Chapter 7
Regulation of Antimicrobial Peptide Gene Expression by Vitamin D

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Abstract In the mid-2000s, several investigators discovered that antimicrobial peptide (AMP) gene expression was regulated by the vitamin D pathway. This revelation provided a potential explanation for the ability of vitamin D to enhance the antimicrobial activity of immune cells like macrophages that had been observed in the 1980s. Further, this finding provided a mechanism that could explain the observed importance of vitamin D in maintaining the epithelial barrier defenses of the skin and gut. As reviewed in this chapter, an abundance of *in vitro* evidence demonstrates the regulation of AMPs by vitamin D. Nevertheless, there is a lack of *in vivo* data that demonstrates just how this regulation plays a role in the immune response against infection. This is due, in part, to the current lack of a viable animal model as the regulation of antimicrobial peptide gene expression occurs only in humans and nonhuman primates. Generation of an appropriate animal model and/or carefully designed human and primate studies should provide a clearer picture of the role that this pathway plays in barrier function and the immune response.

7.1 Introduction

A decade ago three groups simultaneously discovered the regulation of human antimicrobial peptide gene expression by vitamin D. In screening the human genome for vitamin D response elements (VDREs), White and colleagues identified potential VDREs in the cathelicidin antimicrobial peptide (*CAMP*) and β-defensin 4 (*DEFB4*) genes. They demonstrated induction of these two genes by treatment of isolated human keratinocytes, monocytes and neutrophils, and human cell lines with 1α,25-dihydroxyvitaminD_3 [1,25$(OH)_2D_3$] concomitant with increased secretion of bactericidal activity from treated cells (Wang et al. 2004). Ståhle and

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colleagues demonstrated induction of *CAMP* in human keratinocytes *in vitro* by $1,25(OH)_2D_3$ and *in vivo* after topical application of the $1,25(OH)_2D_3$ analog calcipotriol to the skin of human volunteers (Weber et al. 2005). Our group discovered induction of CAMP by $1,25(OH)_2D_3$ in acute myeloid leukemia (AML), immortalized keratinocytes, and colon cancer cell lines as well as normal human bone marrow (BM)-derived macrophages and fresh BM cells from two normal individuals and one acute myelogenous leukemia patient (Gombart et al. 2005). Each group demonstrated the requirement of a VDRE at about 700 base pairs upstream of the transcription start site in the *CAMP* gene by site-directed mutagenesis or deletion (Wang et al. 2004; Weber et al. 2005; Gombart et al. 2005). In the *DEFB4* gene promoter, the VDRE is located about 1200 base pairs upstream of the transcription start site (Wang et al. 2004).

Interestingly, we observed a lack of this regulation in the mouse and discovered from a comparison of various mammalian genomes an evolutionarily conserved VDRE in a short interspersed nuclear element (SINE) in the *CAMP* promoter of primates that was absent in other mammalian genomes (Gombart et al. 2005). Further, we demonstrated that the VDRE in the *CAMP* gene originated from the exaptation of an AluSx SINE in the lineage leading to humans, apes, Old World monkeys, and New World monkeys and remained under purifying selection for the last 55–60 million years (Gombart et al. 2009a). Taken together, these findings revealed a novel activity of $1,25(OH)_2D_3$ and the vitamin D receptor (VDR) in regulation of primate innate immunity due to an evolutionarily fixed, Alu-mediated divergence in steroid hormone nuclear receptor gene regulation between humans/primates and other mammals (Gombart et al. 2009a). Regulation of a murine homolog for *DEFB4* by vitamin D has not been described.

In a series of subsequent *in vitro* experiments, Modlin and colleagues demonstrated that vitamin D was required for the induction of *CAMP* by Toll-like receptor (TLR) signaling, and findings indicated that insufficient serum 25-hydroxyvitamin D [25(OH)D] levels could lead to a lack of *CAMP* gene expression by macrophages in response to infection (Liu et al. 2006). In the current model, TLR signaling induces *CYP27B1* and *VDR* expression. CYP27B1 activity hydroxylates 25(OH)D resulting in the production of the active $1,25(OH)_2D$ that binds to the VDR and thus induces target genes including *CAMP* (Liu et al. 2006).

Activation of the vitamin D pathway by TLR signaling was identified in lung and skin epithelial cells, but was not observed in cell lines derived from the colon (Hansdottir et al. 2008; Schauber et al. 2007; Lagishetty et al. 2010). Wounding of the skin enhanced TLR2 function which enabled keratinocytes to respond to pathogen-associated molecular patterns, activate the vitamin D pathway, and increase CAMP levels in the skin to protect against infection (Schauber et al. 2007). Interestingly, the induction of CAMP gene expression by 1,25(OH)2D3 was observed in keratinocytes and monocytes, but not in epithelial cells of the colon (Schauber et al. 2006). In contrast butyrate induced CAMP in colonic cells, but not significantly in keratinocytes and monocytes suggesting that responses to these stimuli were cell type and microenvironment specific (Schauber et al. 2006). Several studies have demonstrated that butyrate and other histone deacetylase inhibitors

together with $1,25(OH)_2D_3$ cooperatively or synergistically induce CAMP gene expression in monocytes/macrophages, keratinocytes, and lung and colon cells (Gombart et al. 2007; Schauber et al. 2008; Mily et al. 2013; van der Does et al. 2014; Kulkarni et al. 2014).

7.2 The CAMP Gene

A majority of the interest in vitamin D-mediated regulation of antimicrobial peptide (AMP) gene expression has focused on induction of the *CAMP* gene because it is more robustly upregulated as compared to *DEFB4* (Wang et al. 2004). The *CAMP* gene encodes an 18-kDa proprotein called hCAP18. It is processed to release a peptide called LL-37 that is expressed by neutrophils and macrophages for killing bacteria and by epithelial cells in barrier defense (Lehrer and Ganz 2002; Gombart 2009; White 2010). In addition, the LL-37 peptide can chemoattract T cells, dendritic cells, neutrophils, and monocytes (Chertov et al. 1996; Yang et al. 2001), which could allow LL-37 to influence cellular traffic at sites of infection or inflammation. Also, LL-37 affects dendritic cell activation and subsequent priming of T cells when added exogenously (Davidson et al. 2004), demonstrating that adaptive immune responses may be regulated by LL-37. The hCAP18 protein is present in γδ T cells, B cells, monocytes, and NK cells of the peripheral blood (Agerberth et al. 2000) following a general hierarchy of protein expression with neutrophils showing the highest levels, monocytes intermediate levels, and lymphocytes the lowest levels (Lowry et al. 2014). In the lymphocyte population, B cells, NK cells, CD4+ T cells, and CD8+ T cells all have similar levels of hCAP18 expression (Lowry et al. 2014). CAMP is secreted by tissues exposed to the environment and in saliva and seminal fluid (Frohm Nilsson et al. 1999; Murakami et al. 2002; Malm et al. 2000). It is critical for host barrier defense as mice lacking it are susceptible to infection (Gombart 2009; Nizet et al. 2001; Chromek et al. 2006).

7.3 The Vitamin D Pathway

The most effective way to acquire vitamin D is through synthesis in the skin or consumption of a purified supplement as diet, which is a poor source (Holick 2011). Ultraviolet B rays provided by natural or artificial sunlight cleave the B-ring of 7-dehydrocholesterol in the skin to produce cholecalciferol or vitamin D_3. This is absorbed into the blood and hydroxylated in the liver by the cytochrome p450 enzyme CYP27A1 to calcidiol or $25(OH)D_3$. This form (together with $25(OH)D_2$; see below) is measured in the serum as an indicator of vitamin D status (Holick 2011). $25(OH)D_3$ is converted to its bioactive form, calcitriol or $1,25(OH)_2D_3$, by the mitochondrial 1α-hydroxylase enzyme CYP27B1 in the kidney. A fungal-derived form of vitamin D is created by the UVB exposure of ergosterol to generate

ergocalciferol. This form of vitamin D is hydroxylated in the liver to $25(OH)D_2$ and in the kidney to $1,25(OH)_2D_2$ (Holick 2011). Both $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$ bind to the VDR a steroid hormone nuclear receptor/transcription factor that binds to VDREs and recruits cofactors to activate and/or repress the expression of target genes (Mangelsdorf et al. 1995; Christakos et al. 1996).

Synthesis of $1,25(OH)_2D$ in the kidney is essential for efficient uptake of dietary calcium in the gut and to maintain bone health. A drop in circulating Ca^{2+} levels stimulates the production of parathyroid hormone (PTH) which induces CYP27B1 expression by primary renal tubules. Increased $1,25(OH)_2D$ production activates Ca^{2+} transporter expression *via* the VDR in the small intestine, thereby increasing circulating Ca^{2+} and suppressing PTH production (Holick 2011). In a negative feedback loop, activated VDR binds to the *CYP27B1* promoter and represses its expression. Also, VDR induces fibroblast growth factor-23 in osteocytes which inhibits secretion of PTH and represses CYP27B1 expression and induces expression of CYP24A1, a mitochondrial enzyme that catabolizes both $1,25(OH)_2D$ and $25(OH)D$ to limit $1,25(OH)_2D$ levels and prevent hypercalcemia (Paz et al. 2007; Saito et al. 2003; Zierold et al. 1995).

Abundant epidemiological, clinical, and basic research has implicated vitamin D in preventing cancer, autoimmune disorders, cardiovascular disease, and infections (Grober et al. 2013). The synthesis of $1,25(OH)_2D$ in nonrenal tissues and cells likely mediates these additional health benefits (Hewison et al. 2004). The extrarenal synthesis of $1,25(OH)_2D$ occurs in lung, colon, parathyroid glands, bone, skin, and macrophages and is considered important for optimal immune response at sites of infection (Hewison et al. 2004).

7.4 Vitamin D and Immunity

The role of vitamin D in regulating the adaptive immune response is highlighted by numerous lines of evidence. The VDR is expressed in T and B cells, monocytes, macrophages, dendritic cells (DCs), and neutrophils (Provvedini et al. 1983; Bhalla et al. 1983; Deluca and Cantorna 2001; Adorini et al. 2004; Kreutz et al. 1993; Brennan et al. 1987; Takahashi et al. 2002; Mangelsdorf et al. 1984). $1,25(OH)_2D_3$ inhibits Th17 development, increases the frequency of Th2 and regulatory T cells, decreases Th1 development, and modulates T-cell proliferation and cytokine expression (Lemire et al. 1995; Boonstra et al. 2001; Penna and Adorini 2000; Daniel et al. 2008). $1,25(OH)_2D_3$ also promotes tolerance in dendritic cells and T cells and inhibits B-cell differentiation into plasma cells (Adorini et al. 2004; Mathieu and Adorini 2002; Chen et al. 2007). Overall vitamin D mediates an antiinflammatory response and promotes tolerance in the adaptive response.

In addition to responding to circulating $1,25(OH)_2D_3$, dendritic cells, macrophages, and T cells can actively produce it (Hewison 2012). Initially, extrarenal production of $1,25(OH)_2D_3$ by macrophages from some granulomatous disease patients was reported (Barbour et al. 1981; Adams et al. 1983). *In vitro* studies with

normal macrophages indicated that CYP27B1 activity was induced as part of the immune response (Koeffler et al. 1985; Reichel et al. 1986). DCs confer specific homing properties upon T cells during the adaptive immune response, and DCs derived from the skin are able to synthesize $1,25(OH)_2D_3$ from vitamin D_3. This, in turn, induces expression of CC chemokine receptor 10 in T cells and suppresses expression of gut-homing receptors which enable T cells to migrate toward the chemokine CCL27 that is secreted by epidermal keratinocytes. These findings demonstrate that DCs produce locally high levels of $1,25(OH)_2D_3$ to regulate T-cell epidermal tropism (Sigmundsdottir et al. 2007).

The production of potentially high local levels of $1,25(OH)_2D_3$ is most likely important for intracrine and paracrine influences on the interactions between vitamin D, the immune system, and pathogens (Hewison 2012). During the mid-1980s, it was demonstrated that both $25(OH)D_3$ and $1,25(OH)_2D_3$ increased the capacity of human monocytes to control *Mycobacterium tuberculosis* (*Mtb*) growth (Davies 1985; Rook et al. 1986). Nearly 20 years later, as described above, we and others discovered that vitamin D increased expression of the *CAMP* gene (Wang et al. 2004; Weber et al. 2005; Gombart et al. 2005). In addition, the human β-defensin 2 or *DEFB4* gene was identified as a vitamin D inducible antimicrobial peptide gene, but its induction by vitamin D or TLR activation is much less robust than *CAMP* (Wang et al. 2004; Liu et al. 2006). These observations offered a mechanism by which vitamin D could directly enhance killing of *Mtb*.

7.5 Cooperative Induction of Antimicrobial Peptide Gene Expression by Multiple Signaling Pathways

Robust induction of DEFB4 by vitamin D requires activation of additional signaling pathways. Co-treatment of monocytes with IL-1 and $1,25(OH)_2D_3$ induced binding of both NF-κB and VDR to the *DEFB4* promoter and was much more effective in inducing gene expression (Wang et al. 2004; Liu et al. 2009). Also, in the presence of muramyl dipeptide (MDP), the intracellular pattern recognition receptor nucleotide-binding oligomerization domain protein 2 (NOD2) activates NF-κB, and there is a modest induction of the *DEFB4* gene (Voss et al. 2006; Wang et al. 2010); however, treatment with $1,25(OH)_2D_3$ prior to addition of MDP strongly induces the *DEFB4* gene (Wang et al. 2010). It was shown that $1,25(OH)_2D_3$ strongly induced expression of NOD2 in primary human monocytic and epithelial cells which amplified the MDP signal (Wang et al. 2010). In total, studies have shown that the vitamin D pathway alone is insufficient to induce robust expression of *DEFB4,* and activation of additional signaling pathways is required (Liu et al. 2009; Wang et al. 2010).

Several published studies have demonstrated that cytokine expression also modulates vitamin D-mediated *CAMP* and *DEFB4* expression. In human macrophages, TLR2/TLR1 signaling induces IL-15 expression which increases IL-32 which is essential for induction of CYP27B1 and the VDR (Krutzik et al. 2008; Montoya et al. 2014). The subsequent increased conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ by

CYP27B1 activates the VDR and induces *CAMP* expression and antimicrobial activity against *Mtb* (Krutzik et al. 2008; Montoya et al. 2014). In human monocytes, Th1 cytokine IFN-γ upregulates TLR2/TLR1 induction of CYP27B1 and the bioconversion of $25(OH)D_3$ to $1,25(OH)_2D_3$ which enhances induction of *CAMP* (Edfeldt et al. 2010). Further, vitamin D is required for IFN-γ-mediated activity of human macrophages (Fabri et al. 2011). On the other hand, the Th2 cytokine IL-4 induces CYP24A1 expression which leads to the catabolism of $25(OH)D_3$ and downregulation of *CAMP* expression (Edfeldt et al. 2010). In contrast, the Th2 cytokine IL-13 enhances *CAMP* expression by $25(OH)D_3$ due to increased CYP27B1 expression and synthesis of $1,25(OH)_2D_3$ (Schrumpf et al. 2012). No effect is observed with IL-17 in monocytes, but in the presence of $1,25(OH)_2D_3$, IL-17 enhances CAMP expression in human keratinocytes *via* activation of the Act1 and MEK/ERK pathway (Peric et al. 2008). In addition to IL-4, other cytokines can inhibit antimicrobial peptide (AMP) expression. In macrophages, IFN-γ-induced vitamin D-dependent AMP expression was suppressed by IFN-β and IL-10 (Teles et al. 2013). Similarly, in placental cells, IL-10 inhibited β-defensin and *CAMP* expression, while $1,25(OH)_2D_3$ treatment could override the suppression (Olmos-Ortiz et al. 2015). Further, TNF-α and $1,25(OH)_2D_3$ enhanced β-defensin, and TNF-α reduced both basal and $1,25(OH)_2D_3$-induced *CAMP* expression (Olmos-Ortiz et al. 2015). Taken together, the differential effect of T-cell cytokines on CAMP and DEFB4 expression represents mechanisms by which adaptive immune responses can regulate innate immune antimicrobial peptide defenses against pathogens. It remains to be determined how these various signaling pathways work together *in vivo* during infection.

7.6 Fighting Infection Through Increased AMP Expression

Historically, sources of vitamin D were used as treatments for tuberculosis (Martineau et al. 2007). In the 1940s, physicians effectively treated cutaneous *Mtb* infection with high-dose vitamin D_2, but this fell out of favor with the advent of effective antibiotics (Martineau et al. 2007; Dowling 1946; Gaumond 1948). In the 1980s, epidemiological studies pointed to a correlation between higher rates of tuberculosis and vitamin D deficiency (Davies 1985). Further, $1,25(OH)_2D_3$ was shown to enhance intracellular killing by human monocytic cells (Rook 1986). Knockdown of either DEFB4 or CAMP expression in monocytes/macrophages decreased killing of *Mtb* indicating their importance for fighting infection (Liu et al. 2009). The induction of CAMP by vitamin D is required for promoting autophagy to kill *Mtb* (Hoyer-Hansen et al. 2005; Wang et al. 2008; Yuk et al. 2009). Additional findings support a paracrine macrophage-lung epithelial cell signaling pathway that is driven by IL-1β and $1,25(OH)_2D_3$ (Verway et al. 2013). In this model, $1,25(OH)_2D_3$ increased IL-1β secretion in *Mtb*-infected macrophages. The secreted IL-1β induced *DEFB4* expression from airway epithelial cells which enhanced control of *Mtb* growth in co-cultured macrophages *in vitro* (Verway et al. 2013). Taken together

these studies support an important role for vitamin D in modulating the immune response to *Mtb* infection.

These findings have renewed interest in potentially using vitamin D to treat tuberculosis. A review of clinical trials and case series indicates that numerous studies are methodologically flawed (Martineau et al. 2007) or an insufficient vitamin D dose was used (Wejse et al. 2009). Two small randomized studies indicate some benefit from vitamin supplementation of TB patients (Nursyam et al. 2006; Morcos et al. 1998). More recently, pulmonary tuberculosis patients receiving standard therapy and a 100,000 IU dose of vitamin D_3 every 2 weeks showed accelerated sputum conversion if they possessed the *tt* genotype of the vitamin D receptor as compared with placebo (Martineau et al. 2011). Further, vitamin D supplementation accelerated resolution of inflammation during tuberculosis treatment (Coussens et al. 2012). A recent randomized, double-blinded, multicenter, placebo-controlled clinical study involving 258 patients showed that 600,000 IU vitamin D3 once per month for 2 months led to a significant increase in average weight gain and lower residual disease by chest x-ray as compared to placebo (Salahuddin et al. 2013).

Deficiencies in vitamin D are associated with poor outcomes in HIV-infected individual, bacterial vaginosis in the first trimester of pregnancy, increased influenza A infections, and increased respiratory tract infections (Bodnar et al. 2009; Villamor 2006; Aloia and Li-Ng 2007; Sabetta et al. 2010). Supplementation with vitamin D lowered the incidence of seasonal flu in school children, the elderly, and African-American women and lowered the severity of respiratory tract infections (Urashima et al. 2010; Avenell et al. 2007; Aloia et al. 2005; Kenny et al. 2012). In contrast, vitamin D supplementation did not reduce the incidence and duration of severity of upper respiratory tract infection (Li-Ng et al. 2009). In a meta-analysis of 11 placebo-controlled studies involving 5660 patients, vitamin D showed a protective effect against respiratory tract infections with once-daily dosing being better than bolus doses (Bergman et al. 2013). The authors noted that there was significant heterogeneity and evidence of publication bias in the field and warned that results should be carefully interpreted (Bergman et al. 2013). It should be noted that in all of these studies including those with tuberculosis, the role of CAMP induction in these outcomes is unknown. Future studies must optimize dose, dosing frequency, and target populations that are deficient in vitamin D to detect modest effects.

7.7 The Impact of Vitamin D on CAMP Levels

To date, *in vivo* studies demonstrating that vitamin D status or supplementation affects the levels of CAMP/hCAP18 are inconclusive. High levels of hCAP18 are found in the blood; therefore, we hypothesized that vitamin D levels may correlate with hCAP18 levels (Sorensen et al. 1997; Gombart et al. 2009b). In an early study on dialysis patients, we found only a modest positive correlation between hCAP18 and 1,25(OH)2D, but not 25(OH)D levels, but high hCAP18 levels were associated with a significant decrease in 1-year mortality (Gombart et al. 2009b). For sepsis

patients, a positive association between 25(OH)D and hCAP18 levels was observed in all patients (Jeng et al. 2009). In healthy individuals, a positive association between hCAP18 and 25(OH)D levels was observed at levels of 25(OH)D below 32 ng/ml, but not above (Bhan et al. 2011; Dixon et al. 2012). In the elderly and in atopic dermatitis patients and normal controls, a positive correlation was observed without applying a cutoff (Alvarez-Rodriguez et al. 2012; Kanda et al. 2012). On the other hand, in cord blood samples, patients with active TB and patients with pneumonia, a correlation between serum 25(OH)D and hCAP18 was not observed (Yamshchikov et al. 2010; Mandic Havelka et al. 2010; Leow et al. 2011). Supplementation of atopic dermatitis patients with 4000 IU/day vitamin D for 3 weeks increased CAMP in skin lesions and unaffected skin, but a second study with more patients was negative (Hata et al. 2008, 2014). Several studies using high-dose supplementation (50,000–60,000 IU/week) did not observe increased hCAP18 in the blood (Adams et al. 2009; Alvarez et al. 2013; Das et al. 2014). In a randomized controlled trial in patients with severe sepsis, $1,25(OH)_2D_3$ did not increase plasma hCAP18 levels (Leaf et al. 2014). In a study of 15 hereditary vitamin D-resistant rickets patients (possess a nonfunctional VDR) and 17 normal controls, it was shown that VDR is required for induction of CAMP by vitamin D in adherent mononuclear cells cultured for 24 h, but basal expression of CAMP in various cell types, fluids, or tissue samples was not determined (Tiosano et al. 2013). Additional studies are required to determine the effect of vitamin D status or treatment on *in vivo* CAMP expression, particularly on the *in vivo* induction of CAMP in immune cells like macrophages during infection.

7.8 Vitamin D-Mediated Regulation of AMPs: An Animal Model

The difficulty in determining the role CAMP in mediating the effects of vitamin D on the immune response is the lack of a good animal model that replicates the pathway as it is found in humans. As described earlier, vitamin D does not regulate CAMP expression in mice or other mammals (Gombart et al. 2009a). In addition, work from our own group and others revealed a striking difference in the use of vitamin D by human *versus* murine macrophages. As described above, activation of human macrophages by TLR ligands induces expression of CYP27B1 and the bioconversion of $25(OH)D_3$ to $1,25(OH)_2D_3$. This, in turn, leads to the induction of various VDR target genes including *CAMP*. In contrast, TLR activation of murine macrophages does not induce CYP27B1 expression; thus, bioactive $1,25(OH)_2D_3$ is not synthesized by murine macrophages (Kapetanovic et al. 2012; Ooi et al. 2014), and vitamin D target genes are not induced (our unpublished findings). This major difference in the utilization of vitamin D by macrophages highlights the importance of caution when using the mouse model to elucidate the role of vitamin D on immune function in humans. Macrophages are very likely important for producing locally high levels of $1,25(OH)_2D_3$ at sites of infection in humans, but not in mice.

7.9 Conclusion

An abundance of *in vitro* evidence exists to demonstrate the regulation of AMPs, particularly CAMP, by vitamin D. Also, historical, epidemiological, and clinical data is consistent with the vitamin D-CAMP pathway providing protection against infection. Nevertheless, there is a paucity of *in vivo* data that demonstrates that induction of CAMP mediates important antibacterial or viral activities that are attributed to vitamin D. Due to the current lack of a viable animal model, this evidence will need to come from carefully designed human and/or primate studies. Outstanding questions remain on how vitamin D status or supplementation affects CAMP and DEFB4 expression and can active forms of vitamin D increase levels of AMPs to improve immunity.

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