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CUTTING EDGE

Cutting Edge: 1,25-Dihydroxyvitamin D_3 Is a Direct Inducer of Antimicrobial Peptide Gene Expression[1]

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***The hormonal form of vitamin D_3, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), is an immune system modulator and induces expression of the TLR coreceptor CD14. 1,25$(OH)_2D_3$ signals through the vitamin D receptor, a ligand-stimulated transcription factor that recognizes specific DNA sequences called vitamin D response elements. In this study, we show that 1,25$(OH)_2D_3$ is a direct regulator of antimicrobial innate immune responses. The promoters of the human cathelicidin antimicrobial peptide (camp) and defensin β2 (defB2) genes contain consensus vitamin D response elements that mediate 1,25$(OH)_2D_3$-dependent gene expression. 1,25$(OH)_2D_3$ induces antimicrobial peptide gene expression in isolated human keratinocytes, monocytes and neutrophils, and human cell lines, and 1,25$(OH)_2D_3$ along with LPS synergistically induce camp expression in neutrophils. Moreover, 1,25$(OH)_2D_3$ induces corresponding increases in antimicrobial proteins and secretion of antimicrobial activity against pathogens including Pseudomonas aeruginosa. 1,25$(OH)_2D_3$ thus directly regulates antimicrobial peptide gene expression, revealing the potential of its analogues in treatment of opportunistic infections. The Journal of Immunology,* 2004, 173: 2909–2912.**

The innate immune system provides front-line protection against infectious agents (1). Recognition of bacterial LPS by TLR (1) induces expression of antimicrobial peptides (2, 3) that can fend off bacterial and viral infections (2–5) and accelerate wound healing (6). Antimicrobial peptides have generated intense interest because of their therapeutic potential against antibiotic-resistant pathogens such as *Pseudomonas aeruginosa*, the agent responsible for long-term infection and death in many cystic fibrosis patients (7). We are interested in the molecular events underlying signaling by the hormonal form of vitamin D_3, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$).[3] Vitamin D_3 is obtained from limited dietary sources and through the action of UV B light (UVB) on 7-dehydrocholesterol in skin (8). It is a product of the skin's homeostatic system, which acts as a protective barrier and environmental sensor (9). Although initially identified for its role in calcium homeostasis, 1,25$(OH)_2D_3$ is also an immune system modulator (8, 9) and induces expression of the TLR coreceptor CD14 (10, 11).

1,25$(OH)_2D_3$ signals through the vitamin D receptor (VDR), a member of the nuclear receptor superfamily of transcription factors (8, 12) that is widely expressed in epithelial tissues and cells of the immune system (8). Ligand binding induces VDR heterodimerization with related retinoid X receptors and DNA binding to cognate vitamin D response elements (VDREs) composed of direct repeats of consensus PuG(G/T)TCA motifs (8). In this study, we show that 1,25$(OH)_2D_3$ directly induces antimicrobial gene expression and activity through consensus VDREs located in the promoters of the cathelicidin antimicrobial peptide (*camp*) and defensin β2 (*defB2*) genes, pointing to important new therapeutic uses of vitamin D_3 analogues in treatment of opportunistic infections.

Materials and Methods

Recombinant plasmids

camp promoter sequences between −532 or −491 and +124 were cloned by PCR amplification of genomic DNA with primers 5′-agctaactgcaacttctgctt-3′ and 5′-gtgattctcatgcctcagct-3′, respectively, and 3′ primer 5′-cagacatggggac catgaag-3′. *defB2* promoter sequences downstream from −1266 or −1225 were amplified with primers 5′-cagggttcttcagaacctga-3′ and 5′-cagggttcttca gaacctga-3′, respectively, and common 3′ primer (+23) 5′-agactcagctcctggt gaagctc-3′. Fragments were cloned directly into PCR2.1 (Invitrogen, Burlington, Ontario, Canada), then digested with *Bgl*II and *Kpn*I and subcloned into luciferase reporter plasmid pXP2 to make camp-p/pXP2, camp-p(-V)/pXP2, defB-p/pXP2 and defB-p(-V)/pXP2.

Tissue culture

All lines were cultured under recommended conditions. SCC25, Calu-3, and U937 were obtained from American Type Culture Collection (Manassas, VA) and human adult and neonatal primary keratinocytes from BioWhittaker

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[3] Abbreviations used in this paper: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; camp, cathelicidin antimicrobial peptide; CF, cystic fibrosis; ChIP, chromatin immunoprecipitation; defB2, defensin β2; mop, mouse osteopontin; ngal, neutrophil gelatinase-associated lipocalin; VDR, vitamin D receptor; VDRE, vitamin D response element.

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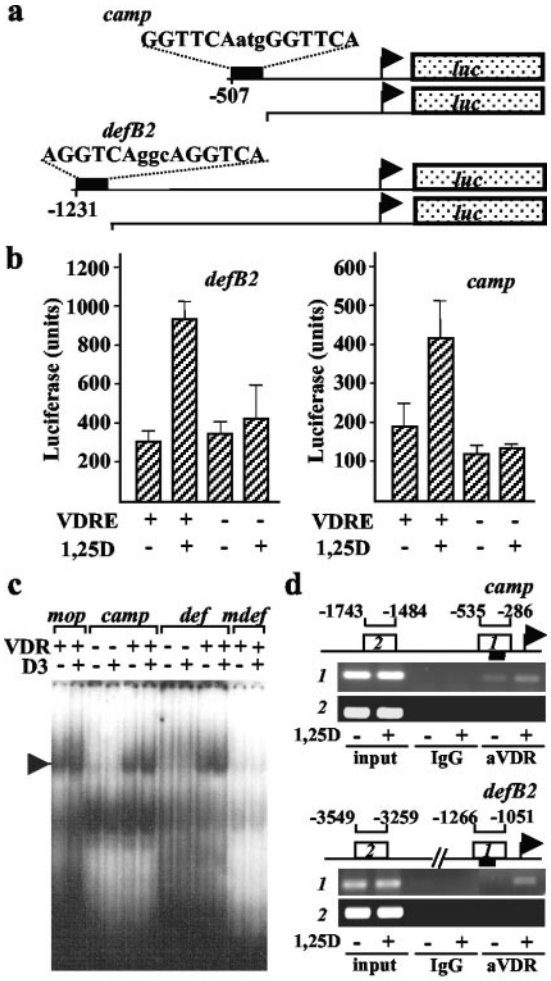

FIGURE 1. The *camp* and *defB2* promoters contain VDREs. *a, camp* and *defb2* promoters cloned into expression vector pXP2. *b,* $1,25(OH)_2D_3$ (1,25D)-dependent luciferase expression in transiently transfected COS-7 cells is dependent on the presence of VDREs. Normalized activities are the means ±SEM from at least three experiments. *c,* EMSAs with extracts of COS-7 cells transfected with a VDR expression vector or empty vector and oligonucleotides containing VDREs from the *camp*, *defB2*, or *mop* promoters, along with a mutant *defB2* VDRE sequence. *d,* ChIP assays of interaction of the VDR with the *camp* and *defB2* promoters in vivo, performed with control IgG or anti-VDR (αVDR) Abs. PCR amplifications were performed on regions of promoters containing VDREs (black bars, *1*) and adjacent control regions (*2*).

(Walkersville, MD). Human monocytes and neutrophils were isolated and cultured as described previously (13, 14). COS-7 cells grown in 6-cm wells in DMEM, supplemented with 10% FBS, were transfected in medium without serum with Lipofectamine 2000 (Invitrogen) with 100 ng of nuclear receptor expression vector pSG5/VDR, 300 ng of camp-p/pXP2, or camp-p(-V)/pXP2, or defB-p/pXP2 or defB-p(-V)/pXP2, and 100 ng of internal control vector pCMV-β-gal. Medium was replaced 6 h after transfection by DMEM, supplemented with 10% FBS. After 24 h, medium was replaced by a medium containing charcoal-stripped serum and ligand (100 nM) for 24 h. Cells were harvested in 200 μl of luciferase reporter lysis buffer (Promega, Madison, WI).

Transcripts were amplified after reverse transcription with Superscript II (Invitrogen) using 5′ and 3′ primers, respectively: 5′-atgaagacccaaaggaatgg-3′ and 5′-gggtacaagattccgcaaaa-3′ for *camp*; 5′-GCTCCTGGTGAAGCTCCC AGCC-3′ and 5′-TGCGTATCTTTGGACACC-3′ for *defB2*; 5′-atgcccctag gtctcctgtg-3′ and 5′-agccgtcgatacactggtcg-3′ for neutrophil gelatinase-associated lipocalin (*ngal*); and 5′-ggtgaaggtcggtgtcaacg-3′and 5′-caaagttgtca ttgatgacc-3′ for *gapdh*. Immunocytochemistry was performed using rabbit anti-LL37 (human) serum (1/400) against CAMP (Phoenix Pharmaceuticals, Belmont, CA) or rabbit anti-HBD-2 antiserum (1/500; Alpha Diagnostic International, San Antonio, TX) and goat anti-rabbit-FITC (1/200) secondary Ab (Sigma-Aldrich, St. Louis, MO) (15).

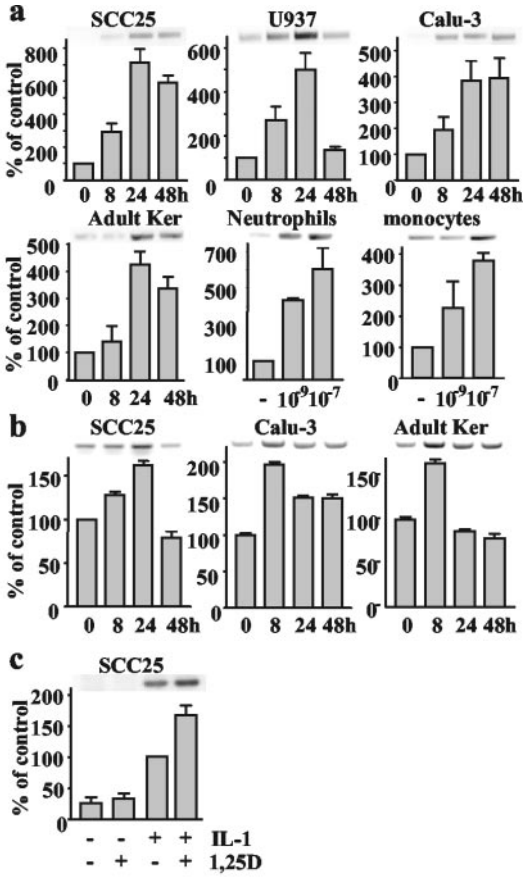

FIGURE 2. $1,25(OH)_2D_3$-regulated expression of *camp* (*a*) and *defB2* (*b*) transcripts in human cell lines and primary cultures. *c,* $1,25(OH)_2D_3$ (10^{-9} M) enhances the stimulatory effect of 8-h incubation with IL-1 (50 ng/ml) on *defB2* expression in SCC25 cells. Results were monitored by RT-PCR and are presented as the means ±SEM from at least three experiments.

EMSAs

Assays were performed with double-stranded oligonucleotides containing VDREs from the *camp* (5′-gatcctcccgggttcaatgggttcaagtgaa-3′), *defB2* (5′-gatc ctgaagaggtcaggcaggtcatgagga-3′), or mouse osteopontin (*mop*) promoters (5′-gatccgtacaaggttcacgaggttcacgtctta-3′) along with a mutant *defB2* VDRE sequence (5′-gatcctgaagagaatggcagaatgtgagga-3′).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays (16) were performed with SCC25 cell lysates immunoprecipitated with either normal rabbit IgG or anti-VDR (C-20) rabbit polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA). PCR were performed with primers: *camp* VDRE region (−535/−286), 5′-ctcagctaactgcaacttctgctt-3′ and 5′-atctc cagctctaggcattg-3′; *camp* outside the VDRE (−1484/−1743), 5′-tatctctcctg gctgtgatc-3′ and 5′-ccatcacataggctcatctg-3′; *defB2* VDRE region (−1266/-1051), 5′-cagggtttcttcagaacctga-3′ and 5′-tgaggtctctggtgtctctc-3′; and *defB2* outside the VDRE (−3549/−3259), 5′-ttctcacaacctttgttgggtc-3′ and 5′-ctgct gttgaagaagggcattgt-3′.

Antimicrobial assays

Escherichia coli and *P. aeruginosa* were grown to early log phase at 37°C in Luria (L) broth. Assays were performed in two ways, with similar results. Cultures (50-μl aliquots) were diluted to 5000 CFU/well in 96-well plates. One hundred fifty microliters of tissue culture medium was added and samples were incubated at 37°C with shaking. A_{600} were measured after 3 h of incubation. Alternatively, 50-μl cultures in L broth were diluted to 500 CFU with 150 μl of regular medium or conditioned medium. Samples were incubated at 37°C with shaking for 2 h, bacteria were plated onto L broth plates, and CFU were counted after 18 h.

Results and Discussion

We have found in a screen of the human genome for VDREs (manuscript in preparation) that the promoters of the *camp*

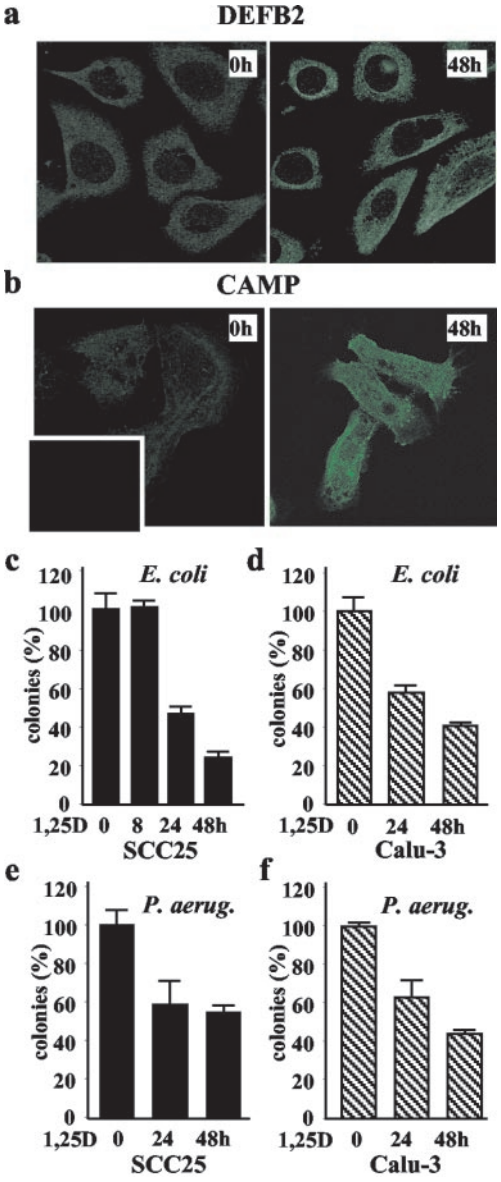

FIGURE 3. $1,25(OH)_2D_3$ (1,25D) induces antimicrobial peptide protein expression and activity. *a* and *b,* Immunocytochemistry was performed using rabbit anti-LL37 (human) serum against CAMP or rabbit anti-HBD-2 antiserum and goat anti-rabbit-FITC secondary Ab. *c* and *d,* Release of antimicrobial activity against *E. coli* from $1,25(OH)_2D_3$-treated SCC25 cells (*c*) or Calu-3 cells (*d*). *e* and *f,* $1,25(OH)_2D_3$ treatment of SCC25 (*e*) or Calu-3 (*f*) cells induces antimicrobial activity against *P. aeruginosa* (means ± SEM from at least three experiments).

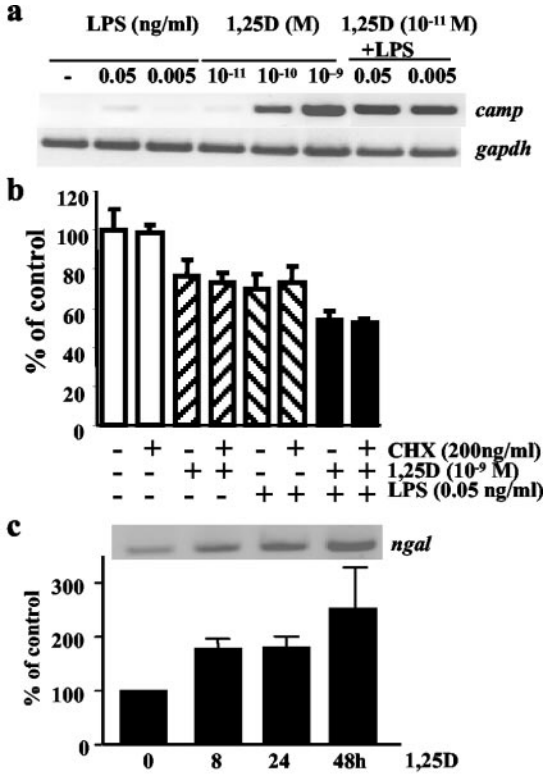

FIGURE 4. Induction of *camp* expression by $1,25(OH)_2D_3$ (1,25D) and LPS in human neutrophils. *a,* Analysis of expression of *camp* and *gapdh* by RT-PCR. *b,* Induction of antimicrobial activity against *E. coli* by individual or combined 4-h treatments of human neutrophils with $1,25(OH)_2D_3$ and LPS in the absence or presence of the protein synthesis inhibitor cycloheximide (CHX). *c,* RT-PCR analysis reveals that $1,25(OH)_2D_3$ treatment of Calu-3 cells induces expression of *ngal*.

(also known as LL37, CAP18, or FALL39) and *defB2* genes contain consensus VDREs 507 and 1231bp upstream of their respective transcription initiation sites (Fig. 1*a*), strongly suggesting that $1,25(OH)_2D_3$ directly regulates their expression. VDRE function was tested with reporter genes driven by cloned *camp* and *defB2* promoters. Sequences containing VDREs mediated $1,25(OH)_2D_3$-dependent expression, whereas deletion of the elements abolished induction by $1,25(OH)_2D_3$ (Fig. 1*b*). Partially ligand-dependent VDR-DNA complexes formed on *camp* or *defB2* VDREs (Fig. 1*c*) were dependent on expression of the VDR and occurred at levels similar to those observed on the consensus *mop* gene element. Binding in vivo in SCC25 cells of the VDR to the *defB2* and *camp* promoters was tested by ChIP assay (Fig. 1*d*), which revealed $1,25(OH)_2D_3$-dependent interaction with VDRE-containing promoter sequences (regions 1), but not adjacent sequences (regions 2) or with the *gapdh* promoter (data not shown). Together, these data show that the *camp* and *defB2* promoters contain functional consensus VDREs.

Expression of the *defB2* and *camp* genes was tested in several $1,25(OH)_2D_3$-sensitive human cells, including adult human keratinocytes primary cultures, neutrophils and monocytes, SCC25 head and neck squamous carcinoma cells (11), a well-differentiated line derived from a floor of the mouth tumor, Calu-3 lung adenocarcinoma cells, which express several markers of upper airway serous cells (17), and U937 myelomonocytic cells (18) (Fig. 2, *a* and *b*), as well as neonatal primary human keratinocytes (data not shown). $1,25(OH)_2D_3$ treatment led to rapid and robust induction of *camp* mRNA in all cells tested (Fig. 2*a* and data not shown). Expression of *defB2* was enhanced by $1,25(OH)_2D_3$ after 24 h in SCC25 and Calu-3 cells and primary cultures of adult keratinocytes (Fig. 2*b*), although the fold induction was substantially lower than that of *camp*. No significant expression of *defB2* was seen in other cells tested (data not shown), consistent with its epithelial expression pattern (2, 3). IL-1 is a robust inducer of *defB2* (19) and stimulated its expression more rapidly (within 8 h) and strongly than $1,25(OH)_2D_3$ (Fig. 2*c* and data not shown). However, 1 nM $1,25(OH)_2D_3$ enhanced the effect of IL-1 on *defB2* over an 8-h period (Fig. 2*c* and data not shown).

$1,25(OH)_2D_3$ treatment of SCC25 cells increased both defB2 and CAMP protein levels as revealed by immunocytochemistry (Fig. 3, *a* and *b*) and Western blotting (data not shown). More importantly, medium from $1,25(OH)_2D_3$-treated cells acquire antibacterial activity indicative of enhanced secretion of functional antimicrobial peptides. SCC25 or Calu-3 cells grown in petri dishes were treated with $1,25(OH)_2D_3$ over 48 h, which led to a time-dependent accumulation in conditioned medium of activity against both *E. coli* and *P. aeruginosa* (Fig. 3, *c–f*), the pathogen responsible for long-term infections in patients with cystic fibrosis (CF) (7). Note that $1,25(OH)_2D_3$ on its own had no antibacterial activity (data not shown).

Signaling by LPS through TLRs induces expression of antimicrobial peptide genes, including *camp* (1). We tested the combined effects of short-term (4-h) incubation with LPS and $1,25(OH)_2D_3$ on *camp* expression in human neutrophils, which revealed a striking synergistic stimulation of expression in the presence of limiting concentrations of the two inducers (Fig. 4*a*) without affecting expression of *gapdh*. In addition, antimicrobial activity was induced in neutrophils treated (4 h) with $1,25(OH)_2D_3$ and LPS individually or in combination (Fig. 4*b*), although the induction was not blocked by cycloheximide under these conditions and was therefore not due to de novo gene expression. These results indicate that $1,25(OH)_2D_3$ alone or in conjunction with LPS can induce *camp* expression and release of antimicrobial activity in neutrophils. Recent microarray studies have shown that LPS can induce multiple and robust changes in gene expression in isolated neutrophils, suggesting that de novo gene expression may contribute to innate immune responses, particularly given data showing that neutrophils are stabilized at sites of infection (20). Finally, we note that the effects of $1,25(OH)_2D_3$ on antimicrobial peptide gene expression are not limited to *camp* and *defB2*, as we have also found that $1,25(OH)_2D_3$ stimulated expression of *ngal* (Fig. 4*c*), which has been shown to have antimicrobial activity (21), although the *ngal* promoter contains no obvious VDREs. Taken together, our data show that $1,25(OH)_2D_3$ has multiple effects on the expression and release of antimicrobial peptides.

The induction of antimicrobial peptide expression by $1,25(OH)_2D_3$ may represent part of a feedback loop to the suppressive effects of UVB on innate immunity and reveals the potential of its analogues in treatment of opportunistic infections. The calcemic activity of $1,25(OH)_2D_3$ has limited its use in treatment of conditions not related to mineral ion homeostasis. However, numerous analogues combine more potent therapeutic activity with weaker calcium mobilization (22). Enhancement of *camp* expression would be of considerable utility, as CAMP restored antimicrobial activity against antibiotic-resistant pathogens in sputum of CF patients (23) and enhanced responses against antibiotic-resistant pathogens in models of CF (24). Moreover, CAMP is a potent antisepsis agent; it blocked macrophage induction and enhanced survival of mice treated with lethal doses of LPS (23, 25). The robust induction of *camp* observed above suggests that $1,25(OH)_2D_3$ analogues may be protective against sepsis. CAMP multifunctionality is further underlined by its acceleration of epithelial wound healing (6), suggesting that enhanced antimicrobial peptide expression would protect against infection after surgery and accelerate healing.

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