Calcitriol Treatment Attenuates Inflammation and Oxidative Stress in Hemodialysis Patients with Secondary Hyperparathyroidism

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Hemodialysis patients with secondary hyperparathyroidism (SHP) suffer from excessive oxidative stress and inflammation. Vitamin D analogues are currently the first line therapy for SHP, but the influence of vitamin D treatment on inflammation and oxidative stress remains unknown. This study investigated the influence of vitamin D therapy on oxidative stress and inflammatory markers in hemodialysis patients with SHP. Twenty-five patients (mean age 58 ± 12 years, 13 males and 12 females) were enrolled in the study to receive calcitriol treatment for 16 weeks. We evaluated changes in the serum biochemical parameters, inflammatory markers [C-reactive protein (CRP) and interleukin-6 (IL-6) levels], serum oxidative stress condition [total antioxidant status (TAS)], and CD4+ T-lymphocyte intracellular cytokines [interferon γ (IFN-γ) and interleukin-4 (IL-4)] before and at the end of the 16-week calcitriol treatment. Correlations between each of these factors were also studied. All patients with SHP had low serum 1,25-dihydroxyvitamin D3 levels and elevated serum levels of intact parathyroid hormone (iPTH), CRP and IL-6. Twenty patients (10 males and 10 females) responded to the calcitriol therapy, with significant decrements in serum iPTH. Our results showed that calcitriol can effectively suppress iPTH secretion, reduce inflammatory markers (CRP and IL-6) and oxidative stress. It can also effectively reduce inflammatory cytokine (CD4+ IFN-γ) and increase anti-inflammatory cytokine (CD4+ IL-4). Interestingly, significant correlations between CD4+ IFN-γ levels and serum iPTH levels, as well as between TAS and iPTH levels were noted. Overall, our study has demonstrated calcitriol treatment significantly attenuates inflammation and oxidative stress in hemodialysis patients with SHP.

Keywords: calcitriol; interferon-γ; interleukin-4; secondary hyperparathyroidism; total anti-oxidative status


Secondary hyperparathyroidism (SHP) is one of the main complications in patients with chronic kidney disease, affecting most patients receiving hemodialysis (HD) (Fraser 2009). The disorder is characterized by parathyroid gland hyperplasia, increased parathyroid hormone (PTH) secretion, and disturbances in bone and mineral metabolism. The development of SHP is attributed to decreased calcitriol production, phosphate accumulation, and hypocalcemia. Increased inflammation and oxidative stress have been proposed to play an important role in the pathogenesis of associated cardiovascular or infectious diseases contributing to low survival rates and quality of life in HD patients (Patel and Singh 2009a). Compared with non-SHP HD patients, patients with SHP may have a higher prevalence of serum inflammatory cytokines and oxidative stress, which are associated with high all-cause and cardiovascular-specific mortality (Lu et al. 2006; Tentori et al. 2008). High PTH levels, inflammation, and oxidative stress may act synergistically in the development and progression of renal osteodystrophy and cardiovascular disease in SHP patients.

Calcitriol and its synthetic analogues suppress PTH secretion and are considered the first-line standard therapy for SHP due to deficient and abnormal vitamin D metabolism in SHP (Bhan and Thadhani 2009; Patel and Singh
2009b). Initially, supplementation with active vitamin D or its analogs was used to focus on calcium/phosphorus homeostasis and renal bone disease. However, vitamin D treatment reduces cardiovascular disease mortality risk in dialysis patients irrespective of PTH levels. Increasing evidence suggests that vitamin D has a wide range of beneficial effects other than PTH suppression, including antiproliferative, pro-differentiative, and immunomodulatory effects (Nagpal et al. 2005; Valdivielso et al. 2009). The biological effect of vitamin D is mediated by its binding to the vitamin D receptor (VDR), which is also widely expressed in most cell types of the immune system, indicating the role of calcitriol in modulating lymphocyte and monocyte activities, as well as cytokine release (Provvedini et al. 1983; Moe et al. 2001). However, the extent of linkage between vitamin D, PTH, inflammation, and oxidative stress in SHP is unknown.

Thus, we designed this prospective study to investigate whether calcitriol treatment in SHP affects inflammatory response, oxidative stress and CD4+ T lymphocytes cytokines beyond calcium effects; in addition, we studied the correlations between each of these factors.

Materials and Methods

Patients

Twenty-five long-term HD patients (13 males and 12 females) diagnosed with renal osteodystrophy at a nephrology unit (Cardinal Tien Hospital) were included in this study after they provided informed consent. Research was carried out according to the principles of the Helsinki Declaration and was approved by the Human and Ethics Committee of the Cardinal Tien Hospital. The mean age of the patients was 58 (12) years, and the mean HD time was 8.8 (3.4) years. All patients had symptomatic disease with muscle weakness and bone and joint pain.

Patients with autoimmune disease, those with evidence of concurrent infection or malignancy, or those on immunosuppressive medications known to interfere with the immune system were excluded from the study. HD patients with SHP enrolled in the study were defined as having symptomatic osteodystrophy (bone and joint pain and muscular weakness) and a serum iPTH level of more than 300 pg/ml. None of the patients had received calcitriol therapy during the previous 6 months or had plasma calcium, phosphorus, or aluminum levels higher than 11.0 mg/dl, 6.0 mg/dl, or 1.0 μmol/l, respectively. Medications, when necessary, included antihypertensive treatments, insulin injection or oral hypoglycemic drugs, diuretics, and/or coronary vasodilators. Blood samples were collected for biochemical analysis and cytokine/chemokine studies. The control group consisted of 20 age- and sex-matched healthy subjects.

Calcitriol treatment

To achieve effective and safe suppression of PTH levels, initial low-dose intravenous calcitriol (1-μg vials; Abbot Laboratories) was administered through an effluent needle at the end of each HD session. Blood was obtained for laboratory tests at the beginning and at every 4 weeks during the 16-week calcitriol treatment. Blood was always collected approximately 44 h after the last treatment. Serum calcium and phosphate levels were monitored every 2 weeks. If serum levels of calcium, phosphorus, and calcium-phosphorus product remained within the acceptable range, calcitriol dosage was adjusted according to the changes in serum alkaline phosphatase (ALP) and iPTH levels, which were monitored monthly (Rodriguez-Garcia et al. 2003). The calcitriol dose was doubled if PTH dropped by less than 15%. The calcitriol dose was maintained if PTH dropped between 15% and 30%. If PTH dropped between 30% and 60%, the dose was reduced by 25%, and, if PTH dropped by more than 60%, the dose was halved.

Fig. 1. Algorithm of calcitriol adjustment in patients with secondary hyperparathyroidism. Low-dose calcitriol 1-μg three times per week during each HD session were initiated. The calcitriol dose was doubled if PTH dropped by less than 15%. The calcitriol dose was maintained if PTH dropped between 15% and 30%. If PTH dropped between 30% and 60%, the dose was reduced by 25%, and, finally, if PTH dropped by more than 60%, the dose was halved. If a decrease of at least 15% in PTH was not observed after 3 increases in the calcitriol dose the treatment was stopped (Fig. 1).

Calcitriol administration was interrupted in the following situations: serum PTH levels at or below 300 pg/ml; calcium-phosphate product, >75; serum phosphate level, >7.0 mg/dl; or serum calcium level, >10.5 mg/dl (Andress 2008). Aluminum hydroxide [Al(OH)3] was administered only to patients who could not maintain their serum phosphate level below 7.0 mg/dl with the maximum allowable dose of calcium carbonate. Patients with serum iPTH levels below 300 pg/ml by the end of the study were deemed responders (n = 20).

Serum measurements

Blood samples were collected and microcentrifuged for measurement. Serum was separated from the blood samples within 1 h of collection and stored at −30°C until analysis. Concentrations of total calcium, inorganic phosphorus, albumin, blood urea nitrogen, creatinine, and total alkaline phosphatase were determined by automated methods (AV 5,000 Chemistry analyzer; Olympus, Tokyo, Japan). High-sensitivity CRP was measured using an ultrasensitive solid phase enzyme-linked immunosorbent assay (ELIZA; DRG instruments GmbH, Marburg, Germany). Serum cytokines (IL-6, IFN-γ, and IL-4) were detected by using commercial ELISA kits (R&D, Minneapolis, MN). Serum 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] was determined by a radio-receptor assay (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). Serum iPTH concentration was measured by radioimmunoaassay using an iPTH Immunoassay kit (Nichols Institute diagnostics, San Juan Capistrano, CA, USA), which is a two-site immunoradiometric assay for the measurement of
the biologically intact 84-amino-acid chain of PTH.

**Flow cytometry and intracellular cytokine staining**

For intracellular cytokine staining, the FastImmune Cytokine System (Becton Dickinson Co., CA, USA) was used. Briefly, freshly isolated peripheral blood mononuclear cells (PBMC) were cultured with a combination of 25 ng/ml phorbol myristate acetate (PMA) plus 1 µg/ml ionomycin in the presence of 10 µg/ml brefeldin A (Sigma Chemical Co., MO, USA) at 37°C in a 5% CO₂ incubator for 4 hours. Cells were stained with monoclonal antibodies directed against allophycocyanin-conjugated CD4 (Pharmingen, Becton Dickinson, San Diego, MN, USA) as a marker for CD4⁺ T lymphocyte. Next, the cells were washed, fixed, and permeabilized followed by incubation with fluorescein isothiocyanate (FITC)-conjugated IFN-γ-specific monoclonal antibodies (MoAb) and phycoerythrin (PE)-conjugated IL-4-specific MoAb (Becton Dickinson). After washing with phosphate buffered saline (PBS) containing 0.1% fetal bovine serum (FBS), cells were fixed with 1% paraformaldehyde and subjected to flow cytometry analysis by using a fluorescence-activated cell sorting (FACS) Caliber cell sorter using Cell Quest software (Becton Dickinson, San Diego, CA, USA). The percentage of cytokine expression in the CD4⁺ T cell population of each patient was then calculated.

**Total antioxidant status assay**

The total antioxidant status (TAS) in 20 µL of serum or distilled water (as a blank) was measured using a TAS kit (Cat. # NX2332, Randox, San Francisco, CA, USA), as previously described (Huang et al. 2003). Briefly, 20 µL of sample was added to 6.1 µmol/L metmyoglobin and 610 µmol/L 2,2′-azino-di-3-ethylbenzthiazoline sulphonate (ABTS); absorbance of the mixture was measured at 600 nm by using an autoanalyzer (Tectron U-240 Plus, Japan) after vortexing, according to the manufacturer’s instructions. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid was used as a standard. The assay principle was that relatively stable blue-green colors are measured at 600 nm when the ABTS is incubated with a peroxidase and H₂O₂ to produce the radical cation ABTS. Antioxidants in the added sample caused suppression of this color production to some degree, which was proportional to their concentration.

**Statistical analysis**

All data are expressed as the mean (s.d.). Statistical analysis was performed by the t test for paired or unpaired values to determine the differences between the 2 groups. Correlation analysis was conducted using tests of linear regression. P < 0.05 was considered to be statistically significant. Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS/PC; SPSS, Inc., Chicago, IL, USA).

**Results**

**Clinical and biochemical parameters**

HD patients with SHP presented with low serum levels of 1,25(OH)₂D₃, high iPTH levels, and elevated inflammatory cytokines level compared to those of non-SHP patients (Table 1). Our results showed that 16-week calcitriol treatment effectively increased serum levels of 1,25(OH)₂D₃, suppressed iPTH secretion, and decreased inflammatory cytokines (CRP and IL-6) in SHP patients (Table 1). Calcitriol also increased TAS (1.63 (0.38) vs. 1.32 (0.25)), as well as modified CD4⁺ T lymphocyte intracellular cytokine activation by decreasing Th1 cytokine IFN-γ levels (3.4 (2.0) vs. 3.6 (2.3)) and increasing Th2 cytokine IL-4 levels (2.2 (1.5) vs. 1.9 (1.3)) (Fig. 2). The side effects of the treatment (hypercalcemia, hyperphosphoremia, or both) were noted in five patients (25%) of the responders.

| Table 1. Comparison of biochemical parameters before and after 16 weeks of intravenous calcitriol treatment in 20 SHP patients. |
|---------------------------------|----------------|----------------|----------------|
| **SHP (n = 20)**                | **Week 0**     | **Week 16**    | **Control (n = 20)** |
| 1,25(OH)₂D₃, pg/ml              | 13.4 (10.3)ₚ   | 18.67 (13.1)ᵡ  | 23.8 (5.7)ᵢ    |
| iPTH, pg/ml                     | 625.8 (207.7)ₚ | 188.5 (54.8)ᵡ  | 23.7 (8.7)ᵢ    |
| TCa, mg/dl                      | 9.59 (0.63)    | 9.76 (0.63)ᵡ  | 9.34 (0.35)ᵢ   |
| Pi, mg/dl                       | 4.78 (0.27)    | 4.91 (0.31)ᵡ  | 4.78 (0.13)    |
| AP, IU/l                        | 5.02 (0.57)ₚ   | 5.17 (0.53)ᵡ  | 4.18 (0.16)ᵢ   |
| Albumin, g/dl                   | 252.4 (165.3)ₚ | 112.8 (57.4)ᵡ | 102.2 (103.3)  |
| IL-6, pg/ml                     | 3.96 (0.36)    | 4.01 (0.38)    | 4.03 (0.41)    |
| CRP, mg/dl                      | 7.54 (3.59)ₚ   | 6.24 (2.81)ᵡ  | 0.45 (0.27)ᵢ   |
| BW, kg                          | 60.1 (10.2)    | 59.8 (10.1)    | 63.7 (9.8)ᵢ    |
| BMI, kg/m²                      | 22.97 (3.28)   | 22.87 (3.18)   | 23.39 (3.18)   |
| KT/V                            | 1.49 (0.26)    | 1.51 (0.28)    |               |
| nPCR, g/kg/day                  | 1.28 (0.31)    | 1.27 (0.29)    |               |

*ₚp < 0.05, *ᵡp < 0.01, vs. control; *ᵢp < 0.05, *ᵢp < 0.01, vs. week 0; *ᵢp < 0.05, *ᵢp < 0.01, vs. week 16.

SHP, Secondary Hyperparathyroidism; Tca, Total calcium; Ica, ionized calcium; PI, inorganic phosphate; AP, alkaline phosphatase.
Fig. 2. Changes in serum iPTH, total antioxidant status, CD4⁺ IFN-γ, and CD4⁺ IL4 levels before, and after calcitriol treatment. Calcitriol treatment effectively suppressed iPTH secretion (A), increased total antioxidant status (B), as well as modified CD4⁺ T lymphocyte intracellular cytokine activation by decreasing Th1 cytokine IFN-γ levels (C) and increasing Th2 cytokine IL-4 levels (D). TAS: total antioxidant status.

Fig. 3. Correlation between serum iPTH levels and CD4⁺ IFN-γ levels before and after calcitriol treatment. There was a positive correlation between iPTH level and CD4⁺ T lymphocyte IFN-γ level during before (A), after (B) and total (C) course of treatment. The positive correlation between iPTH level and CD4⁺ T lymphocyte IL-4 level only significant during before (D), but not after (E) nor total (F) course of treatment.
Correlations among PTH, TAS, and CD4⁺ T lymphocyte intracellular cytokines

The relationships between PTH, TAS, and CD4⁺ T lymphocyte intracellular cytokines were investigated in SHP patients receiving calcitriol treatment. We found that there was a positive correlation between iPTH level and CD4⁺ T lymphocyte IFN-γ level (P < 0.05) and a negative correlation between iPTH level and TAS (P < 0.001) (Fig. 3 and Fig. 4). No other significant relationships were noted.

Discussion

Results of this study showed that calcitriol treatment effectively suppressed iPTH secretion, attenuate inflammatory cytokine (CRP and IL-6) levels, decreased oxidative stress, and modified CD4⁺ T lymphocyte intracellular cytokine activation (IFN-γ and IL-4) in HD patients with SHP. Furthermore, there was a significant correlation between CD4⁺ IFN-γ activity and serum iPTH levels, as well as between TAS and serum iPTH levels. These results confirm the beneficial effects of anti-inflammatory, anti-oxidative, and lymphocyte modulation beyond the PTH-lowering effect of calcitriol treatment for SHP.

Cardiovascular diseases and infections are the leading causes of high mortality in patients with end-stage renal disease (ESRD). T lymphocyte-mediated immunity and oxidative stress both participate in intimal thickening and plaque formation, inducing atherosclerosis susceptibility, as well as contribute to acquired immune dysfunction and infections. We showed that calcitriol treatment effectively suppressed iPTH secretion, decreased inflammation and oxidative stress, and modified CD4⁺ T lymphocyte responses in SHP patients. Calcitriol administration has been used as the primary treatment to prevent parathyroid gland hyperplasia and reduce serum PTH levels in SHP patients. Vitamin D has proven to be much more than a simple “calcium hormone” playing important roles in calcium, phosphorus, and skeletal homeostasis (Nagpal et al. 2005; Valdivielso et al. 2009; Biggar et al. 2011). The vitamin D receptor (VDR) has been identified on macrophages and activated T lymphocytes, suggesting a potential role for vitamin D in regulating the immune system (Bhalla et al. 1986; Matsui et al. 1986; Rigby et al. 1987; Muller and Bendtzen 1996). The VDR ligand-mediated signaling cascade is important in lymphocyte/macrophage activities and cytokine release. Previous epidemiologic studies have revealed that there is a potentially important role for VDR in the survival of patients undergoing dialysis, and vitamin D compounds that can activate the VDR are known to be associated with decreased mortality compared with the absence of any form of vitamin D therapy (Shoji et al. 2004; Melamed et al. 2006; Tentori et al. 2006). Increased intracellular CD4⁺ IFN-γ activity and decreased CD4⁺ IL-4 activity were found during calcitriol treatment in our study, potentially through the ability of VDRs to change the Th1/Th2 balance and influence the production of anti-inflammatory mediators. Vitamin D predominantly decreases the Th1 type immune response and inhibits the production of IFN-γ, which is involved in cell-mediated immunity, and facilitates the development of Th 2 lymphocytes, which are involved in the humoral immune response and have anti-inflammatory and anti-atherogenic properties (Boonstra et al. 2001; Barrat et al. 2002; Staeva-Vieira and Freedman 2002). Furthermore, our results suggested the pleiotropic anti-inflammatory and immunomodulation effects of vitamin D during SHP treatment. Whether the vitamin D treat-
Elevated PTH in ESRD is associated with many cardiovascular, metabolic, hematological, and immunological abnormalities (Patel and Singh 2009a). While the immune defect in CKD appears to be multifactorial, the contribution of PTH remains unclear. There is a direct correlation between PTH levels in uremic patients and the degree of lymphocyte proliferation inhibition in ESRD patients with SHP; PTH has been identified as a possible factor in the development of an acquired immune dysfunction (Shurtz-Swirski et al. 1995). PTH receptors are found on most immunologic cells (neutrophils, B cells, and T cells), suggesting a potential role for PTH in immune system regulation (Geara et al. 2010). Increases in intracellular calcium levels potentially lead to an increase in cellular adenyate cyclase activity; this has been proposed as the mechanism by which PTH influences leukocytes. However, the effects of PTH on T lymphocytes are neither consistent nor conclusive (Geara et al. 2010). Most studies showed that PTH exerts an inhibitory effect on various immune system parameters, while other studies showed that PTH has a stimulatory function. Acute effect of PTH on T lymphocytes results in a dose-dependent increase in cell proliferation in vitro; however, chronic PTH exposure decreases T-lymphocyte proliferation in ESRD patients and changes the CD4/CD8 lymphocyte ratio (Alexiewicz et al. 1990; Kaneko et al. 1997). In our study, we further analyzed the effect of PTH on Th1/2 helper cells and showed a significant correlation among PTH, IFN-γ, and IL-4. Treatment of SHP using parathyroidectomy or medical treatments may reverse the immunologic defects in patients, which may be clinically relevant and potentially translate into a better survival rate. Hence, although these observations could not be definitively linked to excess PTH and immune response modulation, our results indicate that immune system abnormalities in chronic uremia may be partly related to the degree of SHP. Further studies are needed to determine the definite immunomodulatory effects of PTH in SHP.

Beyond the traditional cardiovascular disease risk factors, chronic inflammation and increased oxidative stress are thought to contribute to accelerated atherosclerosis and increased cardiovascular mortality and morbidity (Patel and Singh 2009b). In this study, calcitriol treatment decreased inflammatory cytokines (CRP and IL-6) and oxidative stress and effectively suppressed iPTH secretion in HD patients with SHP. The study results indicate whether these effects originate from suppressed iPTH or are a direct result of calcitriol treatment. PTH is a uremic toxin associated with high inflammation and negative cardiovascular outcomes in ESRD populations; however, primary hyperparathyroidism has also been linked to endothelial dysfunction and increased cardiovascular mortality (Kosch et al. 2000; Melamed et al. 2006). Either SHP treatment using parathyroidectomy or medical treatment may reverse higher inflammatory and oxidative status associated with cardiovascular disease, translating into a better survival. SHP-induced Ca²⁺ loading of PBMC accompanied by oxidative stress induction have been found to play a permissive role in the pro-inflammatory vascular phenotype in aldosteroneism (Chhokar et al. 2005). Our findings suggest a therapeutic role of vitamin D that suppresses PTH in SHP patients. However, VDR agents also induce a survival benefit, which is apparently independent of serum calcium, phosphate, and PTH concentrations. Several studies have shown that vitamin D deficiency is a prominent independent risk factor for mortality and cardiovascular events, and that there is an association between calcitriol levels and survival, indicating a direct role of vitamin D in cardiovascular health (Autier and Gandini 2007; Melamed et al. 2008). Recently, vitamin D replacement was reported to have favorable effects such as reversing hypovitaminosis D-associated endothelial dysfunction and increasing lipid peroxidation, which contributes to atherosclerosis (Tarcin et al. 2009). However, the effects of regulation of vitamin D, calcium homeostasis, PTH, inflammation, and oxidative stress on immune function are complex and poorly understood. We proposed that both direct effects of vitamin D and PTH-lowering effects contribute to anti-inflammatory, anti-oxidative, and lymphocyte modulation during SHP treatment with calcitriol.

Due to the pathogenic complexity between vitamin D, PTH, inflammation, and oxidative stress in SHP, it remains a challenge for physicians to provide protection from the development and progression of cardiovascular and renal osteodystrophy in HD patients. Despite the limited number of subjects and the lack of a randomized control trial in our study, we can still conclude that anti-inflammatory, anti-oxidative, and lymphocyte modulation effects beyond PTH suppression stand important therapeutic effect of calcitriol in SHP. The potential pleiotropic effect of vitamin D is particularly important because the SHP population may have a higher prevalence of inflammation and oxidative stress. We recommend vitamin D therapy as early as possible for every HD patient showing vitamin D deficiency or SHP. Whether it is possible to administer vitamin D to dialysis patients with low/normal PTH levels for anti-inflammatory, anti-oxidative, and immunomodulatory effects requires further investigation (Moe et al. 2001; Gal-Moscovici and Sprague 2010).

In conclusion, calcitriol treatment effectively suppresses iPTH secretion, decreases inflammation and oxidative stress, as well as playing a role in CD4⁺ T lymphocyte modulation in HD patients with SHP. Our study highlights the non-traditional benefits of anti-inflammatory, anti-oxidative, and lymphocyte modulation beyond the PTH-lowering effect observed during SHP treatment with calcitriol.

Conflict of Interest
The authors declare no conflict of interest.
References


