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Vitamin-D regulation of bone mineralization and remodelling during growth

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1. ABSTRACT

Vitamin D status relates to two bone diseases, osteomalacia and osteoporosis which arise from distinct pathophysiogical pathways. They can occur in children as well as adults. Osteomalacia or rickets arises from a delay in mineralization and can be caused by severe vitamin D deficiency where the key to curing osteomalacia is the endocrine action of circulating 1,25-dihydroxyvitamin D to normalize the active intestinal transport of calcium and phosphate. Osteoporosis or sub-optimal bone mineral accretion during growth is a risk factor for fracture in children. Current evidence suggests serum 25-hydroxyvitamin D levels between 20 and 80 nmol/L are associated with decreased bone mineral content as a result, at least partly, of reduced vitamin D metabolism and activity within bone cells. The local synthesis of 1,25-dihydroxyvitamin D within bone is necessary to modulate bone resorption and promote bone formation. Thus an adequate vitamin D status is necessary for vitamin D activity within bone to establish a healthy skeleton.

2. INTRODUCTION

The actions of vitamin D to reduce the risk of a wide range of disease states including osteoporosis, cancer and autoimmune disease continue to attract considerable interest from clinicians and the public alike. This interest has largely arisen from epidemiological studies identifying a strong association between decreased vitamin D status or sunlight exposure (from which vitamin D is derived) and increased incidence of diseases and even all cause mortality (1). There is currently considerable controversy regarding the vitamin D status required to obtain these health benefits (2). Current recommendations largely arise from requirements to resolve the bone disease of rickets in children or osteomalacia in adults. A second issue of interest is the mode of action of vitamin D. Vitamin D_3 was identified in 1919 as the critical factor for resolving the childhood bone disease of rickets (3). It was not until the late 1960's that the biologically active metabolite 1,25-dihydroxyvitamin D_3 (1,25D) (4) was identified as a ligand for a specific nuclear receptor (5). It was then realised that

this biologically active vitamin D metabolite acts as a steroid hormone in an endocrine manner. Thus the paradigm of vitamin D_3 as a pre-prohormone of an endocrine agent has dominated thinking in this area until the end of the 20th century. During the 21st century there has been an increasing quantity and quality of data derived from a variety of organ systems as well as clinical and animal model studies indicating that vitamin D_3 also has autocrine and/or paracrine actions.

There are two bone diseases, rickets/osteomalacia and osteoporosis, for which vitamin D acts, either alone or in combination with calcium, as an effective treatment in both children and adults. The common form of rickets is a defect in the mineralization of growing bone, which in adults is known as osteomalacia. It can arise due to severe vitamin D depletion causing a delay in mineralization although the synthesis of protein matrix of bone (osteoid) by the bone forming cells (osteoblasts) is unimpaired. Osteoid acts as scaffolding for bone mineral deposition. Thus in osteomalacia or rickets there is an excess of osteoid over mineral. The rickets/osteomalacia bone phenotype occurs when vitamin D activity is ablated such as in the vitamin D receptor knockout (Vdr^{KO}) mouse. Thus the further observation that this phenotype could be rescued by feeding a diet containing high levels of calcium and phosphorus indicated that the essential biological activity of vitamin D in the etiology of rickets/osteomalacia is at the level of regulation of intestinal calcium and phosphate absorption and the maintenance of plasma calcium and phosphate homeostasis (6,7).

Vitamin D activity is also important in the prevention of osteoporosis in elderly adults where combined vitamin D and calcium supplementation preserves bone mineral density and reduces the risk of fractures (8,9). The pathophysiology of osteoporosis is different from osteomalacia since it is a condition in which there is less bone tissue in the bone organ compared with healthy bone and the ratio of osteoid to bone mineral is the same as in healthy bone. The risk of fracture at any age is dependent on bone mineral density (10). In children up to 50% of any given population will experience a fracture between the ages of 5 and 18 years (11). Thus optimizing bone mineral accretion during growth is as important for reducing fracture risk during growth as it is for optimizing bone mineral density in adulthood to reduce the risk of fracture in the elderly (12). A similar effect of a depleted vitamin D status in children has been found to impair bone growth and mineralization at appendicular sites and increase the risk for reduced bone gain at other sites (10).

3. VITAMIN D METABOLISM - ENDOCRINE AND AUTOCRINE/PARACRINE ACTIVITIES

Vitamin D is largely derived from sunlight exposure of the skin where UVB light converts 7-dehydrocholesterol by way of two steps to vitamin D, which itself is subject to sequential hydroxylation reactions for bio-activation (13). The first hydroxylation is at the carbon 25 position to produce 25-hydroxyvitamin D (25D) also known as calcidiol or calcifediol. Circulating 25D levels are the best indicator of vitamin D status as a result of the increased solubility of this metabolite in blood compared with vitamin D. Circulating 25D levels are considered to arise from the liver activity of the cytochrome P450 enzyme vitamin D 25-hydroxylase (CYP2R1) (Figure 1) (14). Circulating levels of the biologically active metabolite of vitamin D, 1,25D, also known as calcitriol, arise from hydroxylation at the carbon 1 of 25D in the kidney catalysed by the P450 enzyme 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) (13). A third hydroxylation of the vitamin D metabolites, which has a major effect on vitamin D activity, is at carbon 24. It is catalysed by the P450 enzyme 25-hydroxyvitamin D-24-hydroxylase (CYP24). This reaction is the first step in the inactivation of vitamin D and is responsible for measurable levels of the 24,25-dihydroxyvitamin D and 1,24,25-trihydroxyvitamin D metabolites in blood (13). This is a very important step in vitamin D metabolism since it protects cells from vitamin D toxicity as demonstrated by the generation of a mouse line with the CYP24 gene ablated (15).

The endocrine activity of vitamin D operates through the circulating 1,25D synthesized in the kidney exerting its biological effect at a distant organ. The high positive correlation between serum 1,25D levels and the active transport of calcium by the small intestine as demonstrated in humans and rodents by radiocalcium absorption indicates the intestine as an endocrine target organ (16,17). The expression of the genes for the vitamin D metabolising enzymes in the kidney are strongly related to serum 1,25D levels (18), suggesting that the renal actions of 1,25D must arise from the circulation.

An autocrine activity is defined as biological activity being exerted in the same cells as those in which the agent is synthesised while a paracrine activity occurs in a cell adjacent to those in which the active agent is synthesised. For vitamin D autocrine/ paracrine activities require at least the vitamin D receptor (*VDR*) and the *CYP27B1* genes to be expressed in the same or adjacent cells. The *VDR* gene is expressed in "virtually every tissue of the body" (19) and therefore has the capacity to influence activities within any of these tissues. All cells that express the vitamin D receptor and are biologically responsive to 1,25D are believed to express the *CYP24* gene. As well the CYP27B1 enzyme is also present in a wide variety of tissues. Immunohistochemistry has identified this enzyme in numerous human tissues including kidney, skin, lymph nodes, tonsil, colon, pancreas, adrenal gland, placenta and brain cells (20). An alternative approach has been to study the distribution of tissues which express the *CYP27B1* gene using a transgenic mouse model in which the -1501 nucleotide base pairs upstream of the human *CYP27B1* gene, that is the promoter region regulating expression of this gene, was linked to a luciferase reporter gene (21). Confocal microscopy and immunofluorescence techniques demonstrated that the luciferase enzyme and the endogenous CYP27B1 enzyme were expressed in identical cells in the kidney, testis and brain organs (22). A non-exhaustive survey of tissues found that human *CYP27B1* gene promoter activity was also

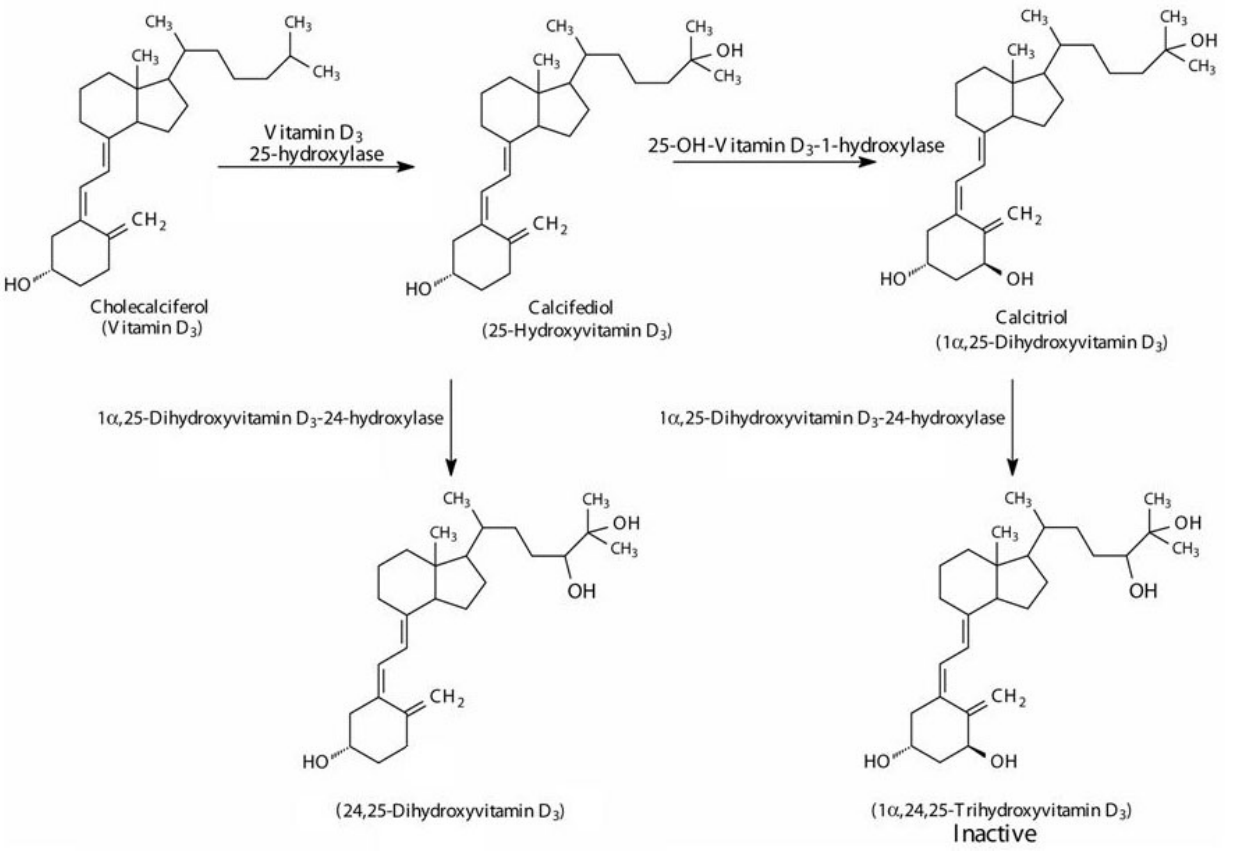

Figure 1. Vitamin D metabolism to the biologically active metabolite 1,25-dihydroxyvitamin D and inactivation through hydroxylation at the carbon 24 position. The serum level of the 25-hydroxyvitamin D metabolite is the indicator of vitamin D status.

identified in skin, bone, bone marrow, spleen, skeletal muscle, heart, distal small intestine, lung and liver (Figure 2). It must be emphasised that circulating 1,25D, in non-pregnant, healthy people, is accounted for only by metabolism of 25D by the kidney and there is no evidence that extra-renal production of 1,25D contributes to the circulating levels other than in disease or following placentation (23).

The quantity of *CYP27B1* promoter activity in the various tissues is also of interest. In 12 week-old male mice fed a normal chow diet containing high levels of calcium (normally 0.8% to 1% calcium) the major tissue of expression was the testis followed by the brain (Figure 1). The kidney exhibited comparable levels of *CYP27B1* promoter activity as skin, bone and bone marrow. Thus under conditions of adequate dietary calcium intake the kidney is not the site of the highest level of CYP27B1 activity. However under conditions of low dietary calcium intake and low vitamin D status marked induction of renal enzyme activity takes place (18,23).

4. ENDOCRINE ACTIVITIES OF VITAMIN D

The major endocrine activity of vitamin D contributes to the maintenance of calcium and phosphate homeostasis involving a coordinated mechanism between the kidney as the site for the synthesis of circulating 1,25D and the intestine, kidney, bone and parathyroid glands where circulating 1,25D is believed to regulate essential activities of calcium and phosphate transport or other factors regulating such activities. The complex interactions of these tissues ensure adequate availability of calcium and phosphate for numerous biological functions, including nerve and muscle activities as well as the maintenance of mineralized tissues.

4.1. Intestine

Arguably the most important role for circulating 1,25D is to stimulate dietary calcium and phosphate absorption in the small intestine. The importance of this action has been convincingly demonstrated by examination of *VDR-null* and *CYP27B1-null* mice. These mouse models replicate two human diseases, vitamin D-dependent rickets type I (VDDR type I) when the *CYP27B1* gene is inactivated, and vitamin D-dependent rickets type II (VDDR type II) when the *VDR* gene is inactivated. In humans and mutant mice alike when all vitamin D biological activity is abolished marked hypocalcemia and hypophosphatemia ensue as a result of intestinal calcium and phosphate malabsorption (24-26). 1,25D increases intestinal calcium absorption through VDR-mediated

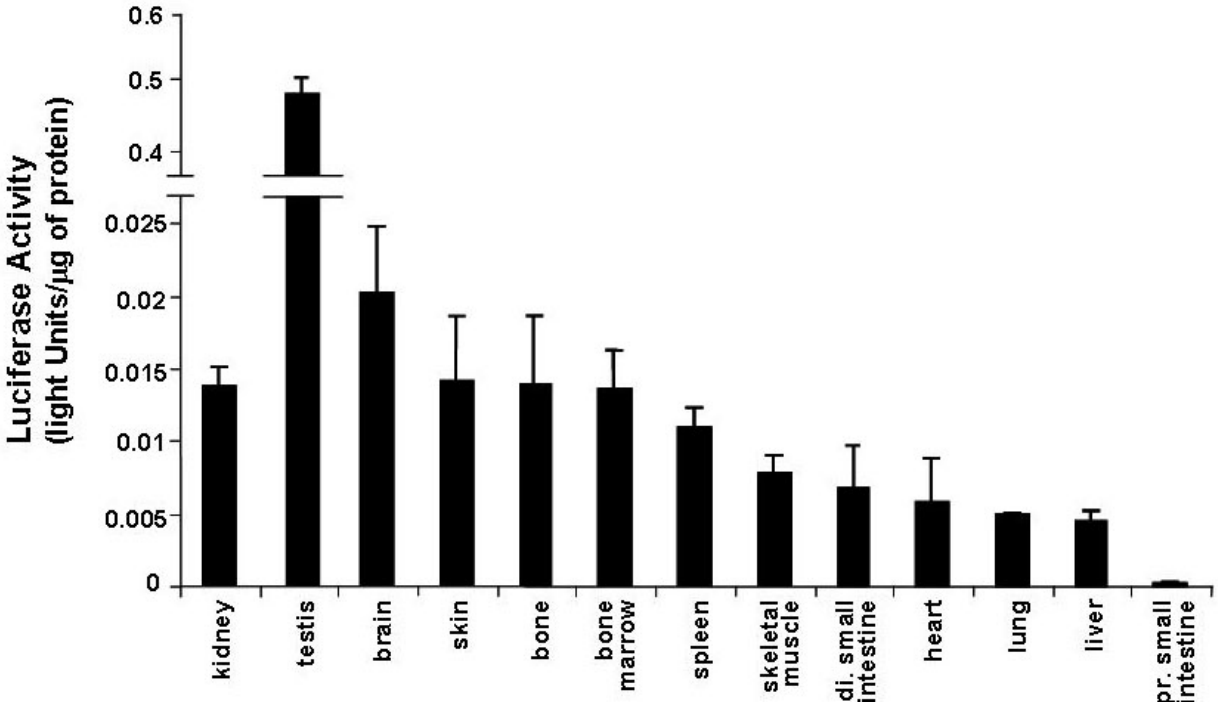

Figure 2. Human *CYP27B1* promoter activity, expressed as luciferase reporter gene activity in various tissues isolated from male 12-week old transgenic mice (n=3) containing the -1501 base pairs human *CYP27B1* gene linked to the luciferase gene fed a laboratory chow diet. (Reprinted from Reference 21 with permission of the publishers.)

increases in the transcription of specific genes involved in active calcium absorption (27). Serum 1,25D correlates positively with the intestinal active transport of calcium as measured by radiocalcium absorption (16). A number of proteins are essential for these processes including those involved in the entry of calcium through the basement membrane into the enterocyte, the movement of calcium through the cytoplasm, and the transfer of calcium across the basolateral membrane into the circulation (28). The cytosolic calcium-binding protein, calbindin-D9k, is thought to be particularly important in the translocation of calcium across the enterocyte. 1,25D is able to up-regulate the expression of calbindin-D9k through a VDRE in the proximal promoter of the gene (29), and mRNA and protein for calbindin-D9k are dramatically reduced in the intestine of VDR knockout mice (30). When the VDR gene is reintroduced only into intestinal cells on the VDR-null background, normocalcemia and normophosphatemia are restored when these transgenic mice are fed a standard diet (31).

4.2. Bone growth, mineralization and remodelling

Vitamin D deficiency in normal animals and humans as well as inactivation of vitamin D by genetic manipulation in mice, as discussed above, produce defects in bone mineralization such as rickets and osteomalacia, characterised by an increase in osteoid (unmineralized bone matrix protein) and impaired calcium-phosphate deposition (32). 1,25D, often in association with other factors including PTH, also induces osteoclastogenesis by stimulating the differentiation of bone marrow-derived pro-myelocytes and monocytes to active osteoclasts (33,34). This process occurs indirectly through increased expression of the osteoblast-derived factor, RANKL, which promotes osteoclastic differentiation (35). Osteoblasts from VDR knockout mice are unable to stimulate the differentiation of osteoclasts, clearly demonstrating the requirement for VDR in the osteoblast for this 1,25D-mediated effect (36). It is also interesting to note that in vitamin D deficiency or *Vdr-null* mice, hypocalcaemia develops despite a high PTH (24,37), suggesting that PTH requires 1,25D in order to stimulate significant bone resorption to normalise extracellular fluid (ECF) calcium.

In children, vitamin D deficiency allows cartilage proliferation to continue for longer with a marked delay in the mineralization of the growth plate (32). This gives rise to the clinical diagnosis of rickets. In adults and in children at sites which do not involve a growth plate such as the vertebral bodies, vitamin D deficiency causes a delay in the mineralization of osteoid, measured as the mineralization lag time. Osteoid thickness declines with increasing age because of a decline in matrix apposition rate with a relatively constant lag time. Humans and mice in which the *VDR* gene is inactive such as the Vdr^{KO} mice when fed a regular diet develop hypocalcemia, hypophosphatemia, secondary hyperparathyroidism and skeletal abnormalities consistent with rickets and osteomalacia. Vdr^{KO} mice are born with the expected Mendelian frequency suggesting that the VDR is not essential for embryonic development and that the pups are phenotypically normal until about 15 days of age. Mouse pups are weaned at about 21 days of age (38). Between approximately 1 month and 8 months of age the body

weight of Vdr^{KO} mice is about 15% lower than sex-matched *wild type* littermates at which time their rate of gain of weight declines further until by 12 months of age they are approximately 50% of the size of the *wild type* mice.

The Vdr^{KO} mice have normal ECF calcium levels until 15 days of age and by 1 month they are significantly hypocalcemic, hypophosphatemic and have developed secondary hyperparathyroidism (39). The skeletons of 2-week old Vdr^{KO} mice are normal although after 21 days of age the extended period of cartilage proliferation in the growth plate is evident with disruption of the columnar alignment of the hypertrophic chondrocytes evident by 35 days of age (40). A delay in mineralization with increased osteoid volume is also evident at this time. These bones demonstrate decreased stiffness and strength when subjected to mechanical testing (7). They reveal a marked increase in osteoblast cell number however osteoclast number is not increased.

A most important finding from the Vdr^{KO} mouse line is the response of young mice to a diet which can normalise calcium and phosphate homeostasis (7). This can be achieved by feeding a "rescue diet" containing 2% calcium and 1.6% phosphate plus 20% lactose which enhances intestinal absorption of calcium and phosphate in the absence of vitamin D activity. When Vdr^{KO} and wild type mice were placed on the "rescue diet" from 16 days to 70 days of age, no delay in mineralization or increase in osteoid volume were detected and none of the histomorphometric variables was different from the wild type littermates.

Significant abnormalities of the growth plate observed in *VDR-null* mice occur prior to the onset of hypocalcaemia (39). In addition, the reduced growth of long bones in *Cyp27b1-null* mice persists after mineral homeostasis is corrected (41), suggesting that 1,25D plays a direct role in endochondral bone formation. Endochondral bone formation involves chondrocytes undergoing an ordered developmental sequence that includes proliferation, hypertrophy and finally apoptosis and replacement by bone. The specific deletion of the *VDR* gene in chondrocytes results in delayed osteoclastogenesis and a transient increase in trabecular bone volume in 15 day old mice (42). More recently, the concept that 1,25D may be necessary for the normal function of cells within the growth plate has been further supported by the study of mouse models in which *Cyp27b1* is either knocked-down or over-expressed within chondrocytes (43). Knock-down of *Cyp27b1* gene expression in chondrocytes resulted in decreased osteoclastogenesis and an increased hypertrophic zone in the embryonic femur. This decreased enzyme activity lead to a transient increase in trabecular bone volume that was apparent at 2 days of age, but had diminished within 2 weeks. Transgenic chondrocytic over-expression of *Cyp27b1* (plus maternal supplementation with 25D) produced a mirror phenotype that included an increased hypertrophic zone and a decrease in trabecular bone volume versus wild-type littermates. The fact that 25D, but not 1,25D, is able to cross the placenta during development (44), suggests that the action of vitamin D to modulate skeletal development in this way may be autocrine in nature.

4.3. Parathyroid Glands

Parathyroid hormone (PTH) produced by the parathyroid gland plays a central role in calcium homeostasis. When the ECF calcium decreases, the transient fall is detected by the calcium sensing receptor (CaR) in the parathyroid gland, leading to induction of PTH synthesis and secretion. PTH can restore ECF calcium to normal by stimulating renal tubular reabsorption of calcium and renal production of 1,25D, and by promoting osteoclastogenesis. 1,25D can exert a negative feedback signal on the parathyroid glands to suppress further synthesis and secretion of PTH and to control parathyroid cell growth (45-47). The *PTH* gene promoter contains a VDRE which acts in a negative fashion (48). However whether this action of 1,25D is acting through an endocrine or autocrine mechanism is controversial since the parathyroid gland also expresses the *CYP27B1* gene and bovine parathyroid gland cells in vitro are capable of metabolising 25D to 1,25D to suppress PTH mRNA levels and secretion (49). The lack of 1,25D feedback on the PTH promoter is pronounced in vitamin D deficiency which, combined with hypocalcaemia, results in parathyroid hyperplasia and secondary hyperparathyroidism. The correction of parathyroid gland growth and serum PTH in the *Vdr-null* mice through dietary calcium supplementation indicates, however, that the feedback of 1,25D on PTH production is not essential and that the ECF calcium concentration is the major regulator of PTH synthesis and parathyroid cell proliferation (30).

4.4. Kidney

Endocrine actions of circulating 1,25D which feedback on the kidney have been well described and reviewed (50). Perhaps the most important effect of circulating 1,25D on the kidney is the negative feedback of the renal synthesis of 1,25D through repression of *CYP27B1* expression and stimulation of *CYP24* expression to reduce circulating 1,25D levels (51,52). 1,25D is also involved in renal calcium reabsorption as demonstrated by studies in the *Vdr-null* mouse (24). 1,25D increases the expression of the renal calcium-transport protein, calbindin-D28k (53,54), and serum 1,25D correlates strongly with the expression of calbindin-28k in the kidney measured in rats over a wide range of age groups (55). Furthermore, 1,25D promotes the PTH-dependent calcium transport in the distal tubule where active calcium reabsorption occurs (56,57). Therefore there are considerable data indicating that the kidney is an endocrine target organ for 1,25D to regulate plasma calcium homeostasis.

The kidney is also an endocrine target organ for the regulation of plasma phosphate homeostasis although in this case the primary agent is FGF23, a polypeptide hormone, which acts to reduce the tubular reabsorption of phosphate from the glomerular filtrate to lower plasma phosphate levels as recently reviewed (58). FGF23 also specifically inhibits renal synthesis of 1,25D through reduction of renal CYP27B1 mRNA levels. The reduction

in circulating 1,25D levels exerts indirect effects to lower plasma phosphate levels. FGF23 is synthesised largely by osteocytes in bone where 1,25D exerts an inhibitory feedback action. This is considered to be an endocrine action of 1,25D since injections of 1,25D into humans or mice reduces circulating FGF23 levels (reviewed in (59)) However as discussed below osteocytes also have the capacity to synthesise 1,25D and initiate biological actions. Currently the interaction between the endocrine and autocrine activities of 1,25D in osteocytes has not been elucidated.

4.5. Neuromuscular and other cellular functions

The vitamin D endocrine system through the action of circulating 1,25D plays a primary role in the maintenance of calcium and phosphate homeostasis. The stringent regulation of ECF calcium concentration within the narrow limits of approximately 10% of the ionised calcium fraction is essential to support normal nerve and muscle function. Calcium is largely an extracellular cation where concentrations of the ionized calcium fraction range between 1.12 to 1.24 mmol/L for the human population while the intra-individual range is considerably less, approximately a total range ±0.02 mmol/L (60). The intracellular calcium levels are in the range of 10^{-7} to 10^{-5} mol/L and it is a highly versatile intracellular signalling agent. Important amongst these actions is the regulation of skeletal and cardiac muscle contraction and relaxation (61). Extracellular calcium levels can effect intracellular levels and modulate biological activities such as muscle contraction which by way of example is affected by hypocalcemia (62). Other cellular functions dependent on intracellular calcium signalling include the release of rennin from the juxtaglomerular cells of the kidney (63). It is considered that the calcium-sensing receptor and voltage-gated calcium channels are important for connecting extracellular calcium levels to intracellular calcium signalling. Thus through the regulation of extracellular calcium levels, the vitamin D endocrine system has the potential to exert major effects on a wide range of physiological activities. When *Vdr* or *Cyp27b1* gene knockout mice are fed a normal diet which leaves the mice in a hypocalcemic state, many physiological functions are disrupted (19). However most but not all of these activities are restored when normocalcemia is achieved with a high calcium "rescue" diet.

A key question is the level of serum 25D required to normalise serum 1,25D and consequently normalise intestinal calcium absorption. Such a level would be expected to resolve osteomalacia given the effect of the "rescue" diet on the *Vdr* and *Cyp27b1* gene knockout mice. Clinical studies in postmenopausal women have clearly demonstrated that as levels of 25D fall below 40 nmol/L down to 10 nmol/L, serum 1,25D levels do not significantly fall until 25D levels are below 20 nmol/L (64). Numerous studies have demonstrated a strong positive relationship between serum 1,25D levels and intestinal radiocalcium absorption which assesses the active transport of calcium in both humans and rats (16,17). Furthermore bone histomorphometry analyses of rodents in whom the vitamin D status was varied using modification of the diet demonstrated that osteomalacia did not develop when the serum 25D level was 20 nmol/L or greater on a moderate dietary calcium intake (65). Thus evidence from clinical and rodent studies strongly indicate that serum 25D levels of greater than 20 nmol/L are adequate to maintain sufficient serum 1,25D to normalise intestinal calcium and phosphate levels which also protects against the development of osteomalacia at least in a rodent model.

There is controversy as to whether the only action of vitamin D necessary for healthy bone is at the intestine or whether actions are required within bone cells. In support of the view that actions of vitamin D are required in bone cells, researchers have investigated over the longer term the effect of the "rescue diet" on the skeleton of Vdr^{KO} mice. When Vdr^{KO} and wild type littermates were fed the "rescue diet" from 21 days until 128 days of age the Vdr^{KO} mice demonstrated a persistent deficiency of bone volume despite this rescue diet (66). Thus while the rescue diet was able to prevent osteomalacia, osteoporosis was evident at 128 days of age with half the cancellous bone volume and marked reduction in mineral apposition rate and ALP activity when compared to wild-type animals. In addition, bone sections stained for osteoclast histological markers, RANKL and tartrate resistant acid phosphatase (TRAP) were reduced in Vdr^{KO} mice, suggesting that bone loss in these animals was not due to bone resorption but rather a failure of osteoblast activity. Consistent with these *in vivo* findings are *in vitro* studies with osteoblast-like cells derived from Vdr^{KO} mice. The results confirm the direct role of vitamin D activity in these cells with regard to proliferation and mineralization. Osteoblast-like cells derived from Vdr^{KO} mouse bone marrow, which is a source of mesenchymal stem cells, demonstrate impaired mineralization when compared to cells derived from wild type littermates (66), probably through an IGF-1 signalling loop (67). In contrast, osteoblasts cultured from Vdr^{KO} cortical or calvarial bone fragments demonstrated enhanced proliferation when compared with WT cells. Thus, the role of VDR-dependent activity in osteoblasts appears to inhibit proliferation and enhance mineralization, as has been previously demonstrated using different *in vitro* cell models (68). These data illustrate that VDR-mediated activity within bone cells can regulate proliferation and mineralization *in vivo* and support the proposal that vitamin D can exert an autocrine/ paracrine action in bone tissue.

5. VITAMIN D METABOLISM AND ACTIVITIES WITHIN BONE CELLS AND BONE TISSUE

A variety of clinical and rodent model studies have demonstrated that at levels of serum 25D between 20 nmol/L and approximately 80 nmol/L serum 25D correlates with various measures of bone cell activity or bone structure independent of the serum 1,25D level. 25D can only activate the VDR at levels around 500 nmol/L (69) thus these effects cannot be attributable to direct activation of the VDR by 25D. They suggest that serum 25D provides a substrate for the synthesis of 1,25D within bone tissue which in turn activates the VDR.

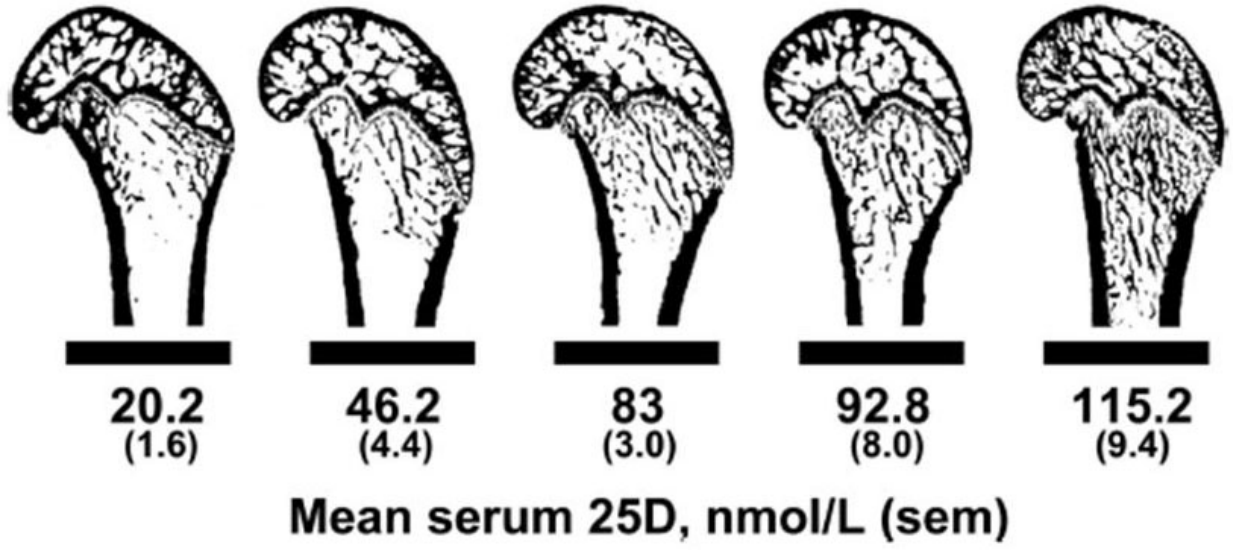

Figure 3. Representative von-Kossa stained sections of the distal femur from 30 week-old rats maintained with serum 25D levels ranging from 20.2 to 115.2 nmol/L for 20 weeks. The distal metaphyseal trabecular bone (bone volume as a ratio to total volume (BV/TV)) significantly increased as the 25D level increased. (Reproduced from reference 61 with permission of the publishers)

In a rodent model of dietary vitamin D depletion when serum 25D levels were reduced from 80 to 20 nmol/L significant bone loss was detected in a dose dependent manner due to increased bone resorption. (Figure 3) The increased resorption is the result of increased expression of the *RankL* gene in bone and increased osteoclastogenesis. Most interestingly no relationship was found between bone volume and either serum 1,25D or parathyroid hormone (65). Further studies have been conducted with 15 month old rats comparing the effects of varying vitamin D status with 0.1% or 1% calcium diets (70). The positive relationship between trabecular bone volume and serum 25D levels was confirmed and extended to a similar relationship between cortical bone volume and 25D levels with a maximum cortical bone volume being achieved at 25D levels of 100 nmol/L or greater. Furthermore these studies indicate that increasing 25D levels to such a level was effective for increasing cortical bone volume only in animals receiving the high calcium diet. Consistent with the increased cortical bone volume, increased bone strength was only achieved in animals fed 1% calcium and serum 25D levels greater than 80 nmol/L.

These *in vivo* data indicating serum 25D level as the major determinant of bone cell activities and bone structure and strength suggest that this pro-hormone is metabolised to 1,25D within bone cells. Is this proposal plausible? It is well established that each of the major bone cell types, osteoblasts, osteoclasts and osteocytes, are capable of metabolising 25D to 1,25D to elicit biological activities. Human and rodent osteoblasts demonstrate significant CYP27B1 enzyme activity which is essential to convert 25D to 1,25D. This action is responsible for increases in expression of key genes associated with maturation and mineralization when 25D is included in the culture media (71). 25D in the media also reduces cell proliferation and stimulates osteoblast maturation and mineralization *in vitro*. Pre-osteoclasts such as in human peripheral blood mononuclear cell preparations also express the *CYP27B1* gene which has been demonstrated to be essential for 25D to optimize the generation of mature osteoclasts in the presence of RANKL and M-CSF *in vitro* (72). (Figure 4) Most interestingly the osteoclasts formed in the presence of 25D demonstrated reduced bone resorbing activity compared with cells matured in the absence of vitamin D metabolites or cells in which the *VDR* gene has been ablated (72). Such data are consistent with the *in vivo* finding described above that osteoclastogenesis was reduced when serum 25D levels were above 80 nmol/L in rat models.

Osteocytes also express the *CYP27B1* gene, mRNA levels increase with differentiation and are associated with the acquisition of mature osteocyte genes including *MEPE (Matrix extracellular PhosphoglycoprotEin), DMP1 (Dentin matrix protein 1), PHEX (Phosphate-regulating gene with Homologies to Endopeptidase on the X chromosome)* (Atkins GJ, unpublished data) and *FGF23 (fibroblast growth factor 23)* (73). Thus *in vitro* data provide strong evidence that the pro-hormone 25D is capable of metabolism by bone cells to the active hormone 1,25D to elicit various activities including the reduction of bone resorption by osteoclasts and to enhance maturation and mineralization by osteoblasts and osteocytes. Each of these activities is consistent with the actions of adequate circulating levels of 25D observed *in vivo*.

As discussed above rodent model studies indicate that the anabolic effects of circulating 25D on the skeleton are dependent on sufficient dietary calcium intake to provide more calcium than is excreted from the body. The question arising from such findings is whether calcium itself exerts any biological activities within the skeletal

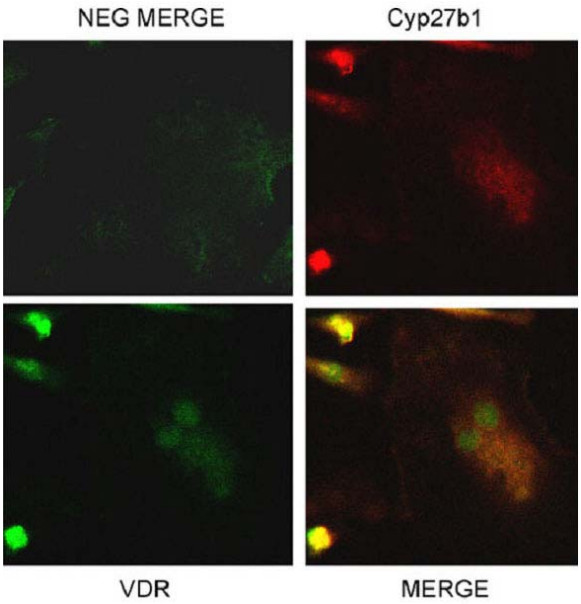

Figure 4. Human peripheral blood mononuclear cells when treated in culture with RANKL (50 ng/ml) and M-CSF (25 ng/ml) for 6 days mature into osteoclasts expressing the necessary components for 25D metabolism and vitamin D activity including the CYP27B1 and VDR proteins as demonstrated by immunofluorescence staining with anti-CYP27B1 antibody, anti-VDR antibody and control IgGs. (Reproduced from reference (87) with permission of the publishers)

system rather than simply providing a raw material, in conjunction with phosphate, for bone mineralization. *In vitro* studies demonstrate that the addition of calcium to culture media enhances the ability of osteoblast-like cells isolated from long bones to mineralize *in vitro* (Yang D, Atkins GJ, Morris HA unpublished results) although such effects may depend on the source of the cells (74). Interestingly a recent report describes that when rats are fed a dietary calcium level sufficient to ensure a positive calcium balance, the levels of *CYP27B1* mRNA are 3-fold higher in bone tissue compared with animals fed a low calcium diet (75). Messenger RNA levels for *CYP24*, which is highly regulated by 1,25D, were also elevated in bone from these animals indicating that 1,25D levels are higher in the bone tissue. Thus, a contributing effect of adequate dietary calcium intake to their positive effects on bone mineral homeostasis may be, at least in part, through increasing the synthesis of 1,25D within bone tissue.

Clinical studies provide critical data to support the hypothesis of an autocrine/ paracrine action of vitamin D in the human skeleton. In a series of ambulant patients (age range 44 to 86 years) histomorphometric analyses on iliac crest bone biopsies demonstrated a significant inverse relationship between serum 25D level and osteoid thickness with the lowest thickness being achieved at 25D levels around 80 to 100 nmol/L. Furthermore the mineralization lag time was similarly negatively related to serum 25D levels (76). No significant relationship was found between any of the bone variables and serum 1,25D levels. Only 3 of these 121 patients had 25D values below 15 nmol/L and the mineralization lag time did not reach a limit to indicate a diagnosis of osteomalacia in any of these patients. Similar data have been reported from a large series of Northern European subjects (675 in total) where osteoid thickness was inversely related to serum 25D levels with an optimal serum level of 75 nmol/L of 25D identified above which osteoid thickness was minimized (77). Once again there was no relationship between bone parameters and serum 1,25D levels. Consistent with these structural data are fracture studies that also indicate that the risk of non-vertebral fractures including fractures of the hip does not reduce until serum 25D levels of 80 nmol/L or greater are achieved (9). Considerable insight into the vitamin D requirement to prevent osteoporosis in the elderly has arisen from studies of patients with hip fractures. The elderly with a mean serum 25D around 40 nmol/L have a markedly increased risk of hip fracture (78,79), as a result of osteoporosis rather than osteomalacia (80,81).

In Australia the National Health and Medical Research Council nutrient reference values recommend an adequate dietary intake of 200 IU (5µg) per day for new-borns up to the age of 50 year (82). The complexity for assessing vitamin D nutrition is that the major source arises from UVB irradiation which varies markedly between winter and summer seasons. Over the last 3 decades many communities throughout the world have been advised to reduce their sunlight exposure, compliance of which has been most effective with school children. Most school children, at least in Australia, are required to wear protective clothing before being allowed to take part in activities outside the classroom. There is evidence that the number of vitamin D-deplete Australians has increased in recent years (83).

Vitamin D status, as indicated by serum 25D levels, is an important determinant of bone mineral accretion in growing children in many, although not all, studies. There is clearly an interaction with dietary calcium intake where threshold values up to 1700 mg of calcium per day for children varying in age between 9 to 17 years of age have been estimated (84). Serum 25D levels between 25 and 40 nmol/L are considered to impair bone growth and mineralization at appendicular sites and increase the risk for reduced bone gain at other sites (10). A school milk intervention program over 2 years in Chinese school girls (aged 10-12 years) found that the combination of vitamin D and calcium improved total body bone mineral density and size-adjusted bone mineral content to a greater extent than milk supplemented by calcium alone (85). This vitamin D supplementation raised the mean serum 25D levels from some 20 nmol/L to approximately 50 nmol/L. A randomised controlled trial in school children (ages 10 to 17 years) increased mean serum 25D levels from 35 nmol/L for those receiving placebo to 95 nmol/L for those receiving the high-dose preparation over a 1-year period. Those receiving the high vitamin D dose achieved higher total bone area and hip bone mineral content compared with either the placebo or low vitamin D dose group (86).

6. CONCLUSIONS

A considerable body of evidence is now available demonstrating a requirement for an adequate vitamin D status and dietary calcium intake to optimize bone mineral accretion during growth which has effects on skeletal health throughout life. This evidence supports the concept that vitamin D acts on bone mineral homeostasis through at least two modes of action, an endocrine activity through renal synthesis of circulating 1,25D to maintain calcium and phosphate homeostasis and an autocrine/ paracrine activity within bone cells to modulate bone remodelling and optimize bone mineral content of the skeleton. The endocrine action of vitamin D protects against the bone disease of rickets in children and osteomalacia in adults. This disease is characterised by a delay in mineralization and is generally observed when the vitamin D status is markedly reduced as indicated by a serum 25D level less than 20 nmol/L.

The autocrine/ paracrine action of vitamin D within bone tissue protects against osteoporosis which is of clinical significance for risk of fracture in both children and adults. The activation of vitamin D in bone cells is clearly associated with reduced bone resorption and retention of mineral during bone remodelling during growth as well as with ageing. However an anabolic effect on bone formation cannot be ruled out. These changes in bone structure are observed with moderate vitamin D depletion as indicated by serum 25D levels between 20 and 80 nmol/L. Optimal bone density in both children and adults has been observed when serum 25D levels are greater than 80 nmol/L combined with an average dietary calcium intake.

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Key Words: Vitamin D metabolism, CYP27B1, CYP 24, Bone Cell Activities, Osteomalacia, Osteoporosis, Vitamin D actvities, Review

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