CHAPTER

THE VITAMIN D RECEPTOR: A Tumor Suppressor in Skin

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Abstract: Cutaneous malignancies including melanomas and non melanoma skin cancers (NMSC) are the most common types of cancer, occurring at a rate of over 1 million per year in the United States. The major cell in the epidermis, the keratinocyte, not only produces vitamin D but contains the enzymatic machinery to metabolize vitamin D to its active metabolite, $1,25(OH)_2D$, and expresses the receptor for this metabolite, the vitamin D receptor (VDR), allowing the cell to respond to the $1,25(OH)_2D$ that it produces. In vitro, $1,25(OH)_2D$ stimulates the differentiation and inhibits the proliferation of these cells and so would be expected to be tumor suppressive. However, epidemiologic evidence demonstrating a negative relationship between circulating levels of the substrate for CYP27B1, 25OHD, and the incidence of these malignancies is mixed, raising the question whether vitamin D is protective in the in vivo setting. UV radiation (UV), both UVB and UVA, as occurs with sunlight exposure is generally regarded as causal for these malignancies, but UVB is also required for vitamin D synthesis in the skin. This complicates conclusions reached from epidemiologic studies in that UVB is associated with higher 25OHD levels as well as increased incidence of cutaneous malignancies. Based on our own data and that reported in the literature we hypothesize that vitamin D signaling in the skin suppresses UVR induced epidermal tumor formation. In this chapter we will first discuss recent data regarding potential mechanisms by which vitamin D signaling suppresses tumor formation, then focus on three general mechanisms that mediate tumor suppression by VDR in the skin: inhibition of proliferation and stimulation of differentiation, immune regulation, and stimulation of DNA damage repair (DDR).

Sunlight, Vitamin D and Skin Cancer, Second Edition, edited by Jörg Reichrath.

INTRODUCTION

Over 1 million skin cancers occur annually in the United States, 80% of which are basal cell carcinomas (BCC), 16% squamous cell carcinomas (SCC), and 4% melanomas, making skin cancer by far the most common cancer afflicting humankind.[1] UV radiation (UVR) from the sun is the major etiologic agent for these cancers. The highest energy UVR, UVC (below 280 nm), does not penetrate the atmosphere. Of the solar radiation that does reach the earth 95% is UVA and 5% UVB. UVB (280–320 nm), although it does not penetrate past the epidermis, is absorbed by DNA in the epidermal cells creating characteristic mutations identified as cyclobutane pyrimidine dimers (CPDs) and pyrimidine(6–4)pyrimidone photoproducts (6–4PP), which if not repaired result in C to T or CC to TT mutations, the UVB "signature" lesion[2,3]. UV wavelengths between 320–400 nm (UVA) are capable of penetrating into the dermis, and do their DNA damage (e.g., 8 hydroxy 2' deoxyguanosine production) primarily by oxidative processes, although at high enough dose levels UVA can produce CPDs.[4] On the other hand UVB is required to convert 7-dehydrocholesterol levels in the skin to pre vitamin D_3, which then isomerizes to vitamin D_3. Moreover, the skin is capable of converting the vitamin D produced to its active metabolite $1,25(OH)_2D$,[5] and this conversion is potentiated by UVR at least in part by cytokines such as TNF-α[6] which are increased by UVR in the epidermis.[7] Both melanocytes[8] and keratinocytes[9] express the vitamin D receptor (VDR) and respond to $1,25(OH)_2D$ with reduced proliferation and increased differentiation.[10,11] Sun avoidance may reduce one's risk of developing skin cancer, but this practice generally results in suboptimal levels of vitamin D in the body. In an analysis by Lucas et al.,[12] the global disease burden due to UVR is substantially less than the disease burden due to vitamin D deficiency. Vitamin D supplementation can compensate, but the skin remains the major site of vitamin D availability for most of the world's population. Moreover, low dose UVR may be protective against skin cancer via the vitamin D signaling mechanisms that will be reviewed in this article, and some epidemiologic evidence is consistent with a potential benefit of low dose UVR. For example, in the study by Armstrong and Kricker,[13] a slight decrease in the incidence of SCC, BCC, and melanomas in 10 US populations was observed when the solar UV measurement was increased from 100 to 110, although higher levels increased the incidence. This same group,[14] evaluating data from the Australian population, did not find a significant increase in SCC with time spent out of doors in the general population. Rosso et al.[15] in a multicenter European study did not find a significant increase in SCC below a threshold of 70,000 accumulated hours of sunshine, although the development of BCC had a lower threshold. In this chapter, after a review of vitamin D metabolism and VDR function, we will examine potential mechanisms that have been proposed for vitamin D induced antitumor mechanisms in general, then focus on those mechanisms that have been shown to be operative in the epidermis.

VITAMIN D METABOLISM

Vitamin D_3 is produced from 7-dehydrocholesterol (7-DHC). Irradiation of 7-DHC with UVB produces pre vitamin D_3, which subsequently undergoes a temperature-dependent rearrangement of the triene structure to form vitamin D_3, lumisterol, and tachysterol. This process is relatively rapid and reaches a maximum within hours[16-18] although both the degree of epidermal pigmentation and the intensity

of exposure influence the time required to achieve this maximal concentration of pre vitamin D_3. With continued UV exposure the biologically inactive lumisterol and tachysterol accumulate eliminating the risk of excessive production of vitamin D. Sunlight exposure increases melanin production, which can absorb UVB, and so provides another mechanism by which excess vitamin D_3 production can be prevented. The intensity of UVR is dependent on latitude and season. In Edmonton, Canada (52°N) very little vitamin D_3 is produced in exposed skin from mid-October to mid-April, while in San Juan (18°N) the skin is able to produce vitamin D_3 all year long.[19] Clothing and sunscreen effectively prevent vitamin D_3 production in the covered areas. Vitamin D_3 produced in the skin can be carried to the liver and other tissues for further metabolism to 25-hydroxyvitamin D (25OHD) and then to the kidney to produce $1,25(OH)_2D$ by the enzyme CYP27B1. However, as noted above, the keratinocyte contains the entire pathway for $1,25(OH)_2D$ production from vitamin D.

The production of $1,25(OH)_2D$ in the skin is under quite different regulation compared with its production by the kidney. In the kidney parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23) and $1,25(OH)_2D$ itself are the principal hormonal regulators: PTH stimulates, whereas FGF23 and $1,25(OH)_2D$ inhibit $1,25(OH)_2D$ production. Keratinocytes respond to PTH with increased $1,25(OH)_2D$ production, but these cells do not have the classic PTH receptor and do not respond to cyclic AMP[5] unlike the kidney. The effect of FGF23 on keratinocyte CYP27B1 expression or function has not been reported. Furthermore, unlike the kidney, $1,25(OH)_2D$ does not directly affect CYP27B1 expression in keratinocytes. Rather, $1,25(OH)_2D$ regulates its own levels in the keratinocyte by inducing CYP24, the catabolic enzyme for $1,25(OH)_2D_3$.[20] In the keratinocyte the major regulators of $1,25(OH)_2D$ production are cytokines such as tumor necrosis factor-α (TNF)[6] and interferon-γ (IFN).[21] These cytokines are activated in the skin by UVB, which of course also increases the substrate via increased vitamin D production.

VITAMIN D RECEPTOR: MECHANISM OF ACTION

The VDR is a member of the nuclear hormone receptor superfamily.[22] These members are characterized by a highly conserved DNA binding domain characterized by two zinc fingers and a structurally conserved ligand binding domain that has at its C-terminal end the AF2 domain to which coactivator complexes bind.[23] The ligand binding domain also serves as the region to which VDR binds to its transcriptional partners, RXR being the major one. In general ligand (ie. $1,25(OH)_2D$) binding is required for the VDR/RXR heterodimer to form and bind to those regions on the DNA called vitamin D response elements (VDRE). Ligand binding also alters the structure of the VDR with major movement of helix 12 (C-terminus) into position to enclose the ligand while exposing sites on the VDR in helices 3, 5 and 12 to which coactivators bind. These coactivators can in turn recruit chromatin modifying enzymes such as histone acetyl transferases (SRC, CBP/p300, pCAF) and DNA demethylases or proteins that bridge the gap between the VDRE and the transcription machinery (Mediator complex) including TATA associated factors, TFIIb, and RNA polymerases (primarily RNA pol II). In the absence of ligand binding sites for corepressors are exposed. These corepressors recruit another set of chromatin modifying enzymes such as histone deacetylases and DNA methyl transferases.[24] The most common VDRE is comprised of two head to tail half sites of hexanucleotides separated by 3 nucleotides, referred to as DR3 VDREs. The sequence of these DR3 half

sites is heterogeneous, with a consensus approximated by RGKTSA where R = A or G, K = G or T, S = C or G. Moreover, many VDREs are not DR3s, although DR3s tend to have the highest affinity for VDR/RXR heterodimers.

The VDREs can be quite distant from the transcription start site of the gene being regulated, occurring in introns, between genes, and in either a 5′ or 3′ relationship to the coding region.[25] Moreover, putative VDREs as demonstrated by techniques such as ChIP-seq, in which binding sites to the genome by VDR are identified using a combination of chromatin immunoprecipitation of the VDR to DNA followed by high throughput sequencing of those binding regions, number in the thousands, with a substantial degree of cell/tissue specificity.[26] Most genes have several VDREs. In a review of two such ChIP-seq studies Carlberg et al.[26] noted that the study in a lymphoblastoid line identified 2776 VDREs for 232 genes whereas the study in THP-1 monocytes identified 1820 VDREs for 638 genes. In the latter study 408 of the genes were upregulated, 230 downregulated. Most of the VDREs for the upregulated genes were within 400kbp of the transcription start site; this was less true for the downregulated genes. Only 93 of the upregulated genes had VDREs within 30kbp of the transcription start site. Moreover, only 31.7% of the VDREs were DR3s. These two studies had a mere 18% overlap of the VDREs identified, but differed not only in cell line but dose and time after $1,25(OH)_2D$ was administered before the cells were analyzed. Earlier microarray studies had likewise demonstrated the many genes regulated by $1,25(OH)_2D$, and the surprising lack of consensus from one study to the next perhaps due to tissue specificity and/or differences in dose and time of $1,25(OH)_2D$ exposure. These studies demonstrate the diversity of vitamin D regulated genes and diversity in type and location of VDREs. Such studies have revolutionized our concepts of the scope and means of vitamin D signaling, and reveal many potential mechanisms by which vitamin D signaling can regulate cancer formation. In this regard an unbiased systems biology approach mapping genetic loci underlying susceptibility to skin cancer put the VDR in the center of a complex set of networks linking regulation of barrier function, inflammation, and tumor formation.[27]

The VDR is essential for nearly all actions of $1,25(OH)_2D$ and its analogs. Tumors that are unresponsive to vitamin D have either lost their ability to produce $1,25(OH)_2D$ (ie. decreased CYP27B1),[28,29] increased their metabolism of $1,25(OH)_2D$ via upregulation of CYP24A1,[30] lost VDR transcriptional activity through post translational alterations in RXR,[31] or decreased their VDR expression. The latter may be secondary to increased activity in tumors of inhibitors of VDR expression such as SNAIL[32] and SLUG,[33] increased methylation of the VDR promoter,[34] or increased expression of miRNA125b, an inhibitor of VDR expression.[35] In melanoma cell lines the administration of 5-aza cytidine (to inhibit DNA methyltransferase) and trichostatin (to inhibit HDAC activity) could restore responsiveness to $1,25(OH)_2D$ by increasing VDR levels and reducing miR125b expression.[36]

MECHANISMS OF TUMOR SUPPRESSION BY VITAMIN D: GENERAL

The demonstration that many genes and pathways are influenced by vitamin D signaling has opened up a large number of potential means by which vitamin D signaling can control tumor growth (recent reviews in refs. 37,38) (Fig. 1).

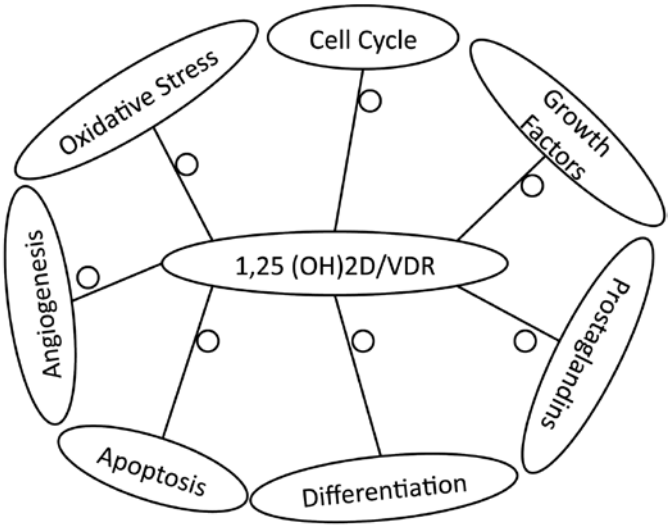

Figure 1. Multiple mechanisms by which $1,25(OH)_2D$/VDR suppress tumor formation. Vitamin D signaling has the potential to suppress tumor formation by affecting a number of pathways. Angiogenesis is suppressed by reducing the expression of VEGF and its receptors. Oxidative stress is reduced by increasing the expression of enzymes that reduce ROS. The cell cycle is inhibited by increasing the expression of cell cycle inhibitors and decreasing the expression of cyclins and cyclin dependent kinases. The expression and/or activation of growth factors is inhibited (for growth factors that promote proliferation) or stimulated (for growth factors that inhibit proliferation). Prostaglandin synthesis is inhibited, the expression of their receptors is reduced, and the expression of enzymes that degrade prostaglandins in increased. On the other hand differentiation of the cells is increased, and apoptosis of damaged cells is stimulated.

Cell Cycle Regulation

Regulation by $1,25(OH)_2D$ of the cell cycle in a number of cells, normal and malignant, has been demonstrated. This results from an upregulation of cell cycle inhibitors such as $p21^{cip}$ and $p27^{kip}$ (cyclin-dependent kinase inhibitors)[39] and retinoblastoma like protein 2 and retinoblastoma binding protein 6[40] and decreased expression of cyclins[41] and cyclin dependent kinases.[42] In addition $1,25(OH)_2D$ increases the interaction of FoxO proteins (tumor suppressors controlling proliferation[43]) with VDR and FoxO regulators Sirt1 and protein phosphatase 1 that maintain FoxO in the nucleus by blocking MAPK phosphorylation.[44] Increased c-MYC expression and activity are frequently found in cancer.[45] c-MYC induces the expression of a number of cell cycle regulatory genes such as cyclin D2 and cdk4. $1,25(OH)_2D$ inhibits the expression of c-MYC,[46] and c-MYC expression is increased in the skin and gut of VDR null mice.[47]

Growth Factors

$1,25(OH)_2D$ and its analogs regulate a number of growth factor pathways. Insulin like growth factor (IGF) stimulated proliferation of breast and prostate cells is reduced by $1,25(OH)_2D$ via its induction of IGF-I binding protein 3.[48,49] TGFβ2 exerts antiproliferative

actions in epithelial cells. 1,25$(OH)_2$D and its analogs increase the expression of TGFβ2 and TGFβ receptors in breast and prostate cancer cells,[40,42,50] while suppressing the expression of the latent TGFβ-binding protein.[42,51] GDF15 (growth differentiation factor 15) is a member of the TGFβ superfamily, and like TGFβ is antiproliferative in prostate cancer cells. Its expression is increased by 1,25$(OH)_2$D.[52,53] Bone morphogenic proteins (BMPs) are also members of the TGFβ superfamily that have been found to be dysregulated in certain cancers.[54] The expression of several BMPs is regulated by 1,25$(OH)_2$D and its analogs in a number of malignant cell lines.[41,42,55] Wnt/β-catenin signaling will be dealt with in depth when we focus on vitamin D regulated pathways in the skin, but this pathway has been extensively studied in the colon based on the frequency of mutations in the adenomatous polyposis coli (APC) gene in colon cancer.[56] In the canonical pathway of wnt/β-catenin signaling the APC complex that would otherwise bind and phosphorylate β-catenin, targeting it for proteosomal degradation, is inactivated, allowing β-catenin to move to the nucleus where it binds to LEF/TCF leading to transcription of genes involved with proliferation. 1,25$(OH)_2$D/VDR binds to β-catenin, preventing its movement into the nucleus and/or binding to LEF/TCF.[57,58] Moreover, by increasing the levels of E-cadherin, which binds β-catenin in the plasma membrane, 1,25$(OH)_2$D can further reduce the translocation of β-catenin into the nucleus.[57,58] Furthermore, 1,25$(OH)_2$D can suppress wnt signaling by stimulating the expression of the wnt antagonist DKK-1.[59] Cystatin D, an inhibitor of several cysteine proteases of the cathepsin family that appear to be involved in wnt signaling, has likewise been shown to be a target gene of 1,25$(OH)_2$D.[60] The induction of cystatin D and other 1,25$(OH)_2$D target genes such as E-cadherin appear to involve a non genomic action requiring calcium activation of RhoA-ROCK-p38MAPK-MSK in colon cancer cells.[61] We have shown that this pathway requires the 1,25$(OH)_2$D induced calcium receptor in keratinocytes.[62] These and other studies point to the interaction between calcium and vitamin D signaling in the regulation of tumor formation,[63] an interaction that to date has received little attention.

Apoptosis

In addition to inhibiting proliferation, 1,25$(OH)_2$D promotes apoptosis in a number of malignant cell lines in part by downregulation of anti-apoptotic genes Bcl-2 and Bcl-X_L[64,65] and upregulation of the proapoptotic gene GOS2 (G_0G_1 switch gene 2).[41,66] Transcripts of other pro-apoptotic genes increased by 1,25$(OH)_2$D include death-associated protein-3, caspase 8 apoptosis-related cysteine peptidase, and fas-associated death domain-like apoptosis regulator as well as a number of caspases.[40] Telomerase is a mechanism that enables cancer cells to escape apoptosis. 1,25$(OH)_2$D suppresses telomerase expression by inducing miRNA498, a transcript in the complementary strand of CTC-360P6.[67] Of interest is this miRNA has its own VDRE.[67]

Oxidative Stress

As noted previously in the discussion of UVA induced effects on the epidermis, oxidative stress can lead to oxidative DNA damage, marked by 8 hydroxy 2'-deoxyguanosine. In VDR knockout mice, 8 hydroxy 2'-deoxyguanosine levels are increased in the colon[68] and reduced by vitamin D supplementation in humans.[69] 1,25$(OH)_2$D induces several antioxidant enzymes in cancer cells including thioredoxin reductase 1,[40,42] superoxide dismutase,[42,52] and glucose-6 phosphate dehydrogenase.[51] The induction of genes associated

with DNA repair will be discussed at greater length when we focus on UVB damage to the epidermis, but the induction by 1,25$(OH)_2$D of GADD45α (growth arrest and DNA-damage inducible α), p53, RAD23B, PCNA, and DAP-1α may all contribute to this aspect of tumor suppression by 1,25$(OH)_2$D/VDR.[40,66,70]

Prostaglandins

Prostaglandins have been shown to stimulate cancer cell growth.[71] 1,25$(OH)_2$D blocks prostaglandin signaling by inhibiting COX2 expression and that of prostaglandin receptors while increasing the expression of hydroxyprostaglandin dehydrogenase 15-NAD, the prostaglandin inactivating enzyme.[53,72]

Angiogenesis

Growing tumors require a blood supply. 1,25$(OH)_2$D inhibits angiogenesis by blocking the expression and function of VEGF (vascular endothelial growth factor),[73-75] and mice lacking VDR had larger and more vascular tumors when implanted with prostate cells from TRAMP mice.[76]

Immune System

The immune system plays an important protective role in cancer protection[77] as evidenced by the increased numbers of malignancies in immunosuppressed hosts including SCCs in immunosuppressed renal transplant patients.[78] This mechanism will be dealt with in more depth when we focus on the skin, as it has not received much study in the context of tumor protection in general by 1,25$(OH)_2$D. In fact by stimulating innate immunity, which would promote inflammation, and suppressing adaptive immunity, which would blunt immune surveillance one might expect 1,25$(OH_2$D to promote rather than block tumor development via its actions on the immune system. As such, this mechanism of action of 1,25$(OH)_2$D and its receptor with respect to cancer development requires further study.

MECHANISMS OF TUMOR SUPPRESSION BY 1,25$(OH)_2$D/VDR IN THE EPIDERMIS

The potential for vitamin D signaling as protection against epidermal tumor formation was demonstrated when Zinser et al.[79] demonstrated that 85% of the VDR null mice but none of the controls developed skin tumors within two months following 7,12 dimethylbenzanthracene (DMBA) administration. These were primarily papillomas. These results have been confirmed using topical administration of DMBA/TPA.[80] However, although only papillomas were seen in the VDR null mice, RXRα null mice developed both BCC and SCC.[80] Subsequently, Ellison et al.[81] and our own group[82]demonstrated that VDR null mice were also more susceptible to tumor formation following UVB, and many of the tumors were SCC and BCC. The appearance of BCC in these studies is surprising since the typical malignancy induced in mouse skin by UVR, ionizing radiation, or chemical carcinogens is SCC not BCC.[83] Given that BCC generally result from increased hedgehog (Hh) signaling,[84] and that lack of VDR results in BCC when β-catenin signaling is increased,[85] we became interested in the relationship between

vitamin D, Hh, and β-catenin signaling in tumor suppression. The lack of a normal innate immune response in CYP27B1 null mice to wounding[86] or infection[87] and the increased numbers of SCC in immunocompromised patients[78] suggested that disruption of the immune system might contribute to the increased susceptibility to tumor formation when vitamin D signaling was impaired. Moreover, we[82] noted a reduction in clearance in CPDs following UVB exposure of the skin of VDR null mice, suggesting that disruption of DNA damage repair was playing a role in tumor susceptibility in these mice. In what follows I will examine three potential mechanisms and pathways within those mechanisms for their contribution to the role of VDR as a tumor suppressor: regulation of proliferation and differentiation with particular attention to the hedgehog (Hh) and wnt/β-catenin pathways, immunoregulation, and DNA damage repair.

Vitamin D Regulation of Epidermal Proliferation and Differentiation

The epidermis is composed of four layers of keratinocytes at different stages of differentiation (reviewed in ref. 10). The basal layer (stratum basale, SB) rests on the basal lamina separating the dermis and epidermis. Within this layer are the stem cells. These cells proliferate, providing the cells for the upper differentiating layers. The basal cells are characterized by keratins K5 and K14 as well as the stem cell marker K15 and integrin α6β4. As the cells migrate upward from this basal layer into the spinous layer (stratum spinosum, SS) they initiate the production of the keratins K1 and K10, the keratins characteristic of the more differentiated layers of the epidermis. Cornified envelope precursors such as involucrin also appear in the spinous layer as does the enzyme transglutaminase K, responsible for the ε-(γ-glutamyl)lysine cross-linking of these substrates into the insoluble cornified envelope. Migrating further into the granular layer (stratum granulosum,{SG}), lying above the spinous layer, the cells acquire the electron-dense keratohyalin granules containing profilaggrin and loricrin that give the SG its name. Loricrin is a major component of the cornified envelope. Filaggrin serves to bundle the keratin filaments, but also when proteolyzed is thought to contribute to the hydration of the outer layers. The granular layer also contains lamellar bodies–lipid, enzyme, and antimicrobial peptide filled structures that fuse with the plasma membrane, divesting their contents into the extracellular space between the SG and stratum corneum (SC). The secreted enzymes process the lipids that contribute to the permeability barrier of the epidermis in conjunction with the keratin bundles and cornified envelope. The antimicrobial peptides provide a barrier against infectious organisms in the SC.

$1,25(OH)_2D$ increases essentially every step of this differentiation process[88-93] while inhibiting proliferation at least at concentrations above 1nM. These actions complement those of calcium,[62] the response to which is enhanced by $1,25(OH)_2D$ via its induction of the calcium receptor,[94,95] and the phospholipase C enzymes[96-98] that regulate intracellular calcium and other signaling molecules critical for the differentiation process. The antiproliferative effects are accompanied by a reduction in the expression of c-myc[99] and cyclin D1[100] and an increase in the cell cycle inhibitors $p21^{cip}$ and $p27^{kip}$. In addition, $1,25(OH)_2D$ and its receptor regulate the processing of the long chain glycosylceramides that are critical for permeability barrier formation[101] and induce the receptors, toll like receptor 2 (TLR2) and its coreceptor CD14, that initiate the innate immune response in skin.[86] Activation of these receptors leads to the induction of CYP27B1 (the enzyme that produces $1,25(OH)_2D$), which in turn induces cathelicidin resulting in the killing of invasive organisms.[86,102] Deletion of either VDR[103,104] or CYP27B1[105] results in defects

in the differentiation process leading to an abnormal barrier and increased proliferation of the epidermis with a defective innate immune response.[86] Two pathways that appear to be important in vitamin D signaling in the epidermis with respect to proliferation and differentiation that we believe underlie the predisposition of the VDR null mouse to tumor formation are the Hh and wnt/β-catenin pathways.

The Hedgehog (Hh) Pathway (Fig. 2). In the skin sonic hedgehog (Shh) is the ligand for patched (Ptch) 1, a 12 transmembrane domain protein that in the absence of Shh inhibits the function of another membrane protein smoothened (Smo). Smo in turn maintains a family of transcription factors, Gli1 and Gli2 in particular, in the cytoplasm bound to Suppressor of fused (Sufu).[106,107] When Shh binds to Ptch 1, the inhibition of Smo is relaxed, Gli1 and 2 are released from Sufu and move into the nucleus where they initiate transcription of a number of factors including each other as well as Ptch 1, the anti apoptotic factor bcl2, cyclins D1 and D2, E2F1, cdc45 (all of which promote proliferation), while suppressing genes associated with keratinocyte differentiation such as K1, K10, involucrin, loricrin and the VDR.[108-112]

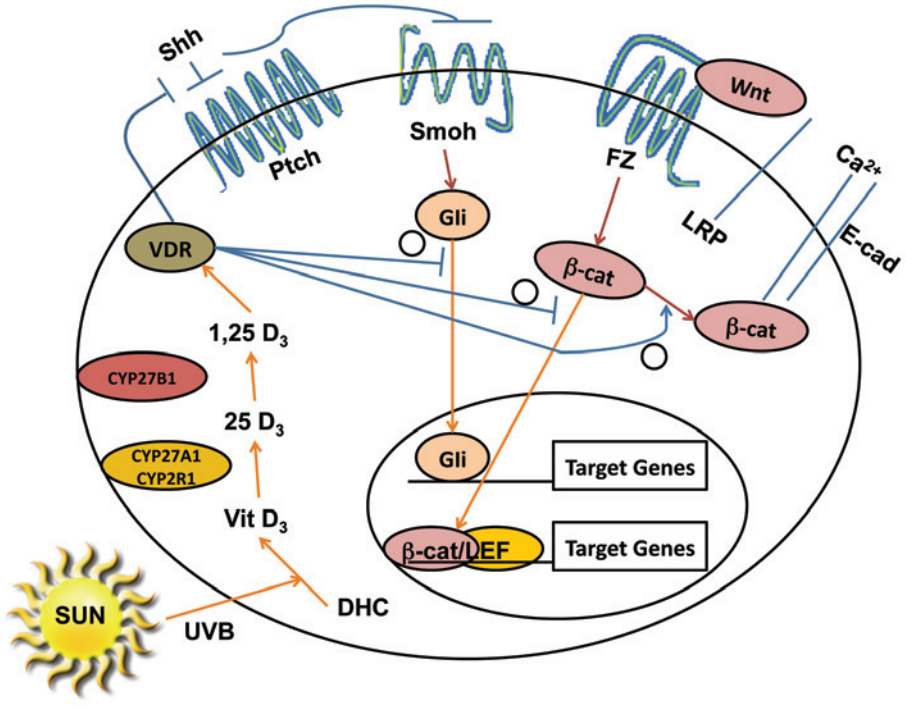

Figure 2. Regulation of Hh and wnt/β-catenin signaling by $1,25(OH)_2D$/VDR. The keratinocyte expresses VDR and is capable of making its own $1,25(OH)_2D_3$ from the vitamin D_3 produced from 7-dehydrocholesterol (DHC) under the influence of UVB, as it has both CYP27A1/CYP2R1 (which convert vitamin D_3 to $25OHD_3$) and CYP27B1 (which converts $25OHD_3$ to $1,25(OH)_2D_3$). $1,25(OH)_2D$/VDR suppresses Shh and gli 1 expression, inhibiting the Hh pathway in keratinocytes. $1,25(OH)_2D$/VDR binds β-catenin and induces E-cadherin expression reducing the amount of β-catenin available for binding to TCF/LEF in the nucleus limiting its transcriptional activity. In combination these actions reduce the proliferative actions of Shh and β-catenin signaling in keratinocytes, limiting their ability to induce tumors in the skin.

The appearance of BCC is characteristic of tumors formed when Hh signaling is disrupted,[113] although activation of Hh signaling also predisposes to UVR induced SCC formation.[114] VDR null animals overexpress elements of the Hh signaling pathway in their epidermis and the epidermal portion (utricles) of the hair follicles.[82] Moreover, $1,25(OH)_2D$ suppresses the expression of all elements of the Hh pathway in a dose dependent fashion that requires the VDR[82,115] and reduces tumor growth in *Ptch* 1null mice. The promoters of Shh and Gli1 have binding sites for VDR[116] suggesting that the effects of $1,25(OH)_2D$ on these genes is direct. However, vitamin D has also been shown to bind to and inhibit the actions of smoothened (Smo) directly without seeming to require further metabolism to $1,25(OH)_2D$.[117,118]

The wnt/β-Catenin Pathway (Fig. 2). Wnt signaling via activation of β-catenin has a complex role in VDR function as discussed briefly earlier. In the canonical pathway the receptor for wnt ligands is a family of seven-transmembrane Frizzled receptors and an LRP5 or LRP6 co-receptor. When wnt binds to this complex disheveled (Dvl) is phosphorylated resulting in disruption of the axin/APC complex and inhibition of glycogen synthase kinase 3β ((GSK-3β)). In the basal state GSK-3β phosphorylates the serine(s) within exon 3 of β-catenin resulting in its degradation by the E3 ubiquitin ligase. Wnt signaling, by blocking this phosphorylation, increases the availability of β-catenin in the nucleus, where it binds to transcription factors of the T-cell factor (TCF) and lymphoid enhancer factor (LEF) families to promote expression of genes such as cyclin D1 and c-myc[119] important for proliferation. β-catenin also forms part of the adherens junction complex with E-cadherin where it may play an important role in keratinocyte differentiation.[120] Tyrosine phosphorylation of E-cadherin, as occurs after calcium administration to keratinocytes, promotes the binding of β-catenin and other catenins to the adherens junction complex[120,121] making it less available for transcriptional activity. $1,25(OH)_2D$ increases E-cadherin expression.[122] Overexpression and/or activating mutations in the β-catenin pathway lead to skin tumors, in this case pilomatricomas or trichofolliculomas (hair follicle tumors).[123-125] As noted earlier VDR binds to β-catenin, and reduces the transcriptional activity of β-catenin in a $1,25(OH)_2D$ dependent fashion.[57] On the other hand binding of β-catenin to VDR in its AF-2 domain enhances the $1,25(OH)_2D$ dependent transcriptional activity of VDR.[58] Palmer et al.[85] evaluated the interaction between VDR and β-catenin in transcriptional regulation in keratinocytes, and identified putative response elements for VDR and β-catenin/LEF in a number of genes. These interactions were either positive or negative, depending on the gene being evaluated. The hypothesis put forward is that genes in which the interaction was positive (ie. stimulated transcription) benefited from β-catenin acting as a coactivator for VDR on VDREs, whereas in situations where the interaction was negative (ie. suppression of transcription) VDR prevented β-catenin from binding to TCF/LEF required for transcription in those genes. We[100] have found in keratinocytes that knockdown of VDR reduces E-cadherin expression and formation of the β-catenin/E-cadherin membrane complex resulting in increased β-catenin transcriptional activity, whereas $1,25(OH)_2D$ administration has the opposite effect. This was associated with increased (with VDR knockdown) or decreased (with $1,25(OH)_2D$ administration) keratinocyte proliferation and cyclin D1 expression. On the other hand Cianferotti et al.[126] found a reduction in proliferation of keratinocytes in the dermal portion of the hair follicle (below the bulge) in VDR null mice, and no stimulation of proliferation when β-catenin was overexpressed in these cells in contrast to the stimulation of proliferation in control animals. Thus VDR/β-catenin interactions

can be positive or negative, depending on the gene/cell/function being evaluated, but in the epidermis in the absence of VDR, the unchecked activity of β-catenin appears to be proliferative and inhibitory of differentiation resulting in BCC.

Vitamin D Regulation of Immune Function in the Skin

VDR and CYP27B1 are found in professional immune cells, namely dendritic cells, macrophages, and lymphocytes[127,128] responsible for both innate and adaptive immune responses as well as in epithelial cells expressing the components of the innate immune response. $1,25(OH)_2D$ regulates the proliferation and function[129] of these cells. Although it is not clear the extent to which dysregulated immune function contributes to cancer development in the skin, a link between inflammation and cancer susceptibility in the skin involving VDR has been established.[27]

Adaptive Immunity. The adaptive immune response involves the ability of T and B lymphocytes to produce cytokines and immunoglobulins, respectively, in response to antigens presented to them by cells such as macrophages and dendritic cells. $1,25(OH)_2D$ suppresses the adaptive immune response by inhibiting proliferation, immunoglobulin production, and differentiation of B-cell precursors into plasma cells.[128] $1,25(OH)_2D$ inhibits T cell proliferation[130] and the differentiation of CD4 cells into Th1 cells capable of producing IFN-γ and IL-2 and activating macrophages[131] and Th17 cells capable of producing IL17 and IL22.[132,133] On the other hand $1,25(OH)_2D$ stimulates IL-4, IL-5, and IL10 production[134] by increasing CD4 cell differentiation into Th2 and regulatory T cells (Treg).[135] The IL-10 so produced is one means by which Treg block Th1 and Th17 development. Part of these effects is mediated by the negative impact of $1,25(OH)_2D$ on the maturation and antigen presenting capability of dendritic cells.[136] It is unclear if this suppression of the adaptive immune system alters tumor surveillance in the skin.

Innate Immunity. The innate immune response involves the activation of toll-like receptors (TLRs)[137] that serve as transmembrane pathogen-recognition receptors detecting specific membrane patterns (PAMP) shed by a wide variety of infectious agents.[138] Activation of TLRs leads to the induction of antimicrobial peptides and reactive oxygen species, which kill the organism. Cathelicidin is the best studied of these antimicrobial peptides. The expression of cathelicidin is induced by $1,25(OH)_2D$ in both myeloid and epithelial cells,[139,140] cells that also express CYP27B1 and so are capable of producing $1,25(OH)_2D$ needed for this induction. Stimulation of TLR2 in macrophages[141] or keratinocytes[86] results in increased CYP27B1 expression, which in the presence of adequate substrate (25OHD) induces cathelicidin expression. Lack of substrate (25OHD), VDR, or CYP27B1 blunts the ability of these cells to respond with respect to cathelicidin production.[86,140,141]

The major cells involved in adaptive immunity in the skin include the Langerhans cells, dendritic cells, and T cells. The Langerhans cells are dendritic like cells within the epidermis that when activated by invading organisms migrate to the lymph nodes serving the skin where they present the antigens to the T cells, initiating the adaptive immune response.[142] Keratinocytes, on the other hand, are equipped with toll like receptors that enable them to respond to invading organisms with elaboration of antimicrobial peptides such as cathelicidin.[102] However, cathelicidin also induces an inflammatory response.[143] UVB leads to a reduction in Langerhans cells and blunts their antigen presenting activity,[144-146]

but stimulates the innate immune function of keratinocytes perhaps as a consequence of UVB induced vitamin D/1,25$(OH)_2$D production in the skin.[147,148]

The potential role of altered skin immunity by UVB with respect to skin carcinogenesis was suggested by Kripke and Fisher.[149] They found that skin tumors originally induced in mice by chronic UVR, would grow when transplanted into mice that had been UV irradiated but not when transplanted into control mice. The role of 1,25$(OH)_2$D production in UVR immunosuppression is not clear. Topical application of high concentrations of 1,25$(OH)_2$D protected against UVR induced suppression of contact hypersensitivity in the mouse,[150] but a study in humans by the same group showed suppression of delayed hypersensitivity (Mantoux test) by topical 1,25$(OH)_2$D.[151] These data are limited, but raise some concern about the balance between innate and adaptive immunity in tumor surveillance, and how that balance is affected by vitamin D.

Vitamin D Regulation of the DNA Damage Response

DNA damage repair (DDR) is the means by which UVR and chemical induced DNA damage is prevented from producing fixed DNA mutations.[152] DDR involves a cascade of damage recognition, repair and signal transduction that coordinates the response of the cell to DNA damage. DDR activates checkpoints that delay the cell cycle, provides time for repair, and directs damaged cells into senescent or apoptotic pathways. DDR involves a number of components, is well orchestrated, tightly controlled, and highly accurate in normal primary cells such that the spontaneous mutation rate is very low, and changes in copy number are negligible.[153-155] With malignant transformation DDR becomes less controlled, and mutation rates and copy number abnormalities increase by orders of magnitude.[153,154,156,157] Nucleotide excision repair (NER) is the principal means by which UVR damage is repaired, enabling repair before DNA replication begins. This is important as NER plays a major role in reducing the amount of damage that becomes fixed as mutations during replication.[158-160] During NER, the DNA damage is recognized, the DNA unwound around the lesion, and 30 base pair portions of DNA containing the lesion are excised by endonucleases such as XPF and XPG followed by fill in with DNA polymerases such as Pol δ,ε,κ.

The NER process has two main branches involving different mechanisms for the initial recognition of DNA damage[161]: transcription coupled repair (TCR) during which DNA polymerases stop replication at the site of the lesion until it is repaired,[162-166] and global genomic repair (GGR), during which non-transcribed regions of the genome are repaired.[167] Keratinocytes in the epidermis of mice lacking VDR are deficient in DDR as demonstrated by a reduced rate of clearing CPDs and 6,4PPs following UVB.[168] Moreover, 1,25$(OH)_2$D increases CPD clearance in VDR intact mice.[169,170] These actions have been demonstrated with 1,25$(OH)_2$D analogs that are not thought to have genomic activity.[169] However, at least part of this enhancement of CPD clearance is due to the upregulation of two genes important for DDR: XPC (xeroderma pigmentosum complementation group C) and DDB2 (damage-specific DNA binding protein 2 also known as XPE).[168,171] Furthermore, 1,25$(OH)_2$D has been shown to increase the levels of p53, which could enhance apoptosis in those cells bearing excess DNA damage,[170] and reduce UVR induced oxidative stress contributing to the DNA damage.[170] As such these actions of vitamin D signaling on DDR contribute to the reduced susceptibility of normal skin to UVB induced tumor formation.

CONCLUSION

The VDR is present in nearly every cell in the body. Moreover, the enzyme, CYP27B1, required for the production of the VDR ligand, $1,25(OH)_2D$, is likewise widely distributed. Because of its abundance of 7-DHC, the epidermis is unique in its capability to produce vitamin D, metabolize it to $1,25(OH)_2D$, and respond to $1,25(OH)_2D$ in a number of ways. Recent data from microarray and ChIP-seq studies have demonstrated hundreds, perhaps thousands of genes regulated by $1,25(OH)_2D$/VDR via VDREs which are located throughout the gene. The selection of the genes regulated by $1,25(OH)_2D$/VDR at any one time is cell specific and most likely dose and time specific with respect to exposure to $1,25(OH)_2D$. As a result of these studies, numerous pathways have been discovered by which $1,25(OH)_2D$/VDR may prevent cancer. In the skin UVB is critical for vitamin D production, but UVB is also the major cause of skin cancer. This chapter examines the question of whether the beneficial effects of UVB on vitamin D production can counter the harmful effects on carcinogenesis. Epidemiologic data suggest that there may be a threshold below which UVR is not carcinogenic, a threshold that would suffice for adequate vitamin D production. Conceivably, vitamin D production at such levels of UVB exposure might even be protective. Three potential mechanisms for such protection were examined. The first mechanism focuses on the role of vitamin D signaling in keratinocyte proliferation and differentiation. In particular, two pathways affecting proliferation and differentiation, namely the hedgehog and wnt/β-catenin pathways, were evaluated. Mice lacking the VDR have increased expression of the hedgehog pathway and increased activation of the wnt/β-catenin pathway. $1,25(OH)_2D$ suppresses both pathways in cells containing the VDR. Overexpression of the hedgehog and wnt/β-catenin pathways lead to increased proliferation and decreased differentiation associated with tumor development. The second mechanism involves the role of vitamin D signaling in the immune system of the skin. The role of the immune system in epidermal carcinogenesis is not clear. However, in an unbiased genomic examination of pathways associated with tumor susceptibility, inflammation, keratinocyte differentiation, and tumor formation were linked through the VDR. $1,25(OH)_2D$/VDR promotes innate immunity but suppresses adaptive immunity. Whether this is beneficial regarding tumor development requires further study. The third mechanism is DNA damage repair (DDR). The epidermis of VDR null mice show impaired DDR following UVR. $1,25(OH)_2D$ accelerates DDR by what appear to be genomic and non genomic actions. On teleologic grounds one might anticipate that the skin has developed mechanisms to protect itself from the harmful effects of UVR. Vitamin D production, metabolism, and regulation of the processes described in this chapter may play a key role in this protection.

ACKNOWLEDGMENTS

I would like to acknowledge the collaborators whose work I have referenced, namely Drs. Zhongjian Xie, Chialing Tu, Arnaud Teichert, Kumar Pillai, Yuko Oda, Yan Jiang, Dennis Oh, James Cleaver; the administrative assistance of Ms Teresa Tong and Victoria Lee; and the funding by a VA Merit Review, NIH RO1 AR050023, PO1 AR39448, and DOD CA110338

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