

## Research Article

# Elevated 1- $\alpha$ Hydroxylase Activity in Monocytes from Patients with Active Tuberculosis

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Received 16 July 2013; Revised 23 October 2013; Accepted 6 November 2013

Academic Editor: Stuart Mannering

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A uremic patient developed hypercalcemia after tuberculosis infection, and his ionized calcium levels correlated with 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) levels. We performed further studies to determine whether monocytes are alternative sites of 1,25(OH)<sub>2</sub>D<sub>3</sub> conversion beyond renal tubular cells. Using an ex vivo bioassay, in this study, we found that 1- $\alpha$  hydroxylase (CYP27B1) activity in monocytes is significantly higher in patients with active tuberculosis (TB) than in those with frequent TB contact. However, when monocytes from patients with active TB were restimulated with antigen derived from *Mycobacterium tuberculosis*, less 1,25(OH)<sub>2</sub>D<sub>3</sub> was observed. In contrast, the level of 1,25(OH)<sub>2</sub>D<sub>3</sub> was unchanged in those with frequent TB contact. We conclude that monocytes may be an alternative source of 1- $\alpha$  hydroxylase that could convert 25-hydroxyvitamin D<sub>3</sub> to the more active 1,25(OH)<sub>2</sub>D<sub>3</sub>.

## 1. Introduction

Tuberculosis has plagued the world since prehistoric times. According to a recent report, one million patients succumb to tuberculosis and related comorbidities annually [1]. In recent decades, tremendous effort has been made to understand the pathophysiological process of the disease. Animal models and human studies have paved the way for the clarification of individual immune responses to *Mycobacterium tuberculosis* (MTB) [2–5]. These studies provide evidence that vitamin D plays an important role in human resistance to tuberculosis. vitamin D has been used to fight tuberculosis for more than 200 years. Cod liver oil and calciferol, major sources of vitamin D, have been used since the 17th century to treat patients with tuberculosis [6]. In normal physiology, after sunshine exposure, 7-dehydrocholesterol stored in the skin was converted to previtamin D<sub>3</sub> followed by thermal isomerization to vitamin D<sub>3</sub> [7]. Next, vitamin D<sub>3</sub> is hydroxylated in the liver to 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>). Then, 25(OH)D<sub>3</sub>

is further hydroxylated to the most active form of vitamin D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub>, by 1- $\alpha$  hydroxylase (CYP27B1) [8, 9]. Renal tubular epithelial cells are a major source of 1- $\alpha$  hydroxylase and play a critical role in determining the concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> in the serum [10]. The presence of CYP27B1 in extrarenal tissues has been recognized for decades [11]. The impact of extrarenal CYP27B1 on the serum concentration of 1,25(OH)<sub>2</sub>D<sub>3</sub> has yet to be determined.

We encountered a 34-year-old patient with uremia who had received regular hemodialysis for 2 years. The patient experienced muscle weakness with fatigue for 2 days before visiting a family physician. Blood chemistry revealed elevated ionized calcium levels (5.44 mg/dL). Despite the hypercalcemia, the patient's symptoms were relieved by conventional treatment. Four months later, the patient experienced progressive muscle weakness accompanied by fever. In the ER, elevated ionized calcium levels were again noted (6.88 mg/dL). In addition, an EKG revealed an abnormal heart rhythm and a short QT interval with widened T wave.

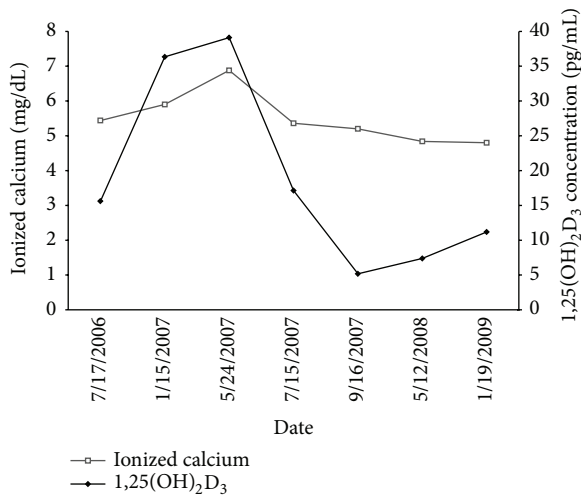


FIGURE 1: Changes in ionized calcium and 1,25(OH)<sub>2</sub>D<sub>3</sub> concentrations in the serum of a patient with active TB and uremia.

A chest X-ray showed right upper lobe pulmonary infiltration that was later determined to be pulmonary tuberculosis. The patient then received 9 months of antituberculosis treatment. Four months posttreatment, the patient's ionized calcium levels normalized (5.2 mg/dL), with a complete resolution of symptoms. To elucidate the pathogenesis of this patient's hypercalcemia, we measured 1,25(OH)<sub>2</sub>D<sub>3</sub> levels in addition to ionized calcium levels. As shown in Figure 1, the levels of 1,25(OH)<sub>2</sub>D<sub>3</sub> were highly correlated with those of ionized calcium.

In addition to renal CYP27B1 expression, macrophages and monocytes are considered important extrarenal sites of CYP27B1 expression. In this study, we evaluated the role of monocytes in the metabolism of vitamin D<sub>3</sub> using an ex vivo bioassay. Furthermore, we stimulated monocytes with antigen derived from MTB to obtain further insight into how monocytes modulate vitamin D<sub>3</sub> metabolism in response to bacterial challenge.

## 2. Materials and Methods

**2.1. Subject Population.** This study was performed at Kaohsiung Medical University. Participants were stratified into two groups: (1) active TB and (2) frequent TB contact. Those with active pulmonary tuberculosis confirmed by a sputum culture and chest film were assigned to the active TB group ( $n = 25$ ). Frequent TB contacts ( $n = 25$ ) included the following: (1) medical staff who had worked at the TB center for at least 10 years and had never been infected and (2) TB patients' family members, clinicians, and nurses who had long-term contact with TB patients and had never been infected. All frequent TB contacts had been vaccinated with BCG and underwent a yearly chest X-ray; when chest films were abnormal, a Mantoux test was performed. We excluded subjects with diabetes, malignancy, or any other disease that could cause immunodeficiency. The two groups were sex and age matched (Table 1).

TABLE 1: Sex and age of study participants.

	Active TB ( $N = 25$ )	Frequent TB contact ( $N = 25$ )	$P$ value
Sex			
Male	13 (52%)	12 (48%)	0.47
Female	12 (48%)	13 (52%)	
Age (mean $\pm$ SD)	44 $\pm$ 6.8	40 $\pm$ 6.2	
<sup>a</sup> Duration of disease (months, mean $\pm$ SD)	3 $\pm$ 1.2		
<sup>b</sup> Duration of exposure (years, mean $\pm$ SD)		13 $\pm$ 2.4	

<sup>a</sup>Duration of disease was defined as the interval from diagnosis to blood collection.

<sup>b</sup>Duration of exposure was defined as the interval from beginning work in a TB center to blood collection.

**2.2. Cell Preparation.** Peripheral blood mononuclear cells (PBMCs) were isolated from heparin-treated blood collected from the 2 groups of donors using a standard Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient. PBMCs ( $1 \times 10^6$  cells/mL) were resuspended in RPMI 1640 medium supplemented with L-glutamine and 10% fetal calf serum. After incubation for 1 hour in a 75 cm<sup>2</sup> flask in a humidified 37°C, 5% CO<sub>2</sub> incubator, the medium containing nonadherent cells was decanted into a conical tube, and the flask was washed twice with serum-free medium to remove any residual nonadherent cells. Adherent monocytes were removed by gentle scraping with a plastic cell scraper. The cells were transferred to a conical tube, centrifuged to remove the wash solution, and resuspended to  $3 \times 10^6$  cells/mL in supplemented medium. The adherent cell population contained more than 85% CD14<sup>+</sup> cells.

**2.3. Antigen.** MTB isolated from patients with tuberculosis was resuspended in phosphate-buffered saline and heat-killed in a water bath at 70°C for 70 minutes. The bacteria were then sonicated using a New Highway sonicator (Farmingdale, NY, USA). The protein concentration of the bacterial homogenate was determined using the Pierce BCA protein assay kit (IL, USA) and stored at -20°C.

**2.4. Cytofluorometric Analysis.** Cytometric analysis was performed using a FACS cytometer (Becton Dickinson). A total of  $3 \times 10^5$  cells were incubated with each monoclonal antibody in saturating quantities in 50  $\mu$ L of staining buffer (Hank's balanced salt solution: 1% BSA and 0.1% sodium azide) for 1 hour at room temperature and then washed three times with PBS. The cells were prepared for analysis by suspension in 500  $\mu$ L of 1% paraformaldehyde in PBS. The monocyte population was gated for analysis based on a side-scatter and forward-scatter dot plot. A total of 5,000 gated cells were used for each analysis. Gated cells were further analyzed by fluorescence staining using fluorescein-isothiocyanate- (FITC-) conjugated anti-CD14 (Ancell).

**2.5. Quantitation of 1,25(OH)<sub>2</sub>D<sub>3</sub>.** Purified monocytes were cultured at a density of  $3 \times 10^5$  cells/mL in 100  $\mu$ L in each

well of a 96-well tissue culture plate with 200 nM 25(OH) $_2$ D $_3$  dissolved in a final concentration of 1% ethanol. After 3 hours of incubation, 1 mL of acetonitrile was added to stop the reaction. The cells and medium were harvested and combined with an equal volume of methanol to remove lipids. The quantity of 1,25(OH) $_2$ D $_3$  was determined using the 1,25-dihydroxyvitamin D  $^{125}$ I RIA kit (INCSTAR, Stillwater, MN, USA). Briefly, 2 mL of water and 5 mL of methanol/water (70:30) were added to a C $_{18}$ OH cartridge to remove salts, polar lipids, and pigments under vacuum. Then, 5 mL of hexane/methylene chloride (90:10) was added to the C $_{18}$ OH cartridge to remove 25(OH) $_2$ D $_3$ , and 5 mL of hexane/isopropanol (99:1) was added to remove 24,25(OH) $_2$ D $_3$ /25,25(OH) $_2$ D $_3$ . Each C $_{18}$ OH cartridge was tightly fitted inside a silica cartridge. After adding hexane/isopropanol (92:8) under vacuum, the C $_{18}$ OH cartridge was removed. Finally, purified 1,25(OH) $_2$ D $_3$  was eluted from the silica cartridge in 5 mL of hexane/isopropanol (80:20). The levels of 1,25(OH) $_2$ D $_3$  were quantitated by competitive radioimmunoassay (RIA) using  $^{125}$ I-labeled anti-1,25(OH) $_2$ D $_3$  and anti-1,25(OH) $_2$ D $_3$  antibodies.

**2.6. Treatment with MTB Antigens.** Monocytes ( $3 \times 10^5$  cells/mL) were incubated with 10  $\mu$ g/mL MTB and 200 ng/mL 25(OH) $_2$ D $_3$ . After 3 hours of incubation, 1 mL of acetonitrile was added to stop the reaction. The resulting 1,25(OH) $_2$ D $_3$  was purified from the cells and the medium and quantitated by RIA. The remainder of the procedure was the same as for the previous assay.

**2.7. Statistical Analysis.** All data were analyzed using Student's *t*-test.

### 3. Results

**3.1. Ionized Calcium and 1,25 Dihydroxyvitamin D $_3$  Concentrations in a Patient with Active TB and Uremia.** Serum ionized calcium and 1,25(OH) $_2$ D $_3$  were determined at different time points from the first visit to one year after the completion of antituberculosis treatment. As shown in Figure 1, the levels of ionized calcium correlated with the concentration of 1,25(OH) $_2$ D $_3$  (Figure 1).

**3.2. Study Population.** Twenty-five individuals were recruited into the frequent TB contact group and 25 patients into the active TB group. The mean age and sex ratio did not differ significantly between the groups. The characteristics of the dataset are presented in Table 1.

**3.3. 1,25(OH) $_2$ D $_3$  Quantitation.** Monocytes were cultured with 25(OH) $_2$ D $_3$  for 3 hours. Then, 1,25(OH) $_2$ D $_3$  was purified from the cells and the medium and measured by RIA. As shown in Figure 2, the amount of 1,25(OH) $_2$ D $_3$  in the active TB group was  $27.4 \pm 12.8$  pg/mL (mean  $\pm$  SD), which was significantly higher than that in the frequent TB contact group ( $15.7 \pm 4.7$ ) ( $P < 0.05$ ). When monocytes were simultaneously incubated with MTB and 25(OH) $_2$ D $_3$  for 3 hours, the amount of 1,25(OH) $_2$ D $_3$  in the active TB group

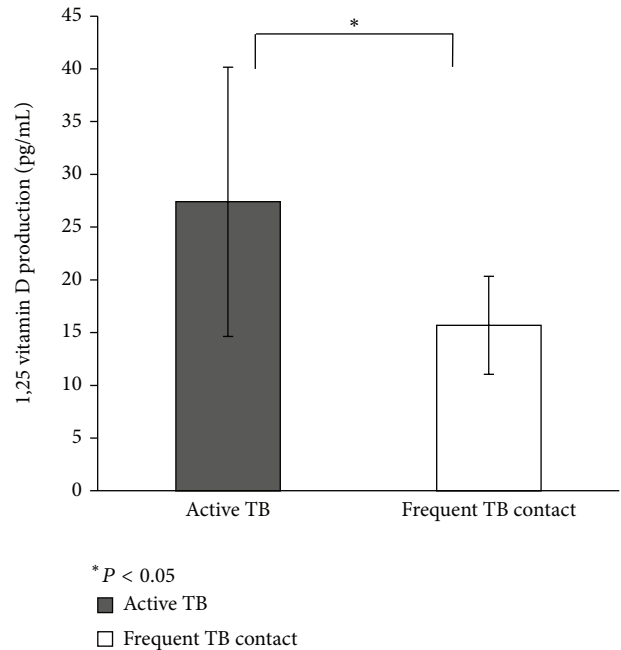


FIGURE 2: Quantitation of 1,25(OH) $_2$ D $_3$  in patients with active TB and frequent TB contacts. RIA measurement of 1,25(OH) $_2$ D $_3$  purified from monocyte suspensions cultured with 25(OH) $_2$ D $_3$  for 3 hours. The amount of 1,25(OH) $_2$ D $_3$  in the active TB group was significantly higher than that in the frequent TB contact group. Each column represents the mean of 1,25(OH) $_2$ D $_3$  quantitation. Error bars represent the standard deviation. \*  $P < 0.05$ .

decreased significantly compared to that with no MTB ( $13.2 \pm 9.6$  and  $27.4 \pm 12.8$ , resp.) ( $P < 0.05$ ) (see Figure 3). There was no difference between monocytes with or without exposure to MTB in the frequent TB contact group ( $14.4 \pm 3.5$  and  $15.7 \pm 4.7$ , resp.).

### 4. Discussion

We observed that 1,25(OH) $_2$ D $_3$  levels correlated with ionized calcium levels in a uremic patient with pulmonary tuberculosis. In active pulmonary tuberculosis, the serum levels of 1,25(OH) $_2$ D $_3$  in this patient were high, which in turn induced high levels of ionized calcium. After treatment, both 1,25(OH) $_2$ D $_3$  and ionized calcium levels decreased. Theoretically, in a uremic patient, renal CYP27B1 activity is trivial. Low serum 1,25(OH) $_2$ D $_3$  levels are often observed in uremic patients [12]. Consistent with what has been observed in humans, in an anephric mouse model, low 1,25(OH) $_2$ D $_3$  levels are also observed [13]. However, an extrarenal source of CYP27B1 has been described for more than 6 decades. Harrell and Fisher were among the first to find extrarenal synthesis of 1,25(OH) $_2$ D $_3$  under pathological conditions [14]. These authors established the association between dysregulated calcium homeostasis and sarcoidosis. Tissue macrophages were later shown to be an extrarenal source of 1,25(OH) $_2$ D $_3$  production in these patient groups [15]. Increasing numbers of tissues were found to express CYP27B1 by different study groups, such as skin melanocytes, tissue macrophages, and

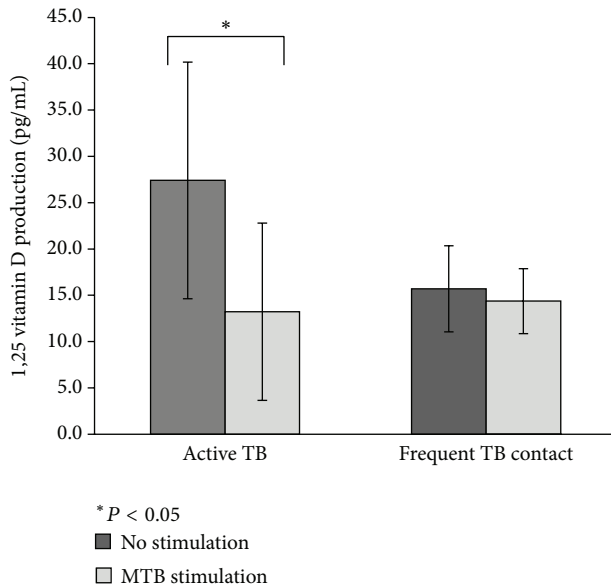


FIGURE 3: Quantitation of  $1,25(\text{OH})_2\text{D}_3$  in patients with active TB and frequent TB contacts with or without *M. tuberculosis* (MTB) antigen. Monocytes were pulsed with  $25(\text{OH})\text{D}_3$  with or without MTB. The amount of  $1,25(\text{OH})_2\text{D}_3$  with MTB exposure decreased significantly compared to that with no MTB in the active TB group. Each column represents the mean of  $1,25(\text{OH})_2\text{D}_3$  quantitation. Error bars represent the standard deviation. \*  $P < 0.05$ .

residual cells of the placenta [11]. Surprisingly, not every cell that expresses CYP27B1 possesses enzymatic activity [16]. To clarify the potential for circulating monocytes to contribute to high  $1,25(\text{OH})_2\text{D}_3$  levels, we utilized an ex vivo bioassay using  $25(\text{OH})\text{D}_3$  as a substrate to determine the activity of CYP27B1. Our results demonstrate that CYP27B1 activity in monocytes from patients with active TB is significantly higher than that in monocytes from individuals with frequent TB contact. Circulating monocytes contribute to the conversion of  $25(\text{OH})\text{D}_3$  to the more active  $1,25(\text{OH})_2\text{D}_3$ . Intriguingly, vitamin  $\text{D}_3$  was traditionally considered to be an endocrine factor;  $1,25(\text{OH})_2\text{D}_3$  produced at local sites (renal tubules) is carried by the blood to affect other tissues or organs, for example, bone. However, in recent decades, vitamin  $\text{D}_3$  was shown to play roles other than endocrine functions. The  $1,25(\text{OH})_2\text{D}_3$  produced by inflammatory cells can stimulate vitamin D receptor expression in both neighboring cells and in the inflammatory cells themselves. By binding to the vitamin D receptor and the retinoid receptor, the ligand-receptor complex can bind to the promoters of many inflammatory genes [17, 18]. Because of these effects, vitamin  $\text{D}_3$  was considered to have both paracrine and autocrine functions. In contrast with the endocrine function of vitamin  $\text{D}_3$  as a calcium regulator, its paracrine and autocrine functions induce inflammatory cells to produce antibacterial peptides and augment the process of autophagy [19–23]. Because  $1,25(\text{OH})_2\text{D}_3$  is produced locally and is not carried to target sites for the regulation of calcium homeostasis, its production does not affect serum calcium levels. Interestingly, in this study, we found that  $1,25(\text{OH})_2\text{D}_3$  produced

by monocytes was a special case. Although  $1,25(\text{OH})_2\text{D}_3$  produced by monocytes can act locally, these cells are carried by the blood to tissues throughout the body. The  $1,25(\text{OH})_2\text{D}_3$  produced by monocytes also behaves as an endocrine factor. Monocytes may be the only cells in our bodies that can orchestrate the endocrine, paracrine, and autocrine functions of vitamin  $\text{D}_3$ . An interesting issue is what is the relative contribution of monocyte source of  $1,25(\text{OH})_2\text{D}_3$  to total  $1,25(\text{OH})_2\text{D}_3$  level. Dusso et al. observed that maximal production of  $1,25(\text{OH})_2\text{D}_3$  from monocyte is trivial (in fmole/hour/microgram DNA) compared to  $1,25(\text{OH})_2\text{D}_3$  concentration in normal (in pmol/mL) [24]. But the data is from an ex vivo experiment, we do not know how many monocytes in the body are stimulated to produce  $1,25(\text{OH})_2\text{D}_3$ . We speculate that the relative contribution of monocyte source of  $1,25(\text{OH})_2\text{D}_3$  to total  $1,25(\text{OH})_2\text{D}_3$  level is small in most circumstances. Even in patients with granulomatous disease, only few of them have clinical symptoms of hypercalcemia resulting from high level of  $1,25(\text{OH})_2\text{D}_3$ . Our indicator case is one of them.

We also found that when monocytes from patients with active TB are cultured with MTB antigen,  $1,25(\text{OH})_2\text{D}_3$  conversion is significantly lower than when no antigen is added. In the frequent TB contact group, there was no difference between being with or without MTB antigen. There are two possible explanations for this observation. First, primed monocytes from patients with active TB can induce more  $24(\text{OH})$  hydroxylase (CPY24) activity than their counterparts, which actively hydroxylates  $1,25(\text{OH})_2\text{D}_3$  to calcitric acid, when they are further stimulated with MTB antigen. vitamin  $\text{D}_3$  induces not only inflammatory gene products but also the expression of CPY24 [25, 26]. CPY24 is a major catabolic enzyme of vitamin  $\text{D}_3$ . CYP27B1 and CYP24 activities are modulated in a diametrically opposite way to control serum levels of  $1,25(\text{OH})_2\text{D}_3$  [26]. Owing to this concerted action, hypercalcemia is seldom observed in patients. The frequent TB contact group demonstrated an even better concerted action, which is why there was no change in  $1,25(\text{OH})_2\text{D}_3$  conversion after stimulation with MTB antigen. A second explanation is that CPY27B1 activity is exhausted when monocytes are restimulated by MTB antigen.

mRNA expression was not determined in this study for the following reasons. First, a large number of monocytes from participants were required for the ex vivo bioassay. If we performed both quantitative mRNA expression and the ex vivo bioassay, we would have needed to collect more than 30 mL of blood from each participant. The collection of this volume of blood was rejected by our ethical committee. Second, as indicated previously, some tissues may express CYP27B1 without detectable enzymatic activity. mRNA levels are not always correlated with enzymatic activity. Despite CYP27B1 expression in some tissues, no enzymatic activity was detected. In future studies, the levels of CYP27B1 and CYP24 and the metabolites of vitamin  $\text{D}_3$ , such as calcitric acid and  $24,25(\text{OH})_2\text{D}_3$ , should be quantified to clarify vitamin  $\text{D}_3$  metabolism in the pathophysiological process of tuberculosis.

## 5. Conclusion

In conclusion, calcium levels correlated with  $1,25(\text{OH})_2\text{D}_3$  levels in a uremic patient infected with tuberculosis. Furthermore, we found that CYP27B1 activity in monocytes is higher among patients with active tuberculosis than those with frequent TB contact.

## Ethical Approval

This study project was approved by the Institutional Review Board of Kaohsiung Medical University Hospital. The approval number is KMUH-IRB-20130103.

## Conflict of Interests

The authors declare that there is no conflict of interests for this paper.

## Authors' Contribution

Yi-Ching Tung and Wen-Chan Tsai performed the experiments and wrote the paper. Tsan-Teng Ou and Wen-Chan Tsai designed the study and collected the specimens. All authors read and approved the final paper.

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