted. Every quarter section defined by a slide’s number was selected for the analysis as an indicator of the parathyroid gland size. Immunohistochemistry analyses were performed followed by inactivation of intrinsic peroxidase by incubation in 3% hydrogen peroxide diluted in methanol. Sections were then treated...
with skim milk to prevent background staining, and incubated with anti-CaR (0.8 μg/ml) or anti-PTH (diluted 1:1000) antibody overnight at 4°C in a humidified chamber. After rinsing, sections were incubated with the biotinylated secondary antibody and peroxidase-conjugated streptavidin, followed by visualization by alkaline phosphatase and counter staining by hematoxylin.

The outside of the parathyroid gland was carefully traced in each slide, and a person who was blinded to the tissue samples calculated the area using Adobe Photoshop v. 5.5 software (Adobe Systems Inc., San Jose, CA, USA). The size given by pixels was changed to a millimeter² value using NIH image 1.62 software (National Institutes of Health, Bethesda, MD, USA) and adjusted for kg body weight (26). The size of each gland was represented by the mean value of three slides. The unstained area was defined as the area apparently stained more faintly than the area of the clearly stained normal parathyroid gland.

Statistical analyses

Biochemical parameters between WT and PC2 mice were analyzed by the Mann-Whitney U test. Serum PTH and Ca levels pre- and post-administration of cinacalcet HCl were analyzed by the Wilcoxon single rank test. Parathyroid gland sizes between WT and PC2 mice were analyzed by the Mann-Whitney U test. Correlations between inhibition rates of PTH secretion and parathyroid gland sizes were analyzed by the Pearson correlation test. All the data are presented as means±S.E.

Results

Effects of cinacalcet HCl on serum Ca levels in WT mice

In WT mice, serum Ca levels were significantly suppressed between 2 and 8 h after the administration of cinacalcet HCl (30 and 100 mg/kg) with a return to basal levels at 24 h (Fig. 1). Serum Ca levels were not significantly affected by the administration of cinacalcet HCl at 10 mg/kg.

Serum parameters in PC2 mice

PC2 mice aged 65–85 weeks showed significant increases in serum Ca, PTH, and 1,25(OH)₂D levels and a significant decrease in serum phosphate levels compared with age-matched WT mice, indicating that the PC2 mice had developed biochemical primary hyperparathyroidism, as expected, at this age (Table 1). PC2 mice grew normally, and showed no difference in weight and SUN values compared with WT mice. Older PC2 mice aged 93–99 weeks showed more severe hyperparathyroidism with significantly higher serum Ca and PTH levels than younger PC2 mice aged 65–85 weeks. Older WT mice (93–99 weeks old) had similar levels of these parameters as the younger mice (65–85 weeks old) (data not shown). No increment in SUN was observed in the mice used in this study, confirming that hyperparathyroidism was generated primarily and not secondarily by renal failure.

Administration of cinacalcet HCl to PC2 mice

Oral administration of 30 mg cinacalcet HCl/kg significantly suppressed serum Ca levels in both 65- to 85-week-old PC2 and WT mice by 2 h after administration (Fig. 2A). Serum PTH levels were also suppressed significantly by 30 mg cinacalcet HCl/kg, from 66±7 pg/ml to 11±3 pg/ml, and from 138±20 pg/ml to 89±15 pg/ml in WT and PC2 mice respectively. The inhibitory rates of PTH secretion (%) were 82±6 and 36±7 in WT and PC2 mice respectively, indicating that the suppressive effect of cinacalcet HCl on PTH was significantly weaker in PC2 mice than in WT mice (Fig. 2B). Serum phosphate and ALP levels showed a tendency to decrease after administration of cinacalcet HCl, although this was not statistically significant (data not shown).

CaR expression on parathyroid glands by immunohistochemical analysis and its correlation with the effect of cinacalcet HCl administration

The parathyroid gland sizes of 65- to 85-week-old PC2 mice were 3.14 times larger than those of age-matched WT mice (Fig. 3). Areas of reduced CaR expression were also larger in parathyroid glands from PC2 mice compared with those from WT mice (Fig. 3). In parathyroid glands from WT mice, no focal regions of reduced CaR expression areas existed such as those seen in PC2 mice (Fig. 4c); however, small numbers of individual cells, which were not clustered as in PC2 mice, were also regarded as hypo-expression regions in WT mice. Reduced CaR expression areas had an adenomatous appearance with a multilobular, tubular pattern, which was encapsulated on hematoxylin-eosin staining stabilization.

Figure 1 Serum Ca levels in WT mice after bolus administration of vehicle or estimated doses of cinacalcet HCl. Values represent means±S.E., n = 3 or 4/group. **P < 0.01, ***P < 0.001 compared with vehicle (control) (Mann–Whitney U test).
In these reduced CaR expression areas, PTH expression, examined by simultaneous immunostaining with anti-PTH antibody, was upregulated in PC2 mice (Fig. 4c, f). The sizes of the reduced CaR expression areas exhibited significant negative correlations with the ability of 30 mg cinacalcet HCl/kg to inhibit PTH secretion in PC2 mice only ($r = -0.717$, $P = 0.011$) and also in total mice examined including PC2 and WT mice ($r = -0.751$, $P < 0.001$) (Fig. 5).

The effect of cinacalcet HCl on advanced hyperparathyroid PC2 mice

Older PC2 mice aged 93–99 weeks, which developed more severe hyperparathyroidism, were also examined for the suppressive effect of cinacalcet HCl. In these mice, both the size of the parathyroid glands and the size of reduced CaR expression areas were larger than those of 65- to 85-week-old PC2 mice (data not shown). The administration of 30 mg cinacalcet HCl/kg failed to suppress either serum Ca or PTH levels (Fig. 6). The same mice were given 100 mg cinacalcet HCl/kg after a one week washout period during which serum Ca and PTH returned to basal levels (data not shown). Cinacalcet HCl at a dose of 100 mg/kg significantly suppressed both serum Ca and PTH levels by 2 h post treatment in the mice that had been resistant to 30 mg cinacalcet HCl/kg.

**Discussion**

Cinacalcet HCl, an allosteric modulator of the calcium-sensing receptor (CaR), suppresses PTH secretion and has recently been approved for the treatment of patients with secondary hyperparathyroidism on maintenance dialysis. Although the efficacy of cinacalcet in patients with primary and secondary hyperparathyroidism has been reported, the crucial relationship between the potency of calcimimetics on PTH secretion and CaR expression level on the parathyroid glands is as yet unknown. In this study, as CaR expression on parathyroid glands became less, the effect of cinacalcet HCl on the suppression of PTH was weaker in PC2 mice, a mouse model of primary hyperparathyroidism. In addition, resistance of cinacalcet HCl in mice with severe hyperparathyroidism induced by extraordinary hypo-expression of CaR expression, was overcome by increasing the dose of cinacalcet HCl administered.

In this study, we demonstrated a negative correlation between the potency of cinacalcet HCl on the parathyroid glands and on parathyroid CaR expression. In addition, a relative resistance to cinacalcet HCl was found in mice with severe hyperparathyroidism, which was overcome by increasing the dose administered.

This is the first study to examine the effect of the calcimimetic, cinacalcet HCl, in an *in vivo* model of primary hyperparathyroidism, in contrast to the

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<th>Table 1 Serum biochemistries of WT and PC2 mice. Values are means±s.e.</th>
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<td>(WT 65–85 weeks $\ (n = 9)$)</td>
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<td>(PC 65–85 weeks $\ (n = 11)$)</td>
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<td>(Old PC 93–99 weeks $\ (n = 8)$)</td>
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<td>ALP (K-A)</td>
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Ca, total calcium; P, phosphate; ALP, alkaline phosphatase; K-A, King–Armstrong unit; SUN, serum urea nitrogen.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus WT mice; # $P < 0.05$ versus PC2 mice (Mann–Whitney U test).
well-documented effects of these agents in secondary hyperparathyroidism. In uremic 5/6 NX rat models, hypocalcemia is observed due to decreased 1,25(OH)\textsubscript{2}D levels and suppressed intestinal Ca absorption (27). In such relative hypocalcemic to normocalcemic status, calcimimetics effectively suppress PTH secretion and parathyroid cell proliferation (28 –30). In patients on maintenance hemodialysis, cinacalcet HCl successfully reduced serum PTH resulting in a decrease in the Ca × phosphorus product (22).

Recently, the beneficial effect of cinacalcet HCl in normalizing serum Ca levels by suppressing serum PTH levels was demonstrated in patients with primary hyperparathyroidism, who exhibited mild to moderate hypercalcemia (range 9.4–12.7 mg/dl) (25). Notwithstanding the fact that this previous clinical study (25) reported a suppressive effect of calcimimetic on serum Ca in patients with primary hyperparathyroidism, it is crucial to investigate its action since the effect of these compounds might be attenuated because the decreased parathyroid CaR may have already been activated by hypercalcemia. Notably, in this murine study we demonstrated a correlation between the effect of cinacalcet and the expression level of CaR in parathyroid glands. However, it is of interest that increasing the dose of cinacalcet HCl could overcome the resistance of CaR activation.

A previous study (18) reported that, in normal rats, administration of cinacalcet HCl at 3 and 10 mg/kg significantly suppressed ionized Ca levels between 1 and 8 h after administration, and levels returned to basal values by 24 h. Although ionized Ca levels did not return to basal levels by 24 h at a dose of 30 mg/kg in rats (18), 100 mg cinacalcet HCl/kg could not suppress Ca levels at 24 h in mice in this study, indicating that the elimination rate of cinacalcet HCl in mice appears to be faster than in rats. A larger dose of cinacalcet HCl was necessary to suppress serum Ca levels in mice compared with rats, so we decided to administer cinacalcet HCl at both 30 and 100 mg/kg to mice in our experiments, this being enough to decrease serum levels 2 h after administration.

The suppressive effect of cinacalcet HCl on serum Ca in 65- to 85-week-old PC2 mice was observed using a single administration of 30 mg cinacalcet HCl/kg, despite the decrease in parathyroid CaR expression and putatively ongoing CaR-mediated signaling due to hypercalcemia in these mice. These data indicated that cinacalcet HCl could be an effective drug for primary hyperparathyroidism even under hypercalcemic conditions. Type II calcimimetic compounds such as
cinacalcet HCl are allosteric modulators that bind to a different site on the CaR than that used by ionized Ca, allowing cinacalcet HCl to increase CaR sensitivity to Ca ions and to suppress PTH secretion.

To analyze whether the expression of CaR, the target of cinacalcet HCl, on parathyroid glands might be involved in the potency of cinacalcet HCl’s suppression of serum Ca and PTH, immunohistochemical examination of CaR expression was performed on the parathyroid glands of mice that had been subjected to cinacalcet HCl administration. In the parathyroid glands of PC2 mice, which had mild to moderate primary hyperparathyroidism, tiny areas of reduced CaR expression, containing a couple of parathyroid cells, were dotted within their parathyroid glands (Fig. 4b,e). Some mice with moderate to severe hyperparathyroidism exhibited asymmetrical parathyroid glands. Such asymmetrical parathyroid glands contained focal regions encapsulated like adenomas. On such adenomatous regions, CaR expression was reduced in a reciprocal manner with the increased expression of PTH (Fig. 4c,f). The inhibition of PTH secretion by 30 mg cinacalcet HCl/kg exhibited significant and negative correlation with the size of the area of reduced CaR expression (Fig. 5), suggesting the importance of reduced CaR expression in the development of resistance of parathyroid glands to calcimimetics.

Older PC mice (93–99 weeks old) exhibited primary hyperparathyroidism in a more severe form than younger PC mice (65–85 weeks old) (Table 1). In these mice with advanced hyperparathyroidism, administration of 30 mg cinacalcet HCl/kg was no longer effective, as evidenced by the lack of its effect on the suppression of serum Ca and PTH levels (Fig. 6). The mechanism by which 30 mg cinacalcet HCl/kg lost its effect in these mice might be explained by the remarkable reduction in CaR in the parathyroid glands. Although these mice were resistant to 30 mg cinacalcet HCl/kg, an increased dose of 100 mg/kg significantly suppressed both serum Ca and PTH (Fig. 6). The suppressive ratio on PTH secretion of 30 mg cinacalcet HCl/kg administration in younger mice was similar as that of 100 mg/kg in older mice. These phenomena strongly suggested that increasing the dose of cinacalcet HCl could activate the signal transduction pathway to suppress PTH secretion via CaR, even though the expression of CaR on a parathyroid cell per se, the target of this compound, was extremely reduced. Further study of this in vivo model is desirable to examine the long-term effect of this compound in potentially preventing parathyroid cell proliferation and improving skeletal and cardiovascular complications.

In summary, a negative correlation between the potency of cinacalcet HCl and CaR expression in parathyroid glands suggested that cinacalcet HCl exerts its effect via CaR and that the potency of cinacalcet HCl depends on the degree of CaR expression. Even in the CaR hypo-expression state induced by severe hyperparathyroidism, the resistance to CaR activation could be overcome by increasing the dose of cinacalcet HCl.

In conclusion, the CaR is a potentially useful target for a therapeutic agent like cinacalcet HCl to suppress PTH secretion, even though the CaR expression was reduced in parathyroid glands, which was observed in advanced primary and secondary hyperparathyroidism.

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References


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