Journal of Steroid Biochemistry & Molecular Biology 121 (2010) 228–233

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

1α-Hydroxylase and innate immune responses to 25-hydroxyvitamin D in colonic cell lines☆,☆☆

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ARTICLE INFO

Article history:
Received 23 October 2009
Received in revised form 1 February 2010
Accepted 3 February 2010

Keywords:
Vitamin D
1α-Hydroxylase
Epithelial cell
Macrophage
Cathelicidin
Defensin

ABSTRACT

Vitamin D-insufficiency is a prevalent condition in populations throughout the world, with low serum levels of 25-hydroxyvitamin D (25OHD) linked to a variety of human health concerns including cancer, autoimmune disease and infection. Current data suggest that 25OHD action involves localized extra-renal conversion to 1,25-dihydroxyvitamin D (1,25(OH)2D) via tissue-specific expression of the enzyme 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase). In cells such as macrophages, expression of 1α-hydroxylase is intimately associated with toll-like receptor (TLR) recognition of pathogens. However, this mechanism may not be exclusive to extra-renal generation of 1,25(OH)2D. To investigate the relationship between TLR-mediated pathogen recognition and vitamin D-induced antibacterial activity, intracrine responses to 25OHD metabolism were explored in vitro using the established colonic cell lines Caco-2 and Caco-2 clone BBe. Analysis of antibacterial factors such as cathelicidin (LL37) and β-defensin-4 (DEFB4) was carried out following co-treatment with TLR ligands. Data indicate that, unlike macrophages, Caco-2 and BBe colonic cell lines are unresponsive to TLR-induced 1α-hydroxylase. Alternative activators of 1α-hydroxylase such as transforming growth factor β were also ineffective at priming intracrine responses to 25OHD. Thus, in common with other barrier sites such as the skin or placenta, colonic epithelial cells may require specific factors to initiate intracrine responses to vitamin D.

1. Introduction

Interaction between vitamin D and the immune system has been recognized for over a quarter of a century. Despite this, the potential importance of vitamin D to normal human innate immunity has only gained recognition in the last few years. Two key developments have underpinned this change in perspective. The first is that our perception of what constitutes adequate vitamin D status has changed. The observation that serum levels of the inactive form of vitamin D, 25-hydroxyvitamin D (25OHD) as high as 75 nM correlate inversely with parathyroid hormone [1] has prompted introduction of the term "vitamin D-insufficiency". This is defined by serum levels of 25OHD that are sub-optimal (<75 nM) but not necessarily rachitic (<20 nM) [2]. Unlike serum concentrations of active 1,25-dihydroxyvitamin D (1,25(OH)2D), which are primarily defined by renal regulators of the enzyme, 25-hydroxyvitamin D-1α-hydroxylase (1α-hydroxylase), circulating levels of 25OHD are a direct reflection of individual exposure to sunlight or dietary intake of vitamin D. As a consequence of these new parameters it is clear that the numbers of people throughout the world classified as vitamin D insufficient is much greater than originally thought [3].

The second important development to alter our perception of vitamin D concerns recent studies highlighting its potent effects on innate immunity [4–7]. Previous experiments by Rook and colleagues first demonstrated the ability of 1,25(OH)2D to suppress proliferation of pathogens such as Mycobacterium tuberculosis (M. tb) in macrophages [8]. However, it was not until much later that similar actions were described for 25OHD. In studies to define monocyte responses to M. tb, Modlin and colleagues demonstrated induction of the vitamin D receptor (VDR) and 1α-OHase in monocytes treated with ligand to TLR2/1, the principal pathogen-recognition receptor for M. tb [9]. They were then able to demonstrate activation of monocyte target genes as a consequence of local synthesis of 1,25(OH)2D from 25OHD. The key immune target of this intracrine metabolism was cathelicidin (LL37), an antimicrobial protein known to express a functional vitamin D response element in its gene promoter [10,11]. Induction of LL37 appears to be a crucial step in facilitating successful phagolyzosomal killing of bacteria, particularly M. tb [12]. Significantly, ex vivo induction of LL37 in monocytes following TLR activation

☆ Special issue selected article from the 14th Vitamin D Workshop held at Brugge, Belgium on October 4–8, 2009.

☆☆ Grant support: NIH grant AR050626 to MH.

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doi:10.1016/j.jsbmb.2010.02.004

has been shown to correlate closely with serum levels of 25OHD, underlining the importance of intracrine vitamin D metabolism to this facet of innate immunity [9,13].

Induction of LL37 by 1,25(OH)2D does not appear to be restricted to monocytes and macrophages. Similar activation of the antimicrobial factor has been reported in bronchial epithelial cells [14], myeloid cell lines [10], as well as decidual [15], and trophoblastic [16] cells of the placenta. However, this effect does not appear to be universal despite the ubiquitous expression of VDR [17]. The question therefore arises as to whether different parameters are required to promote vitamin D-induced innate immunity within different tissues. In the following manuscript we review reported mechanisms for regulation of 1α-OHase and LL37 in 'barrier' tissues such as the skin, placenta and gastrointestinal (GI) tract. In addition we present data from studies using colonic cell lines, which provide a further perspective on the role of localized synthesis of 1,25(OH)2D in promoting antimicrobial activity in cells not conventionally involved in immune responses.

2. Materials and methods

2.1. Reagents

Unlabeled 1,25-dihydroxyvitamin D_3 (1,25(OH)2D) and 25-hydroxyvitamin D_3 (25OHD) were purchased from Biomol (Plymouth Meeting, PA). Toll-like receptor (TLR) 2 ligand 19 kDa lipopeptide (19 kDa, Invivogen, San Diego, CA, USA). TLR4 ligand lipopolysaccharide (LPS, Invivogen). [^{3}H]25-hydroxyvitamin D_3 ([^{3}H]25OHD_3, specific activity, 187 Ci/mmol) was purchased from Amersham Biosciences, Piscataway, NJ.

2.2. Cell culture

The Caco-2 colonic cell line and the BBe clone of Caco-2 (both kind gifts of Dr. J.C. Fleet, Purdue University) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS as described previously [18]. Cell treatments were for 24 h and included 1,25(OH)2D (10 nM), 25OHD (100 nM), 19 kDa (1 ng/ml) and LPS (100 ng/ml). Ficoll-isolated peripheral blood mononuclear cells (PBMCs) derived from anonymous donors were obtained from the Center for AIDS Research Virology Core/BSL3 Facility (supported by the National Institutes of Health award AI-28697 and by the UCLA AIDS Institute and the UCLA Council of Bioscience Resources). Monocytic cells were isolated from PBMCs by adherence and then maintained for 7 days in 24-well plates using RPMI 1640 medium supplemented with 10% FCS and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Immunex, Seattle, WA). The resulting cells showed increased expression of macrophage makers such as CD14 [19].

2.3. Quantification of 1α- and 24-hydroxylase activity

Synthesis of 1,25(OH)2D by Caco-2 and BBe cells was assessed by quantifying the conversion of radiolabeled 25OHD to 1,25(OH)2D in serum-free cultures of these cells. For each assay 50 nM [^{3}H]25OHD_3 (Amersham Biosciences, Piscataway, NJ) was added

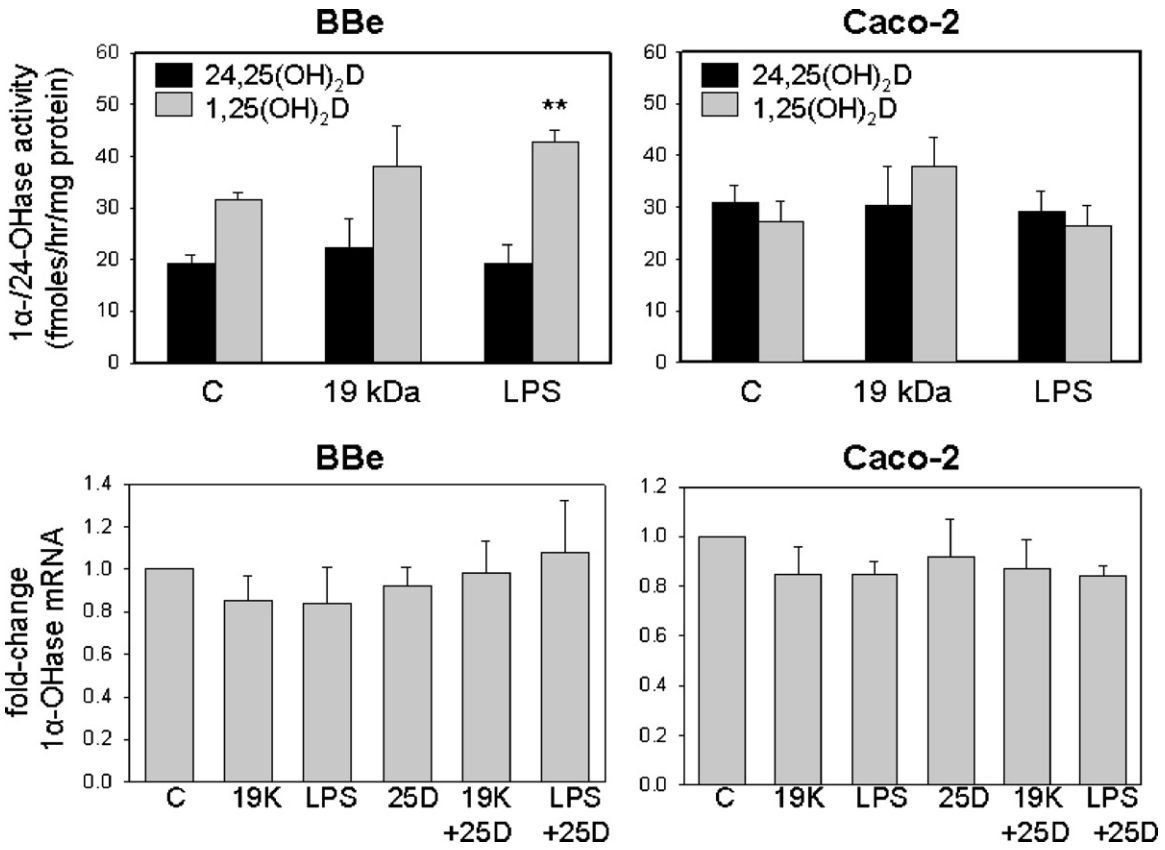

Fig. 1. Toll-like receptor regulation of vitamin D metabolism and 1α-hydroxylase expression in colonic cell lines. BBe and Caco-2 colonic cells were treated with vehicle (control, C), ligand to TLR2 (19 kDa lipoprotein, 19 kDa) or ligand to TLR4 (lipopolysaccharide, LPS), or combinations of TLR ligand with 100 nM 25-hydroxylvitamin D (25D) for 24 h and then assessed for: (A) 24-hydroxylase activity (synthesis of 24,25(OH)$_2$D) and 1α-hydroxylase activity (synthesis of 1,25(OH)2D). Data are shown as fmoles metabolite produced/h/mg protein ± SD for $n = 4$ separate assays. (B) Expression of mRNA for 1α-hydroxylase (1α-OHase). RT-PCR data are shown as fold-change in 1α-OHase mRNA relative to vehicle-treated cells. **Statistically different from vehicle-treated cells, $p < 0.01$.

to cells in 200 μl of serum-free medium and then incubated for 5 h at 37 °C, with the reaction being terminated by freezing at −20 °C. Protein from these samples was initially precipitated with added acetonitrile (1:1). Vitamin D metabolites were then extracted from the reaction mixtures by elution on C18-OH columns according to manufacturer's instructions (Diasorin, Stillwater, MN). The resulting eluent was resuspended in 25 μl of elution solvent hexane:methanol:isopropanol (90:5:5), vortexed for 15 s, and individual metabolites separated by HPLC using a Beckman System Gold system with an Agilent Technologies Zobax Sil normal-phase column (Agilent Technologies, Paolo Alta, CA) eluted at a rate of 1.5 ml/min for 20 min. Elution profiles for standard vitamin D metabolites ($25OHD_3$, $24,25(OH)_2D_3$, $1,25(OH)2D_3$) were determined by UV absorbance at 264 nm. Elution of metabolites of ^{3}[H]-$25OHD_3$ was assessed using a β-Ram Model 4 in-flow detector (IN/US, Tampa, FL) in conjunction with Ultima-Flo M scintillation fluid (Perkin-Elmer, Boston, MA) at a 2:1 ratio with a 5 s dwell time to designate the increments for data collection. Lauralite 3 software (LabLogic, Sheffield, UK) was used to quantitate peaks of radioactivity corresponding to $25OHD_3$, $24,25(OH)_2D_3$ or $1,25(OH)2D_3$. Data were reported as mean fmoles metabolite synthesized/h/mg cellular protein ± SD following $n = 3$ separate incubations.

2.4. Quantitative PCR analysis of gene expression

RNA was extracted from mouse tissues using the RNeasy™ Total RNA extraction kit as detailed by the manufacturer (Qiagen, Valencia, CA). RNA was eluted in RNase-free elution solution and aliquots (1.5 μg) were reverse-transcribed using Powerscript™ MMLV reverse transcriptase as described by the manufacturer (ABI, Foster City, CA). Expression of target gene mRNA was quantified using an ABI 7700 sequence detection system (ABI, Foster City, CA) as described previously [20]. Approximately 50 ng of cDNAs were used per reaction. All reactions were multiplexed with the housekeeping gene 18S rRNA, provided as an optimised control probe labeled with VIC™ fluorochrome (ABI), enabling data to be expressed in relation to an internal reference to allow for differences in sampling. The fluorogenic probes for target genes were labeled with five-carboxy fluorescein (FAM). Data were obtained as Ct values (the cycle number at which logarithmic PCR plots cross a calculated threshold line), and used to determine ΔCt values (Ct of target gene − Ct of housekeeping gene, 18S rRNA). All reactions were performed in triplicate and expressed as a mean of these values. PCR amplification of target gene cDNA was carried out using the following Taqman human gene expression assays: 1α-OHase, Assays-on-Demand™ (ABI) primer and probe mix Hs01096149; LL37, Hs00189038_m1; DEFB4 Hs00175474_m1; 24-OHase, Hs Hs00167999_m1 (CYP24A1). cDNAs were amplified under the following conditions: 50 °C for 2 min; 95 °C for 10 min; followed by 44 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.5. Statistics

Where indicated, experimental means were compared statistically using one-way analysis of variance (ANOVA) with the Holm–Sidak method used as a post hoc multiple comparison procedure and Sigmaplot Software (Systat Inc., San Jose, CA, USA). Statistical analyses were carried out using raw ΔCt values.

3. Results

Studies were initially carried out to assess metabolism of 25OHD in two colonic cell lines, Caco-2 and a sub-clone of Caco-2 (BBe) able to develop an enterocyte-like phenotype with features of mature, small-intestinal epithelial cells [18]. Both

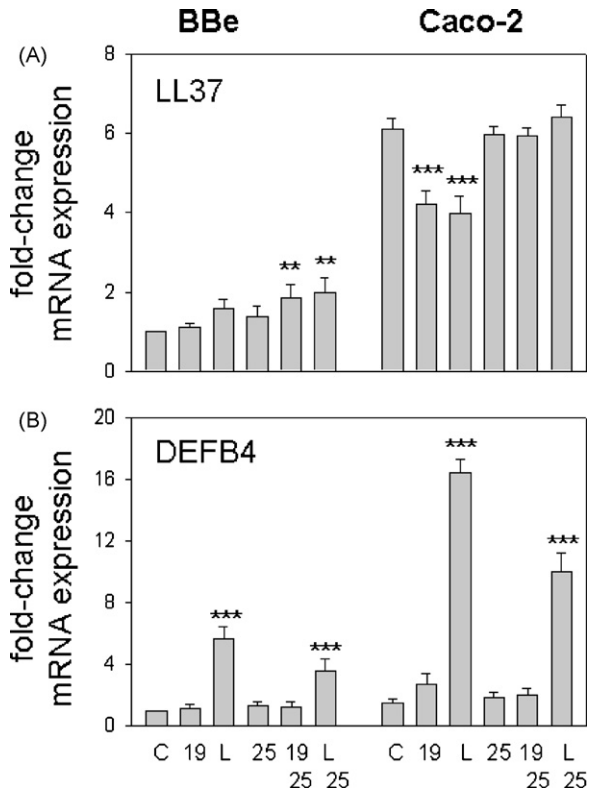

Fig. 2. Toll-like receptor regulation of intracrine induction of antimicrobial agents in colonic cell lines. BBe and Caco-2 colonic cells were treated with vehicle (control, C), ligand to TLR2 (19 kDa lipoprotein, 19) or ligand to TLR4 (lipopolysaccharide, L), or combinations of TLR ligand with 100 nM 25-hydroxylvitamin D (25) for 24 h and then assessed for: (A) expression of mRNA for cathelicidin (LL37); (B) β-defensin-4 (DEFB4). RT-PCR data are shown as fold-change in mRNA relative to vehicle-treated cells. **Statistically different from vehicle-treated cells, $p < 0.01$. ***Statistically different from vehicle-treated cells, $p < 0.001$.

Caco-2 and BBe colonic cell lines demonstrated the capacity to synthesize 1,25(OH)2D and $24,25(OH)_2D$ from 25OHD (Fig. 1A). For Caco-2 cells, neither enzyme activity was influenced by exposure to ligand for TLR2 (19 kDa) or TLR4 (LPS). BBe cells showed a small but significant increase in 1,25(OH)2D production when treated with LPS, although 24-OHase activity was unaffected by the TLR4 ligand. The capacity for synthesis of 1,25(OH)2D in untreated Caco-2 (27 ± 4 fmole/h/mg protein) and BBe cells (32 ± 1 fmole/h/mg protein) was lower than that observed for human macrophages (135 ± 16 fmole/h/mg protein for peripheral blood monocytes cultured for 7 days). Likewise, 24-OHase activity in untreated Caco-2 (31 ± 3 fmole/h/mg protein) and BBe cells (19 ± 2 fmole/h/mg protein) was lower than that observed for human monocytes (117 ± 19 fmole/h/mg protein). For both BBe and Caco-2 cells none of the treatments had any significant effect on expression of mRNA for 1α-hydroxylase (Fig. 1B).

Previous studies have shown that both Caco-2 and BBe cells are sensitive to 1,25(OH)2D. Further studies were therefore carried out to determine whether endogenous 1α-OHase activity in Caco-2 or BBe cells was able to support induction of the antimicrobial proteins LL37 and DEFB4, which are both characterized by the presence of vitamin D response elements in their gene promoters [10,11]. Data in Fig. 2A indicated that there was low baseline expression of LL37 in untreated BBe cells and this was unaffected by treatment with 19 kDa, LPS or 25OHD alone. However, co-treatment of BBe cells with 25OHD and 19 kDa or LPS significantly elevated LL37 expression. Treatment with 1,25(OH)2D induced LL37 to levels equivalent to LPS/19 kDa in combination with 25OHD (data not shown). By

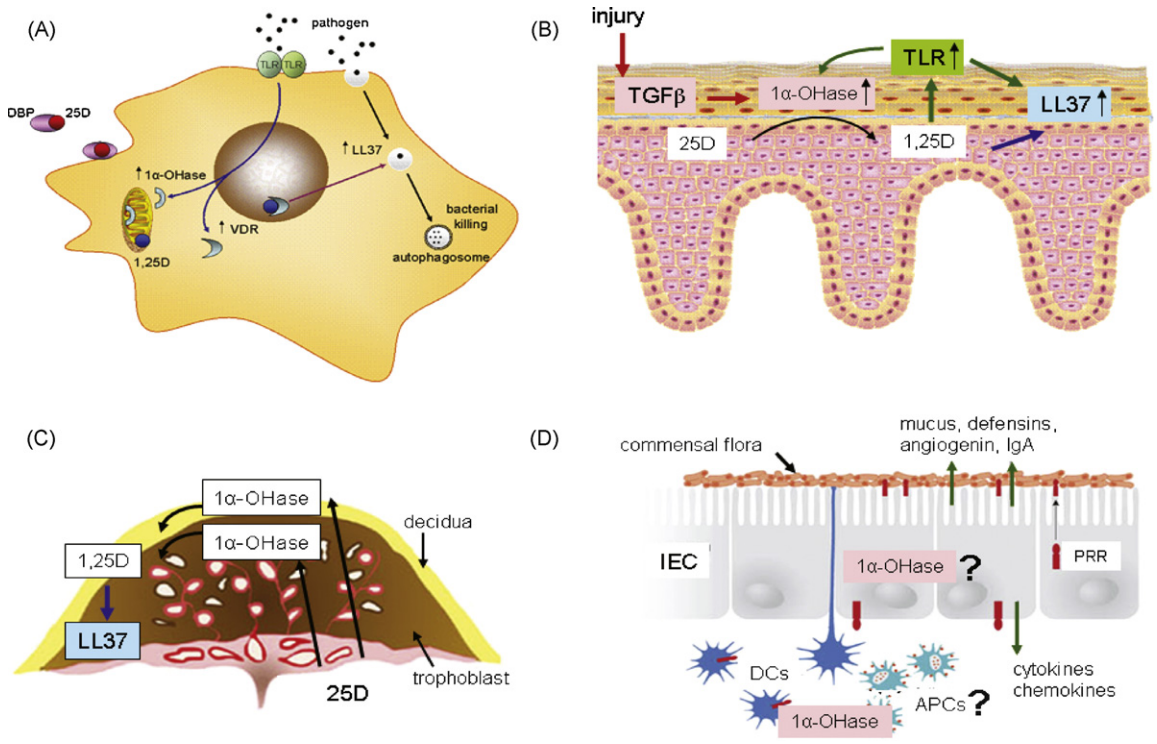

Fig. 3. Mechanisms for involvement of 1α-hydroxylase in antimicrobial responses within different barrier tissues. Schematic representation of mechanisms for intracrine induction of antimicrobial agents such as cathelicidin (LL37) in: (A) monocytes; (B) skin; (C) placenta; (D) gastrointestinal tract. *Abbreviations*: TLR (toll-like receptor); DBP (vitamin D binding protein); $25D_3$ (25-hydroxyvitamin D_3); $1,25D_3$ (1,25-dihydroxyvitamin D_3); 1α-OHase (25-hydroxyvitamin D-1α-hydroxylase); VDR (vitamin D receptor); LL37 (cathelicidin); TGF-β (transforming growth factor β); IEC (intestinal epithelial cell); PRR (pathogen-recognition receptor); DCs (dendritic cells); APCs (antigen presenting cells).

contrast, 1,25(OH)2D stimulated a 250-fold induction of 24-OHase mRNA in BBe cells, whilst 25OHD in combination with LPS produced only a 10-fold induction of 24-OHase (data not shown).

In untreated Caco-2 cells expression of LL37 was 6-fold higher than corresponding BBe cells. However, in contrast to the BBe cells, treatment with the TLR2 or TLR ligands acted to suppress expression of LL37. Treatment with 25OHD alone had no effect on Caco-2 LL37 but the suppressive effects of 19 kDa and LPS were not observed when used in combination with 25OHD. As with BBe cells, treatment of Caco-2 with 1,25(OH)2D (10 nM, 24 h) induced LL37 to levels equivalent to combined LPS/19 kDa and 25OHD (data not shown). However, these responses to 1,25(OH)2D were paralleled by a 500-fold induction of 24-OHase mRNA in Caco-2 cells, whereas treatment with 25OHD in combination with LPS produced only a 7-fold induction of 24-OHase (data not shown).

Low baseline expression of DEFB4 was also observed in the colonic cell lines, with no apparent difference between Caco-2 and BBe cells (Fig. 2B). In both cell lines LPS potently induced DEFB4, whilst 19 kDa and 25OHD had no effect. Interestingly, combined treatment with 25OHD attenuated the stimulatory effects of LPS on DEFB4 in both cell lines.

4. Discussion

TLR induction of intracrine metabolism of vitamin D appears to be central to bacterial killing by monocytes. However, given the widespread expression of both 1α-OHase and VDR in cells at extra-renal sites [21,22], it is likely that similar induction of innate immunity will occur in these tissues. Nevertheless, studies to date indicate that although VDR-mediated induction of LL37 is characteristic of several cell types [10,14–16], it is not universal. Thus, although many cells may exhibit the potential for localized synthesis of 1,25(OH)2D from 25OHD, the in vivo impact of this on key physiological mechanisms such as LL37 expression may be dependent on tissue-specific factors. Data presented here indicate that although both Caco-2 and BBe cell lines express 1α-OHase and produce endogenous 1,25(OH)2D, only BBe cells showed a modest intracrine induction of LL37 using 100 nM 25OHD as substrate. No intracrine induction of LL37 was observed in Caco-2 cells, consistent with previous studies [17]. The differential responsiveness of colonic cells with respect to LL37 does not appear to be due to altered sensitivity to 1,25(OH)2D, as BBe cells appear to express lower levels of VDR than Caco-2 cells [18]. Thus, it seems likely that antimicrobial responses at sites such as the GI tract will involve complex microenvironmental factors that either influence vitamin D responsiveness or act independently on agents such as LL37.

Barrier site elaboration of vitamin D-induced innate immunity has been most well studied using epidermal keratinocytes which, like monocytes, demonstrate synthesis of 1,25(OH)2D and intracrine induction of LL37 [23]. As with monocytes, these effects can be stimulated following TLR2-activation. However, because keratinocytes express low levels of TLR, initial induction of these receptors is required to facilitate pathogen-sensing [23], a function that can be fulfilled by 1,25(OH)2D. Basal expression of 1α-OHase by keratinocytes is stimulated by transforming growth factor beta 1 (TGF-β_1), and this alternative activation pathway appears to be sufficient for 1,25(OH)2D upregulation of TLR2 leading, in turn, to amplification of LL37 production similar to that described for macrophages [23]. In human skin, expression of TGF-β_1 is a feature of tissue injury and thus vitamin D-mediated LL37 expression may be part of a mechanism linking wound repair with enhanced innate immune surveillance [23]. The precise integration of mechanisms associated with vitamin D and innate immunity in the skin versus immune cells such as monocytes remains to be elucidated. In particular, it is interesting to contrast the positive action of 1,25(OH)2D on

keratinocyte TLR expression [23], with the 1,25(OH)2D-mediated suppression of TLR2 and TLR4 reported for monocytes [24].

In contrast to monocytes, colonic epithelial cells and keratinocytes, 1,25(OH)2D appears to be constitutively synthesized by cells from the placenta [25,26]. Expression of 1α-OHase and VDR is profoundly elevated in maternal decidua and fetal trophoblast early in the first trimester of pregnancy, remaining elevated until the third trimester [20]. Initially, this was linked to the rise in maternal serum 1,25(OH)2D that occurs at the end of the first trimester. However, studies of 1α-OHase-deficient animals and anephric pregnant women indicate that this is not likely to be the case [27]. Instead, the presence of VDR in the placenta suggests that vitamin D functions in an autocrine fashion at the fetal–maternal interface [28]. One possible explanation is that 1,25(OH)2D functions as a regulator of placental calcium transport [28], but an immunomodulatory function has also been proposed [29]. The latter stems, in part, from studies highlighting immunological, barrier function of the heterogeneous cells that make up the placenta. Maternal and fetal cells are able to mediate innate and adaptive immune responses. In primary cultures of human decidual cells we showed that both 1,25(OH)2D and 25OHD (the latter metabolized endogenously to 1,25(OH)2D) induced expression of LL37, whilst suppressing inflammatory cytokine production [15]. More recently we have demonstrated that cultured trophoblastic cells also show potent induction of LL37, suggesting that similar innate immune responses to locally metabolized 25OHD occur in both maternal and fetal tissues [16]. In view of the fact that infection during pregnancy is a prevalent cause of preterm birth and fetal mortality, it is tempting to speculate that the constitutive elevation of 1α-OHase in the placenta provides an optimal system for maintaining innate immune surveillance during pregnancy. As yet, the mechanism for induction of 1α-OHase in decidua and trophoblasts remains unclear. However, in common with monocytes, keratinocytes and colonic cells, the efficacy of this intracrine system will be highly dependent on available 25OHD, in other words vitamin D status.

In summary, studies over the last 5 years have shown that local conversion of 25OHD to 1,25(OH)2D potently induces innate antimicrobial effects in a variety of tissue types. As outlined in Fig. 3, the precise mechanism involved in upregulating 1α-OHase activity is likely to vary depending on the cell type involved. The roles of TLR signaling and injury-induced TGF-β expression have been well documented for peripheral blood monocytes and epidermal keratinocytes respectively (Fig. 3A and B). By contrast, the factors associated with regulation of 1α-OHase expression and activity in barrier tissues such as the placenta and GI tract (Fig. 3C and D) are less clear. To address this issue, future studies will need to consider the site-specific microenvironmental factors that impact on these tissues. In the colonic epithelial cells, it is important to recognize that the GI tract is routinely exposed to billions of enteric bacteria acting as commensals within the endogenous microbiota. Thus, regulation of the vitamin D system and antimicrobial activity may be very different from that observed in the skin or placenta where exposure to bacteria is normally minimized. Prospective studies will need to consider the possibility that immunoregulation of the vitamin D system within the GI tract involves pathogen-recognition receptors other than TLR2 and TLR4. In particular, TLR5, which plays a pivotal role in directing colonic mucosal discrimination between pathogenic and commensal bacteria [30] and TLR3, which protects against experimental colitis when activated in the GI tract [31], have yet to be extensively studied with respect to the vitamin D axis. Further characterization of these pathogen-recognition receptors, together with the use of in vivo models for controlled bacterial colonization will help to shed new light on the factors that control the vitamin D metabolism/signaling axis within the GI tract.

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