

Control of cutaneous antimicrobial peptides by vitamin D3

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Abstract Constant exposure to a wide variety of microbial pathogens represents a major challenge for our skin. Antimicrobial peptides (AMPs) are mediators of cutaneous innate immunity and protect primarily against microbial infections. Cathelicidins were among the first AMPs identified in human skin and recent evidence suggests that they exert a dual role in innate immune defense: At first, due to their antimicrobial activity they kill pathogens directly. In addition, these peptides initiate a potent host response to infection resulting in cytokine release, inflammation and a cellular response. Disturbed cathelicidin expression and function was observed in several common inflammatory skin diseases, such as psoriasis where cathelicidin peptide converts inert self-DNA and self-RNA into an autoimmune stimulus. In atopic dermatitis decreased levels of cathelicidin facilitating microbial superinfections have been discussed. Furthermore, abnormally processed cathelicidin peptides induce inflammation and a vascular response in rosacea. Until recently, the molecular mechanisms underlying cathelicidin regulation were unknown. Recently, the vitamin D3 pathway was identified as the major regulator of cathelicidin expression. Consequently, vitamin D3 entered the spotlight as an immune modulator with impact on both innate and adaptive immunity. Therapies targeting vitamin D3 signaling may provide new approaches for infectious and inflammatory skin diseases by affecting both innate and adaptive immune functions.

Keywords $1\alpha,25$ -Dihydroxyvitamin D3 · Antimicrobial peptides · Alarmins · Cathelicidin · Psoriasis · Skin

The non-classical actions of vitamin D3

Since the beginning of the twentieth century it has become evident that sufficient intake of vitamin D3 is essential for the maintenance of calcium homeostasis and bone health [36]. In addition to this original role, vitamin D3 has now become the subject of intensive research as an immune regulator with the impact on innate and adaptive immunity [5, 77]. Several cell types of the adaptive immune system were found to be influenced by vitamin D3 signaling e.g. dendritic cells (DCs) and T cells [77, 78]. In a distinct immune regulatory role, vitamin D3 affects the innate antimicrobial defence at epithelial barriers, such as the airway epithelium or the skin [69, 86]. Either role might be of clinical importance as vitamin D3 deficiency is common especially in the elderly population. Hypovitaminosis D is assumed to contribute to increased susceptibility to infection and a higher mortality risk [2]. Sparse sun exposure (often in an effort to prevent skin cancer) and an unbalanced and insufficient diet are thought to be reasons for low vitamin D3 serum levels [63]. Insistent recommendations to prevent sun exposure complicate this situation [51].

Nevertheless, under normal circumstances the human body is able to synthesize sufficient vitamin D3 from precursors under the influence of UVB irradiation [8]. $1\alpha,25$ -dihydroxyvitamin D3, the hormonally active form of vitamin D3, is synthesized by the interplay of different cell types. The synthesis of $1\alpha,25$ -dihydroxyvitamin D3 from the precursor 7-dehydrocholesterol (provitamin D3) occurs in the basal and suprabasal layers of the skin and requires UVB

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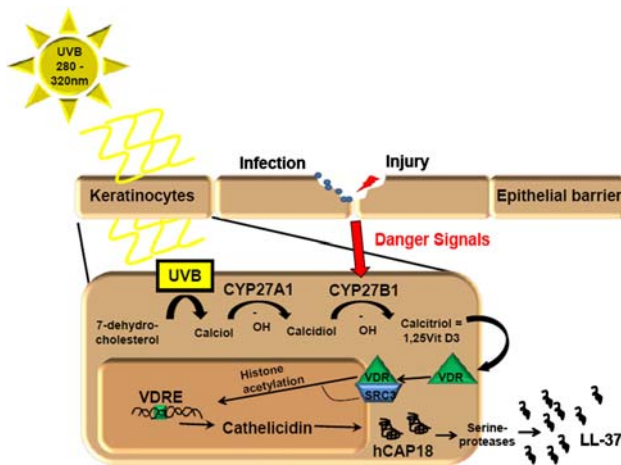


Fig. 1 Model for UVB triggered vitamin D3 activation and cathelicidin response in the skin. Photochemical conversion of 7-dehydrocholesterol to calcidiol in the skin requires UVB irradiation. In keratinocytes, two subsequent hydroxylation steps mediated by CYP27A1 and CYP27B1 result in active calcitriol/1 α ,25-dihydroxyvitamin D3 (1,25VitD3) that binds to and activates the vitamin D receptor (VDR) in an autocrine manner. Steroid receptor coactivator 3 (SRC3) complexes with the VDR and recruits histone acetyltransferases which open up chromatin and facilitate access to the cathelicidin gene. VDR binds to the vitamin D response element (VDRE) in the cathelicidin promoter region and activates transcription. Cathelicidin is synthesized as an inactive pro-peptide (hCAP18) which is cleaved upon release to active LL-37 by serine proteases

radiation in the range of 280–320 nm [43]. The product of this fast photochemical process is calcidiol (previtamin D3) which is an inactive form of vitamin D3. Hydroxylation of calcidiol by vitamin D 25-hydroxylase (CYP27A1) in the liver and by 25-hydroxyvitamin D3 1 α -hydroxylase (CYP27B1) in the kidney leads to active 1 α ,25-dihydroxyvitamin D3 (calcitriol) [61]. Keratinocytes express both CYP27A1 and CYP27B1 and can therefore activate vitamin D3 independent of renal and hepatic hydroxylation steps (Fig. 1) [6]. This is especially important as vitamin D3 is required for normal keratinocyte proliferation, differentiation and function [4, 43]. Thus, a disturbed or insufficient vitamin D3 metabolism can very likely influence various keratinocyte and thus, skin functions [7].

Vitamin D3 and adaptive immunity

The vitamin D receptor (VDR) is a nuclear receptor expressed by almost all cells of the adaptive immune system which renders them responsive to vitamin D3 [77]. It has been shown earlier that vitamin D3 suppresses adaptive immunity by inhibition of antigen-presenting cells and by the generation of a T_{H2} microenvironment [9, 30, 64]. Upon vitamin D3 treatment of T cells T_{H2} cytokines, such as IL-4, IL-5 and IL-10 were increased while IL-2 and IFN- γ —typical T_{H1} cytokines needed for T cell proliferation and

activation—were decreased [9, 45, 46]. In addition, T_{H17} cell functions were inhibited while regulatory T cells (Tregs) were increased upon vitamin D3 treatment in mice with experimental inflammatory bowel disease [18, 29]. Recently, T_{H17} cells were found to be involved in psoriasis pathogenesis which might explain in part the beneficial effect of vitamin D analogs in this disease [88]. In addition, vitamin D3 affects homing of T cells in the skin: the expression of the chemokine receptor CXCR10 on T cells was increased upon vitamin D3 treatment which enables these cells to react upon the skin-specific chemokine CCL27 secreted by keratinocytes [74]. In contrast, vitamin D3 decreased the expression of cutaneous lymphocyte-associated antigen (CLA) on T cells [82]. In this context, treatment with vitamin D3 analogs could attenuate T cell mediated skin diseases through effects on this homing receptor, but would enhance CXCR10-positive cell infiltrates.

Besides T cells, B cell proliferation and immunoglobulin production was shown to be inhibited by vitamin D3. However, only pre-activated, proliferating B cells were found to be affected, whereas initial B-cell division was not [16]. DCs are further targets of vitamin D3 and activation of the VDR in these cells inhibits cell differentiation and maturation [60]. In addition, vitamin D3 decreased the expression of costimulatory molecules and cytokine secretion leading to an impaired antigen-presenting capability of DCs [17, 90].

These suppressive effects of vitamin D3 on adaptive immunity might be beneficial in autoimmune driven diseases, such as inflammatory arthritis, inflammatory bowel disease and skin diseases, such as psoriasis [20]. Actually, multiple studies demonstrate that treatment of psoriatic plaques with vitamin D3 analogs significantly ameliorates cutaneous inflammation [15, 23]. Vitamin D3 is produced in keratinocytes and its analogs are applied topically to the skin. Thus, the regulation of immune functions of resident cells such as keratinocytes by vitamin D3 and its analogs has to be considered as well and these functions might be equally relevant in inflammatory skin diseases.

The skin's innate defense mechanisms

Our skin constitutes an initial defense barrier against invading microbial pathogens. Epidermal keratinocytes shape the physical barrier of the skin (such as the stratum corneum) and contribute to innate immunity. In addition, professional innate immune cells, such as DCs and macrophages reside in the skin ready to react to infections [54]. Small cationic peptides classified as antimicrobial peptides (AMPs) contribute to the chemical shield on the surface of the skin and other epithelia. AMPs were first defined as endogenous antibiotics due to their capability to kill various pathogens,

e.g. gram-positive and gram-negative bacteria, viruses and fungi. However, AMPs were found to be more than that: today it is well established that they act as immune modulators with impact on innate and adaptive immune functions. Therefore, they are considered important players in the host defense system [65]. Keratinocytes and other resident cells in the skin, such as cells in eccrine glands, mast cells and sebocytes produce and secrete AMPs. Beyond that invading immune cells (e.g. neutrophils, NK cells) contribute to the pool of AMPs in the skin [14, 22, 52, 65].

AMPs constitute a diverse group of structurally distinct peptides with similar functions. Today, several dozens of different peptides with antimicrobial function in the skin are known [11]. Often these AMPs are better known for other biological activities as their antimicrobial function was later identified. An example is the leukocyte protease inhibitor or the α -melanocyte-stimulating hormone [11]. Also, no clear definition for antimicrobial activity exists and the experimental assays to define antimicrobial activity are not standardized so that the list of skin-derived AMPs is expected to grow [66].

Two important and well studied AMP gene families in the skin are the defensins and cathelicidins [72, 89]. The first skin-derived AMP found in humans was β -defensin 2 (HBD2) [31]. HBD2 is most effective against gram-negative bacteria, whereas HBD3 from the same AMP family has a broader spectrum of antimicrobial action [32]. HBD2 is induced in skin inflammation and infection [71]. Only recently, it was also shown by Glaser et al. that HBD2 and 3 are inducible in vitro and in vivo by UVB irradiation [26], whereas HBD1 is constitutively expressed [90].

Another well studied AMP in the skin is cathelicidin, often referred to its peptide forms hCAP18 or LL-37. Similar to HBD2 and 3, hCAP18 mRNA is also inducible in human skin by UVB irradiation in vivo [49]. The human genome contains only one cathelicidin gene (CAMP) and cathelicidin is produced as a pro-peptide which is composed of an N-terminal cathelin domain and a C-terminal 37 amino acid sequence with antimicrobial activity. This part can be cleaved from the inactive precursor by serine proteases of the kallikrein family to form active LL-37 [13, 83, 89]. LL-37 can be further processed to smaller peptides with different functions [53]. In aqueous solution, LL-37 forms an α -helix which enables the peptide to disrupt both bacterial membranes and viral envelopes [11]. Even antifungal activity of LL-37 in *Candida* infections was reported [48]. Besides, LL-37 interacts with mammalian cells in a ligand-receptor mediated or a receptor-independent manner resulting in a host response which is referred to as the “alarmin” function of LL-37 [57]. Alarmin functions include the direct interaction with formyl peptide receptor like 1 or G-coupled receptors [55, 85]. Furthermore, LL-37 influences ATP-receptor P2X7 and TLR signaling in immune cells,

EGF receptor transactivation and intracellular Ca^{2+} mobilization [21, 75, 76, 91]. Also, chemokine and cytokine release was induced by LL-37 [91]. In synergy with the immune modulator IL-1 β , LL-37 enhances innate immune responses by multiple pathways [65, 87]. Thus, the dual role of cathelicidin, namely the antimicrobial and the alarmin function, suggests an important role for this peptide in cutaneous innate immune defense.

Regulation of cathelicidin expression by vitamin D3

Similar to other AMPs, human cathelicidin is expressed by keratinocytes and cells of the adaptive immune system [1, 12]. Cathelicidin expression is upregulated in infected and injured skin [22, 24] and cathelicidin promotes neovascularization, wound healing, but also chemokine expression and migration of immune cells (neutrophils, mast cells, monocytes and T cells) [1, 19, 34, 39]. Although LL-37 is induced in these conditions the regulation of cathelicidin gene expression was long unclear, since mediators of infection did not influence gene expression [68].

Several recent reports suggest a connection between vitamin D3 and AMP expression in keratinocytes. Wang et al. [79] demonstrated that vitamin D3 has a pivotal role in antimicrobial immunity of the skin when they identified a vitamin D3 response element (VDRE) in the promoter region of the cathelicidin gene. Subsequently other groups confirmed that cathelicidin is a direct target of vitamin D3 in keratinocytes [27, 80]. Furthermore, conservation of cathelicidin gene regulation by the vitamin D3 pathway in humans and non-human primates during evolution has been demonstrated recently [28]. Until present several elements in the molecular mechanisms of vitamin D3 mediated cathelicidin expression have been characterized: Epigenetic changes, such as histone acetylation and co-activator activity enable the control of cathelicidin gene expression by vitamin D3 in keratinocytes [67, 70]. Furthermore, it was shown by our group that IL-17 and vitamin D3 pathways act in synergy to enhance the expression of cathelicidin in keratinocytes [58]. In particular, vitamin D3 enables IL-17A to increase cathelicidin expression through activation of Act1 and MEK-ERK.

In cutaneous wounds or bacterial infections the expression of cathelicidin LL-37 is increased as is TLR2 and its co-receptor CD14. In addition, it was shown that vitamin D3 is responsible for the increased expression of TLR2 and CD14 in vitro and in vivo [69]. However, a sudden change in the vitamin D3 concentration in the wound milieu seemed unlikely. As mentioned above, vitamin D3 is activated through hydroxylation by CYP27B1 in keratinocytes and monocytes in the skin [69]. In addition, the expression

of CYP27B1 is under the control of danger signals that are active during skin infection and injury [47, 69]. Thus, the local increase of CYP27B1 in infected or injured tissue and the subsequent activation of vitamin D3 induce cathelicidin expression and function in cutaneous wounds (Fig. 1). Therefore, vitamin D3 predominantly suppresses adaptive immunity, whereas innate immune mechanisms such as cathelicidin expression in wounds and infections are activated.

Role of cathelicidin in skin diseases

Under physiological conditions cathelicidin and other AMPs protect the skin from microbial infection. In healthy skin only very low amounts of cathelicidin LL-37 are detectable. Upon infection or wounding cathelicidin expression is strongly increased [22, 24, 69]. Yet in some skin diseases, the barrier function of the skin and control of inflammation is disturbed and cathelicidin levels are altered compared with healthy skin. In atopic dermatitis, for example, viral or bacterial superinfections are common and this might be due to disturbed expression of AMPs [56]. An altered cytokine milieu was suggested to be the reason for the diminished inducibility of cathelicidin expression and other AMPs, such as HBD2 in the course of this disease [37]. In particular, T_H2 cytokines like IL-4 and IL-13 suppressed the induction of AMPs. However, this view has been challenged recently by other authors [3], who found increased cathelicidin transcript and protein in lesional skin from patients with atopic eczema when compared with non-lesional skin or skin from healthy controls. Harder et al. [81] reported increased functional HBD2 and other AMP expression in lesional skin in atopic eczema when compared with healthy controls. Still, a third group reported downregulated cathelicidin expression in injured skin in atopic eczema following acute wounding in contrast to enhanced expression in wounds in healthy skin [50].

Another disease with possible involvement of cathelicidin LL-37 is rosacea which is characterized by centrofacial skin inflammation and vascular reactivity. It was observed that patients suffering from this disease express abnormally high levels of LL-37 peptide, whereas in healthy individuals smaller, processed forms predominate [84]. These processed peptides mediate antimicrobial rather than immunomodulatory functions. Furthermore, in rosacea the processed forms of LL-37 peptide were different from the peptides found in healthy individuals due to an abnormal and increased activity of cutaneous protease activity [84]. Thus, in rosacea an unknown molecular mechanism leading to increased LL-37 expression and abnormal protease activity results in skin inflammation.

Psoriasis is a third example of an inflammatory skin disease associated with abnormal AMP activity. Psoriasis is suggested to be an autoimmune disease with T cell involvement and an overreacting innate immune response resulting in chronic cutaneous inflammation [10, 54, 62]. Still, the auto-antigen triggering the immune response remains unknown. Recently, Lande et al. [41] showed that plasmacytoid dendritic cells (pDCs) in psoriasis are activated by self-DNA complexed with LL-37. Cathelicidin LL-37 expression is increased in lesional skin [56] and self-DNA-LL-37 complexes signaled through TLR9 resulting in IFN- α release by pDCs. Consecutively, secreted IFN- α activates a T cell response that leads to cutaneous inflammation [41]. The self-DNA needed for the initiation of this inflammatory cascade might come from dead and dying cells in the psoriatic plaque. Thus, in psoriasis overexpression of cathelicidin together with a second factor (self-DNA) which under healthy conditions is not present in the skin might lead to skin inflammation [41]. Another recent study published by Ganguly et al. [25] revealed that LL-37 not only forms complexes with self-DNA, but also with self-RNA. In addition, the LL-37–nucleic acid complexes activated pDCs and additionally myeloid DCs (mDCs) via TLR7 and TLR8 signaling, respectively. Thus, the transformation of inert nucleic acids (self-DNA, self-RNA) into immunologically relevant immune activators by cathelicidin is thought to drive an autoinflammatory response in psoriasis.

Therapeutic potential of vitamin D3 and its analogs

As cathelicidin expression and function is altered in several common inflammatory skin disorders, such as psoriasis, rosacea and atopic dermatitis new therapeutic approaches to restore normal cathelicidin expression and function might prove beneficial for the treatment of these skin diseases. Targeting the vitamin D3 pathway—so far the only known pathway that directly influences cathelicidin expression in keratinocytes—might be such a therapeutic strategy. In the following paragraphs possible approaches will be discussed.

Atopic dermatitis

Skin inflammation in atopic dermatitis can be ameliorated by UVB irradiation. So far, the positive effects of this therapy were attributed to the effect of UVB on T cells. However, the beneficial effects could also be a result of the UVB induced activation of AMPs, such as HBD2 and 3 [26] and/or the UVB induced vitamin D3 synthesis and subsequent cathelicidin expression in the skin [44]. T_H17 cells capable of producing IL-17A were reported to be increased in the skin and

blood of patients with atopic dermatitis [40]. It was shown by Peric et al. [58] that IL-17A enhances vitamin D3 induced expression of cathelicidin in keratinocytes. Thus, topical treatment or oral supplementation of vitamin D3 might prove effective in this disease [92]. Malbris et al. [50] showed recently that the vitamin D3 activated pathways leading to induced cathelicidin in keratinocytes in atopic skin are functional. Indeed, Hata et al. [33] showed increased cathelicidin in skin biopsies in patients suffering from atopic eczema following a course of oral vitamin D supplementation. Thus, increasing vitamin D3 synthesis and elevating vitamin D3 levels in the serum could help in strengthening the innate defence barriers to prevent cutaneous infections which trigger skin inflammation in atopic dermatitis.

Rosacea

In rosacea, blockade of cathelicidin expression and LL-37 processing might ameliorate skin inflammation. A VDR gene polymorphism was described in patients with severe rosacea indicating that vitamin D signaling is involved in disease pathogenesis [38]. By targeting the vitamin D3 pathway, cathelicidin overexpression could be blocked and the production of the disease aggravating cathelicidin fragments inhibited. Another possible therapeutic approach could be the inhibition of the increased proteolytic activity in the skin in rosacea by blocking serine protease activity.

Psoriasis

In psoriasis, blocking or decreasing cathelicidin expression could disrupt the vicious circle of cutaneous inflammation driven by high levels of LL-37 complexed with self-DNA or self-RNA released from dead cells resulting in pDC activation [25, 41]. Vitamin D3 analogs have been used in the topical treatment of psoriasis for a long time although the molecular mechanisms behind their anti-psoriatic action are not completely understood. Vitamin D3 analogs bind to VDR which in turn should bind to the vitamin D responsive element in the promoter region of cathelicidin gene and thus increase LL-37 expression and skin inflammation [59]. However, the opposite is true: Vitamin D analogs decrease inflammation and reverse morphological changes in psoriatic plaques [42]. In particular, we could show that calcipotriol treatment of psoriatic skin leads to a decrease in inflammatory cytokine expression (IL-17A, IL-17F and IL-8) as well as to a decrease in HBD2 and 3. Surprisingly, the expression of cathelicidin hCAP18 was upregulated by calcipotriol in keratinocytes in vitro and in vivo [59]. So far this discrepancy could not be explained and further studies are needed to elucidate the role of cathelicidin in psoriasis

pathogenesis. Possible explanations for this observation include effects of vitamin D3 analogs on cathelicidin peptide processing and activation or effects of these compounds beyond the vitamin D3 pathway.

Chronic wounds

In normal intact skin, cathelicidin LL-37 is expressed at barely detectable levels. Disruption of the epithelial barrier leads to various events aiming to restore the skin integrity. LL-37 is rapidly upregulated in cutaneous wounds and participates in the re-epithelialization process of the skin [22, 34, 73]. Recently it was shown by Heilborn et al. [35] that LL-37 is increased in acute and chronic non-healing wounds upon treatment with the vitamin D3 analog calcipotriol. In addition, CYP27B1, the enzyme responsible for hydroxylation of 25-hydroxyvitamin D3 to active 1 α ,25-dihydroxyvitamin D3, was found to be upregulated in acute, but not in chronic wounds. This suggests that wound healing can possibly be improved by increasing cathelicidin LL-37 levels via vitamin D3 treatment or activation of vitamin D3 metabolism.

Conclusion

In summary, influencing cathelicidin expression by targeting the vitamin D3 pathway might present a novel therapeutic approach for the treatment of skin diseases with disturbed hCAP18/LL-37 expression, such as psoriasis, rosacea or atopic dermatitis. However, a deeper insight and understanding of the molecular mechanisms of AMP biology and their regulation by the vitamin D3 pathway is needed before this knowledge can be translated into therapeutic use. Nevertheless, increasing data on immune functions of AMPs and their molecular regulation by different signaling pathways underscores their importance in skin physiology and recommends them as targets for pharmacological therapy.

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Conflict of interest statement The authors declare that they have no conflict of interest.

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