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Effect of vitamin D_3 on antimicrobial peptide gene expression in the neutrophils of pulmonary tuberculosis patients

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We studied the effect of 1α 25 dihydroxy vitamin D_3 ($1\alpha,25(OH)_2D_3$), the active form of vitamin D_3, on the expression of cathelicidin (CAMP), defensin-α3 (DEF-α3) and vitamin D receptor (VDR) genes in neutrophils cultured with or without *Mycobacterium tuberculosis* (*Mtb*) from pulmonary tuberculosis patients and healthy controls. Vitamin D_3 significantly enhanced the CAMP, DEF-α3 and VDR mRNA expression in the neutrophils of both the study groups ($P < 0.05$). Moreover, significantly upregulated CAMP expression was observed in vitamin D_3-treated neutrophils infected with *Mtb* ($P < 0.05$) in both the study groups. These findings suggest that vitamin D_3 enhances innate immunity by increasing the expression of the CAMP gene, which might inhibit the growth of *Mtb* at the site of infection.

Keywords: Antimicrobial peptide genes, cathelicidin, neutrophils, tuberculosis, vitamin D_3.

TUBERCULOSIS (TB) is a granulomatous disease caused by *Mycobacterium tuberculosis* (*Mtb*). During mycobacterial infection, polymorphonuclear cells (PMNs) or neutrophils leave the blood vessels and infiltrate the site of infection and get involved actively in the inflammatory response[1]. Neutrophils have different types of granules and secrete an array of antimicrobial peptides that play an important role in innate immunity. They express four alpha defensins, also known as human neutrophil peptides (HNPs1-4), that can attract the monocytes and help macrophages kill intracellular pathogens[2,3]. Moreover, human neutrophils also express cathelicidin antimicrobial peptide (hCAP-18/CAMP)[4], which undergoes further cleavage by protease-3 to form LL37, the active form of CAMP[5].

Adequate serum concentration of vitamin D_3 is critical for optimal innate immune response. Rapid sputum clearance of mycobacteria and radiological improvement were observed in Indonesian pulmonary tuberculosis (PTB) patients, who received vitamin D supplementation[6]. Several studies have shown that 1,25-dihydroxyvitamin

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D_3 ($1,25(OH)_2D_3$), the active form of vitamin D, induces higher expression of CAMP[7–9], which can act against intracellular *Mtb*[8]. Vitamin D_3 induces antimicrobial peptide synthesis through vitamin D receptor (VDR), which is expressed by all immune cells, including neutrophils, macrophages and dendritic cells[10,11]. The promoter regions of *camp* and *defb2* genes are reported to have vitamin D response elements that mediate vitamin D_3-dependent gene expression[12].

The effect of vitamin D_3 on the expression of antimicrobial peptides and VDR in macrophages infected with *Mtb* has been studied. Studies are lacking on the effect of vitamin D_3 on the immune functions of neutrophils. In the present study, we have studied the effect of vitamin D_3 on the expression of CAMP, defensin-α3 (DEF-α3), and VDR genes in the neutrophils of PTB patients and healthy controls (HCs).

Twenty PTB patients (mean age ± SD, 36 ± 11.19) and 23 HCs (mean age ± SD, 27.26 ± 6.04) were recruited for the study. Patients were clinically and radiologically diagnosed and PTB was confirmed by sputum smear positivity for *Mtb*. All patients were HIV-negative and recruited before the commencement of anti-tuberculosis treatment from the clinics of the National Institute for Research in Tuberculosis (NIRT), Chennai and the Institute of Thoracic Medicine, Chennai. HCs consisted of students and staff of city colleges and the Madras University. Informed consent was obtained from all the study subjects, and the study was approved by the Institutional Ethics Committee of NIRT, Chennai.

Polymorphonuclear cells were isolated from 20 ml of heparinized venous blood by Ficoll-Hypaque gradient centrifugation followed by sedimentation in 3% Dextran (Sigma, USA). The neutrophil-rich supernatant was collected and the residual erythrocytes were removed by hypotonic lysis, washed in Hank's balanced salt solution (HBSS) and suspended in 1 ml RPMI 1640 tissue culture medium (Sigma). This preparation contained more than 95% neutrophils as judged by flow cytometry with anti-CD16 FITC antibody and viability was >95% as assessed by the trypan blue exclusion method. Two million neutrophils/ml were cultured in RPMI-1640 medium with 10% autologous serum in the presence and absence of vitamin D_3 at 10^{-7} M concentration in a 24-well culture plate (Costar, Cambridge, MA, USA). Our earlier studies have shown that 10^{-7} M concentration of 1α 25 dihydroxy vitamin D_3 (1α,$25(OH)_2D_3$); the active form of vitamin D_3, is known to exert optimal effects and hence was used in the present study[13]. The cultures were infected with live *Mtb* H37Rv at 1 : 10 multiplicity of infection (MOI) and incubated for 18 h at 37°C and 5% CO_2 in an incubator (Heraeus, Kendro Laboratories, Germany). Since 1,25 dihydroxyvitamin D_3 (calcitriol) (Sigma) was dissolved in 95% ethanol, ethanol (ETOH) was used as a control and the final concentration of ethanol did not exceed 0.05% in the cultures.

Total RNA was extracted from the neutrophils and converted to cDNA as described earlier[9]. The expression of the target genes was quantified relatively using TaqMan assay method. Validated TaqMan assay primers and probes and TaqMan Universal polymerase chain reaction (PCR) master mix (Applied Biosystems, CA, USA) were used for the expression analysis of target genes – CAMP (Hs00189038_m1), DEF-α3 (Hs00414018_m1), VDR (Hs01045840_m1) and housekeeping gene, β-actin. All analyses were performed in triplicates in an ABI Prism® 7500 sequence detection system. Target genes were normalized to β-actin content and fold induction of target genes relative to the unstimulated cultures was calculated using the $2^{-\Delta\Delta Ct} = \Delta Ct_{(stimulated)} - \Delta Ct_{(unstimulated)}$, ΔCt is $Ct_{(target)} - Ct_{(beta\text{-}actin)}$, and Ct is the cycle at which an arbitrary detection threshold is crossed. The expression of CAMP was estimated from culture supernatants using human cathelicidin antimicrobial peptide ELISA kit (My Biosource, San Diego, California, USA). The assay was done according to the manufacturer's protocol.

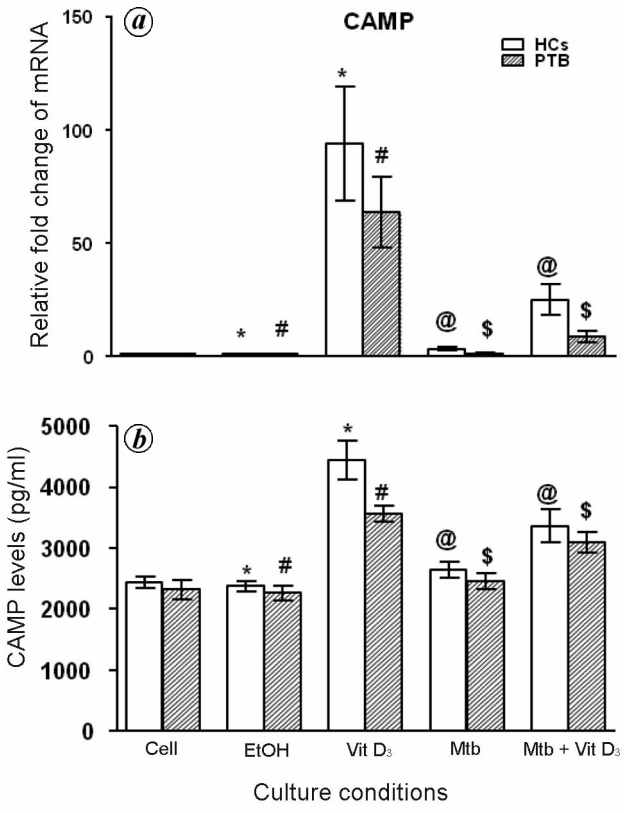

Figure 1. ***a***, Effect of vitamin D_3 on the relative expression of CAMP mRNA in neutrophil cultures of PTB patients and normal HCs. CAMP mRNA expression was relatively quantified by real-time PCR and normalized to housekeeping gene, β-actin and fold induction over unstimulated cultures was calculated by $2^{-\Delta\Delta Ct}$ method. Results are expressed as mean ± SE. Ethanol (EtOH) versus vitamin D_3: $^{*}P < 0.0014$, $^{\#}P < 0.0007$. In *Mtb* versus *Mtb*+ vitamin D_3: $^{@}P = 0.0002$, $^{\$}P < 0.0001$. ***b***, Effect of vitamin D_3 at 10^{-7} M concentration on CAMP protein expression. EtOH versus vitamin D_3: $^{*,\#}P = 0.0078$. In *Mtb* versus *Mtb*+ vitamin D_3, $^{@,\$}P = 0.0078$.

The results are expressed as mean ± standard error (SE) in the figures. Wilcoxon signed rank test was used for paired comparison. A *P*-value less than 0.05 was considered to be statistically significant. All computations were done using GraphPad Prism software (version 4).

Vitamin D_3 significantly enhanced the CAMP mRNA expression when compared to ethanol-treated control cells in HCs and PTB patients ($P < 0.0001$). Similarly, a significant difference in the expression of CAMP mRNA was observed in cells cultured with *Mtb* and vitamin D_3, when compared to those infected with *Mtb* alone in HCs ($P = 0.0002$) and PTB patients ($P < 0.0001$) (Figure 1 *a*). Vitamin D_3 significantly induced the CAMP protein levels in the culture supernatant of both the study groups ($P = 0.0078$) compared to ethanol-treated control cells. A similar effect was also observed in cells cultured with *Mtb* and vitamin D_3, when compared to those infected with *Mtb* alone in HCs and PTB patients ($P = 0.0078$; Figure 1 *b*).

Approximately about one-fold difference in the expression of defensin-α3 mRNA was observed in neutrophils cultured with vitamin D_3 compared to ethanol-treated control cells in HCs ($P = 0.0033$) and PTB patients ($P = 0.0004$). There was no significant difference in the expression of defensin-α3 mRNA in neutrophils cultured either with *Mtb* alone or *Mtb* with vitamin D_3 in both the study groups (Figure 2).

VDR expression was significantly increased in cells cultured with vitamin D_3 in controls ($P = 0.0169$) and PTB patients ($P = 0.0018$) compared to ethanol-treated cells. In HCs, higher VDR mRNA expression was observed in neutrophils infected with *Mtb* on vitamin D_3 treatment compared to cells infected with *Mtb* alone ($P = 0.024$; Figure 3).

Neutrophils play a crucial role in the immune response against *Mtb* by antimicrobial activity[14] and also by shaping the adaptive immune response[15]. The present study investigates the influence of vitamin D_3 on the expression of genes that are crucial for mediating the innate immune response against *Mtb* in neutrophils. The results suggest that vitamin D_3 significantly upregulated the expression of CAMP in neutrophils in the absence as well as presence of *Mtb*. Our observations in neutrophils are consistent with the results from other studies done with macrophages, which reveal that vitamin D_3 strongly upregulates the expression of cathelicidin[7–9]. In the present study, similar to CAMP, vitamin D_3 was also found to enhance the expression of DEF-α3 in both the study groups when compared to ethanol-treated control cells. DEF-α3 is another antimicrobial peptide expressed in neutrophils. It has been shown that about 10% of the TB patients lack DEF-α3 and MDR-TB patients have low plasma concentration of HNP1-3 when compared to control[16]. Increased production of CAMP and DEF-α3 on vitamin D_3 stimulation might help restrict *Mtb* growth and development of MDR-TB. An *in vitro* study has shown that exogenous addition of LL-37, the active form of CAMP, to *Mtb* infected THP-1 cells led to 39% killing of bacteria after 24 h and 60% killing after 72 h. Hence it is possible that increased expression of CAMP might lead to the decreased survival of *Mtb*[17].

In the present study, increased expression of VDR mRNA was observed in the presence of vitamin D_3 in both study groups. On *Mtb* infection, vitamin D_3 significantly upregulated the VDR expression in HCs. It has been shown that *Mtb* ligation with TLR-2 triggers the vitamin D conversion from 25(OH)D_3 (inactive form) to 1,25$(OH)_2D_3$ (active form) through the activation of VDR and subsequent induction of CYP27B1 (enzyme associated with vitamin D_3 biosynthesis pathway) gene expression. CYP27B1-mediated formation of active 1,25$(OH)_2D_3$ upregulates the expression of antimicrobial

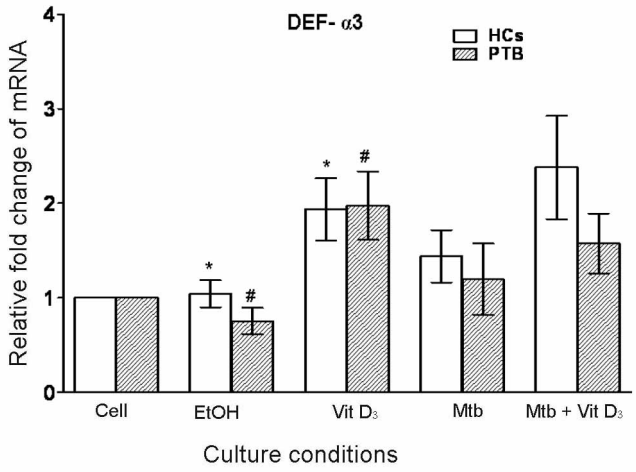

Figure 2. Effect of vitamin D_3 on the relative expression of DEF-α 3 mRNA in the neutrophil cultures of HCs and PTB patients. In ETOH versus vitamin D_3. $^{*}P = 0.0048$, $^{\#}P = 0.0010$.

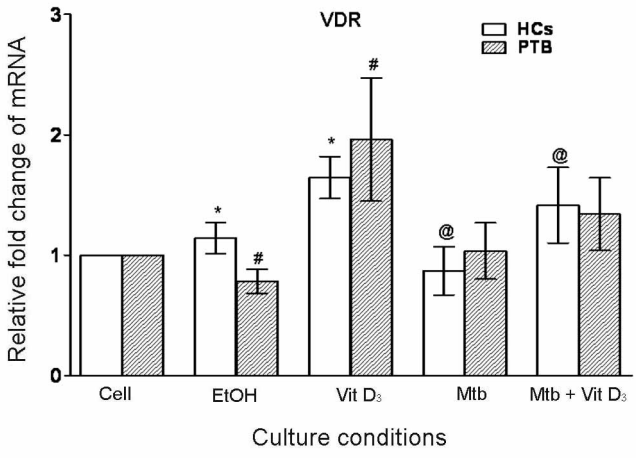

Figure 3. Vitamin D_3 and relative expression of vitamin D receptor (VDR) mRNA in the neutrophil cultures with *Mtb* in HCs and PTB patients. In EtOH versus vitamin D_3: $^{*}P = 0.0169$, $^{\#}P = 0.0018$. In *Mtb* versus *Mtb*+ vitamin D_3: $^{@}P = 0.024$.

peptides such as CAMP[18]. Thus, our results suggest that upregulated VDR expression during *Mtb* infection in HCs triggers the increased expression of CAMP and other antimicrobial peptides. Increased expression of cathelicidin has also been reported to activate the transcription of autophagy-related genes, *Beclin-1* and *Atg5*. Autophagy is reported to be an important host defence mechanism against *Mtb*[8]. Thus, vitamin D_3 might serve to restrict the growth of *Mtb* through multiple mechanisms.

In conclusion, the results suggest that vitamin D_3 upregulates the antimicrobial peptides CAMP and DEF-α3 during *Mtb* infection, which may be detrimental to *Mtb* and may probably be eliminated by neutrophils through the mechanism of autophagy. Moreover, administration of vitamin D_3 as a nutritional supplementation during anti-TB treatment may be an useful adjunct therapy for the disease.

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In situ formation of silver nanowire networks on paper

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Simple, universally adaptable techniques for fabricating conductive patterns are required to translate laboratory-scale innovations into low-cost solutions for the developing world. Silver nanostructures have emerged as attractive candidates for forming such conductive patterns. We report here the *in situ* formation of conductive silver-nanowire networks on paper, thereby eliminating the need for either cost-intensive ink formulation or substrate preparation or complex post-deposition sintering steps. Reminiscent of the photographic process of 'salt printing', a desktop office printer was used to deposit desired patterns of silver bromide on paper, which were subsequently exposed to light and then immersed in a photographic developer. Percolating silver nanowire networks that conformally coated the paper fibres were formed after 10 min of exposure to light from a commercial halogen lamp. Thus, conductive and patterned films with sheet resistances of the order of 4 Ω/□ can be easily formed by combining two widely used processes – inkjet printing and photographic development.

Keywords: Conductive patterns, inkjet printing, paper electronics, photographic development, silver nanowire.

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