Genomic and proteomic approaches for probing the role of vitamin D in health¹–⁴

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ABSTRACT

Although we have learned a great deal about vitamin D metabolism and function since it first became apparent that this factor was required for bone health, there are still many gaps in our understanding, and the paradigm for future discovery-based research, and I demonstrate how these and other tools might be used to better define optimal vitamin D status. The advances in technologies that permit whole-transcriptome analysis and large-scale proteomic analysis permit us to conduct unbiased evaluations of physiologic states and responses to treatments and interventions. In this review, I briefly demonstrate several instances in which genomic or proteomic analysis has expanded our understanding of vitamin D actions, I present a paradigm for future discovery-based research, and I demonstrate how these and other tools might be used to better define optimal vitamin D status.

INTRODUCTION

Traditionally, vitamin D metabolism has been viewed as an endocrine system that responds to changes in serum calcium concentrations (1). Low dietary calcium intake is reflected by a decrease in serum calcium concentrations, which is in turn a signal for the increased production and release of parathyroid hormone (PTH). Among its functions, PTH stimulates renal 1α-hydroxylase activity, leading to increased conversion of 25-hydroxyvitamin D₃ [25(OH)D₃] to 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]. Elevated serum 1,25(OH)₂D₃ concentrations then stimulate the expression of vitamin D-responsive genes within the primary target tissues that control calcium homeostasis (ie, TRPV6 and calbindin D₉k in intestine, osteocalcin and RANKL in bone, and TRPV5 and calbindin D₂₈k in kidney) through activation of the vitamin D receptor (VDR). Although this system is clearly functional and biologically important during periods of calcium stress, it may not be sufficient to explain all of the biological actions of vitamin D.

As several recent reports and other reviews in this conference demonstrated, improved bone health and cancer chemoprevention may be more closely related to changes in serum 25(OH)D₃ concentrations than to serum 1,25(OH)₂D₃ concentrations. For example, increases in serum 25(OH)D₃ concentrations were associated with both maximal suppression of PTH (a proresorptive agent) (2–4) and increased efficiency of calcium absorption (5–8). There is also evidence that regular, high-dose, vitamin D supplementation decreased calcium absorption rates for common osteoporotic sites (9). This finding and other data suggest that local production of 1,25(OH)₂D₃, rather than endocrine signaling attributable to renal production, is critical for optimal bone health and cancer prevention (10–12). In addition, as we have come to understand more about the details of the molecular mechanisms controlling VDR function (13, 14), researchers have identified 1,25(OH)₂D₃ as an activator of various signal transduction pathways leading to the stimulation of protein kinases such as Src kinase, protein kinase C, protein kinase A, and the mitogen-activated protein kinases (15). These examples suggest that strict adherence to the accepted concepts of vitamin D biological mechanisms and actions are not likely to explain fully the health benefits of vitamin D.

In light of these and other recent findings, I contend that the field of vitamin D research would be well served by the use of several new technologies in an attempt to better describe the full range of vitamin D actions. The advances in technologies that permit whole-transcriptome analysis and large-scale proteomic analysis permit us to conduct unbiased evaluations of physiologic states and responses to treatments and interventions. In this review, I briefly demonstrate several instances in which genomic or proteomic analysis has expanded our understanding of vitamin D actions, I present a paradigm for future discovery-based research, and I demonstrate how these and other tools might be used to better define optimal vitamin D status.

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GENOMIC AND PROTEOMIC APPROACHES: WHAT DO THEY OFFER TO THE FIELD OF VITAMIN D RESEARCH?

General approach

In the past decade, advances in the areas of genomics and proteomics created a revolution in science. These approaches are reviewed in detail elsewhere (16, 17) but are summarized briefly in Figure 1. Our traditional approach to understanding biological processes has been reductionist. We look for proteins that modulate functions of cells and tissues, and we study the regulation of their production and activity. There is no question that this approach has been very fruitful and will continue to be so. However, while this approach focuses our attention on testable hypotheses, it also provides virtual blinders, ie, we look only for the things we have already been studying. Moving beyond this approach can lead to dramatic advances in our understanding of scientific areas. For example, studies of the known proteins involved in iron metabolism failed to identify the cause of the iron-overload disease hemochromatosis. Only after the gene that is mutated in hemochromatosis was identified through a large-scale sequencing effort (genomics) did researchers find that the mutated gene encoded a protein with features similar to the major histocompatibility complex proteins, rather than a protein that was previously thought to be involved in iron metabolism (18). Similarly, studies have revealed that bone mass and metabolism can be regulated by genes that we would classically associate with obesity, such as leptin (19) and LDL receptor-related protein 5 (20). "Omic" approaches give us the opportunity to see beyond our expectations and discover new gene targets for vitamin D actions and functions.

Studies identifying potential, new, vitamin D-regulated targets with genomics

Several transcript-profiling studies with microarrays have been conducted to elucidate the biological role of 1,25(OH)₂D₃. Because of the high cost of microarray-based gene expression profiling, most of the studies reported in the literature used very restricted conditions [eg, a single dose and time of treatment with 1,25(OH)₂D₃] and did not include replicates. This limits the utility of the approach and decreases our confidence in the results. However, more carefully controlled experiments were conducted and addressed some of the experimental design points noted in Figure 2. Figure 2A demonstrates that, depending on the time point examined after 1,25(OH)₂D₃ treatment and the time course of primary responses to the treatment, the changes in transcript levels could include both primary responses (eg, those resulting from direct, VDR-mediated interactions with gene promoters) and downstream responses that are indirect. Detection of a change in expression does not prove that a gene is a direct vitamin D target gene.

This phenomenon of differential patterns of responses to vitamin D is evident in the work of Lin et al (21). Those authors examined the time course of the response to 100 nmol/L 1,25(OH)₂D₃ or the vitamin D analog EB1089 in squamous cell carcinoma cells (SCC25) and identified 152 genes as being regulated by vitamin D (89 up and 63 down), with the use of the Affymetrix FL array (Affymetrix, Santa Clara, CA) and a 2.5-fold cutoff for determining a meaningful change in expression.
Clustering was performed on the basis of the pattern of expression or the functional classification of the transcripts. Figure 3 shows the diversity of the vitamin D responses in these cells. Even within the genes with documented, functional, vitamin D response elements (VDREs) in their promoters, there was heterogeneity in responses. For example, the CYP24 transcript levels were rapidly increased by vitamin D treatment (significantly increased in 1 h), whereas osteopontin transcript levels increased more slowly in response to treatment (maximal expression at 12 h). This suggests that similar VDREs are differentially regulated, depending on the promoter context (22), but it also demonstrates the difficulty of discerning a direct transcriptional response solely on the basis of a time course, i.e., even later responses can be attributable to direct effects. Another interesting finding from that study was that the vitamin D–induced responses in the transcript profile were much more diverse than might have been predicted previously. For example, several transcripts coding for proteins involved in protection from oxidative stress were gradually up-regulated by the vitamin D analog, including glucose-6-phosphate dehydrogenase (generating NADPH), glutathione peroxidase, and selenoprotein P. In addition, the thioredoxin reductase transcript was increased by 1 h after treatment, with peak induction by 6 h. Rapid suppression of transcripts for a variety of signaling peptides (e.g., PTH-related protein and galanin) and induction of intracellular cell signaling proteins (e.g., Cox-2, phosphoinositide-3-kinase, and p85 subunit) were also observed after treatment. It is not clear which of these responses is primary; none of these genes was previously shown to be regulated by vitamin D or to contain a functional VDRE. However, because 1,25(OH)₂D₃ promotes cellular differentiation, the up-regulation of some transcripts may represent a vitamin D–induced shift to a more differentiated phenotype. In any case, these data suggest that the traditional approach of examining only the expression of genes controlling cell cycle proteins in an attempt to explain the prodifferentiating action of vitamin D may provide limited information regarding the biological mechanisms of 1,25(OH)₂D₃ actions in proliferating or cancer cells.

To identify direct vitamin D actions, scientists often conduct multiple complementary experiments and compare the results for consistency. In Figure 2B, 3 complementary mouse experiments are illustrated. Treatment of normal mice with 1,25(OH)₂D₃ would be expected to up-regulate or down-regulate specific genes in a target tissue, whereas the examination of target tissue transcript profiles in mice that lack essential components of the vitamin D signaling system (e.g., VDR or 1α-hydroxylase) would be expected to demonstrate opposite effects on vitamin D target genes [e.g., a gene that is activated with 1,25(OH)₂D₃ injection would be down-regulated in VDR- or 1α-hydroxylase-null mice]. A preliminary attempt at this approach with a small group of animals was recently reported by Li et al (23). By comparing the gene expression profile changes that occurred in kidney after 1,25(OH)₂D₃ injection with those that resulted from loss of the VDR (wild-type mice compared with VDR knockout mice), those authors identified 95 genes for which the response attributable to vitamin D injection was the opposite of the response attributable to loss of the VDR. Twenty-eight of those transcripts (including 1α-hydroxylase mRNA) were up-regulated in VDR-null mice and down-regulated in vitamin D–treated mice, whereas 67 of the transcripts (including 24-hydroxylase mRNA) were down-regulated in VDR-null mice and up-regulated in vitamin D–treated mice. Like the study by Lin et al (21), this study identified many potential vitamin D target genes. Although neither of these studies definitively identified new targets, they narrowed the list of candidates considerably and provided clear guidance for investigators who wish to conduct careful reductionist experiments involving these genes.

Studies identifying new protein complexes necessary for vitamin D actions

Very few studies have taken a proteomics approach to the examination of vitamin D actions in cells. One notable study was recently conducted with keratinocytes by Oda et al (24). Previously, Rachez et al (25) developed an in vitro assay to assess the complex of proteins that associates with the VDR during vitamin D–mediated gene transcription. By using a VDR ligand-binding domain–glutathione S-transferase fusion protein, they were able to identify the VDR-interacting protein (DRIP) complex, a complex of 16 proteins that is essential for vitamin D–mediated gene transcription because of its ability to recruit RNA polymerase II to vitamin D–responsive genes (14, 25). Oda et al (24) used this approach to identify the proteins associated with the VDR in nuclear extracts from proliferating and differentiated keratinocytes. Although they identified a similar complex of proteins interacting with the VDR in proliferating keratinocytes (e.g., DRIP complex members and RXR), they found that key members of the complex had changed in differentiated keratinocytes. Proteomic analysis of the proteins associated with the VDR in proliferating and differentiated nuclear extracts showed that at least 5 members of the DRIP complex were lost with differentiation but 2 new proteins, SRC-2 and SRC-3, became prominent members of the complex. That study suggested that the complex mediating VDR-mediated gene expression might not be uniform across vitamin D target tissues or even within the cells of a tissue at different stages of their life spans. This could account for the observed diversity of sensitivity of various cell types/tissues to 1,25(OH)₂D₃ treatment or the ability of a vitamin D analog to work in one tissue but not another.

At least one other line of vitamin D research might also be improved with a proteomics approach. Specifically, it is now clear that vitamin D stimulates rapid activation of signal transduction pathways, e.g., it activates several protein kinases, leading to phosphorylation of various proteins (15). The final targets of
the kinases activated by 1,25(OH)\textsubscript{2}D\textsubscript{3} have not yet been identified but it is clear that these targets of phosphorylation are critical for understanding the biological importance of these rapid vitamin D actions. It is apparent that new technologies developed for assessment of the phosphoproteome (26) are likely to identify not only the signal transduction pathways used by 1,25(OH)\textsubscript{2}D\textsubscript{3} but also the terminal proteins whose biological functions are either activated or inhibited through stimulation of these phosphorylation cascades.

**Identification of functional biomarkers of vitamin D actions**

A major issue in the area of vitamin D research is defining the amount of vitamin D needed for optimal health. Although there are some concerns about the reliability and cross-comparison of various assays for measuring 25(OH)\textsubscript{D}\textsubscript{3} concentrations as an index of vitamin D status (27), the main issue is how 25(OH)\textsubscript{D}\textsubscript{3} concentrations relate to function, ie, bone biological processes related to the risk of osteoporosis or epithelial cell biological processes related to cancer risk. The data from several studies suggest that the cutoff value for adequate serum 25(OH)\textsubscript{D}\textsubscript{3} concentrations may be higher than simply the concentration that prevents rickets (2–4). This idea is based on the association of high vitamin D concentrations with suppression of serum PTH concentrations, a measurement that is being used as a surrogate marker of bone resorption (with the assumption that maximal suppression of PTH is necessary for maximal protection of bone). However, is the PTH concentration an appropriate marker for bone resorption? Or would linking serum 25(OH)\textsubscript{D}\textsubscript{3} concentrations to a more relevant functional endpoint or using a biomarker that is correlated directly with a functional endpoint be better for defining optimal vitamin D status? And what about cancer risk? Should we assume that the serum 25(OH)\textsubscript{D}\textsubscript{3} concentration that is optimal for the protection of bone is optimal for the prevention of cancer? In a perfect world, scientists would be able to directly relate vitamin D status to factors such as fracture incidence, changes in bone density with time, or the development of prostate, breast, or colon cancer. However, such studies would require very large populations and a long study period (as well as being ethically questionable). An alternative might be to correlate vitamin D status with an intermediate endpoint; for bone health, for example, active bone resorption could be measured for a smaller, more controlled, study population. Unfortunately, the means to study active bone resorption (and early functional indices of cancer risk) are currently inadequate for this task. Bone density changes require long periods for reliable observation, and current serum markers are highly variable and thus less reliable. This indicates that new biomarkers of relevant functional endpoints important for bone health and vitamin D biological processes are needed.

Cancer researchers are leading the way toward using the techniques of genomics and proteomics to provide diagnostic markers. For example, Sorlie et al (28) used gene expression profiling of breast tissue biopsies to define the discriminating diagnostic signatures for 6 distinct classes of breast cancer, which suggests that a molecular signature could be used instead of a more subjective histologic evaluation. Although that study and other cancer-profiling studies made great use of gene expression profiles for tumor biopsies as a classification system, the need for a tissue biopsy is a serious limitation to the use of gene expression profiling as a general screening tool for healthy people. Effective biomarkers for assessing healthy people are likely to come from readily available, minimally invasive sampling of blood, blood cells, serum, or urine. The use of serum proteomic profiles, such as that used for ovarian cancer diagnosis, as reported by Petricoin et al (29), may be a more fruitful approach for issues related to nutrient status and health.

In the “omic” approach to identifying and defining biomarkers of disease or of physiologic deficits, the basic idea is to compare the profiles of individuals in ≥ 2 well-defined groups (eg, vitamin D-replete and vitamin D-deficient subjects) and then define the changes in the profiles that are correlated best with changes in the condition of interest. For continuous variables such as vitamin D status, the markers may be continuous or they may exhibit breakpoints. Although this approach may provide us with assessment parameters that may be easier to measure than vitamin D metabolite concentrations, without functional correlates this would be no more informative than serum 25(OH)\textsubscript{D}\textsubscript{3} concentrations. Functional assessment could be included to strengthen the relationship; this general scheme is presented in **Figure 4**. For example, bone resorption could be measured directly through assessment of the release of calcium from bone. Ongoing work in Dr. Connie Weaver’s laboratory at Purdue University suggests that a new technique for labeling bones in vivo with small amounts of the natural isotope \textsuperscript{41}Ca may provide an objective accurate measure of bone resorption (30). \textsuperscript{41}Ca is a long-lived isotope of calcium that can be produced inexpensively through neutron activation. Because minuscule amounts of \textsuperscript{41}Ca can be measured with atomic mass spectroscopy, radiologically benign amounts of the isotope can be administered to human subjects to label their bones. After \textsuperscript{41}Ca has been cleared from soft tissues (∼100 d), the appearance of \textsuperscript{41}Ca in the serum or urine is a direct reflection of calcium lost from bone. Because of the long half-life of \textsuperscript{41}Ca and the relatively low turnover of bone, subjects can be examined repeatedly for > 10 y (permitting assessment of multiple treatments or multiple levels of a treatment for the same subject). With the exception of the long study period, this is consistent with work that was previously conducted with animals and high concentrations of \textsuperscript{45}Ca or tetracycline (31).

A discordance in the relationship between the effects of vitamin D status on bone resorption and on the serum proteome could have important physiologic implications. For example, if serum proteins changed as vitamin D status was reduced but this was not accompanied by coordinate changes in bone resorption (\textsuperscript{41}Ca release), then this could be an indication that the vitamin D

**FIGURE 4.** Scheme for the identification and cross-validation of serum biomarkers related to vitamin D (VD) status and bone resorption.
biomarker is related more to cancer risk (or one of the other potential functions now being proposed for vitamin D, such as diabetes mellitus risk). This could prove to be the basis for future work on the vitamin D–non-bone disease connections.

CONCLUSIONS

The field of vitamin D biology has a long history. However, we are now being confronted with both basic questions related to vitamin D actions and applied issues regarding how to use our fundamental understanding of vitamin D biological processes. Advances in chromatographic techniques have permitted us to better understand vitamin D metabolism, and the revolution in molecular biology has helped us explain the mechanistic basis for vitamin D actions on cells. In this review, I have attempted to explain how the new approaches of genomics and proteomics have the potential to expand our understanding of vitamin D biological processes and the role of vitamin D in human health.

REFERENCES