New insights into mineral and skeletal regulation by active forms of vitamin D

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Recent studies in mice using genetic approaches have shed new light on the physiological effects of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) and the vitamin D receptor (VDR) in skeletal and mineral homeostasis, and on their interaction with calcium. These studies in mice with targeted deletion of the 25-hydroxyvitamin D-1α-hydroxylase (1α(OH)ase), and of the VDR or of double mutants, have shown the discrete effects of calcium in inhibiting parathyroid hormone secretion and in enhancing bone mineralization, but overlapping effects of calcium and $1,25(OH)_2D$ on inhibiting parathyroid growth and on normal development of the cartilaginous growth plate. The $1,25(OH)_2D$/VDR system is essential, however, in enhancing intestinal calcium absorption and in optimally increasing osteoclastic activation. In addition, the $1,25(OH)_2D$/VDR system has important anabolic effects on bone, thus defining a dual role for this system in bone turnover. These studies are revealing functions of the vitamin D/VDR system which have relevance for new concepts of the pathophysiology of renal bone disease and, in particular, of the adynamic bone disorder, and for the development of new analogs of the active form of vitamin D, which have less calcemic activity and greater skeletal anabolic effects.

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Circulating concentrations of the active metabolite of vitamin D, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), derive from 1α-hydroxylation of 25-hydroxyvitamin D (25(OH)D) by the renal mitochondrial enzyme, 25(OH)D-1α-hydroxylase (1α(OH)ase).[1–3] $1,25(OH)_2D$ circulates bound to the vitamin D binding protein. After being taken up by target cells and bound to intracellular binding proteins, the sterol interacts with the vitamin D receptor (VDR), a member of the nuclear receptor superfamily.[4] In the nucleus of target cells, the ligand-activated VDR heterodimerizes with the retinoid X receptor and the dimer binds to response elements on target genes.[5] Coregulators are recruited to link the dimer to the basal transcriptional machinery and thereby modulate gene transcription. Differential interactions with the binding proteins involved in transport or intracellular chaperoning of $1,25(OH)_2D_3$, mobilization of discrete VDR coregulators, differential effects on VDR turnover, or distinct rates or pathways of elimination of vitamin D analogs are among the mechanisms which contribute to the specific actions of newer vitamin D analogs. In addition to the canonical pathway of action described above, a number of pathways involving rapid, nongenomic effects have been described. These include interaction of the ligand with a membrane receptor, either a novel receptor or the known VDR, thereby activating cell signaling pathways.[6,7] Both the VDR and extrarenal 1α(OH)ases have been described in a variety of tissues, beyond those related to skeletal and mineral homeostasis, implicating vitamin D action in a broad range of physiologic functions.

RENAL OSTEODYSTROPHY

Circulating concentrations of $1,25(OH)_2D$ have profound effects on parathyroid function, mineral metabolism, and skeletal function, which are severely disordered in chronic kidney disease (CKD). $1,25(OH)_2D$ synthesis is impaired in CKD in part due to suppression of the 1α(OH)ase by retained phosphate and in part because of loss of renal parenchyma. As a result, low levels of calcitriol develop in stage 3 CKD. This results in reduced calcium absorption, which, with hyperphosphatemia due to impaired renal tubular function and decreased bone formation, leads to hypocalcemia. Hypocalcemia, low $1,25(OH)_2D$, and phosphate retention can lead to the development of secondary

hyperparathyroidism and disordered skeletal remodeling, which may be accentuated by decreased levels of VDR and diminished levels of the calcium sensing receptor in hyperplastic parathyroid tissue. The skeletal consequence of secondary hyperparathyroidism is a disorder of bone remodeling referred to as osteitis fibrosa. Secondary hyperparathyroidism also contributes to the reduced longitudinal bone growth that occurs in CKD, affecting the pediatric population due to effects of the derangements of Ca, Pi, and vitamin D on the cartilaginous growth plate.

GENETIC ANALYSIS OF THE 1,25(OH)$_2$D/VDR SYSTEM AS A PROBE FOR FUNCTIONALITY

Several 'knockout' mouse models, with targeted disruption of either the *1α(OH)ase*[8,9] or the *VDR*[10–12] gene have been generated (1α(OH)ase$^{-/-}$ and VDR$^{-/-}$ mice, respectively). These represent murine models of the inherited human disorders, vitamin D-dependent rickets type I and type II, respectively.[13,14] We have used these models and double null mutants (1α(OH)ase$^{-/-}$VDR$^{-/-}$) to examine the effects of the 1,25(OH)$_2$D/VDR system on mineral, parathyroid, and skeletal homeostasis.[15] The study of the mechanisms underlying the 1,25(OH)$_2$D- and VDR-deficient phenotypes in these mouse mutants has been facilitated by the correction of the hypocalcemia, which these animals exhibit, by a 'rescue' diet containing high concentrations of calcium, phosphorus, and lactose.[16–18] These genetic models and the environmental manipulations of the models have therefore provided powerful probes to dissect *in vivo* the discrete and overlapping contributions of vitamin D, calcium, and parathyroid hormone (PTH) to the endocrine control of skeletal and mineral metabolism.[19]

Effects on calcium homeostasis and parathyroid function

Optimal dietary calcium absorption in the 1α(OH)ase$^{-/-}$ and the VDR$^{-/-}$ mice required an intact 1,25(OH)$_2$D/VDR system. In more recent studies in young 1α(OH)ase$^{-/-}$ mice and mice with targeted ablation of the PTH gene (*PTH*$^{-/-}$), we found that both 1,25(OH)$_2$D and PTH could independently and co-operatively modulate renal calcium transporters including the luminal epithelial channel transient receptor potential-vanilloid-5 (TRPV5), the intracellular calcium-binding proteins calbindin-D$_{28K}$ and calbindin-D$_{9K}$, and the basolateral membrane sodium–calcium exchanger, NCX1.[20] These results are consistent with the actions of 1,25(OH)$_2$D reported in other 1α(OH)ase$^{-/-}$ models.[21] In the 1α(OH)ase$^{-/-}$ and VDR$^{-/-}$ models, secretion of intact PTH was modulated primarily by ambient serum calcium in that normalization of serum calcium with the rescue diet normalized circulating PTH even in the absence of 1,25(OH)$_2$D or the VDR. Nevertheless, the parathyroid gland hyperplasia, which the mutants exhibited, persisted when calcium alone was normalized. The combination of normal extracellular calcium and 1,25(OH)$_2$D (apparently independently of the VDR) was required for normalization of parathyroid gland size. The results indicate that calcium cannot entirely substitute for deficient vitamin D in maintaining parathyroid homeostasis, and that the two agents act co-operatively in modulating parathyroid cell proliferation.

Development of the cartilaginous growth plate

On a normal- or high-calcium lactose-free intake, all three hypocalcemic mutant mouse models, the 1α(OH)ase$^{-/-}$, VDR$^{-/-}$, and 1α(OH)ase$^{-/-}$VDR$^{-/-}$ mice, develop characteristic skeletal rachitic changes of enlarged and distorted cartilaginous long bones with widened hypertrophic zones.[8–10,11,15,18] These abnormalities appear less severe in VDR$^{-/-}$ mice with elevated endogenous 1,25(OH)$_2$D levels than in the other two mutants, suggesting that 1,25(OH)$_2$D may modulate cartilage function independently of the VDR. Nevertheless, 1,25(OH)$_2$D *per se* cannot normalize the growth plate if hypocalcemia is not normalized and, conversely, elimination of hypocalcemia with the rescue diet does not completely normalize the growth plate in the mouse models that have deficient endogenous 1,25(OH)$_2$D. Consequently, both calcium and 1,25(OH)$_2$D together appear necessary for normal development of the cartilaginous growth plate, analogous to the effect on parathyroid growth. Previous studies in double-null mutants of the VDR and of the retinoid X receptor γ have suggested the existence of a novel VDR in the cartilaginous growth plate with which 1,25(OH)$_2$D may interact.[22] In addition, rapid, nongenomic effects have been reported in cartilage cells.[6,7] Our findings are consistent with one or other or both of these possibilities.

Skeletal mineralization

Mineralization of both cartilage and bone is severely impaired in all mutants (the 1α(OH)ase$^{-/-}$, VDR$^{-/-}$, and 1α(OH)ase$^{-/-}$, VDR$^{-/-}$ models) that are hypocalcemic on either a lactose-free-normal or high-calcium intake.[15] However, cartilage mineralization and bone mineralization are normalized in all models when hypocalcemia is eliminated by the rescue diet. Administration of exogenous 1,25(OH)$_2$D$_3$ normalizes mineralization only when serum calcium is normalized, that is, in the 1α(OH)ase$^{-/-}$ model.[15] Consequently, the major determinant of skeletal mineralization appears to be the ambient concentration of extracellular calcium (and phosphate) and the 1,25(OH)$_2$D/VDR system appears to play no direct role in this process.

Bone remodeling

Osteoblast numbers, bone formation, and bone volume are markedly increased in all hypocalcemic animals (1α(OH)ase$^{-/-}$, VDR$^{-/-}$, and 1α(OH)ase$^{-/-}$ VDR$^{-/-}$ mice) on either a lactose-free, normal-calcium intake,[8–10] or a lactose-free, high-calcium intake.[15] This appears to be due to the anabolic effect of PTH, which is markedly elevated in association with the severe secondary hyperparathyroidism in these animals. Increased serum alkaline phosphatase reflects the increased osteoblastic stimulation by PTH and is normalized when PTH is normalized by eliminating the

secondary hyperparathyroidism. The increased bone volume is largely due to increased unmineralized bone matrix however, as a result of the ambient hypocalcemia. Interestingly, a sustained elevation of increased PTH is generally associated with increased osteoclastic bone resorption as well as increased bone formation. Nevertheless, osteoclast number and resorbing surface are not appreciably elevated in these models compared to wild-type, suggesting an inappropriate response to the increased PTH. Furthermore, in our studies,[15] a decrease in the average osteoclast size was seen in the $1,25(OH)_2D$- and/or VDR-deficient mutant animals with secondary hyperparathyroidism. This suggests that there is uncoupling of bone turnover in the presence of a defective $1,25(OH)_2D$/VDR system and the relatively low resorption may contribute, with increased osteoblast activity, to the increased bone volume. Indeed, previous studies with osteoclast-generating models *in vitro* have shown that osteoblastic cells from $VDR^{-/-}$ mice in culture with normal spleen cells could not sustain $1,25(OH)_2D$-stimulated osteoclast production, although PTH could.[23] Therefore, although PTH and local modulators of bone resorption may sustain a normal level of osteoclastic resorption in these models, an intact $1,25(OH)_2D$/VDR system is required for an optimal osteoclastic response to increased PTH.

In view of the fact that osteoclast/chondroclast production at the chondro-osseous junction may also be defective, diminished removal of hypertrophic chondrocytes may occur in this region, leading to altered cartilage growth plate remodeling. Therefore, the enlargement of the cartilaginous growth plate, and notably the hypertrophic zone, may also be in part due to reduced activity of the $1,25(OH)_2D$/VDR system on the chondroclast/osteoclast system.[17]

In our studies we also found that in all three mutant models, that is, in the absence of $1,25(OH)_2D$, VDR, or both $1,25(OH)_2D$ and VDR, when serum calcium was normalized by the rescue diet and secondary hyperparathyroidism was prevented, osteoblast numbers, mineral apposition rate, and bone volume were suppressed below the levels seen in wild-type mice.[15] This showed that, when the $1,25(OH)_2D$/VDR system is deficient in the presence of normal PTH and normal calcium, a requirement of the $1,25(OH)_2D$/VDR system for baseline bone anabolism can be unmasked. The $1,25(OH)_2D$/VDR system therefore appears to exert an anabolic effect, which is necessary to sustain basal bone-forming activity. This inhibition of bone formation was not previously observed in either $VDR^{-/-}$ or $1\alpha(OH)ase^{-/-}$ mice on the rescue diet and may reflect the older age of our mice at the time of analysis. Furthermore, other studies have also pointed to an anabolic effect of $1,25(OH)_2D$,[24–26] supporting the observations in our model. Our studies therefore suggest that $1,25(OH)_2D$ may exert effects on both bone resorption and bone formation analogous to those of PTH.

Comparative skeletal effects of $1,25(OH)_2D$ and PTH

To better understand the relative contributions of $1,25(OH)_2D$ and PTH to bone anabolism, we recently compared $1\alpha(OH)ase^{-/-}$ mice to $PTH^{-/-}$ mice.[20] Reduced osteoblastic bone formation in the metaphyseal region was seen in both mutants, confirming our findings of a requirement for endogenous $1,25(OH)_2D$ as well as for endogenous PTH for bone anabolism. PTH deficiency however caused only a slight reduction in long bone length but a more marked reduction in trabecular bone volume in these models, whereas $1\alpha(OH)$ase ablation caused a smaller reduction in trabecular bone volume but a significant decrease in bone length. Therefore, although both $1,25(OH)_2D$ and PTH can affect long bone growth and trabecular bone growth, PTH plays a predominant role in appositional bone growth, whereas $1,25(OH)_2D$ acts predominantly on endochondral bone formation. We subsequently confirmed these findings by administering exogenous $1,25(OH)_2D3$ (calcitriol) and PTH to these animals.[27] Consequently, both $1,25(OH)_2D$ and PTH exert anabolic effects that are discrete but complementary.

Overall therefore, these genetic models have provided important new insights into the role of the active form of vitamin D in mineral and skeletal homeostasis (Table 1). The actions on parathyroid growth, on regulating the development of the epiphyseal growth plate, and on regulating bone formation seem of particular relevance for the mineral and skeletal alterations which occur in both pediatric and adult kidney disease, and improved knowledge of the physiology of the vitamin D system may lead to improved design and use of vitamin D analogs in CKD.

VITAMIN D ANALOG THERAPY IN CKD

Calcitriol and adynamic bone disorder

In patients with CKD, placebo-controlled trials have demonstrated that administration of daily oral calcitriol

Table 1 | Effects of 1,25(OH)2D, calcium, and PTH on selected parameters of mineral and skeletal metabolism based on *in vivo* studies in genetic models

	Effectors[a]			
Parameters	$1,25(OH)_2$ D/VDR	Ca	$1,25(OH)_2$ D+Ca	PTH
Calcium absorption	+			
Renal calcium transporters	+			+
Parathyroid secretion		−		
Parathyroid growth			−	
Cartilaginous growth plate development			+	
Endochondral bone formation	++			
Bone mineralization		+		
Appositional bone formation	+			++
Bone volume				+
Bone resorption – osteoclastic activity	+			+
Bone formation – osteoblastic activity	+			+
1α-(OH)ase activity	−	−		+
24-(OH)ase activity	+	+		−

[a]+, stimulatory effect; −inhibitory effect; no symbol indicates no effect or effect not examined.

reduced PTH levels and improved bone biopsy findings.[28,29] Concerns regarding the safety of such therapy related to deterioration of kidney function were addressed, demonstrating that the rate of decline in glomerular filtration rate did not differ between placebo- and vitamin D-treated patients.[28,30] Nevertheless, despite oral daily regimens of calcitriol or alfacalcidol, secondary hyperparathyroidism remained the predominant bone lesion observed, especially in dialysis patients and the younger age groups.[31–33]

The introduction of intravenous calcitriol was associated with marked reductions in PTH levels and bone turnover in hemodialysis patients.[34] Oral intermittent regimens were subsequently shown to be as effective despite the greater bioavailability of intravenous calcitriol.[35–37] Some authors concluded from their studies of intermittent calcitriol therapy that the reduction in bone formation rate appeared to be greater than that of PTH levels.[38,39] Such findings led authors to suggest a direct negative effect of vitamin D on osteoblastic activity. In patients on peritoneal dialysis, high-dose intermittent oral or intraperitoneal calcitriol therapy was associated with a marked decline in bone formation rates, leading to the development of the adynamic bone disorder (ABD) in a substantial proportion of the patients.[38,40,41] Calcium-based phosphate binders were given to all of the patients and the serum calcium levels increased during the period of the studies. This prompted the conclusion that both the use of active vitamin D sterols and the exogenous calcium load played a role in the pathogenesis of the ABD (Figure 1).[41]

Alternative pathogenic mechanisms of the ABD have, however, been recently demonstrated in translational studies, which are in agreement with the genetic analysis of the $1,25(OH)_2D$/VDR system functionality in bone remodeling. Lund *et al.*[42] have shown in mice subjected to renal ablation and proportional reduction in dietary phosphorus with maintenance of normal Ca, Pi, PTH, and calcitriol levels that the ABD resulted. These data strongly indicate that renal injury directly impairs skeletal anabolism. The data were confirmed in another animal model with the metabolic syndrome of obesity, hypertension, dyslipidemia, and type II diabetes (low-density lipoprotein receptor-deficient mice fed high-fat/cholesterol diets), where induction of CKD resulted in the ABD despite secondary hyperparathyroidism.[43] Thus, reinterpretation of the human studies is possible, with the conclusion that reversal of secondary hyperparathyroidism uncovers the effect of renal disease on the skeleton. This produces the concept that secondary hyperparathyroidism is an adaptation to loss of skeletal anabolism produced by kidney disease, especially early in CKD before hyperphosphatemia and calcitriol deficiency are uniformly detected.

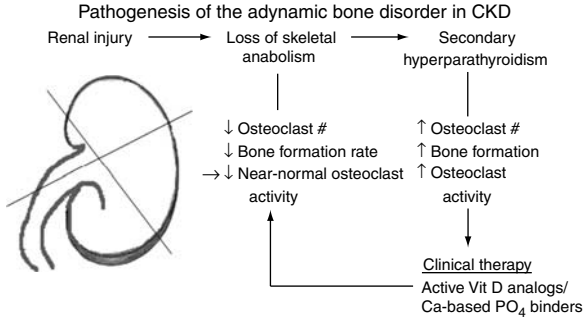

Figure 1 | In CKD, renal injury produces loss of skeletal anabolism possibly through decreases in bone morphogenetic protein influence. The reduction in the bone mineralization front represents a decrease in the exchangeable phosphorus pool contributing to stimulation of PTH secretion and secondary hyperparathyroidism. Thus, the latter, especially early in CKD, is an adaptation to loss of skeletal anabolism. When the adaptation is removed by correction of secondary hyperparathyroidism using active vitamin D analogs and Ca-based PO_4 binders, the effect of kidney disease on the skeleton is uncovered – the ABD.

Calcitriol and extra-skeletal calcification

Calcitriol therapy increases intestinal calcium and phosphorus absorption, with the subsequent development of hypercalcemia and hyperphosphatemia in some patients with CKD. The increase in calcium–phosphorus product limits therapy with vitamin D analogs and may play a role in the development of extraskeletal and vascular calcification.[44] Milliner *et al.*[45] found postmortem evidence of soft tissue and vascular calcification in 60% of 120 children with end-stage kidney disease.[45] The strongest correlation with calcinosis among the calcium phosphate ($Ca\text{-}PO_4$) product, sex, age of onset of kidney disease, and vitamin D therapy was with the latter. Current evidence suggests that abnormalities in $Ca\text{-}PO_4$ mineral metabolism, use of calcium-containing phosphate binders, and therapy with active vitamin D sterols play a role in the development of vascular calcification.[46,47] Vascular calcification and hyperphosphatemia are independent risks for cardiovascular disease and excess mortality in the end-stage kidney disease (ESKD) population.[48–51] Even among the young ESKD patients, cardiovascular disease is the leading cause of death and a substantial proportion of such patients demonstrate evidence of coronary artery calcification.[52] The role of calcitriol and active vitamin D analogs in the pathogenesis of vascular calcification is unclear despite the discussion above. Several epidemiologic studies suggest that active vitamin D analog therapy is associated with reduced vascular calcification,[53,54] while translational studies[55,56] and studies *in vitro*[57,58] indicate that they may be causative of vascular calcification.

Vitamin D analogs

New vitamin D analogs have been designed to minimize the increase in intestinal calcium and phosphorus absorption while continuing to suppress hyperparathyroidism as effectively as calcitriol at least in animal studies.[59–61] Three new vitamin D analogs are available for clinical use in CKD: 22-oxacalcitriol (OCT) in Japan, and 19-nor-1,25-dihydroxyvitamin D_2 (paricalcitol) and 1α-hydroxyvitamin D_2 (doxercalciferol) in the USA. Interestingly, in a series of repeat bone biopsies, OCT did not decrease bone formation rates.[62]

Studies with 19-nor-1,25-dihydroxyvitamin D_2 demonstrate fewer episodes of hypercalcemia and a lower calcium-phosphorus product with equal suppression of PTH during 8 weeks of the study.[63,64] The long-term effects of these therapeutic approaches have yet to be determined on vascular calcification and mortality. However, in a large dialysis cohort analyzed retrospectively, greater survival rates were described in patients treated with paricalcitol compared to patients treated with calcitriol.[65]

In translational studies using our murine models of the ABD described above,[42,43] we have examined the ability of therapy with calcitriol or 19-nor-1,25-dihydroxyvitamin D_2 to rescue the ABD.[66] Treatment with 10 and 20 ng/kg of calcitriol or 50 and 100 ng/kg of paricalcitol for 12 weeks following the induction of CKD and institution of a reduced phosphorus diet produced an increase in the trabecular bone volume, a reduction in osteoid surface, and an increase in mineralizing surfaces in the paricalcitol-treated animals compared to the calcitriol-treated animals. Calcitriol, but not paricalcitol, increased osteoclast surfaces, suggesting, perhaps, differential modulation of the VDR function in the bone cell transcriptional machinery. These results were recapitulated in the $LDLR^{-/-}$ mice with CKD fed high-fat diets,[67] and, while further studies are required to clarify these preliminary results, the possibility of differential modulation of skeletal VDRs needs to be further investigated. The actions of the skeletal VDR in our translational studies in CKD are therefore in keeping with genetic studies of 1α(OH)ase deficiency and VDR deficiency, to not inhibit but rather to stimulate osteoblastic function. These results require further analysis in the context of the ABD in dialysis patients and the need to stimulate skeletal anabolism. Since we have demonstrated that renal osteodystrophy contributes to hyperphosphatemia in CKD, and that hyperphosphatemia is a direct stimulus to osteogenic differentiation within the vascular tunica media, stimulation of bone formation and skeletal mineral deposition may have a lasting benefit in avoiding the vascular calcification that otherwise complicates CKD and ESKD. Overall therefore, newer vitamin D analogs with more favorable properties based on novel scientific insights may substantially improve mineral and skeletal homeostasis in CKD.

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