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The Biology and Pathology of Vitamin D Control in Bone

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ABSTRACT

Vitamin D is a steroid pro-hormone, whose active metabolite binds the vitamin D receptor (VDR) which, in turn, binds to DNA sequences on target genes as a heterodimer with the retinoid-X receptor, resulting in regulation of gene expression. The vitamin D pro-hormone can be synthesized in the skin, in response to ultraviolet radiation; however, dietary sources have become increasingly important as a result of cultural changes over the past few centuries. Based on its initial discovery as an anti-rachitic factor, studies of the role of vitamin D and its receptor have largely focused on the skeleton. Investigations into the pathophysiologic basis and therapeutic responses of skeletal disorders associated with impaired vitamin D action have led to the identification of the molecular pathways involved in hormone activation and regulation of gene expression by the liganded VDR. These studies have also demonstrated that the skeletal actions of the VDR and its ligand are largely redundant if normal mineral ion homeostasis can be maintained by other means. However, investigations in animal models with tissue-specific ablation of the VDR or the enzyme required for hormone activation have demonstrated novel actions in skeletal tissues. The active vitamin D metabolite has been shown to have both paracrine and endocrine actions in other tissues as well. J. Cell. Biochem. 111: 7–13, 2010.

KEY WORDS: VITAMIN D; RICKETS; OSTEOMALACIA; BONE

Vitamin D is a steroid hormone that acts as a ligand for the vitamin D receptor (VDR), a classic transcription factor, which exerts its effects via the formation of a complex with the retinoid X receptor (RXR). The VDR-RXR heterodimer then interacts with DNA response elements on target genes [MacDonald et al., 2001]. Vitamin D levels are a reflection of the synthesis of vitamin D in response to ultraviolet B (UV-B) exposure of the skin and dietary intake of vitamin D [Holick et al., 1980; Norman, 1998]. In the skin, UV-B light converts 7-dehydrocholesterol to previtamin D_3 [Holick et al., 1980]; with adequate UV-B exposure the skin can synthesize as much as 80–100% of the daily vitamin D requirement [Glerup et al., 2000b]. There are two forms of dietary vitamin D: ergocalciferol, which is plant based, and cholecalciferol, which is animal based. Intestinal absorption of vitamin D occurs primarily in the small intestine and is facilitated by the presence of bile salts [Greaves and Schmidt, 1933]. Absorption efficiency is approximately 50%. After its synthesis or absorption, vitamin D is metabolically activated by an initial hydroxylation in the liver to form 25-hydroxvyitamin D (25OHD). 25OHD, or calcidiol, is the more stable vitamin D metabolite, thus evaluation of its circulating levels is used to assess vitamin D stores [Holick, 2007]. In the kidney, 25OHD undergoes a second hydroxylation step to produce 1,25-dihydroxyvitamin D ($1,25(OH)_2D$), or calcitriol, the active circulating metabolite [Boyle et al., 1972; Holick et al., 1972; Wong et al., 1972; Fraser et al., 1973]. Calcitriol can also be generated in non-renal tissue, including macrophages and muscle [Liu et al., 2006]. This non-renal activation of 25OHD to $1,25(OH)_2D$ is thought to contribute to our increasing recognition of a number of important actions of this steroid hormone that are dependent on local activation. There is accumulating evidence suggesting that vitamin D deficiency increases the risk of diabetes mellitus [Hypponen et al., 2001; Pittas et al., 2007], hypertension [Krause et al., 1998; Li et al., 2002; Judd et al., 2008], malignancy [Martinez et al., 1996; Tangpricha et al., 2001; Chen et al., 2009; Krishnan and Feldman, 2010], musculoskeletal dysfunction [Glerup et al., 2000a; Pfeifer et al., 2002; Bischoff-Ferrari et al., 2004a], infection [Wilkinson et al., 2000; Liu et al., 2006], autoimmune disease [Munger et al., 2006], and all-cause mortality [Dobnig et al., 2008; Melamed et al., 2008]. The first and best characterized phenotype of vitamin D deficiency is the development of skeletal disorders, notably rickets and osteomalacia.

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As the skeleton matures, the epiphyses and metaphyses fuse resulting in cessation of longitudinal bone growth. From this point, mineralization and bone turnover are employed to maintain skeletal strength and integrity. Vitamin D deficiency in the adult skeleton manifests as osteomalacia. Histologically, osteomalacia is characterized by areas of unmineralized osteoid, which may be apparent radiologically as Looser's zones (pseudofractures) [Pettifor, 2005b]. The earliest descriptions of bony deformities consistent with rickets are found in the ancient texts of Homer (900 BC) and the Roman physician Soranus Ephesius (130 AD). Soranus described the classic deformities of rickets in infants residing in both Rome and Greece [Pettifor, 2005a].

The discovery of Vitamin D and its role in preventing rickets is attributed to Elmer McCollum, a nutritional biologist at Johns Hopkins, and John Howland, a pediatrician [McCollum et al., 1921; Shipley et al., 1921; Howland, 1933]. The therapeutic use of cod liver oil, early in the 20th century, led to a dramatic resolution of the rachitic phenotype in affected individuals. With a decline in the cases of rickets due to vitamin D deficiency, other forms of rickets began to emerge; these were termed "vitamin D resistant rickets." Characterization of the molecular basis for these disorders has elucidated important steps in the regulation of vitamin D activation and function. Pseudovitamin D deficiency rickets (PDDR) is an autosomal recessive disorder characterized by impaired or absent activity of the 25OHD 1-α hydroxylase, leading to impaired conversion of 25OHD to $1,25(OH)_2D$ [St-Arnaud et al., 1997; Kitanaka et al., 1998]. Hereditary vitamin D-Resistant rickets (HVDRR) is due to mutations in the vitamin D receptor [Hughes et al., 1988]. Circulating $1,25(OH)_2D$ levels are very high in this disease, however they are essentially ineffective due to lack of a functional VDR [Kristjansson et al., 1993]. X-linked hypophosphatemia (XLH) is an X-linked genetic disorder that causes hypophosphatemia due to decreased reabsorption of phosphate in the renal tubule [DiMeglio, 2000]. Renal function is typically preserved and $1,25(OH)_2D$ levels are either low or inappropriately normal given the degree of hypophosphatemia [DiMeglio, 2000]. These mineral ion and hormonal abnormalities in XLH are due to elevated circulating levels of the phosphaturic hormone, fibroblast growth factor 23 (FGF23) [Jonsson et al., 2003]. The genetic mutation is that of an endopeptidase, PHEX [HYP-Consortium, 1995]; however, the link between this mutation and the resultant phenotype has eluded molecular characterization. In spite of this dramatic increase in our knowledge over the past century, which is based on important clinical and physiological observations, the exact role of vitamin D, its active metabolite, and its receptor in the skeleton is still being elucidated.

BASIC SCIENCE STUDIES OF VITAMIN D ACTION

The receptor-dependent actions of $1,25(OH)_2D$ regulate the expression of bone matrix proteins and promote osteoclast differentiation by inducing the expression of the ligand for the receptor activator of NF-κB (RANK). In addition, $1,25(OH)_2D$ has been shown to regulate chondrocyte maturation and gene expression. While $1,25(OH)_2D$ has several actions that contribute to the regulation of skeletal and mineral ion homeostasis, identification of direct actions of $1,25(OH)_2D$ on the skeleton remains an area of active investigation.

As discussed, our understanding of the role of vitamin D in skeletal growth and maturation has been largely based on studies aimed at identifying the pathophysiological basis for rickets and osteomalacia in human and animal models. The availability of genetically engineered mice has permitted in depth analyses of the molecular basis for the phenotypes observed when vitamin D action is impaired. Mice lacking a functional VDR phenocopy the human disorder, HVDRR [Li et al., 1997]. They are normal at birth, but develop abnormalities in mineral ion homeostasis due to impaired intestinal calcium absorption. The increase in parathyroid hormone (PTH) that ensues, in an effort to maintain normal calcium levels, leads to hypophosphatemia due to PTH-dependent urinary phosphate losses. The skeletal manifestations observed in both mice and humans with VDR mutations, mimic those seen in vitamin D deficiency [Thomas and Demay, 2000]. Rickets, characterized by an expanded and hypomineralized growth plate is observed, as is osteomalacia. However, preventing the development of abnormal mineral ion homeostasis results in a histologically and biomechanically normal skeleton in growing mice [Amling et al., 1999]. Similarly, parenteral calcium administration has been shown to heal osteomalacia and rickets in children with VDR mutations [Balsan et al., 1986]. Thus, in the presence of normal mineral ions, the receptor dependent effects of $1,25(OH)_2D$ are redundant. What other factors are called in to play, to compensate for the absence of the VDR, have not been completely elucidated. It has, however, been demonstrated that in the absence of a functional VDR, osteoclastogenesis and RANK ligand synthesis in response to PTH is preserved [Takeda et al., 1999].

Interestingly, when removed from potential endocrine and paracrine signals and placed in culture, osteoblasts lacking the VDR exhibit phenotypic differences from normal osteoblasts. Calvarial osteoblasts lacking the VDR demonstrate an acceleration in the onset of osteoblast differentiation in culture. This is manifested by an earlier onset and increased magnitude of alkaline phosphatase activity, as well as an increase in mineralized matrix formation. In addition, the number of osteoblast colony forming units is also increased [Sooy et al., 2005]. In contrast, bone marrow stromal cells isolated from the VDR null mice demonstrate normal osteoblast differentiation, but enhanced adipogenesis. When cultured under adipogenic conditions, the expression of PPARγ is enhanced in the absence of the VDR, leading to an increase in the number and size of adipocytes compared to cultures from wildtype mice. Absence of the VDR results in enhanced expression of two inhibitors of canonical Wnt signaling, DKK1 and SFRP2. No in vivo evidence of enhanced adipogenesis of bone marrow stromal cells is observed in the VDR null mice, suggesting that other paracrine factors compensate for the lack of VDR in vivo [Cianferotti and Demay, 2007]. Studies in normal murine calvarial osteoblasts demonstrate that the actions of $1,25(OH)_2D$ are dependent upon the stage of osteoblast differentiation and duration of treatment [Owen et al., 1990]. The expression of mRNAs encoding osteopontin and matrix gla protein are induced by acute treatment with $1,25(OH)_2D$ at all stages. However, chronic treatment impairs both proliferation and differentiation of osteoblasts.

These studies raise the interesting question as to whether the effects observed in VDR null mice are due to the absence of receptor per se rather than lack of ligand-dependent receptor action. The generation of mice lacking the enzyme that activates vitamin D by 1-α hydroxylation (*Cyp*27b1) revealed a skeletal phenotype that is indistinguishable from that of mice lacking the VDR [Dardenne et al., 2001]. Although restoration of normal mineral ion homeostasis corrects the skeletal abnormalities, normal growth is observed only when the mice are treated with $1,25(OH)_2D$, suggesting that the receptor dependent actions of this hormone contribute to bone growth [Dardenne et al., 2004]. Interestingly, overexpression of the VDR by mature osteoblasts leads to an increase in both cortical and trabecular bone in vivo [Gardiner et al., 2000].

The VDR is also expressed in chondrocytes and $1,25(OH)_2D$ has been shown to modulate gene expression and differentiation of growth plate chondrocytes in vitro. However, studies in humans with VDR mutations and in VDR null mice demonstrate that normalization of mineral ion levels or prevention of abnormal mineral ion homeostasis leads to a normal growth plate [Balsan et al., 1986; Amling et al., 1999]. Investigations in VDR null mice demonstrate that, within 2 days of the development of hyperparathyroidism, an expansion in the hypertrophic chondrocyte layer is observed [Donohue and Demay, 2002]. Although extracellular calcium has been shown to promote expression of markers of terminal chondrocyte differentiation [Chang et al., 2002], studies in additional murine models of rickets, including the murine model for the human disease XLH (*hyp* mouse), demonstrated that hypophosphatemia is the underlying pathophysiologic basis for rickets [Sabbagh et al., 2005]. Low circulating phosphate levels lead to impaired apoptosis of hypertrophic chondrocytes, resulting in expansion of the growth plate, characteristic of rickets. Phosphate has been shown to induce apoptosis of avian chondrocytes in a dose dependent manner [Mansfield et al., 1999; Adams et al., 2001; Mansfield et al., 2001]. Further characterization of this programmed cell death in primary murine chondrocyte cultures demonstrates that phosphate treatment of hypertrophic chondrocytes activates caspase-9, a mediator of the mitochondrial apoptotic pathway, in a cell type and differentiation stage-specific manner. Analysis of the growth plate phenotype of wildtype mice treated with a caspase-9 inhibitor confirms that activation of the mitochondrial apoptotic pathway is critical for hypertrophic chondrocyte apoptosis in vivo, demonstrating a role for the mitochondrial apoptotic pathway in growth plate maturation in vivo.

While these investigations point to phosphate as a critical regulator of growth plate maturation, the VDR also has important paracrine effects in the growth plate. Targeted ablation of the VDR in proliferating chondrocytes (using Col II-Cre) results in normal growth plate morphology, associated with a transient increase in bone volume prior to weaning. This latter observation was shown to be secondary to a decrease in chondrocyte production of RANK ligand, leading to a decrease in osteoclastogenesis, and was accompanied by a decrease in VEGF expression, resulting in a decrease in vascular invasion. An intriguing observation in these mice was the presence of elevated circulating phosphate and $1,25(OH)_2D$ levels prior to weaning. This was thought to be due to a decrease in FGF23 expression in osteoblasts, a direct consequence of chondrocyte-specific VDR ablation, implicating an important paracrine loop between the chondrocyte and the osteoblast/osteocyte in the regulation of FGF23 expression as well as in the regulation of vascular invasion [Masuyama et al., 2006]. Studies in mice with chondrocyte-specific ablation of *Cyp*27b1, and thus no local $1,25(OH)_2D$ production, demonstrate that paracrine and endocrine actions of locally produced hormone play a role in maturation of the growth plate. Similar to the mice with chondrocyte specific ablation of the VDR, mice lacking *Cyp*27b1 in chondrocytes have a decrease in RANK ligand and VEGF expression. This was associated with an increase in the hypertrophic chondrocyte zone associated with a delay in vascular invasion during embryonic development and an increase in bone volume in neonatal long bones due to a decrease in osteoclastogenesis [Naja et al., 2009]. Similar to the mice with chondrocyte-specific ablation of the VDR, circulating levels of FGF23 were significantly decreased in these mice.

Treatment of cells with $1,25(OH)_2D$ leads to rapid responses, such as increases in intracellular calcium levels and activation of protein kinase C. The former effects are not observed in osteoblasts lacking the VDR, suggesting that they are receptor-dependent [Erben et al., 2002]. In support of this hypothesis, VDR protein, as well as ligand binding, has been shown in caveolae enriched plasma membranes and is markedly reduced in membranes isolated from VDR knockout mice [Huhtakangas et al., 2004]. These latter investigations demonstrated co-localization of the VDR with caveolin-1; however, other studies failed to show this co-localization. Rather, an association between this latter protein and ERp60, a membrane associated receptor implicated in the rapid actions of $1,25(OH)_2D$ was found [Boyan et al., 2006]. Thus, the contribution of ERp60 to the rapid actions of $1,25(OH)_2D$, and the in vivo relevance of these rapid effects have not yet been resolved.

CLINICAL SKELETAL EFFECTS OF VITAMIN D

In addition to its essential role in mineral ion absorption and skeletal growth, vitamin D is critical for the maintenance of skeletal homeostasis. Vitamin D deficiency leads to decreased intestinal calcium absorption, secondary hyperparathyroidism, hypophosphatemia and increased bone turnover [Holick, 2007; Viljakainen et al., 2009]. Altogether these alterations in mineral metabolism due to vitamin D deficiency result in lower bone mineral density [Bischoff-Ferrari et al., 2004b, 2009b] and an increased risk of bone loss or fracture in both men and women [Bouillon et al., 2008; Cauley et al., 2008; Ensrud et al., 2009; Cauley et al., 2010]. The serum 25OHD level defining sufficiency has increased to >75 nmol/L (30 ng/ml) [Adams and Hewison, 2010]. This is due to the association of improved mineral absorption and bone mineral density with higher circulating 25OHD levels and the epidemiologic data that higher 25OHD levels are associated with reduced risk of a number of chronic illnesses and of overall mortality. Using this definition, both children and adults are at high risk for vitamin D deficiency with prevalence rates in the USA being as high as 72% [Looker et al., 2002; Nesby-O'Dell et al., 2002; Gordon et al., 2004, 2008; Weng

et al., 2007; Orwoll et al., 2009]. Populations that shield themselves from solar exposure or who have pigmented skin are at increased risk for vitamin D deficiency [Looker et al., 2002]. Some of these groups may experience greater deleterious effects of low 25OHD levels on bone mineral density [Araujo et al., 2009]. In addition to these aforementioned effects in children and adults, there is increasing data that maternal vitamin D deficiency can affect in utero skeletal development. In a prospective cohort of 424 pregnant women in England, mothers with vitamin D deficiency were more likely to have fetuses with femoral bones that had rachitic features on high resolution three-dimensional ultrasound, based on an increase in distal metaphyseal cross-sectional area and a higher femoral splaying index [Mahon et al., 2010].

Vitamin D has been studied as a potential treatment for osteoporosis in both men and women [Chapuy et al., 1992; Ooms et al., 1995; Dawson-Hughes et al., 1997; Vieth, 2004]. Since treatment of vitamin D deficiency is associated with an increase in bone mineral density due to mineralization of osteoid, it remains unclear whether vitamin D has any effect on osteoporotic bone, or if its benefits are a reflection of resolution of osteomalacia and secondary hyperparathyroidism. Meta-analysis by Bischoff-Ferrari et al. [2005] of seven clinical trials that included 9,820 patients, suggests that a higher daily dose of vitamin D (700–800 international units (IU)), than that recommended by the Institute of Medicine as of 2009, is required to achieve the serum 25OHD level of 100 nmol/L (40 ng/ml), which is associated with 26% and 23% reduction in hip and non-vertebral fracture risk, respectively. This threshold 25OHD level and the daily vitamin D intake needed to achieve fracture reduction may explain the overall lack of anti-fracture efficacy of vitamin D observed in the Randomized Evaluation of Calcium or Vitamin D (RECORD), Women's Health Initiative (WHI), and vitamin D Individual Patient Analysis of Randomized Trials (DIPART) trials [Grant et al., 2005; Jackson et al., 2006; DIPART-Group, 2010]. In the RECORD trial, 5,292 participants were randomized to vitamin 800 IU, calcium 1,000 mg, vitamin D plus calcium, or placebo for 3 years. The groups did not differ in incidence of new fracture, however, the study vitamin D dose resulted in a post-treatment mean 25OHD level of only 62 nmol/L (25 ng/ml); based on the Bischoff-Ferrari et al. meta-analysis this post-treatment 25OHD level may have been too low to achieve anti-fracture efficacy [Bischoff-Ferrari et al., 2005; Grant et al., 2005]. In the WHI, 36,282 women were randomized to calcium 1,000 mg with vitamin D 400 IU or placebo for 7 years. While there was no overall benefit in the treatment group, there was a 29% reduction in risk of hip fracture in those individuals who adhered to the calcium and vitamin D supplementation regimen [Jackson et al., 2006]. Consistent with this, the DIPART study, which examined data from 68,517 patients in seven trials, demonstrated that vitamin D given in doses of 400–800 IU/day is only effective in fracture prevention when combined with calcium, and not when given as monotherapy [DIPART-Group, 2010].

Low vitamin D levels may prevent patients from achieving the maximal beneficial response to anti-resorptive medications used to treat or prevent osteoporosis, although this detrimental effect is not seen with the currently available anabolic agent, teriparatide [Dawson-Hughes et al., 2007; Adami et al., 2009]. In one study that assessed 1,515 postmenopausal women receiving anti-resorptives (alendronate, risedronate, and raloxifene), those patients with 25OHD below 50 nmol/L (20 ng/ml) were more likely to have smaller annualized gains in bone mineral density and 1.7 times more likely to have new fractures [Adami et al., 2009]. This potential negative effect of vitamin D deficiency on anti-osteoporosis therapy is further compounded by the high prevalence of low 25OHD levels in these patients. Prevalence of 25OHD levels $<$50 nmol/L (20 ng/ml) and $<$75 nmol/L (30 ng/ml) in patients either receiving osteoporosis therapy or advice is as high as 30% and 70%, respectively [Holick et al., 2005; Guardia et al., 2008].

Understanding the clinical benefits of vitamin D on the skeleton is made more difficult by the following issues. Isolating the direct effects of vitamin D on fracture reduction is challenging due to the fact that vitamin D is often combined with calcium in clinical trials [Chapuy et al., 1992; Dawson-Hughes et al., 1997; Bischoff-Ferrari et al., 2005; Grant et al., 2005; Jackson et al., 2006; Avenell et al., 2009]. Furthermore, it is difficult to distinguish the direct effects of vitamin D on bone from its beneficial effects on muscle that result in decreased falls, and thereby, fracture risk [Bischoff-Ferrari et al., 2009a]. While, initially there was concern that plant-based ergocalciferol was not as effective at increasing 25OHD levels as animal-based cholecalciferol [Trang et al., 1998; Houghton and Vieth, 2006], subsequent studies have shown that both formulations are effective at treating vitamin D deficiency [Holick et al., 2008; Pietras et al., 2009].

CONCLUSIONS

Investigations into the pathophysiological basis for rickets and osteomalacia have led to a number of seminal discoveries in the past century. The observation that rickets could be cured by sunlight and by ingestion of fish oils led to the identification of the vitamin D pro-hormone as a critical regulator of mineral ion homeostasis. While supplementation with vitamin D effected a cure in the vast majority of affected individuals, the identification and characterization of the molecular defects in those resistant to this treatment elucidated the molecular pathway involved in hormone activation and receptor-dependent hormone action. Further investigations into the pathophysiologic basis of rickets and osteomalacia associated with renal phosphate wasting led to the identification of FGF23, an important phosphaturic hormone secreted by osteocytes, special cells of the osteoblast lineage which are thought to function as skeletal mechanosensors. While studies in humans and animals with impaired vitamin D action have demonstrated that the effect of $1,25(OH)_2D$ and the VDR in the skeleton are largely redundant, investigations in tissue-specific knockout and transgenic mice continue to identify novel actions of the VDR and its ligand in the skeleton. The increasing awareness of the ability of peripheral tissues to activate 25OHD has led to a number of new fields of investigation into the role of vitamin D and its receptor in these "non-traditional" target tissues. In addition, it has led to the development of guidelines for repletion of, not only the active vitamin D metabolite, but also its pro-hormone, in patients with chronic kidney disease [K/DOQI-Work-Group, 2003]. Lastly, the

data generated by clinical trials, examining the vitamin D intake and circulating levels that are required for optimal skeletal health have led to a reevaluation of the recommended daily intake of vitamin D by the Institute of Medicine, which will likely result in the revision of the current guidelines [National-Academy-of-Sciences, 2009].

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