Vitamin D and cancer

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The impact of dietary intake upon cell and tissue physiology, as well as pathophysiology, has emerged as being highly significant to the etiology of a number of high-profile malignancies. The vitamin D receptor (VDR) is a member of a large transcription factor family of nuclear receptors and responds specifically to a hormonal micronutrient ($1\alpha25(OH)_2D_3$). A central endocrine role for this receptor in bone health was established at the beginning of the 20th century. An alternative role has been established over the last 25 years for the VDR to regulate cell growth and division, and promote differentiation through autocrine and paracrine mechanisms. These findings from *in vitro* and *in vivo* experiments have generated considerable interest in the potential to target the VDR in either chemoprevention or chemotherapy cancer settings. As with many potential cancer therapeutics, it has become equally clear that cancer cells display *de novo* and acquired mechanisms of resistance to these actions. Consequently, researchers are developing a range of experimental and clinical options to bring about more targeted actions, overcome resistance and enhance the efficacy of VDR-centered therapeutics.

Expert Rev. Endocrinol. Metab. 1(2), 219–231 (2006)

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KEYWORDS:
1α-hydroxylase, 24-hydroxylase, chemoprevention, chemotherapy, *cytochrome P24, cytochrome P27b1* GADD45α, $p21^{waf/cip1}$, vitamin D receptor

Role of diet in cancer

Common epithelial cancers arise in self-renewing tissues

The underlying causes for high-profile cancers, such as those of the prostate, breast and colon, are still not clearly understood. Only the minority of cases are determined by strongly deterministic genetic factors. For example, only approximtely 5% of breast cancer cases are linked to high penetrant genetic mutations at the breast cancer *BRCA1* and *BRCA2* gene loci. Historically, this exclusive genetic causality provided a paradigm for investigating the mechanisms and etiology of cancer, although, in the post-genomic era, other strong penetrance genes have not been readily identified. An alternative contemporary view is that cancer etiology includes a contribution from an ill-defined combination of genetic factors with weak penetrance interacting with a multitude of environmental factors [1]. Reflectively, the single greatest risk factor for most cancers is age, with the average age of onset of breast, prostate and colon cancer being in the sixth and seventh decades of life. The sporadic, temporal acquisition of a cancer phenotype is compatible with multifactorial models that require disruption of mechanisms of cell restraint and tissue organization [2].

Epithelial linings of the prostate, mammary glands and the gastrointestinal tract all typify self-renewing tissues that contain stem cell populations. These cells give rise to committed progenitors and, in turn, the multiple cell lineages required for tissue function [3–5]. Stem cells are relatively rare and long-lived, but are frequently quiescent. Furthermore, they are uniquely able to undergo asymmetric division and to give rise to both other stem cells and transiently amplifying populations of progenitor cells, which in turn give rise to the differentiated cell types. By contrast, these differentiated epithelial cells are functional, but short-lived, and are lost through programmed cell death processes, being replaced by newly differentiated transiently amplifying cells. Cellular control of the intricate balance of the processes of division, differentiation and programmed cell death includes common roles for Wnt, Hedgehog and other developmental signal

10.1586/17446651.1.2.219

ISSN 1744-6651

transduction processes [3,6]. Convergent targets for these signals include key regulators of cell proliferation, such as the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$.

As a result of their long life cycle and high proliferative capacity, stem cells, rather than the differentiated cells, are candidates for tumorigenesis. To counter this, there appear to be a range of mechanisms in place within stem cells to maintain genomic integrity [7]. These controls, notwithstanding the transformation of stem cells, have given rise to the concept of cancer stem cells. Accumulating evidence supports the presence of these cells in prostate, breast and colon cancers [3–5].

Emerging roles for diet impacting on malignancy

Recently, a significant appreciation of the impact of diet on the initiation or progression of cancer has come to light. The WHO has now stated that bad diet is the second most preventable cause of cancer (after smoking). This impact will increase further owing to demographic factors and, possibly, owing to changing dietary habits worldwide. Aspects of these relationships are found in breast, prostate and colon cancer, where the etiology of the disease reflects the cumulative impact of dietary factors over an individual's lifetime. Equally, these relationships have the potential to be exploited clinically through chemoprevention, for example, in the Selenium and Vitamin E Cancer Prevention Trial (SELECT) that assesses the chemoprevention potential of vitamin E and selenium in prostate cancer [8].

Despite the significant and potential clinical benefit of these relationships, the critical time frame during which dietary factors may be protective against cancer development remains unclear; for example, during embryogenesis, childhood development or adult life. Understandably, resolving this is highly challenging. Considerable resources were required to elucidate what is now established as a clear causal relationship between cigarette smoke and lung cancer. To address these issues, the emerging field of nutrigenomics aims to dissect the impact of dietary factors on genomic regulation and, thereby, physiology and pathophysiology, using a range of postgenomic technologies [9].

Vitamin D receptor & other nuclear receptors allow a local, integrated response to lipophilic nutrients

The nuclear receptors form one of the largest human families of transcription factors and bind with a range of affinities to lipid-derived hormonal, dietary and environmental factors to regulate gene targets; they can be classified broadly according to ligand affinities. The first group of receptors bind ligands with high affinity, typified by the sex steroid hormone estrogen receptors (ERα and β). Equally, a number of micronutrient ligands are also bound with high affinity by specific receptors. For example, the all *trans* and 9-*cis* retinoic acid and $1\alpha25(OH)_2D_3$ are bound by the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [10], and vitamin D receptor (VDR), respectively [11]. The second group of receptors bind with broader affinity to more abundant liphillic compounds, such as macronutrients. For example, the peroxisome proliferator-activated receptors (PPARs) [12,13], liver X receptors (LXRs) [14] and farnesoid X receptor (FXR) [15] recognize polyunsaturated fatty acids and bile acids (TABLE 1). Finally, a group of orphan receptors exists for which no ligands have been identified. Phylogenetic classification, by contrast, has defined seven subfamilies and, within these, the VDR is in the group 1 subfamily and shares homology with the LXRs and FXR, and more distantly the PPARs.

Both high- and broad-affinity receptors appear to work in concert. For example, the VDR can also respond to the secondary bile acid lithocholic acid (LCA), and, equally, the RXRs can mediate a local response to fatty and bile acids [16]. Examination of VDR, RARs, PPARs, FXR and LXR signaling reveals that they share common target genes [17], such as those that regulate the cell cycle (e.g., $p21^{waf1/cip1}$ [18–22]) and also xenobiotic clearance via cytochrome (CY)P450s (e.g., *CYP3A4* [23,24]). Furthermore, there appears to be co-regulation of the receptors. FXR induces the related nuclear receptor, PPARα and the VDR induces PPARδ [25], thus providing molecular evidence for a local, paracrine cross-talk between the receptors. The postgenomic description of the nuclear receptor superfamily conjoined with profiling approaches [26] reveals that not only colon epithelial cells but also breast epithelial and prostate epithelial cells express a rich cohort of nuclear receptors, including LXRs, FXR, PPARs, RXR and VDR [10–12,14,27]. The co-expression of these receptors suggests a broader and integrated network in the local sensing of dietary-derived lipid molecules, providing a functional link between hormonal, environmental and dietary cues and tissue homeostasis [28].

Local remodeling of chromatin is central to nuclear receptor transcriptional functions

The nuclear receptors share a common architecture, which includes defined regions for DNA recognition, ligand binding and cofactor interactions. The DNA binding domain recognizes specific response elements (REs) in target gene enhancer/promoter regions. Most receptors preferentially form homo- or heterodimeric complexes; RXR is a central partner for VDR, PPARs, LXRs and FXR. Therefore, simple REs are formed by two recognition factors and their relative distance and orientation contributes to receptor binding specificity, although more recently, composite elements have been identified, suggesting more integrated control.

In the absence of a ligand, VDR–RXR dimers exist in an *apo* state as part of large complexes (~2.0 MDa) [29], associated with co-repressors (e.g., NCoR2/SMRT) and bound to RE sequences. These complexes actively recruit a range of enzymes that post-translationally modify histone tails, for example, histone deacetylases (HDACs) and methyltransferases, and, thereby maintain a locally condensed chromatin structure around RE sequences [30]. Ligand binding induces a so-called *holo* state, facilitating the association of the VDR–RXR dimer with co-activator complexes. A large number of interacting co-activator proteins have been described, which can be divided into multiple families including the p160 family, the non-p160 members and members of the large bridging VDR-interacting protein (DRIP)–tri-iodothyronine receptor auxillary protein (TRAP)–activator-recruited cofactor (ARC) complex, which links the receptor complex to the co-inte-

Table 1. Nuclear receptors bind with varying affinites to a range of dietary-derived factors to regulate target genes involved with the control of cell metabolism, proliferation and differentiation, and programmed cell death.

Nuclear receptors	Dietary-derived ligand	Example target genes
High affinity		
ERα	Lignan (e.g., secoisolariciresinol diglucoside) isoflavones (e.g., genistein)	*Progesterone receptor*
ERβ		
RARα	All *trans* retinoic acid	*CYP26A1* *$p21^{waf1/cip1}$*
RARβ		
RARγ		
RXRα	9-cis retinoic acid dietary lipids (e.g., docosahexanoic acid)	
RXRβ		
VDR	$1\alpha25(OH)_2D_3$ and bile acids (e.g., lithocholic acid)	*CYP24*, *$p21^{waf1/cip1}$*, *IGFBP-3*, *E-cadherin*, *GADD45α*, *TGF-β_2*, *PPARδ*, *CYP3A4*
Broad affinity		
PPARα	Eicosapentaenoic acid	*$p21^{waf/cipi1}$*, *CYP4A1*
PPARδ,	Omega 3 fatty acids (e.g., linoleic acid)	
PPARγ1	15-deoxy-$D^{12,14}$-prostaglandin J_2 (15d-PGJ_2) Omega 6 fatty acids (e.g. 5,8,11,14-eicosatetraenoic acid)	
PPARγ2		
LXRβ	27-hydroxycholesterol	*CYP7A1*
FXR	Chenodeoxycholic acid	*CYP3A4*, *LRH-1*
CAR/PXR	Xenobiotics	*CYP3A4*

CAR: Constitutive androstane receptor; CYP: Cytochrome P450; ER: Estrogen receptor; FXR: Farnesoid X receptor; GADD: Growth arrest and DNA damage; IGF: Insulin-like growth factor; IGFBP: Insulin-like growth factor binding protein; LXR: Liver X receptors; PPAR: Peroxisome proliferator-activated receptor; PXR: Pregnane X receptor; RXR: Retinoid X receptors; VDR: Vitamin D receptor.

grators CREB binding protein (CBP)/p300 and basal transcriptional machinery [31–34]. These receptor co-activator complexes coordinate the activation of an antagonistic battery of enzymes, such as histone acetyltransferases, and thereby induce the reorganization of local chromatin regions at the RE of the target gene promoter. The complex choreography of this event has emerged recently and involves cyclical rounds of promoter-specific complex assembly, gene transactivation, complex disassembly and proteosome-mediated receptor degradation [32].

The expression, localization and isoforms of corepressor complexes have emerged as critical to determine the spatio-temporal equilibrium between the antagonistic actions of the *apo* and *holo* nuclear receptor complexes and, thus, determine target gene promoter responsiveness in a range of physiological and pathological settings. For example, in regulating nuclear receptor function during neural cell differentiation, in determining cell-specific responses to estrogenic hormones and in the inappropriate silencing of nuclear receptor actions associated with cancer [35–37].

It remains unclear to what extent the various histone modifications, initiated by the *apo* and *holo* nuclear receptor megacomplexes around target gene response elements, influences the subsequent transcriptional responsiveness of the promoter. It has been proposed that these modifications may form a stable and heritable histone code that determines the assembly of factors upon the chromatin template and controls individual promoter transcriptional responsiveness [38]. The SW13/ADA2/NCoR/TFIIIB (SANT) motif contained in the co-repressor NCoR2/SMRT recognises and sustains specific histone modifications, thereby supporting this latter idea [39].

VDR signaling *in vivo* & *in vitro*

Autocrine versus paracrine VDR signaling

Vitamin D status is predominantly dependent upon cutaneous synthesis initiated by solar ultraviolet (UV) radiation, although a minor component is derived through dietary intake. The correct and sufficient level of serum vitamin D is currently a matter of considerable debate. Existing recommendations for daily intake are in the range of 150–200 IU/day. More recently, workers examining the impact on the $1\alpha25(OH)_2D_3$ on the prevention of osteoporosis have suggested that the correct level may be as high as 3000 IU/day [40,41].

Vitamin D is converted in the liver to 25-hydroxyvitamin D_3 ($25\text{-}OH_2D_3$) and circulating levels of this metabolite serve as a useful index of vitamin D status. Subsequent hydroxylation

steps at the carbon 1 position by 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase encoded by *CYP27b1*) produce the biologically active metabolite $1\alpha25(OH)_2D_3$. A second mitochondrial CYP450 enzyme 25-hydroxyvitamin D_3 24-hydroxylase (24-hydroxylase encoded by *CYP24*), can use both 25-OH-D and $1\alpha25(OH)_2D_3$ as substrates, and is the first step in the inactivation pathway for these metabolites.

1α-hydroxylase is expressed in a wide range of tissues, including prostate, breast and colon epithelial cells, and thus, circulating $25(OH)D_3$ may enter into an intracellular VDR signaling axis that coordinates the local synthesis, metabolism and signal transduction of $1\alpha25(OH)_2D_3$, forming a classical negative-feedback loop. Thus, $1\alpha25(OH)_2D_3$ is regulated in an endocrine manner, principally associated with its calcemic function and, locally at an autocrine level, probably associated with its role in regulation of cell proliferation and differentiation.

VDR actions in noncalcemic normal tissues

These actions have been the subject of intensive investigation and a consistent theme that emerges is the regulation of target genes, which subsequently control cell growth, differentiation and programmed cell death. *In vitro*, $1\alpha25(OH)_2D_3$ is able to regulate proliferation of a wide range of normal tissues, including epithelial cells from the prostate, breast and colon. There is evidence that $1\alpha25(OH)_2D_3$, acting through the VDR, plays a role in augmenting development, differentiation and milk production in the mammary gland. Recently, the use of murine knockout approaches has revealed that disruption of the VDR results in profound calcemic phenotypes associated with the disruption of duodenal calcium absorption and bone mineralization [42]. Interestingly, mammary gland formation and function are also disrupted, supporting a negative proliferative and prodifferentiative role for $1\alpha25(OH)_2D_3$ to govern ductal outgrowth. The accelerated ductal growth seen in $VDR^{-/-}$ animals is exacerbated further during the pregnancy-associated proliferative burst, and moreover, the widespread postlactation apoptosis associated with involution is delayed [43,44]. Thus, the mammary gland represents an intriguing area where the endocrine (calcemic) and autocrine (cellular) effects of the VDR converge.

In vivo *VDR anticancer actions*

A clear difficulty in investigating the chemoprevention effects of the VDR is that mice are not humans; their spectrums of age-associated malignancies are different to humans and other key metabolic differences also exist. Recapitulating these lifetime effects are further compounded by the need to establish the window in which chemoprevention effects may play a role in either tumor initiation or progression.

The VDR-deficient animals have become extremely useful tools to elucidate more clearly the role for the VDR acting in a chemopreventative manner. A series of animals have been generated in which the VDR-ablated background has been crossed into animals with tumor disposition phenotypes. Thus, crossing the VDR-deficient and heterozygote mice with mouse mammary tumor virus (MMTV)–neu transgenic mice has generated animals that show a degree of VDR haplosufficiency. The mammary tumor burden in the crossed mice is reduced with the presence of one wild-type VDR allele and further with two wild-type VDR alleles [45]. Alternatively, the $VDR^{-/-}$ animals demonstrate greater susceptibility to carcinogen challenge. For example, challenging these mice with 7,12-dimethylbenz[a]anthracene (DMBA) induced more preneoplasic lesions in the mammary glands than in wild-type mice [46].

A parallel and larger series of studies have examined the ability of dietary or pharmacological addition of vitamin D compounds to either prevent tumor formation or inhibit the growth of exogenously added xenograft tumors. Investigators have focused on modifying dietary regimes that demonstrate a tumor predisposition. Long-term studies on mice fed with a western-style diet (e.g., high fat and phosphate, and low vitamin D and calcium content) increased epithelial cell hyperproliferation. Equally acute exposure to these diets, for example, over 12 weeks, proved sufficient to induce colon-crypt hyperplasia; effects that could be ameliorated through the addition of calcium and vitamin D [47].

Another important model in which to test chemoprevention and chemotherapy capacity is the Apc_{min} mouse. Adenomatous polyposis of the colon (APC) is a key negative regulator of β-catenin action and is commonly disrupted in humans who develop colon cancer. The rate of polyp formation in these Apc_{min} mice was increased significantly in mice fed a western diet compared with animals on standard chow. Only moderate effects of $1\alpha25(OH)_2D_3$ on polyp formation were found in this model and were associated with potent side effects (e.g., hypercalcemia). However, the effects were more pronounced and significant when a potent analog of $1\alpha25(OH)_2D_3$ was used, with reduced toxicity [48].

The efficacy of $1\alpha25(OH)_2D_3$ and its analogs has also been tested extensively in carcinogen-induced models *in vivo,* and established a range of protective effects against tumor initiation, progression and invasion, supporting chemoprevention and chemotherapy applications for the VDR. Equally, immunodeficient mice injected with human breast and other cancer cell lines demonstrated tumor suppression and reduced angiogenesis in response to $1\alpha25(OH)_2D_3$ [49–53].

A complimentary approach to these studies has been to examine the capacity of $1\alpha25(OH)_2D_3$ to interact with other dietary components, which are known to be chemoprotective. One such strategy has focused on the ability to enhance local autocrine synthesis and signaling of $1\alpha25(OH)_2D_3$. For example, phytoestrogens, such as genestein, are known to be protective and *in vivo* soy or genestein feeding appears to increase the local expression of *CYP27B1* and reduce *CYP24* expression in the mouse colon, resulting in locally elevated levels of $1\alpha25(OH)_2D_3$ [54]. These results appear to support the concept that Asian diets, which are rich in phtyoestrogens and vitamin D may, in part, explain the traditionally low rates of breast, prostate and colon cancer in this region.

In vitro *anticancer effects*

In 1981, 1α,25$(OH)_2D_3$ was shown to inhibit human melanoma cells significantly *in vitro*, and subsequently, 1α,25$(OH)_2D_3$ was found to cause differentiation in cultured mouse and human myeloid leukemic cells. Following these studies, 1α,25$(OH)_2D_3$ has been demonstrated to have a range of antiproliferative effects in a wide panel of cancer cell lines, including MCF-7 breast cancer cells, LNCaP prostate cancer cells and CaCo2 colon cancer cells [11,55–60].

Comprehensive genome-wide *in silico* and transcriptomic screens to elucidate the VDR transcriptome have revealed broad consensus on certain targets, but have also highlighted variability. In part, these studies may reflect experimental design, cell line differences and genuine tissue-specific differences of cofactor expression, which alter the magnitude and the extent of VDR transcriptional actions [61–64].

The common antiproliferative VDR functions are associated with arrest in G_0/G_1 of the cell cycle, associated with the upregulation of a number of cell cycle inhibitors including $p21^{waf1/cip1}$ and $p27^{kip1}$. Promoter characterization studies have demonstrated a series of vitamin D response elements (VDREs) in the promoter/enhancer region of $p21^{waf1/cip1}$ gene, indicating that it is a primary 1α25$(OH)_2D_3$ responding gene [18,19]. By contrast, $p27^{kip1}$ protein levels appear to be regulated by a range of post-transcriptional mechanisms, such as enhanced mRNA translation and attenuating mechanisms that mediate its degradation, often in a cell type-specific manner [65–67]. The upregulation of the $p21^{waf1/cip1}$ and $p27^{kip1}$ principally mediate G_1 cell cycle arrest, but 1α25$(OH)_2D_3$ mediates a G_2/M cell cycle arrest in a number of cancer cell lines through the direct induction of a growth arrest and DNA damage gene (*GADD45α*) [37,62,68]. GADD45α inhibits the activation of mitosis-promoting B/cyclin dependent kinase (CDK)1 complexes. Again, however, this regulation appears to combine direct gene transcription and a range of post-transcriptional mechanisms. These studies highlight the difficulty of establishing strict transcriptional effects of the VDR and the range of post-transcriptional effects that act in concert to regulate target protein levels. Concomitant with these events is a downregulation of cyclins, such as A, D1 and E, decreases in kinase activities associated with activated complexes and, ultimately, the dephosphorylation of the retinoblastoma protein and sequestration of E2F family members in a repressive complex [69].

It is interesting to note that the levels of $p21^{waf/cipi1}$ and $p27^{kip1}$ mRNA expression play roles in the terminal differentiation of committed progenitor cells and, thus, 1α25$(OH)_2D_3$ may play an integrated role, with other transcription factors, in regulating self-renewal.

Programmed cell death

A common feature of certain cells, notably MCF-7 breast cancer cells, is a profound and rapid induction of apoptosis, irrespective of p53 content. This may reflect the role that the VDR plays in the involution of the postlactating mammary gland. The direct transcriptional targets that regulate these actions remain elusive to an extent, although there is growing evidence for the involvement of the Bcl-2 family of proteins [70,71]. Induction of programmed cell death following 1α25$(OH)_2D_3$ treatment is also associated with an increased generation of reactive oxygen species (ROS). 1α25$(OH)_2D_3$ treatment upregulates vitamin D-upregulated protein (VDUP)1, which binds to the disulfide-reducing protein thioredoxin and inhibits its ability to neutralise ROS, thereby potentiating stress-induced apoptosis [72,73].

Interestingly, the apoptotic responses in other cells, for example, LNCaP, appear to be delayed and less pronounced, occurring up to 6 days post-treatment. In which case, the apoptosis probably reflects less direct effects, but rather the integration of VDR signaling with other systems. Similarly in other cell systems, including myeloid cells, 1α25$(OH)_2D_3$ appears to mediate antiproliferative and prosurvival effects through the regulation of antiapoptotic target genes, such as *MCL-1*. In particular, myeloid cells undergo a profound monoctyic differentiation in response to 1α25$(OH)_2D_3$ treatment [58,74]. Taken together, these data suggest the extent and timing of apoptotic events arises through integration of VDR signaling with other cell signaling systems.

Adhesion & migration

A number of investigators have highlighted the effect of 1α25$(OH)_2D_3$ in regulating cellular homotypic adhesion and thereby suppress the invasive capacity of cells; many of these effects are associated with a more differentiated phenotype. A number of workers have demonstrated in colon cancer cell lines, such as CaCo-2 and HT29 cells, that 1α25$(OH)_2D_3$ treatment elevates expression of a number of brush-border-associated enzymes, such as alkaline phosphatase, as well as intermediate filaments, vinculin, ZO-1, ZO-2, desmosomes and E-cadherin [75]. E-cadherin is a major component of the adherent junctions and is essential for the maintenance of the epithelial phenotype, both through maintaining homotypic cell adhesion and by sequestrating β-catenin and, thereby, attenuating the mitogenic effects of Frizelled/Wnt signaling. The promoter/enhancer region of the *E-caderin* gene is a frequent target of epigenetic silencing of promoter CpG island methylation, reflecting this important dual role. *E-cadherin* mRNA is also regulated in other cell types, such as LNCaP and MCF-7, and may account for the suppression of the invasive phenotype displayed upon treatment with 1α25$(OH)_2D_3$ [76].

In an elegant series of studies, Munoz and colleagues have dissected the inter-relationships between the VDR, E-cadherin and the Wnt signaling pathway in colon cancer cell lines and primary tumors. In these studies, the induction of *E-cadherin* was seen in subpopulations of SW480 colon cancer cells, which express the VDR and respond to 1α25$(OH)_2D_3$. Thereby, the VDR limits the transcriptional effects of β-catenin by physically and directly binding it in the nucleus, and by upregulating E-cadherin to sequestrate β-catenin in the cytoplasm. In malignancy, these actions are corrupted through the downregulation of

VDR mRNA, which appears to be a direct consequence of binding by the transcriptional repressor SNAIL; itself a key regulator of the epithelial–mesenchyme transition, which is overexpressed in colon cancer [75,77,78].

Genomic integrity & DNA repair

An important and emergent area, both in terms of physiology and therapeutic exploitation, is the apparent role of the liganded VDR in maintaining genomic integrity and facilitating DNA repair. There appears to be close cooperation between VDR actions and the p53 tumor suppressor pathway. Correlative data suggest that cells that respond to $1\alpha25(OH)_2D_3$ generally have wild-type p53 and, at the molecular level, several target genes are shared by both signaling pathways, such as *p21*$^{waf1/cip1}$ and *GADD45α* [18,19,68–80]. Together, these findings suggest that signaling systems monitor and respond cooperatively to dietary and environmental signals to regulate mitosis negatively. Although this area has only emerged recently, there are a number of functional studies that support such cooperation; for example, $1\alpha25(OH)_2D_3$ enhances ionizing radiation-induced apoptosis of LNCaP cells, which retain wild-type p53 [81].

The antiproliferative effect of $1\alpha25(OH)_2D_3$ in MCF-7 and LNCaP cells has also been associated with the induction of *BRCA1* mRNA and protein via transcriptional activation, again supporting a role in genomic surveillance [82].

Integrated signaling

Collectively, these studies suggest an integrated aspect of VDR and other cell signaling systems, as well as the importance of cell context to determine the phenotypic response. The cooperative actions with other nuclear receptors and with receptor tyrosine kinases will be examined to illustrate these concepts further.

Cross-talk with other nuclear receptors

There is considerable evidence in the literature that the VDR cross-talks with other members of the NR1 subfamily of nuclear receptors. These range from direct physical interactions, to co-regulation of target gene promoters. Greater complexity has emerged as the RE, because the VDR have been found arranged in clusters, combined with the binding sites of other nuclear receptors to form more complex and integrated responsive regions, as found in the promoters of the CYP450 enzyme genes *CYP3A4*, *CYP24* and *CYP27* [23,24,83,84]. Also, there is evidence for more transient interactions through the exchange of cofactors, such as the central dimeric RXR partner, and the coordinated exchange of co-activators and co-repressors. The cellular readout of these molecular interactions can been seen in a number of studies that demonstrate cooperativity among $1\alpha25(OH)_2D_3$ and retinoids and PPAR ligands [10,25,79,85–87].

More broadly, a range of cooperative actions have been identified between the VDR and the principal sex steroid hormone receptors in breast and prostate cells. There appears to be reciprocal signaling between the VDR and ERα in breast cancer cells; for example, phytoestrogens, such as genistein, induce the VDR. Furthermore, $1\alpha25(OH)_2D_3$ and genistein cooperate to increase the stability of the VDR protein and to upregulate p21$^{waf1/cip1}$ in breast cancer models *in vitro* and to cooperate *in vivo* to regulate gut epithelial turnover and differentiation [88–90].

Cross-talk with receptor tyrosine kinases

Another concept to emerge is the integrated actions of the VDR with cell membrane-located receptor tyrosine kinases. These studies have revealed a high degree of co-regulation with members of the ERBB, transforming growth factor (TGF) and insulin-like growth factor (IGF) families. These signals are highly contextual and include both downregulation of growth-promoting signals, such as *IGF-I* or *ERBB1*, as well as upregulation of negative growth regulation and an increase in *IGFBP-3* [91,92]. Similarly, other pathways, such as those mediated by the TGF family, appear to be targeted with $1\alpha25(OH)_2D_3$; for example, by upregulating the *TGF-β2* receptor [93]. Internally, the VDR enhances a number of signal transduction pathways, with proteins on the p38 stress response pathway appearing to be both modulated by, and co-operatively acting with, the VDR [94,95]. Reflecting the contextual biology of these signal transduction pathways, the final phenotype response is divergent.

Mechanisms of suppression & resistance to the actions of the VDR

Reduced environmental availability of $1\alpha25(OH)_2D_3$

Epidemiological studies by Garland and colleagues have demonstrated that the intensity of local sunlight is correlated inversely with the risk of certain cancers, including breast, prostatic and colorectal carcinoma [96–101]. In support of these findings, levels of 25OH-D, the major circulating metabolite of vitamin D, are significantly lower in breast cancer patients than in age-matched controls [102]. Furthermore, there are reduced *CYP27b1* mRNA, as well as protein levels in breast cancer cell lines and primary tumors. Comparative genome hybridization studies have found that *CYP24* is amplified in human breast cancer and *CYP24* elevation is seen in primary breast tumors in relation to paired normal tissue, associated with altered patterns of $1\alpha25(OH)_2D_3$ metabolism [103,104]. Therefore, overexpression of 24-hydroxylase may further abrogate growth control mediated by $1\alpha25(OH)_2D_3$, via target cell inactivation of the hormone. Thus, the authors and others have proposed that cancer is associated with low circulating concentrations of 25OH-D, arising as a result of reduced exposure to sunlight, altered dietary patterns and impaired generation of $1\alpha25(OH)_2D_3$ within breast tissue [104–109].

Parallel epidemiological studies have also linked the incidence of prostate cancer to vitamin D insufficiency as a result of either diet or the environment. In 1990, Schwartz and colleagues suggested a role for vitamin D in decreasing the risk for prostate cancer based on the observation that mortality rates in the USA are inversely related to incident solar radiation [97]. Recently, a study of men in the San Francisco Bay area reported a reduced risk of advanced prostate cancer associated with high sun exposure and similar relationships have been established in

UK populations [99,110]. As with breast cancer, the proposed mechanism for the protective effects of sunlight on prostate risk involves the local generation of $1\alpha25(OH)_2D_3$ from circulating 25OH-D in prostate epithelial cells. Cancerous prostate cells express reduced 1α-hydroxylase activity. Prediagnostic serum levels of 25OH-D have been assessed in several prospective studies, with some reporting an increased risk among men with low circulating levels of the vitamin D metabolite, and a suggestion of an inverse relationship with advanced disease [96,107,111,112].

As with breast and prostate cancer, some epidemiological studies have noted that colon cancer risk and mortality increase with increasing latitude; for example, adjusted death rates from colon cancer in Caucasian males in the USA were nearly three-times higher in north eastern than sunnier more southerly states [113].

Cellular resistance to the actions of the VDR

A major limitation in the therapeutic exploitation of $1\alpha25(OH)_2D_3$ in cancer therapies is the resistance of cells to $1\alpha25(OH)_2D_3$, as cancer and leukemic cell lines often display a spectrum of sensitivities, including complete insensitivity to $1\alpha25(OH)_2D_3$, irrespective of VDR expression. One research focus to overcome this has involved the development of analogs of $1\alpha25(OH)_2D_3$, and multiple studies have demonstrated that these compounds have some enhanced potency, although resistance remains an issue. The molecular mechanisms for $1\alpha25(OH)_2D_3$ insensitivity in cancer are emerging. The VDR is neither mutated nor is there a clear relationship between VDR expression and growth inhibition by $1\alpha25(OH)_2D_3$ [114].

Genetic resistance

The gene encoding the VDR protein is known to display polymorphic variation. Thus, polymorphisms in the 3′ and 5′ regions of the gene have been described and variously associated with risk of breast, prostate and colon cancer, although the functional consequences remain to be established clearly. For example, a start codon polymorphism in exon II at the 5′ end of the gene, determined using the *fok*I restriction enzyme, results in a truncated protein. At the 3′ end of the gene, three polymorphsms have been identified that do not lead to any change in either the transcribed mRNA or the translated protein. The first two sequences generate *Bsm*I and *Apa*I restriction sites and are intronic, lying between exons 8 and 9. The third polymorphism, which generates a *Taq*I restriction site, lies in exon 9 and leads to a silent codon change (from ATT to ATC). Both insert an isoleucine residue at position 352. These three polymorphisms are linked to a further gene variation, a variable length adenosine sequence within the 3′ untranslated region (UTR). The polyA sequence varies in length and can be segregated into two groups: long (L) sequences of 18–24 adenosines or short (S) sequences [96,115–117].

Multiple studies have addressed the association between VDR genotype and cancer risk and progression. In breast cancer, the *Apa*I polymorphism shows a significant association with breast cancer risk, as indeed have *Bsm*I and the L polyA variant. Similarly, the *Apa*I polymorphism is associated with metastases to bone [118,119]. The functional consequences of the *Bsm*I, *Apa*I and *Taq*I polymorphisms are unclear but, due to genetic linkage, they may act as a marker for the polyA sequence within the 3′UTR, which in turn determine transcript stability. Interestingly, combined polymorphisms and serum 25OH-D levels compound breast cancer risk and disease severity further [120].

Earlier studies suggested that polymorphisms in the *VDR* gene might also be associated with risk factors of prostate cancer. Ntais and colleagues performed a meta-analysis of 14 published studies with four common gene polymorphisms (*Taq*1, polyA repeat, *Bsm*1 and *Fok*1) in individuals of European, Asian and African descent. They concluded from the study that these polymorphisms are unlikely to be major determinants of susceptibility to prostate cancer on a wide population basis [121]. Equally, studies in colon cancer have yet to reveal conclusive relationships and may possibly be dependent upon the ethnicity of the population studied.

Epigenetic resistance

To date, no cytogenetic abnormalities of the *VDR* gene have been reported. Therefore, the authors and others have begun to explore epigenetic mechanisms that disrupt VDR signaling. The lack of an antiproliferative response is reflected by a suppression of the transcriptional responsiveness of antiproliferative target genes, such as $p21^{waf/cip1}$, $p27^{kip1}$, *GADD45α* and *BRCA1* [37,82,122,123]. Paradoxically, VDR transactivation is sustained or even enhanced, as measured by induction of the highly $1\alpha25(OH)_2D_3$-inducible *CYP24* gene [124,125]. Together, these data suggest that the VDR transcriptome is skewed in cancer cells to disfavor antiproliferative target genes and the lack of functional VDR alone cannot explain resistance. The authors have proposed that apparent $1\alpha25(OH)_2D_3$ insensitivity is the result of epigenetic events, which skew the promoter responsiveness to suppress responsiveness of specific target gene promoters.

In support, the authors found frequently elevated co-repressor mRNA expression, most commonly involving *NCoR2/SMRT*, in malignant prostate primary cultures and cell lines, with reduced $1\alpha25(OH)_2D_3$ antiproliferative response. These data indicated that the ratio of VDR to co-repressor may be critical to determine $1\alpha25(OH)_2D_3$ responsiveness in cancer cells. The authors reasoned that this molecular lesion could be targeted by co-treatment of ligand [$1\alpha25(OH)_2D_3$] plus the (HDAC) inhibitors, such as trichostatin A. These approaches restored the $1\alpha25(OH)_2D_3$ response of the androgen-independent PC-3 cells to levels indistinguishable from control normal prostate epithelial cells. This reversal of $1\alpha25(OH)_2D_3$ insensitivity was associated with re-expression of gene targets associated with the control of proliferation and induction of apoptosis, notably *GADD45α*. A small interfering (si)RNA approach towards *NCoR2/SMRT* demonstrated the significant role that this co-repressor plays in regulating this response, with its repression resulting in profound

enhancement of the induction of *GADD45α* in response to $1\alpha25(OH)_2D_3$. These data support a central role for elevated *NCoR2/SMRT* levels in suppressing the induction of key target genes, resulting in a loss of sensitivity to the antiproliferative action of $1\alpha25(OH)_2D_3$ [37,82,122].

In parallel studies, the authors have demonstrated a similar spectrum of reduced $1\alpha25(OH)_2D_3$ responsiveness between nonmalignant breast epithelial cells and breast cancer cell lines. Again, this was not determined solely by a linear relationship between the levels of $1\alpha25(OH)_2D_3$ and VDR mRNA expression. Rather, elevated co-repressor mRNA levels, notably *NCoR1*, in ERα-negative breast cancer cell lines, and primary cultures were associated with $1\alpha25(OH)_2D_3$ insensitivity. Again, targeting this molecular lesion through cotreatments of $1\alpha25(OH)_2D_3$ with HDAC inhibitors coordinately regulated VDR targets, such as $p21^{waf/cipi1}$ and *GADD45α*, and restored antiproliferative responsiveness [126,127].

Together, these data support the concept that altered patterns of co-repressors inappropriately sustain histone deacetylation around the VDRE of target gene promoter/enhancer regions, and shift the dynamic equilibrium between *apo* and *holo* receptor conformations to favor transcriptional repression of key target genes, such as $p21^{waf1/cip1}$ or *GADD45α*. Thus, VDR gene targets are less responsive in $1\alpha25(OH)_2D_3$ insensitive cancer cells compared with nonmalignant counterparts. Furthermore, targeting this molecular lesion with cotreatments of vitamin D_3 compounds plus HDAC inhibitors generates a temporal window where the equilibrium point between *apo* and *holo* complexes is shifted to favor a more transcriptionally permissive environment.

These findings compliment a number of parallel studies undertaken by others, which have established cooperativity between $1\alpha25(OH)_2D_3$ and butyrate compounds, such as sodium butyrate (NaB) [128–133]. These compounds are short-chain fatty acids produced during fermentation by endogenous intestinal bacteria and have the capacity to act as HDAC inhibitors. Stein and colleagues have identified the effects in colon cancer cells of $1\alpha25(OH)_2D_3$ plus NaB cotreatments to include the coordinate regulation of the VDR itself. In the authors' studies, in the time frame studied (0–24 h), no evidence for changes in VDR mRNA levels upon cotreatment with $1\alpha25(OH)_2D_3$ plus trichostatin (TS)A was seen. However, these studies together underscore further the importance of the dietary-derived milieu to regulate epithelial proliferation and differentiation beyond sites of action in the gut.

Expert commentary

Proliferation and differentiation of a number of normal cell types, such as prostate and breast epithelial cells and also myeloid cell types, has encouraged a number of workers to undertake clinical evaluation of vitamin D compounds. However, the results of these trials have been largely equivocal, that is, there have been few clear sustained clinical responses that recapitulate the encouraging *in vitro* studies. More recently, researchers have taken two alternative approaches with clearer clinical responses. Many of these studies are being driven by teams in the USA. Thus, Beer and colleagues have championed dosing regimes with far higher doses than previously thought tolerated in prostate cancer patients and delivered more sustained clinical responses. Trump and colleagues have also considered the option of cotreatments of $1\alpha25(OH)_2D_3$ in combination with established chemotherapy regimes or novel agents, such as HDAC inhibitors [134–138].

It would seem that $1\alpha25(OH)_2D_3$ and derivative compounds will be used in a more focused manner, either in a chemoprevention or chemotherapy context with active monitoring of disease. It will be important to diagnose patients who have a $1\alpha25(OH)_2D_3$-responsive molecular profile, for example, of receptor and cofactors, and to measure the responsiveness of the VDR transcriptome. Equally, combination therapies with $1\alpha25(OH)_2D_3$ compounds are most likely to demonstrate the most potent profiles. It is also likely that the understanding generated from VDR biology will inform the clinical translation of ligands that target the related receptors, for example, PPARs, FXR or LXR.

Five-year view

Historically, researchers have studied the abilities of single nuclear receptors, such as the VDR, to regulate a discrete group of gene targets and influence cell function. This has led to substantial knowledge concerning many of these receptors individually. Cell and organism function, however, depend on the dynamic interaction of a collection of receptors through the networks that link them. The current lack of an integral view of how these interactions bring about function and dysfunction, for example, in the aging human individual, can be attributed to the relatively recent limitation of available techniques and tools to undertake such studies. The implementation of the new postgenomic techniques, together with bioinformatics and systems biology methodology, will generate such an integral view of the processes, thereby revealing and quantifying the mechanisms by which cells, tissues and organisms interact with diet [139]. This transition will allow VDR processes to be described in the dynamic interaction with other nuclear receptors, as well as the cell signal transduction pathways to identify critical nodes of control. Ultimately, this will deliver a predictive, preventative and personalized understanding of the dietary interactions in the individual.

The analyses of adult stem cell components in tissues, such as the colon, prostate and breast, are currently being dissected intensively. A number of basic questions regarding the cellular process in these cells and the committed progenitors they give rise to remain to be answered. For example, it is becoming clear that the stem cells in these tissues do not express the critical sex steroid nuclear receptors, thus, breast stem cells neither express the ERα nor respond directly to estrogenic ligands, which has profound implications for the diagnosis and treatment of breast cancer [140]. It remains unknown at what point the VDR and other dietary-sensing receptors are expressed and are responsive in the models of tissue self-renewal. Clearly, understanding these basic aspects will have significant implications for the understanding of dietary signaling capacity in human health and disease.

Key issues

- Diet forms one of the most significant factors that influences the initiation and/or progression of common cancers, such as those of the gastrointestinal tract, prostate and mammary glands.
- Resolving these relationships has the potential to transform acute, potentially lethal diseases, such as cancer, to chronic conditions that can be contained more readily.
- The nuclear receptor superfamily plays a key role in sensing a diverse range of macro- and micro-nutrients and, therefore, is being investigated intensively for its potential to mediate anticancer therapies.
- Within this family, the vitamin D receptor (VDR) plays a central role in controling serum calcium levels. More recently, it has emerged that a diverse range of cell types express this receptor and it regulates a range of cell fate decions.
- Exploitation of these relationships in cancer is being explored, but several hurdles will need to be cleared to achieve full clinical translation. The correct level of serum vitamin D remains an area of controversy, and the requirement for ultraviolet radiation to catalyze its synthesis has the potential to contradict skin-care health compaigns. Equally, cellular mechanisms of resistance towards the VDR appear to either be selected for or emerge in cancer cells.

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