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Profiling 1,25-dihydroxyvitamin D_3-regulated gene expression by microarray analysis☆

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Abstract

Vitamin D was discovered as a preventive agent of nutritional rickets, a defect in bone development due to inadequate uptake of dietary calcium. However, a variety of studies over the last several years has revealed that Vitamin D controls much more than calcium homeostasis. In particular, there is widespread evidence that the hormonal form of Vitamin D, 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], is an important regulator of cellular differentiation and proliferation. Direct genomic signaling by 1,25$(OH)_2D_3$ occurs through the Vitamin D receptor, which is a nuclear receptor and a ligand-activated regulator of gene transcription. 1,25$(OH)_2D_3$ can therefore directly regulate patterns of gene expression within a target cell. The development of high throughput genomics technologies have greatly enhanced our capacity to identify the genetic and biochemical changes associated with the physiological actions of 1,25$(OH)_2D_3$. Microarray analyses of expression profiles in 1,25$(OH)_2D_3$-treated cells have underlined its widespread effects on cellular differentiation and proliferation. They have provided a molecular basis for the accumulating epidemiological and preclinical evidence indicating that 1,25$(OH)_2D_3$ can act as a chemopreventive agent against several malignancies including cancers of the prostate and colon. In addition, they have underlined the therapeutic potential of 1,25$(OH)_2D_3$ analogues as modulators of immune system function.

Keywords: 1α,25-Dihydroxyvitamin D_3; Vitamin D receptor; Genomics; Microarray; Gene expression profiling; Cancer chemoprevention; Immune system

1. Introduction to Vitamin D signaling

Naturally occurring Vitamin D3 is found in a limited number of dietary sources, and is produced by the photochemical/thermal conversion of cutaneous 7-dehydrocholesterol by ultraviolet light [1]. Vitamin D3 is one of several factors produced by the skin's complex homeostatic system, which functions as a protective barrier against the environment, and is directly connected to the body's immune and neuroendocrine functions [2]. Vitamin D3 is 25-hydroxylated in the liver and converted into the hormonal 1α,25-dihydroxy form [1,25$(OH)_2D_3$] in the kidney and peripheral organs [2,3]. Vitamin D compounds are catabolized via 24-hydroxylation by the cytochrome P450 CYP24, giving rise to 1α,24,25-trihydroxyvitamin D_3, which is 10 times less potent than 1α,25$(OH)_2D_3$ [4]. Further oxidation leads to progressive loss of biological activity and to production of water-soluble calcitroic acid, which is excreted [5].

Much of 1,25$(OH)_2D_3$ signaling occurs through its cognate nuclear Vitamin D receptor (VDR) [6], which is a member of the nuclear receptor family and a direct regulator of gene transcription. The domain structure of the VDR is typical of nuclear receptors, with highly conserved DNA binding and ligand binding domains (LBDs). Similar to several nuclear receptors, the VDR functions as a heterodimer with members of the retinoid X receptor (RXR) family of receptors. Strong interactions between VDR and RXR LBDs are essential for stable dimerization and high affinity DNA binding. Nuclear receptors regulate transcription in part by binding specific DNA sequences known collectively as hormone response elements, which are generally composed of tandem hexameric motifs, and normally located in the 5′-flanking region of target genes [7]. Vitamin D response elements (VDREs) are composed of tandem motifs with the consensus PuG(G/T)TCA, which are often arranged as direct repeats separated by 3 bp (DR3-type). However, VDR/RXR heterodimers can also recognize everted repeats of hexameric motifs (also known as inverted palindromes, in which the upstream motif is flipped through 180°) spaced by either 6 or 9 bp [8–11]. Nuclear receptors regulate transcription of target genes by ligand-dependent recruitment of accessory proteins known collectively as coregulators. A detailed discussion of coregulator recruitment and their function in regulation of transcription is beyond the scope of this

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review. For more detail, please see reviews on coregulators in general by McKenna and O'Malley [12], and those regulating the VDR in particular by Rachez and Freedman [13].

2. The pleiotropic physiological consequences of $1,25(OH)_2D_3$ signaling

Signal transduction by $1,25(OH)_2D_3$ has a broad range of physiological effects [2,3]. Primarily, $1,25(OH)_2D_3$ controls calcium transport in the intestinal epithelia, and modulates bone resorption. Several studies over the years have revealed the widespread effects of $1,25(OH)_2D_3$ on cellular proliferation and differentiation ([3] and Refs. therein). $1,25(OH)_2D_3$ also inhibits cell proliferation in several models of cancer, including myeloid leukemia, melanoma and carcinomas of the breast, prostate, colon and head and neck [3]. Moreover, $1\alpha,25(OH)_2D_3$ analogues display chemopreventive actions in animal models of colon, hamster cheek pouch, hepatocellular, gastrointestinal and skin carcinogenesis ([14] and Refs. therein). These preclinical studies provide support for epidemiological data correlating the prevalence of certain cancers, particularly prostate and colon cancers, and exposure to sunlight, consistent with chemopreventive effects of $1\alpha,25(OH)_2D_3$ [14].

Growing evidence also indicates that $1\alpha,25(OH)_2D_3$ is an important modulator of the immune responses, consistent with broad expression of the VDR in cells of the immune system and the capacity of $1\alpha,25(OH)_2D_3$ to regulate cellular differentiation. Indeed, mice in which the VDR gene had been ablated displayed abnormal pro-inflammatory T helper 1 (Th1) cell development [15], and mice rendered $1\alpha,25(OH)_2D_3$ deficient by knockout of the gene encoding 25-hydroxyvitamin D3 1α-hydroxylase were deficient in peripheral T lymphocytes [16]. Moreover, $1,25(OH)_2D_3$ inhibits dendritic cell maturation, which is critical for T cell-mediated immune responses [17–19], and reduces expression of the cytokine interleukin-12 (IL-12), whose signaling is critical for Th1 maturation.

3. Genomic analysis of signaling by $1,25(OH)_2D_3$ and its analogues

Given that $1,25(OH)_2D_3$ signals through a nuclear receptor and is thus a direct regulator of gene transcription, its signaling is ideally suited for study by microarray analysis. We have been interested in studying the anticancer properties of $1,25(OH)_2D_3$ analogues, and particularly their potential as chemopreventive agents for head and neck squamous carcinoma (HNSCC). Early stage HNSCC can be successfully treated with surgery and/or radiation therapy. However, primary tumors are often associated with areas of dysplastic epithelia, which lead to the development of second primary carcinomas [20]. Thus, it is important to identify chemopreventive agents in HNSCC. While the limiting factor for use of $1,25(OH)_2D_3$ in cancer therapy has been hypercalcemia, many potent analogs have been developed with reduced calcemic effects [14,21]. The analogue EB1089 contains a side chain modified to render it less susceptible to catabolic degradation [22]. In vivo studies of HNSCC, prostate and breast carcinomas using therapeutic EB1089 dosages showed no clinically significant hypercalcemia [22,23].

We have performed a series of microarray analyses of $1,25(OH)_2D_3$ and EB1089 signaling in human SCC25 cells [24,25], which although derived from a floor of the mouth/base of the tongue tumor, display a relatively differentiated phenotype [26], and thus approximate the dysplastic epithelia that would be the target of HNSCC chemoprevention. In contrast to less differentiated HNSCC cell lines, SCC25 cell proliferation is completely arrest in G0/G1 by $1,25(OH)_2D_3$ and EB1089 treatment [24]. Our studies to date have identified almost 200 $1,25(OH)_2D_3$/EB1089 target genes in SCC25 cells. Analysis of a time course of EB1089-regulated gene expression over 48 h in SCC25 cells identified 89 up- and 63 downregulated genes [25]. Clustering analyses distributed upregulated genes into five groups based on their kinetics of induction. Similarly, downregulated genes were distributed into groups that essentially mirrored those of upregulated genes. Although the regulation of several genes was relatively slow (not peaking after 48 h), analysis of the effects of the protein synthesis inhibitor cycloheximide on expression indicated EB1089 directly regulated the vast majority of target genes identified ([25] and unpublished results). Notably, we also found that two upregulated genes, CYP24 and osteopontin, whose promoters contain well-characterized VDREs [27,28] fell into different clusters. CYP24, whose induction peaked after 6 h of EB1089 treatment, was among the most rapidly regulated genes, whereas regulation of the osteopontin gene was significantly slower, peaking at 24 h. This indicates that the kinetics of gene induction by the EB1089-activated VDR bound to different VDREs is strongly promoter-specific.

It is also noteworthy that a comparison of several upregulated and downregulated targets did not reveal any evidence for gene-specific differences in the efficacy of regulation by $1,25(OH)_2D_3$ and EB1089 [25]. However, in a number of instances regulation by $1,25(OH)_2D_3$ was more transient that that of EB1089, returning to or near baseline within 48 h. The enhanced duration of EB1089-regulated gene expression was ascribed to its insensitivity to catabolism by CYP24 [29]. Treatment of cells with $1,25(OH)_2D_3$ and the cytochrome P450 inhibitor ketoconazole produced gene expression profiles that were essentially identical to those seen in cells treated by EB1089 alone [25].

4. Genomic profiling provides a molecular basis for the chemopreventive action of $1\alpha,25(OH)_2D_3$ analogues

Microarray analyses, coupled with other experiments, have revealed some of the molecular events underlying

the chemopreventive effects of $1\alpha,25(OH)_2D_3$ analogues. Collectively, these studies have shown that EB1089 performs three key functions of a chemopreventive agent; it is antiproliferative, it induces cellular differentiation, and has potential genoprotective effects. It is unlikely that regulation of a single gene controls the antiproliferative effects of $1\alpha,25(OH)_2D_3$ analogues. Antiproliferative activity has been associated with enhanced transforming growth factor-β signaling [30], amphiregulin [31] and to cell-specific induction of cyclin-dependent kinase (CDK) inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ at both transcriptional and post-transcriptional levels [3,14,23,30,32–34]. Increased levels of 27^{KIP1} in $1\alpha,25(OH)_2D_3$-treated HNSCC cells were associated with reduced protein turnover rather than enhanced gene transcription [34], and correlated with $1\alpha,25(OH)_2D_3$-dependent repression of cks1 and $p45^{skp2}$ genes, which encode components of the SCF^{SKP2} ubiquitin ligase that regulates $p27^{KIP1}$ turnover [34]. In addition, microarray studies revealed that EB1089 downregulated expression of several mitogenic factors in HNSCC cells. These included VEGF-related protein, which is mitogenic in Kaposi's Sarcoma and hematopoietic cells [35], Cyr61, which encodes a growth factor implicated in angiogenesis and tumorigenesis, whose expression is induced by estrogen in breast cancer cells [36], and midkine (MK), a mitogenic factor overexpressed in several carcinomas [37].

Several target genes identified provided evidence that EB1089 treatment drives SCC25 cells towards a more differentiated state, thereby reversing their malignant phenotype (Fig. 1). EB1089 repressed the expression of several markers associated with cancer progression (e.g. N-cadherin, squamous cell carcinoma antigen (SCCA), tenascin C, tumor antigen L6, carcinoma associated antigen GA733-2) and induced the expression of several genes associated with epithelial cell differentiation (cystatin M, protease M, type XIII collagen, desmoglein 3). For example, SCCA is a serum marker of uterine cervix, head and neck, lung and esophageal cancers, and ablation of its expression inhibits

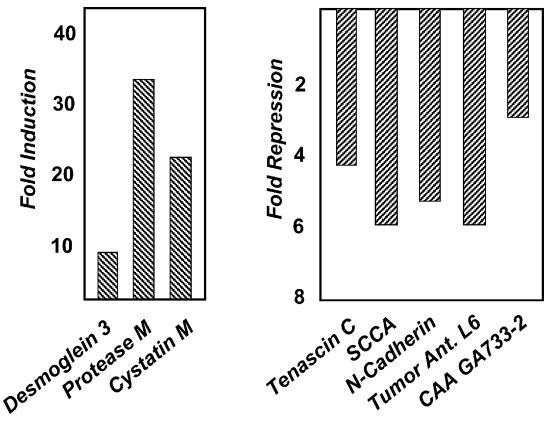

Fig. 1. Regulation of markers of epithelial cell differentiation (left panel) and cancer progression (right panel) by EB1089 in human SCC25 HNSCC cells. See Ref. [25] for details.

growth and induces natural killer cell infiltration of tumors [38]. Overexpression of N-cadherin in HNSCC cells is consistent with the phenomenon of "cadherin switching" in seen in carcinomas. Other work has shown that repression of overexpressed N-cadherin in HNSCC is associated with restoration of an epithelial phenotype [39]. In addition, the inhibition of $p45^{SKP2}$ expression observed in $1\alpha,25(OH)_2D_3$-treated HNSCC cells [34] is noteworthy because its overexpression is associated with a poor prognosis in HNSCC [40].

The potential genoprotective effects of $1,25(OH)_2D_3$ analogues were underlined by the observation that EB1089 induced expression of the growth arrest and DNA damage gene (GADD45α) in HNSCC cells [23,24], and that EB1089 induced GADD45α expression in tumor xenografts of a mouse model of HNSCC [23]. Recent studies have also shown that $1,25(OH)_2D_3$ induced GADD45α expression in insulinoma cells [41]. The identification of GADD45α as a $1\alpha,25(OH)_2D_3$ target gene is important as it is directly implicated in DNA repair and is required for maintenance of global genomic stability [42]. EB1089 also induced expression of several genes and corresponding enzymes that are implicated in control of redox balance. These included glucose-6-phosphate dehydrogenase, which lies at the head of the pentose phosphate shunt, a source of reducing equivalents, glutathione peroxidase and thioredoxin reductase ([25] and unpublished results). Significantly, induction of thioredoxin reductase activity has also been observed in microarray analyses of $1\alpha,25(OH)_2D_3$-treated prostate and breast carcinoma cells [43,44]. These findings are consistent studies showing that treatment of leukemic cells with $1\alpha,25(OH)_2D_3$ reduces intracellular levels of reactive oxygen species (ROS) [45]. It can be speculated that the antioxidant effects of $1\alpha,25(OH)_2D_3$ signaling may represent a physiological feedback loop to the photochemical synthesis of Vitamin D in skin by ultraviolet light, which is a DNA damaging agent and an inducer of ROS.

EB1089 also stimulated expression of the gene encoding the NRF2 transcription factor [25]. NRF2 is induced by a number of chemopreventive agents, and in turn stimulates expression of several phase II detoxifying enzymes. Ablation of the nrf2 gene in mice rendered them more resistant to carcinogenesis and eliminated the beneficial effects of chemopreventive agents [46]. An enhancement of xenobiotic metabolism by $1\alpha,25(OH)_2D_3$ is also consistent with its direct induction of several genes encoding members of the cytochrome P450 family of oxidative enzymes [9–11].

5. Insights from microarray studies into $1,25(OH)_2D_3$-dependent regulation of immune system function

Keratinocytes are considered to be an integral part of the immune system of the skin [2], and treatment of SCC25 cells with EB1089 provided multiple insights into the im-

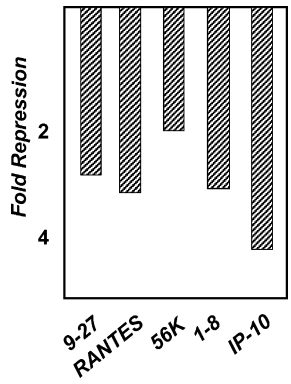

Fig. 2. Downregulation of IFNγ-regulated genes by EB1089 in SCC25 HNSCC cells. See Ref. [25] for details.

munomodulatory effects of $1,25(OH)_2D_3$ [25]. The role of $1,25(OH)_2D_3$ in controlling the function of epithelial cells in innate immunity was emphasized by the strong induction of the gene encoding the pattern receptor CD14, which is also a $1,25(OH)_2D_3$ target gene in monocytic HL60 cells [47]. Significantly, EB1089 downregulated interferon γ (IFNγ)-regulated genes encoding 9–27, 1–8D, interferon-inducible 56 K protein, the T cell chemokine IP-10, and the chemokine RANTES (Fig. 2), consistent with an inhibition of IFNγ signaling. Notably, $1,25(OH)_2D_3$ also strongly induced expression of T1/ST2, a member of the interleukin-1 receptor family. Gene ablation studies have revealed that T1/ST2 signaling is essential for normal T helper 2, Th2, cell differentiation [48]. These results are consistent with EB1089 stimulating Th2 responses, and inhibiting a number of genes associated with proinflammatory Th1 responses.

Data from microarray analyses have helped provide a molecular basis for the therapeutic effects of $1\alpha,25(OH)_2D_3$ analogues in treatment Th1-stimulated autoimmune diseases. Studies in mice have shown that $1\alpha,25(OH)_2D_3$ can inhibit progression of systemic lupus erythematosus, the multiple sclerosis-like disease experimental autoimmune encephalomyelitis (EAE), collagen-induced arthritis, inflammatory bowel disease and autoimmune diabetes [3]. EAE is induced by treatment of mice with myelin basic protein. Dietary $1\alpha,25(OH)_2D_3$ inhibited the onset of EAE and the progression of established disease [3,49]. $1\alpha,25(OH)_2D_3$ analogues account for 50% of all drugs used to treat mild to moderate psoriasis, a Th1-driven chronic inflammatory skin disease psoriasis, which affects 2% of the population. $1\alpha,25(OH)_2D_3$ analogues are used topically, and one of the most thoroughly tested is the compound calcipotriol, which is effective either alone or when administered in combination with anti-inflammatory steroids [50]. Enhanced IFNγ signaling and overexpression of IP-10, both of which inhibited in EB1089-treated SCC25 cells [25], underlie the inflammatory reactions in psoriasis.

6. Conclusions

The broad expression pattern of the VDR, and the widespread effects of its cognate hormone on cellular differentiation and proliferation have opened up a number of new fields of investigation for researchers interested in $1\alpha,25(OH)_2D_3$ action over and above its effects on calcium homeostasis. While considerable work remains to done to fully understand the potential of $1\alpha,25(OH)_2D_3$ analogues as anticancer agents, and immune system modulators, microarray analyses have generated important data that have helped provide a molecular basis for their therapeutic actions. Some of the numerous novel genetic markers of $1\alpha,25(OH)_2D_3$ signaling identified in microarray studies will undoubtedly be useful in assessing the therapeutic potential of analogues.

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