Evolution of our understanding of vitamin D

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The development of our understanding of the function of vitamin D from its discovery in the second and third decades of the 20th century to its hormonal activation of its nuclear receptor and to its present position of an important factor in public health has been traced. The key discoveries of the conversion of vitamin D to its hormonal form, its regulation, and the evolving picture of its molecular mechanism of action are presented. The recognition of its role beyond mineralization of the skeleton to its role in skin, the immune system, and its protective role in some forms of malignancy represent more recent developments. The evolution of derivatives of 1α,25-dihydroxyvitamin D₃ as therapeutic agents suggests a richness of therapeutic potential. All of this nevertheless illustrates that much more remains to be discovered and applied to our armaments for preventing and treating disease.

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INTRODUCTION

In the past several years, vitamin D has received an enormous rebirth of attention, primarily at the public health level, because of vitamin D’s many unexpected benefits. There is currently, especially in the United States and Canada, an attempt to increase not only awareness of the benefits of vitamin D but also to increase the recommended daily allowance of vitamin D.¹ Much of this renewed interest lies in the realization that vitamin D not only is important for mineralization and skeletal growth but has many other roles in regulation of the parathyroid gland, in the immune system, in skin, in cancer prevention, in xenobiotic metabolism, and in cellular development and differentiation. This is particularly satisfying because, following the initial discovery of vitamin D in the early part of the 20th century and the elimination of rickets as a major medical problem, vitamin D achieved major medical and nutritional importance. However, following World War II, an outbreak of idiopathic hypercalcemia and related arterial supravascular stenosis was attributed to food fortification with vitamin D.² This resulted in the elimination of vitamin D fortification of foods in many parts of the world, especially Europe. Thus, vitamin D received an undeserved bad reputation, which remained until recent years, and rickets has reappeared in certain urban areas.

Certainly, vitamin D is potentially toxic when provided in large amounts.³ Toxicity results from hypercalcemia, nephrocalcinosis, aortic calcification, and other unwanted deposits of calcium and phosphorus in soft tissues.³ Unwanted calcification is believed to result from the high calcium times phosphorus product in the serum. Today, this danger still lurks. Thus, it is very important that, in making recommendations to increase D intake, the total burden of vitamin D from both skin and ingestion will not cause vitamin D intoxication in even a few of our population. Very recently, a report appeared that indicates 10,000 units of vitamin D₃ per day is safe for 6 months.⁴ Certainly this is an important start, but we must remember that vitamin D itself is stored and the danger of it accumulating in the adipose tissue and possibly resulting in toxicity in the future must be investigated. Nevertheless, the importance of vitamin D nutrition and/or...
production in skin is clearly evident and, as outlined here, there is a molecular basis for this belief.

**IS VITAMIN D A VITAMIN?**

It is well understood that the first evidence of the existence of vitamin D was obtained by Sir Edward Mellanby in 1919, who was able to produce rickets in dogs by feeding them a diet of oat meal and maintaining them indoors.5 He was able to cure that disease with cod liver oil but was uncertain as to whether the cure was due to the newly discovered vitamin A6 or whether it was due to a new factor. In 1922, McCollum clearly demonstrated that cod liver oil contained another vitamin that he named “vitamin D” that is responsible for mineralization of the skeleton and hence healing of rickets.7 Confounding this discovery is that of Huldshinsky in 1919 that rickets in children could be cured by ultraviolet light.8 This provided the first clue that vitamin D is, in fact, not a vitamin, but it somehow equated ultraviolet light to fish liver oil. Steenbock and Black9 and Hess and Weinstock10 independently demonstrated very clearly that ultraviolet light is capable of converting an inactive substance found in foods, skin, and elsewhere into a substance that could heal rickets. In short, ultraviolet light could yield vitamin D. Thus, the skin is of major importance in our daily production of vitamin D. Although a quantitative picture is not fully available, much information is now at hand especially from the work of Holick and his group that the production of vitamin D in skin has up to this time been the most important source of vitamin D.11 That being true, we must depart from the idea that vitamin D is, in fact, a vitamin and must embrace the idea that it is a prohormone normally produced in skin by photolysis of 7-dehydrocholesterol, a steroid produced by the dehydrogenation of cholesterol. This subject is addressed in a separate article in this supplement.

**METABOLIC ACTIVATION OF VITAMIN D**

For decades it had been assumed that vitamin D3 is biologically active itself and needed no further conversion. This was reinforced as late as 1960 by Egan Kodicek, a prominent investigator in Britain who prepared the first radiolabeled vitamin D3,12 and more recently in 1967 by Haussler and Norman13 who claimed that vitamin D3 was the active substance in the small intestine. However, with the synthesis and use of radiolabeled vitamin D of high specific activity, our group learned that with physiologic amounts, the radiolabeled vitamin D disappears rapidly and the label appears in more polar compounds. Upon isolation, the polar metabolites proved to be more biologically active and acted much more rapidly than the parent vitamin.14 This was followed by the isolation and chemical identification of 25-hydroxyvitamin D3 (25-OH-D3) in 196815 and the final hormone in 1971 termed 1α,25-dihydroxyvitamin D3 [1,25-(OH)2D3].16 These structures were confirmed by chemical synthesis, which made them and an analog, 1α-hydroxyvitamin D3 (1α-OH-D3), available to the medical world; they have since appeared in various forms as therapeutic agents.17,18

The two-step hydroxylation of vitamin D to produce the final active hormone was found in animals19 but was very quickly confirmed by examining kin of patients with a disease called “vitamin D-dependency rickets”, which was discovered by Prader in 1961.20 Fraser et al.21 showed that this disease could be cured with physiologic amounts of synthetic 1,25-(OH)2D3 and not its precursor, 25-OH-D3. This demonstrated that the 1α-hydroxylation activation of vitamin D is required for function. This, together with studies of nephrectomized animals, proved unequivocally that vitamin D itself is biologically inactive and must be converted to a hormonal form in a two-step process before it can function as the hormone 1,25-(OH)2D3.19

A tremendous amount of activity has been expended in elucidating the enzymes responsible for the two hydroxylations resulting in activation of vitamin D. Two enzymes have been studied as being responsible for 25-hydroxylation, which occurs not only in the liver but in other tissues, at least to some degree, as well.22,23 A liver mitochondrial enzyme can 25-hydroxylate vitamin D when presented at elevated concentrations.24 This enzyme (CYP27A1) has been cloned25 and a null mutant has been prepared.25 The mutation has little or no effect on serum 25-OH-D3 levels, suggesting it plays no role in producing physiologic amounts of 25-OH-D3. A microsomal enzyme that appears to operate at the physiologic level26 has only recently been cloned by the Manglesdorf group and, so far, a knockout has not yet appeared.27 Whether it is the key enzyme in initial activation of vitamin D or whether there are several enzymes, some of which remain to be discovered, remains uncertain at the present time. In any case, 25-hydroxylation does not appear to be a highly regulated step in the activation of vitamin D and seems to reflect primarily the vitamin D status of the animal.28 In fact, measurement of 25-OH-D3 is universally accepted as one of the very best biomarkers to determine the vitamin D status of patients or populations. Currently, a normal level of 25–35 ng (62–88 nmolar) per ml appears to be a normal range; however, for protection against some of the degenerative diseases, levels of up to 75–90 ng per ml appear to be desirable.29

Despite enormous efforts in identifying some 33 metabolites of vitamin D3,16 1,25-(OH)2D3 appears to be the functional form of vitamin D in biology, although many reports that 24,25-dihydroxyvitamin D3 is a
functional metabolite have appeared. Blocking of the 24-position of 25-OH-D$_3$ with fluoro groups prevents 24-hydroxylation, but this compound can clearly support a normal healthful phenotype for two generations, eliminating the possibility that 24-hydroxylation is an important functional metabolic pathway.$^{31,32}$

**CLONING OF THE CYTOchrome P450 27B1 (25-OH-D$_3$-1$\alpha$-HYDROXYLASE): ITS POSSIBLE ROLE IN PARACRINE AND AUTOCRINE FUNCTION AND IDENTIFICATION OF THE MOLECULAR BASIS OF VITAMIN D-DEPENDENCY RICKETS TYPE I**

Three groups successfully cloned the cytochrome P450 27B1 (CYP27B1), which opened the door for understanding the molecular basis of the human disorder, vitamin D-dependency rickets type I.$^{33-35}$ Of interest is that often the mutations in humans are heterozygous in which one allele is mutated at one site and another allele is mutated at another, resulting in an inactive or deleted CYP27B1.$^{36}$ This work verifies the early metabolic and clinical work carried out by Fraser et al.$^{32}$ delineating this disease as a defect in 1$\alpha$-hydroxylase.

One of the major developments has been the idea that the 1$\alpha$-hydroxylase is expressed at extrarenal sites.$^{37}$ Reports are now abundant of the existence of the CYP27B1 expression in cultures of skin, bone, colon, parathyroid glands, and virtually every tissue in the body.$^{37}$ This appears to argue that there is a paracrine/autocrine function of 1,25-(OH)$_2$D$_3$ in a variety of tissues. The most convincing evidence for extrarenal production of 1,25-(OH)$_2$D$_3$ is in the disease sarcoidosis in which a patient having no kidneys had high levels of 1,25-(OH)$_2$D$_3$ in the circulation, obviously originating from an extrarenal site.$^{38}$ Other reports of hypercalcemia in lymphoma and certain malignancies may signal the presence of extrarenal expression of this highly calcemic hormone.$^{35}$

However, the question remains: to what extent is there autocrine and paracrine activity for 1,25-(OH)$_2$D$_3$ at extrarenal sites under normal conditions? Two groups carried out experiments in acutely nephrectomized animals in which radiolabeled 25-OH-D$_3$ of high specific activity (160 Ci/mmol) was administered; the results in these animals were compared to those in uremic animals retaining their kidneys.$^{39,40}$ It was clearly shown that, in the absence of kidneys, no radioactivity in the plasma in the form of 1,25-(OH)$_2$D$_3$ could be demonstrated, nor could it be demonstrated in certain other tissues. Thus, the idea of normal paracrine production of 1,25-(OH)$_2$D$_3$ did not appear to be a reasonable expectation, yet reports have continued to appear. Recently, our research group has produced a CYP45027B1 knockout mouse in which the $\beta$-galactosidase gene was inserted in place of the 1$\alpha$-hydroxylase gene using the 1$\alpha$-hydroxylase promoter.$^{35}$

Thus, expression of the $\beta$-galactosidase gene driven by the CYP27B1 promoter of the 1$\alpha$-hydroxylase could be studied in other tissues. We found that $\beta$-galactosidase in hypocalcemic animals is abundantly expressed in the renal cortex, as expected, whereas it is not found in the wild type and is found to a lesser extent in the kidneys of the heterozygote.$^{35}$ Further work has shown that the placenta in the pregnant female is another site of 1$\alpha$-hydroxylation and was confirmed with the $\beta$-galactosidase experiment. However, no $\beta$-galactosidase activity could be detected in skin, raising the question of whether the paracrine function of vitamin D is of any physiologic importance at this site. Obviously, additional work is needed to evaluate the significance of extrarenal 1$\alpha$-hydroxylation under normal circumstances but, at present, its importance seems doubtful.

**THE FUNCTION OF 1,25-(OH)$_2$D$_3$**

In bringing into focus the many functions of 1,25-(OH)$_2$D$_3$, it is essential that we consider how it carries out its basic functions in causing mineralization of the skeleton or the healing of rickets and osteomalacia. It has been clearly established that the rachitic skeleton can be normally mineralized by simply maintaining blood calcium and phosphorus in the normal range by infusion of these mineral elements.$^{41}$ Thus, a function of vitamin D on the mineralization process is unlikely, but vitamin D causes mineralization by the elevation of plasma calcium and phosphorus concentrations to levels that result in mineralization of skeleton on one hand and prevention of hypocalcemic tetany on the other.$^{42}$ Plasma calcium concentration and phosphorus concentration is maintained by action of the vitamin D hormone acting at three sites. Vitamin D activates the active transport of calcium against the concentration gradient in the enterocyte of the small intestine. It also independently causes the active transport of phosphate in the enterocyte.$^{43}$ When dietary calcium is insufficient, calcium must come from some other source. That source is the skeleton and improved retention by the kidney. Thus, the hormonal form of vitamin D stimulates osteoclast-mediated bone resorption; however, this action also requires the parathyroid hormone (PTH).$^{43}$ Furthermore, the renal reabsorption of calcium in the distal tubule requires the presence of both 1,25-(OH)$_2$D$_3$ and PTH.$^{44}$ These then represent sources of calcium that raise blood calcium for support of mineralization and prevention of hypocalcemic tetany, as reviewed previously.$^{45}$

The physiologic factors that are known to regulate serum calcium have also been reviewed previously$^{45}$ and have not appreciably changed at the physiologic level. Briefly, as shown in Figure 1, a calcium-sensitive protein in the parathyroid cells induces secretion of the PTH in
response to even slight hypocalcemia. The parathyroid hormone binds to the epithelial cells of the renal tubule and to osteoblasts of bone. In the proximal tubule cells, PTH stimulates the CYP27B1 gene, producing the 1α-hydroxylase that causes synthesis of 1,25-(OH)2D3. 1,25-Dihydroxyvitamin D3 then turns on the three sites of calcium mobilization. Calcium rises in the plasma, shutting down the parathyroid sensor and thus shutting down parathyroid secretion, completing the feedback loop. We now know that an important function of the vitamin D hormone is to suppress the preproparathyroid gene and to prevent proliferation of parathyroid cells in response to hypocalcemia. Under conditions of high serum calcium, the peptide hormone calcitonin is secreted from the "C" cells of the thyroid and functions to block the mobilization of calcium from bone and calcium reabsorption in the kidney. It is of some interest that calcitonin can also stimulate CYP27B1 in the kidney, which provides small amounts of 1,25-(OH)2D3 for functions of vitamin D not related to calcium homeostasis. The mechanisms whereby the CYP27B1 is regulated are not clearly known, although a PTH-sensitive site in the promoter of the CYP27B1 gene has been identified.

DEGRADATION AND ELIMINATION OF 1,25-(OH)2D3

24-Hydroxylation of 1,25-(OH)2D3 and 25-OH-D3 was discovered very early when 24,25-(OH)2D3 was discovered as a major metabolite of vitamin D. Major efforts by the Japanese scientist, Okuda, resulted in cloning of the 24-hydroxylase. This enzyme, known as the CYP24A1, is the major effector in the degradation of 1,25-(OH)2D3 and 25-OH-D3. In the absence of vitamin D, this gene is silent and the 24-hydroxylase cannot be found in any of the tissues. This enzyme is induced by 1,25-(OH)2D3 and its analogs working through the VDR, as described below. This enzyme is known to not only carry out the 24-hydroxylation but to cause metabolism of 1,25-(OH)2D3 all the way to calcitroic acid. Calcitroic acid is eliminated primarily through the bile into the fecal pathway and can account for almost all of the 1,25-(OH)2D3 metabolism in the body. There are other minor pathways of metabolism but they do not appear to be of great physiologic importance.

The 24-hydroxylase has been knocked out and these null mice were at first thought to have a phenotype of failure of chondrocyte mineralization, but this was later shown to be due to the accumulation of 1,25-(OH)2D3 to levels that interfere with chondrocyte function. In any case, it is quite clear that 24-hydroxylation is not a functional pathway but a pathway of vitamin D hormone elimination, as originally demonstrated on the basis of experiments using 24,24-difluoro 25-OH-D3. Rats raised for two full generations on 24,24-difluoro-25-OH-D3, a compound that is easily 1-hydroxylated but cannot be 24-hydroxylated, showed no phenotype, demonstrating that 24-hydroxylation is not required for optimal growth and reproduction.

To illustrate the importance of this enzyme to metabolism of 1,25-(OH)2D3, VDR-null mice were used. In these animals, the receptor is not present and, therefore, the 24-hydroxylase gene remains silent. Figure 2 shows that injected radiolabeled 1,25-(OH)2D3 remains untouched for hours, and actually days, in the wild-type animal and appeared primarily as water-soluble metabolites, presumably calcitroic acid.

CLONING OF THE VDR PROTEIN AND A STUDY OF ITS MOLECULAR MECHANISMS

In 1987, Baker et al. cloned the human receptor, whereas our group cloned the rat receptor, which differs by having four amino acids less than the human receptor. There is an enormous amount of literature available now on the VDR. However, it is clear that the VDR requires a retinoid X receptor (RXR) to form a heterodimer to bind to the vitamin D-responsive elements in target genes (VDRE). The VDREs are almost without exception repeat hexamers separated by three unspecified nucleotides found primarily in the promoters of target genes. VDR is a member of the nuclear receptor family and is closely related to the retinoic acid receptor and the thyroid hormone receptor.

The essentiality of the VDR for the function of vitamin D was first demonstrated very clearly by an experiment of nature. Vitamin D-resistant rickets type II, which was discovered in children who presented with severe rickets and high blood levels of 1,25-(OH)2D3 and who were resistant to the vitamin D hormone.
children are now known to have a mutation in the VDR gene resulting in either a lack of expression of any receptor or an expression of a defective receptor or a partially defective receptor; hence, the range of dependency varies depending upon the amount of functional receptor present. These children, if they are null mutants, cannot be treated with any form of vitamin D but require the infusion of calcium and phosphorus to help in their phenotype; of course, the prognosis is poor because of the many functions of vitamin D.

Vitamin D-null mice that have been generated by two research groups have clearly confirmed that the receptor is essential for vitamin D function. Of considerable importance is that these mice are fully able to reproduce if they are placed on what is called a rescue diet of high calcium, phosphorus, and 20% lactose, which allows for normal absorption of calcium and phosphorus and results in normal blood levels of calcium and phosphorus. While these animals are not completely normal, their skeletons certainly appear to be normally mineralized. So far, no difference has been found among vitamin D deficiency, receptorless mice, and vitamin D-sufficient receptorless mice, which questions the possibility of a heretofore unknown VDR or the physiologic significance of non-genomic actions of vitamin D.

A great deal has been learned about how the vitamin D hormone works in transcription, far too much to be presented here. However, the current thinking is according to the model illustrated in Figure 3. It seems clear that the first event is the binding of the ligand to the VDR, which causes the rejection of a co-repressor, although the evidence of this is not strong at this stage. The altered VDR bound to the ligand now is able to bind to the 3′ segment of the VDRE together with RXR on the 5′ segment and a number of other proteins such as CBP/p300, pCAF, and SRCs that have histone acetylase activity. This causes an alteration of the chromatin structure. At least some of the ancillary proteins leave and another set of proteins, including DRIP 208 and RNA polymerase II, now bind to the receptor complex to bring about transcription. There is an associated bending of the DNA and a phosphorylation but it is not clear whether these events are essential. This then results in vitamin D causing either stimulation of transcription or suppression of transcription. Much remains to be learned about the molecular mechanisms involved in vitamin D action at the transcriptional level, but there has been considerable progress.

Very recently, using CHIP assays, DREs 70 Kb upstream from the transcription start site were found in the RANK ligand gene. These likely function as enhancers and represent a relatively new development.

In microarray studies performed in vivo in the intestine, for example, at least 50 genes are upregulated and 50 are downregulated by vitamin D. Thus, the actually cellular response to the vitamin D hormone is, indeed, a complex one and a great deal of work will be required in the next decades to understand fully how vitamin D alters the cellular activity of target cells and, very importantly, how different target cells respond differently to the vitamin D hormone.

The VDR gene has been cloned and a great deal is known about it in man, rats, and mouse. The receptor gene in man is considerably more complex than that in the mouse in that it has at least two promoters and has approximately two exons that are non-coding. The mouse gene is illustrated in Figure 4. It is an SP1-driven...
gene, not having a TATA box but having several AP1 sites. There is no VDRE in the promoter, but there appear to be VDREs, as revealed by ChIP-chip analysis, in the intron between exons 2 and 3 as well as two more between exons 3 and 4. These are believed to act as enhancer elements and could explain the autoregulation of the VDR gene that will be described below.

A great deal needs to be learned in regard to the VDR. The literature is confusing, but it is very clear that some tissues do not express the VDR at any meaningful level, whereas others express large amounts in an uncontrolled fashion. In the literature, one will find references to VDR in a large number of tissues, which may or may not be true. In our hands and with anti-
bodies that are extremely selective, we have failed to find any evidence of receptor protein in liver, adult heart muscle, skeletal muscle, smooth muscle, or most cells of the brain. The highest concentration of receptor was found in the duodenum, somewhat less in the colon, and even less, but nevertheless measurable, in kidney and skin. A major question is why the receptor is not expressed in some tissues but is expressed in others and why it is regulated in some tissues but not in others. The kidney and PTH receptors appear to be very clearly regulated. We also found two factors that are required for expression of the VDR in the kidney. Restriction of calcium and hence hypocalcemia together with vitamin D deficiency results in relatively little expression of the VDR. Higher levels of dietary calcium still result in little expression of the receptor in the kidney but, if given with 1,25-(OH)₂D₃, the receptor level is markedly elevated (Figure 5). Calcium and 1,25-(OH)₂D₃ are both required for maximal expression of the VDR in the kidney and parathyroid glands. At this time, it is not clear if this is true in other tissues or not; nevertheless, this identifies in vivo two major regulators of the VDR. In clear contrast, zero calcium in the diet or absence of vitamin D has little effect on expression of the VDR in the duodenum. If the receptor is not present in the parathyroid glands or in the kidney, it is clear that the actions of 1,25-(OH)₂D₃ on those organs cannot take place.

Since the direct demonstration that an important function of vitamin D is to increase intestinal calcium absorption, there have been many contributions to our current view. David Schachter’s group showed this to be an active transport system, as confirmed by Brautbar et al. Wasserman’s group identified the calbindin D₂₈k in chick intestine and the calbindin D₉k in mammals as a calcium binding protein induced by vitamin D. This group suggested this cytosolic protein could be a calcium carrier. Hoenderop et al. identified the brush border trans membrane proteins TrPV5 and TrPV6 as vitamin D-dependent calcium channel proteins. A basal membrane calcium ATPase has also been shown to be stimulated by vitamin D. A sodium/calcium exchange system has also been suggested to play a role in calcium extrusion at the basal membrane. Furthermore, the paracellular transport of calcium has been suggested by Wasserman and by Kutuzova and DeLuca.

The current model for transepithelial transport of calcium can be found in the report of Hoenderop et al. This model has now been tested using calbindin D₉k-null mice. The absence of calbindin D₉k does not affect either the absorption of calcium brought about by 1,25-(OH)₂D₃ nor its ability to raise serum calcium. Clearly, calbindin D₉k is not required for these functions of vitamin D. Benn et al. have examined intestinal calcium transport in the double knockout of calbindin D₉k and the TRPV6. A modest change in calcium transport was found. We are clearly very far from understanding the vitamin D-induced transepacellular transport of calcium, but calbindin D₉k can certainly no longer be considered an essential step in the process.

RENAL OSTEODYSTROPHY AND THE USE OF VITAMIN D THERAPIES

Figure 6 is a diagrammatic representation of the physiologic events occurring during chronic renal failure in terms of developing secondary hyperparathyroidism and the resultant renal osteodystrophy. The loss of renal mass means loss of the endocrine organ expressing the vitamin D endocrine system. Furthermore, the kidney is the primary organ for unloading phosphate from the body, since we live in a phosphate-rich environment; vitamin D induction of the FGF-23 is another possible way in which phosphate is eliminated from the body through the kidney. One of the first physiologically negative events in renal failure is the accumulation of phosphate in the serum; this causes hyperphosphatemia, which suppresses ionized calcium. This signals the parathyroid gland to secrete PTH in an attempt to raise the ionized calcium concentration. Hypocalcemia also results in loss of the
VDR in the parathyroid gland. This then reduces the responsiveness of the gland to the vitamin D hormone. Diminished renal mass also reduces production of 1,25-(OH)\textsubscript{2}D\textsubscript{3}; together with the lack of VDR in the parathyroid gland, this results in the parathyroid gland being resistant to suppression by the vitamin D hormone. There is increased synthesis of PTH and increased proliferation of the parathyroid cells causing excessive secretion of PTH. The large amounts of PTH together with even small amounts of VDR activator will cause erosion of the skeleton, giving rise to renal osteodystrophy.

The accepted practice has been primarily to begin phosphate restriction and phosphate binding to take care of the major problem resulting from loss of renal mass. Once the phosphate is controlled, the administration of a vitamin D compound is indicated to 1) correct any hypocalcemia and 2) suppress the parathyroid gland. The presence of both the VDR activator and adequate amounts of calcium in the serum causes the VDR to be in the gland in significant quantities that can then suppress PTH by the exogenous administration of the vitamin D hormone or analog. This process has been relatively successful for controlling renal osteodystrophy. However, in the United States there are three possible candidates for therapy: 1α,25-dihydroxyvitamin D\textsubscript{3} (known as Calcijex\textsuperscript{®} from Abbott Labs); 1α-hydroxyvitamin D\textsubscript{2} (known as Hectorol\textsuperscript{®} from Genzyme); and 19-nor-1,25-dihydroxyvitamin D\textsubscript{2} (known as Zemplar\textsuperscript{®} from Abbott Labs). All three of these compounds arose from our laboratory and were put into clinical practice. These compounds differ markedly in their basic biological properties. In calcemia, 19-nor-1,25-dihydroxyvitamin D\textsubscript{2} was designed to be low and should thus be able to replace 1,25-(OH)\textsubscript{2}D\textsubscript{3} in suppression of parathyroid with much less impact on hypercalcemia. Hectorol\textsuperscript{®} is a prodrug for 1α,25-dihydroxyvitamin D\textsubscript{3}, which should be the biological equivalent of 1,25-(OH)\textsubscript{2}D\textsubscript{3}. Figure 7 shows the relative abilities of these compounds to raise serum calcium in a vitamin D-deficient rat, which

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**Figure 6** Factors leading to the erosion of bone in patients with chronic renal failure or renal osteodystrophy.

**Figure 7** A comparison of vitamin D analogs currently in use in the treatment of renal osteodystrophy. This experiment was carried out on vitamin D-deficient rats that are severely hypocalcemic and serum calcium was measured in response to the indicated analog. The graph represents the amount of analog required to raise serum calcium by 1 mg\% in these animals.
provides a picture of their potential danger in causing hypercalcemia. In fact, when one measures the use of these compounds for the treatment of secondary hyperparathyroidism versus the calcium phosphorus product produced in the plasma, it is clear that 19-nor-1,25-dihydroxyvitamin D$_3$ has very low activity on the calcium phosphorus product, whereas 1α-hydroxyvitamin D$_2$ raises the calcium/phosphorus product as the dose is increased. Thus, it has been reported, and adequately supported by additional case reports, that the longevity of dialysis patients is increased by approximately 24% with the use of Zemplar™.97 These findings clearly justify the design of analogs with low calcemic activity. However, since it is evident that Zemplar™ still raises serum calcium and phosphorus, we have been developing compounds with an even greater reduction in their calcemic activity. Two of the most promising are shown in Figure 8. Most important is 2-methylene-19-nor-(20S)-1α-hydroxy-bishomopregnacalciferol (2MbisP), which is currently undergoing preclinical trials.98 This compound, which is modified on carbon-2 with the methylene group, has an absence of the 10,19-double bond and an unnatural or 20S-configuration on carbon 20. Most importantly, it has virtually no side chain, which one would have predicted would result in no biological activity. It is one-fifth as active as 1,25-(OH)$_2$D$_3$ in binding to the receptor and in transcriptional activity. This is surprising since it was believed that the 25-hydroxyl is essential to receptor binding. However, unlike 1,25-(OH)$_2$D$_3$, it has virtually no activity in raising intestinal calcium transport or bone calcium mobilization with doses as high as 180 micrograms/kg body weight. At the same time, if one examines its ability to suppress PTH in the plasma of vitamin D-sufficient rats, it is clear it is having a profound suppressive effect on PTH in the blood at much lower concentrations (7.9 micrograms/kg), which gives an enormous safety margin to the physician. This compound has been tested in the laboratories of Eduardo Slatopolsky with identical results in the 5,6-nephrectomy model, where this compound can suppress PTH without changing serum calcium.99 However, it must be clearly emphasized that in the design of analogs the primary and most important action of 1,25-(OH)$_2$D$_3$ is to raise serum calcium in order to mineralize the skeleton. In designing analogs, it is very difficult to get rid of its primary activity and to emphasize the activity in other systems.

**FUNCTIONS OF VITAMIN D BEYOND MINERALIZATION OF THE SKELETON**

The first true evidence of non-calcium- and phosphorus-related activities of the vitamin D hormone was the demonstration of its receptor in tissues not in any way related to this process.45 We have already discussed the presence of the receptor in the parathyroid glands but it is also found in the keratinocytes, promyelocytes, monocytes, lymphocytes, ovarian cells, islet cells of the pancreas, etc. It did seem that the presence of the receptor signals some function of vitamin D compounds in those organs. The first important breakthrough in this area was by Dr. Tatsuo Suda, who demonstrated that terminal differentiation of promyelocytes to the monocyte could be engendered by the vitamin D hormone.100,101 While it caused differentiation of the promyelocytes, it also suppressed proliferation of those cells. Evidence has been brought forth that this is related to suppression of certain significant cell cycle proteins102; nevertheless, this remains to be determined. The fact that cancer cell growth suppression and differentiation could be induced by 1,25-(OH)$_2$D$_3$ raised the hope that 1,25-(OH)$_2$D$_3$ or an analog, might be useful in the treatment of malignant disease.103 However, the application of vitamin D to cells with rapid proliferation became immediately apparent, indicating that the vitamin D hormone and its analogs could be used to treat...
the disease psoriasis. Continued pursuit of the promyelocyte differentiation to the monocyte revealed that vitamin D plays an important role in the development of the giant osteoclast, which plays a major role in bone remodeling, modeling, and bone resorption. A diagrammatic representation of how this occurs is shown in Figure 9. Both vitamin D and the PTH, as well as other factors, stimulate the transcription and production of RANKL, the ligand for the NFκB receptor. When this occurs, the osteoclast precursors are converted to mononuclear prefusion osteoclasts that differentiate into polykaryon and become activated osteoclasts. The vitamin D hormone through RANKL functions throughout this cycle. Thus the bone resorption process and the role of vitamin D and PTH has, at least in part, been determined.

The suppression of keratinocyte proliferation in culture led to the idea that vitamin D hormone could be used to suppress plaque psoriasis, and although calcitriol is being used in some parts of the world for psoriasis treatment, a compound designed by Leo Pharmaceuticals (Dovonex®) is one of the current methods of choice for treating plaque psoriasis. Undoubtedly, better analogs for this disease will become available, which will reduce the side effects and be even more effective.

One area of interest is autoimmune disease. Early work of our group demonstrated that vitamin D could suppress delayed hypersensitivity response. Thus, it became clear that the vitamin D hormone might possibly be useful for suppressing autoimmune disease. Using an experimental model of multiple sclerosis, it could be shown that treatment with super physiologic amounts of 1,25-(OH)2D3 can completely block experimental autoimmune encephalomyelitis induced by myelin basic protein in B10.PL-susceptible mice. Furthermore, rheumatoid arthritis can be suppressed by 1,25-(OH)2D3 and the vitamin D hormone can block type I diabetes in the non-obese diabetic (NOD) mouse – a very important finding. Exactly how these suppressions occur is unknown, although there is an increase in the number of Th2 anti-inflammatory T-cells, which produce a number of cytokines that suppress the Th1 or inflammatory cells. However, the molecular mechanism of its action in the immune system remains ill-defined. Furthermore, the blocking of autoimmune diseases in experimental models is always complicated by the presence of hypercalcemia. Thus, once again, vitamin D functions to suppress autoimmune disease in vivo, but it does so at the cost of hypercalcemia. Can analogs be developed for use in the treatment of these diseases without causing hypercalcemia?

Other components of the vitamin D system can be approached by analog synthesis. Using the PTH and 1α-hydroxylase double-knockout mouse, it was shown
that 1,25-(OH)\textsubscript{2}D\textsubscript{3} physiologically has, in fact, anabolic activity in inducing synthesis of new bone.\textsuperscript{111} Thus, the vitamin D hormone, which was believed to have the sole function of causing mineralization in the skeleton, has at least a small component of anabolic activity that stimulates bone synthesis. Our group has been successful in developing analogs that possess anabolic activity on bone.\textsuperscript{112,113} Two that are currently under development are shown in Figure 10. Of these two, the 2MD is currently in phase 2 trials for the treatment of osteoporosis because of its anabolic activity.\textsuperscript{114,115} By focusing on 2MD, it can be noted that it differs from the vitamin D hormone by the presence of a 2-methylene group, the absence of a 10,19-methylene group, and the presence of an unnatural configuration on the 20-carbon. These changes have resulted in a compound that is selective for bone and which causes anabolic activity.

**DEVELOPMENT OF A BONE-SELECTIVE ANABOLIC FORM OF VITAMIN D**

The 2MD is equal in binding to the receptor as the hormone but it is 10 times more active in causing transcription and it is 10 times more active in causing cellular differentiation in HL-60 cells.\textsuperscript{112} Because of its equal binding activity, it has equal activity to 1,25-(OH)\textsubscript{2}D\textsubscript{3} in intestinal calcium absorption, as expected, but it is 30–100 times more active than 1,25-(OH)\textsubscript{2}D\textsubscript{3} on bone calcium mobilization in vivo, signaling a selective activity on bone. When tested in vitro on bone marrow cultures, 2MD is two orders of magnitude more effective than 1,25-(OH)\textsubscript{2}D\textsubscript{3} in stimulating osteoclastogenesis and in stimulating the activity of the osteoclast itself. Most surprising was that when one incubates human osteoblasts for 1 week in the presence of the vitamin D hormone or 2MD and then incubates them for an additional week with \(\beta\)-glycerol phosphate and ascorbic acid, the vitamin D hormone has very little activity on forming bone nodules in culture, whereas 2MD, even at \(10^{-12}\) M, was extremely effective in bone formation.\textsuperscript{113} This prompted us to test its activity in retired female breeder rats that had been ovariectomized and had lost their bone.\textsuperscript{113} Trabecular bone was largely gone following ovariectomy, even though it was low already, because these were retired female breeders. When treatment is administered for 23 weeks with an amount of 2MD that does not raise calcium above the normal range, not only is the trabecular bone density at the distal ends of the femur restored fully, but the thickness of the cortical bone is increased markedly.\textsuperscript{114} Bone histomorphometry confirmed this as a stimulation of bone formation.\textsuperscript{114} In a 13-week study conducted by Drs. Ke and Brown at Pfizer, 2MD was tested in young ovariectomized rats. They found very clear anabolic activity of 2MD illustrated by micro CT images of the distal femur.\textsuperscript{115} Note that as little as one-half microgram per day results in a clear increase in trabecular bone and this is dose-response related up to 10 ng/day. Again, there is no doubt that selective anabolic activity in bone has been achieved, and we believe it will be possible to develop analogs selective for a number of applications in human disease.

**MOLECULAR BASIS OF SELECTIVE ANALOG ACTIVITY**

Many factors are involved in analog selectivity in vivo. Certainly, rapid clearance of the analog from the circulation and elimination can be one mechanism of lowering calcemic activity. It is also certain that MC903 (Dovonex\textsuperscript{®}) and 22-oxa-calcitriol are cleared rapidly, which accounts for their very low calcemic activity.\textsuperscript{45} Nevertheless, they are useful for topical treatment of psoriasis and, if entering the circulation from skin, they are rapidly eliminated, reducing concerns about hypercalcemia.

Failure to bind to the vitamin D plasma binding protein (DBP) has been suggested as an important factor.

![Figure 10 Analogs currently under development that are anabolic on bone.](image-url)
governing analog activity. This is certainly true for in vitro experiments, where the absence of DBP markedly increases the in vitro activity of 1,25-(OH)₂D₃ but does not change the activity of analogs that do not bind DBP. Interestingly, DBP-null animals present no vitamin D-related phenotype.

Selectivity certainly does take place at the cellular level, but exactly how remains to be determined. Failure of an analog to support coactivator binding or to increase coactivator binding has been suggested but not proved. An assessment of which genes are activated or suppressed by selective analog may provide clues but that information is not yet available.

Attempts to determine if selective analogs can alter the crystal structure of the LBD of VDR have been made by Moras et al. and ourselves. The crystal structure of the LBD remains unchanged by selective analogs, but the selective analogs are distored in the crystal structure. It is still possible that, in solution, the selective ligands may alter the 3D structure of the VDR, but that remains to be determined using techniques such as high-resolution NMR.

CONCLUSION

Our understanding of vitamin D has increased greatly in a period of 80 years, but we have a long way to go to complete the picture and to use what we have learned to improve the health of our population and treat disease.

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