Vitamin D_3 elicits calcium response and activates blood monocyte-derived macrophages from patients with vitamin D dependent rickets type II

Eiji Takeda[a,*], Kazumi Takata[a], Hisami Yamanaka[a], Yutaka Taketani[a], Kyoko Morita[a], Ken-ichi Miyamoto[a], Masayuki Shono[b], Shigetada Teshima[c], Kazuhito Rokutan[c]

[a] *Department of Clinical Nutrition, School of Medicine, The University of Tokushima, 3-18-15 Kuramoto-Cho, Tokushima City, Tokushima 770, Japan*
[b] *General Laboratories for Medical Research, The University of Tokushima, 3-18-15 Kuramoto-Cho, Tokushima City, Tokushima 770, Japan*
[c] *Nutritional Physiology, School of Medicine, The University of Tokushima, 3-18-15 Kuramoto-Cho, Tokushima City, Tokushima 770, Japan*

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Abstract We studied the effects of vitamin D_3 metabolites on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and the respiratory burst of monocyte-derived macrophages (MDM) from patients with vitamin D dependent rickets type II. Treatment of MDM from the patients and healthy donors with 1 nM $1,25(OH)_2D_3$ produced a rapid elevation of $[Ca^{2+}]_i$ and similarly primed both types of cells for enhanced capacity for O_2^- release with phorbol diester. These results suggest that macrophages may have distinct non-genomic pathways of vitamin D_3, which partly explain the absence of immunodeficiency and the disappearance of rickets after treatment with vitamin D_3 in the patients.

Key words: Vitamin D dependent rickets type II; Vitamin D_3; Non-genomic action; Monocyte-derived macrophage; Calcium response; Superoxide anion release

1. Introduction

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is the major regulator of calcium homeostasis and is now considered to be a true steroid hormone acting via intranuclear vitamin D receptor (VDR)-binding regulation of gene transcription [1]. This classical receptor-mediated pathway of $1,25(OH)_2D_3$ action is not sufficient to account for all of the known effects of the seco-steroid. For example, $1,25(OH)_2D_3$ rapidly stimulates Ca^{2+} transport and cyclic guanosine monophosphate (cGMP) production in several cell systems, and some of these actions have been suggested to be independent of the genomic action [2]. In contrast, it was also reported that $1,25(OH)_2D_3$ did not produce rapid accumulation of cGMP in cells with deficient or mutant VDRs [3,4]. Thus, there is some controversy about the requirement of $1,25(OH)_2D_3$ binding to the VDR in the initiation of these rapid effects. To address this question, we prepared blood monocyte-derived macrophages (MDM) from patients with vitamin D dependent rickets type II (VDDR II), who had already been characterized to have defects in VDR-mediated pathways [5–7], and studied the response of these cells to $1,25(OH)_2D_3$.

VDDR II consists of a spectrum of intracellular VDR defects and is characterized by the early onset of severe rickets and associated alopecia. Without treatment, patients suffer inanition, severe skeletal deformity, recurrent respiratory infections, and death by 8 years of age. We characterized the VDR mutations in six Japanese patients and successfully treated them with massive doses of vitamin D_3 [5]. They did not show any symptom of immunodeficiency. Furthermore, skeletal abnormality with total alopecia did not recur after the cessation of the therapy during 7–25 years of follow-up, despite their cells continuing to harbor the VDR mutation [6]. We preferentially used MDM from peripheral blood in this study, since $1,25(OH)_2D_3$ is one of the inducers of differentiation of a monocyte-macrophage lineage and is also a potent activator for macrophage functions. The activation of macrophages by $1,25(OH)_2D_3$ results in enhanced capacity for production of microbicidal oxygen metabolites [8,9]. In this paper, we studied the effects of $1,25(OH)_2D_3$ on calcium response and superoxide anion (O_2^-) release in MDM from patients with VDDR II to assess the role of VDR in macrophage functions.

2. Materials and methods

2.1. Chemicals and media

The vitamin D_3 metabolites, $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, $25(OH)_2D_3$, and 22-oxa-$1,25(OH)_2D_3$, were generous gifts from Chugai Pharmaceutical Co. (Tokyo). Stock preparations were dissolved and diluted in absolute ethanol, and the concentration of the metabolites in each solution was verified by measuring the absorbance at 264 nm. Solutions were made so as to keep the final concentration of ethanol in the culture medium below 0.1% (v/v). Superoxide dismutase (SOD, from horse heart), ferricytochrome *c*, phorbol 12-myristate 13-acetate (PMA), and lipopolysaccharide (LPS, from *Escherichia coli* K-235) were obtained from Sigma Chemical Co. (St. Louis, MO). Fura-2 acetoxymethyl ester (fura-2/AM) was purchased from Dojin Chemical Co. (Tokyo). Dulbecco's modified Eagle's medium (DMEM) was from Flow Laboratories (McLean, VA). Hanks' balanced salt solution without phenol red (HBSS) was purchased from Nissui Pharmaceutical Co. (Tokyo). All reagents to which MDM were exposed in culture were determined to be free from detectable LPS by the *Limulus* ameobocyte lysate assay (E-toxiate, Sigma).

2.2. Preparation of monocyte-derived macrophages

Two patients with VDDR II were subjected to this study. The clinical manifestation and identical point mutation in VDR cDNA were reported previously (patents 1 and 3 in [7]). We had already confirmed that neither binding activity of the VDRs to vitamin D responsive element nor induction of 25(OH)D-24-hydroxylase with $1,25(OH)_2D_3$ was observed in several types of cells from those patients, and that these abnormalities still continued to be observed after treatment with vitamin D_3 [10,11]. The two patients were treated with massive doses of vitamin D_3 for 12 (patient 1) and 36 months (patient 3), and the therapy had been discontinued for 6 (patient 1) and 10 years (patient 3) without recurrence of any clinical skeletal symptom. The two patients were completely free from vitamin D_3 treatment when they donated their peripheral blood for the present study.

MDM were prepared by the method of Marodi et al. [12] with

*Corresponding author. Fax: (81) (886) 33-7094.

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minor modifications. Heparinized venous blood from healthy donors and the patients with VDDR II was overlaid on a density gradient medium composed of 13.8% sodium metrizoate and dextran 500 with osmolality 460 mOsm (Nycomed Pharma, Oslo, Norway), and a mixed mononuclear layer was obtained by centrifugation at $450 \times g$ for 30 min [13]. After centrifugation and washes in DMEM, mononuclear cells were suspended in DMEM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated autologous serum and adjusted to a final concentration of 1×10^6 cell/ml. The cells were incubated in teflon beakers (Savillex, Minnetonka, MN) at 37°C in 5% CO_2-95% air for 3 days. The cultured cells were detached by gentle pipetting and collected in centrifuge tubes. After washing twice with DMEM, the cells were cultured in DMEM on glass coverslips, placed in 35-mm diameter culture dishes, or in 24-well culture plates for 2 h, and then non-adherent cells were removed by vigorously washing with ice-cold saline. The adherent cell population contained >85% macrophages by microscopic examination after Wright-Giemsa or esterase stain; other cells appeared to be lymphocytes. Viability of the cells remained >96% (trypan blue exclusion).

2.3. Measurement of $[Ca^{2+}]_i$ in single cells

$[Ca^{2+}]_i$ in single cells was determined by microfluorometry, as described previously [14]. Briefly, glass coverslips were placed in 35-mm diameter culture dishes. After culturing the MDM on the coverslips overnight in DMEM, they were incubated in the dark in DMEM containing 2.5 mM fura-2/AM and 1% bovine serum albumin at 37°C. After incubation for 20 min, the cells were washed four times with HBSS and then placed in a bath (volume 0.5 ml) on the stage of a fluorescence microscope (Optiphoto, Nikon) equipped with a photomultiplier tube (R649; Hamamatsu Electronics, Shizuoka, Japan), a photon counter (545A; NF Circuit Design, Hiroshima, Japan), and an appropriate combination of filters (Nihon Shinkukogaku, Osaka, Japan), in which alternating excitation at wavelengths of 340 and 380 nm was repeated. The solution (HBSS) at 37°C was continuously circulated at a rate of 1 ml/min. The intensity of emission at 510 nm with excitation at 340 and 380 nm was monitored every 3 s. Absolute values of $[Ca^{2+}]_i$ were calculated by the method of Grynkiewicz et al. [15] using a personal computer (NEC).

2.4. Assay of O_2^- release

The cells cultured in teflon beakers for 3 days were washed and resuspended in DMEM, and 1×10^6 cells were placed in each well of a 24-well culture plate. After removal of non-adherent cells, MDM were cultured overnight in DMEM supplemented with 1 nM $1,25(OH)_2D_3$ or 50 ng/ml LPS or without any reagent. O_2^- release from MDM was assayed by measuring the SOD-inhibitable reduction of ferricytochrome *c*, corrected for the protein content, as described previously [16].

3. Results

3.1. Calcium response to vitamin D_3 metabolites in MDM from normal subjects

Single cell analysis showed that the extent of calcium response to $1,25(OH)_2D_3$ varied among the single cells. It has been shown that externally added ATP elicits the mobilization of Ca^{2+} in guinea pig peritoneal macrophages [17]. Addition of 0.3 M ATP induced rapid and transient elevation of $[Ca^{2+}]_i$, as shown in Fig. 1A. Therefore, ATP was always used as a positive control for calcium response, and data from cells unresponsive to ATP were omitted. The final concentration of ethanol in the circulating medium was less than 0.1%, at which concentrations ethanol did not promote any $[Ca^{2+}]_i$ change in MDM.

Exposure of MDM from normal subjects to 1 nM $1,25(OH)_2D_3$ rapidly increased $[Ca^{2+}]_i$ from the basal level of 209 ± 5 nM (mean ± S.D., $n = 5$) to 413 ± 23 nM ($n = 5$). A typical response is shown in Fig. 1A. Both ATP- and $1,25(OH)_2D_3$-induced increases of $[Ca^{2+}]_i$ were almost completely inhibited when extracellular Ca^{2+} was removed with EDTA (Fig. 1B), indicating that these agents evoke extracellular Ca^{2+} influx across the plasma membrane.

MDM from healthy volunteers similarly responded to other vitamin D_3 metabolites with various affinities to VDR and rapidly increased $[Ca^{2+}]_i$, as shown in Fig. 1C–E. Exposure to 1 nM $25(OH)D_3$, $24,25(OH)_2D_3$, or 22-oxa-$1,25(OH)_2D_3$ increased $[Ca^{2+}]_i$ to a maximum level of 416 ± 21 ($n = 5$), 310 ± 30 ($n = 5$), or 349 ± 32 nM ($n = 5$), respectively, suggesting that the calcium response might not be mediated by VDR.

3.2. Effect of $1,25(OH)_2D_3$ on calcium response of MDM from patients with VDDR II

When MDM, prepared from patients with VDDR II, were treated with 1 nM $1,25(OH)_2D_3$, they also responded to $1,25(OH)_2D_3$ and rapidly increased $[Ca^{2+}]_i$ to a maximum level of 408 ± 47 nM ($n = 5$), thus demonstrating that VDR-dependent processes may not be necessary to evoke the calcium transient (Fig. 1F).

3.3. Effect of $1,25(OH)_2D_3$ on O_2^- release of MDM from patients with VDDR II

In response to bacterial LPS or cytokines, macrophages markedly enhance their capacity for production of oxygen intermediates, which have been postulated to play a central role in microbicidal and cytocidal activities. When MDM were prepared and cultured under LPS-free conditions, they were quiescent; control MDM released 98 ± 28 (mean ± S.D., $n = 5$) nmol O_2^-/mg protein per h with PMA (Table 1). This value increased about 3-fold after treatment with LPS. Treatment of control MDM with $1,25(OH)_2D_3$ also augmented the release similarly with LPS. MDM from the patients with VDDR II responded to LPS and increased the amount of O_2^- release. Furthermore, these cells were also primed by $1,25(OH)_2D_3$ for enhanced capacity for O_2^- release with PMA, suggesting that macrophages defective in VDR functions have distinct non-genomic pathways that promote functional activation of macrophages in response to $1,25(OH)_2D_3$.

Table 1
Effect of $1,25(OH)_2D_3$ on O_2^- release from MDM

Treatment	O_2^- generation (nmol/mg protein per h)	
	Control MDM	VDDR II MDM
None	98 ± 28	148 ± 14
LPS (50 ng/ml)	303 ± 7	445 ± 11
$1,25(OH)_2D_3$ (1 nM)	244 ± 28	377 ± 31

MDM were untreated or treated overnight with 50 ng/ml LPS or 1 nM $1,25(OH)_2D_3$. O_2^- release of MDM stimulated by 500 ng/ml was measured by the SOD-inhibitable reduction of ferricytochrome *c*, as described previously [16]. Values are mean ± S.D., $n = 5$.

4. Discussion

Vitamin D_3 exerts its biological action through binding to its nuclear receptor, VDR, that activates the transcription of target genes. In addition to this classical genomic action, vitamin D_3 has been suggested to have distinct non-genomic pathways. $1,25(OH)_2D_3$ can rapidly induce phosphoinositide breakdown, activation of several enzymes, Ca^{2+} mobilization, and accumulation of cGMP in multiple tissues [2]. These rapid actions have been postulated to be non-genomic actions of $1,25(OH)_2D_3$ [2]. The $[Ca^{2+}]_i$ is a critically important intracellular messenger for regulating cellular functions [18].

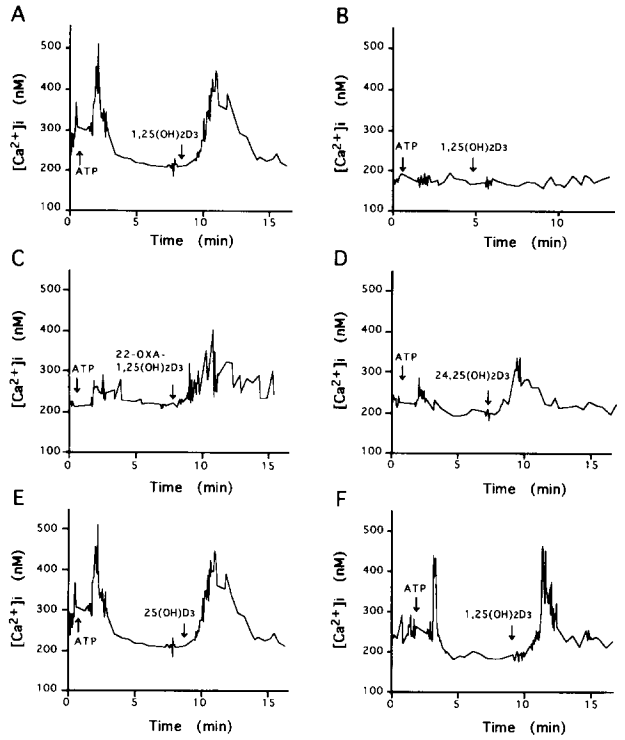

Fig. 1. Effect of vitamin D_3 metabolites on $[Ca^{2+}]i$ in single MDM. MDM were prepared from healthy donors (A–E) or patients with VDDR II (F) and attached on glass coverslips as described in Section 2. The fura-2/AM-loaded cells were placed on a bath on the stage of a fluorescence microscope. Cells were first stimulated by 0.3 M ATP and then exposed to 1 nM $1,25(OH)_2D_3$ (A,B,F), 1 nM 22-oxa-$1,25(OH)_2D_3$ (C), 1 nM $24,25(OH)_2D_3$ (D), or 1 nM $25(OH)D_3$ (E). In B, ATP and $1,25(OH)_2D_3$ were added in the presence of 1.5 mM EDTA. Circulation of HBSS containing ATP or one of the vitamin D_3 metabolites was started at the times indicated by the arrows. Medium containing the stimulants reached the bath at 1 min. Changes in $[Ca^{2+}]i$ were measured as described in Section 2. Results shown are representative of those in 5 separate experiments.

$1,25(OH)_2D_3$ increased $[Ca^{2+}]_i$ by stimulating influx of extracellular Ca^{2+} in both osteoblastic ROS 17/2.8 cells and ROS 24/1 cells, in the presence and absence of VDR, respectively [19], suggesting that the calcium transient induced by $1,25(OH)_2D_3$ is independent of VDR. In the human bipotential leukemic cell line, HL-60, $1,25(OH)_2D_3$ evoked a calcium transient, but its synthetic analogue (1α,3β,5*Z*,7*E*)-9,10-secocholesta-5,7,10(19)-triene-1,3,25-triol (22-oxa-$1,25(OH)_2D_3$) did not [20]. However, as shown in Fig. 1, normal macrophages derived from blood monocytes similarly responded to various vitamin D_3 metabolites ($1,25(OH)_2D_3$, $24,25(OH)_2D_3$, $25(OH)_2D_3$, 22-oxa-$1,25(OH)_2D_3$) and showed the same time course of $[Ca^{2+}]_i$ rise immediately after exposure to these agents, whereas each affinity of vitamin D metabolites for VDR is markedly different. Furthermore, MDM from patients with VDDR II showed a rapid elevation of $[Ca^{2+}]_i$, which was identical to that of control MDM. These results support the concept that $1,25(OH)_2D_3$-induced calcium response is not mediated by VDR.

Civitelli et al. [19] and Farach-Carson et al. [21] have suggested the existence of a plasma membrane receptor for $1,25(OH)_2D_3$ that is distinct from the traditional VDR. However, the receptor has not yet been characterized, and several investigators suggested that some of the rapid effects of $1,25(OH)_2D_3$ may be mediated by the VDR [3,4].

Macrophages play a central role in defense against microbial infection by presenting antigen to lymphocytes during the development of specific immunity and by serving as accessory cells to lymphocytes. Macrophages also carry out fundamental protective functions in ingesting and killing invading organisms. In this study, we examined the effects of $1,25(OH)_2D_3$ preferentially on macrophages, since $1,25(OH)_2D_3$ is known as an inducer of differentiation of a monocyte-macrophage lineage and a potent activator of macrophage functions [22–25]. $1,25(OH)_2D_3$ enhances the competence of MDM for secretion of oxygen intermediates and dramatically augments the microbicidal and tumoricidal activity of these cells [24]. These actions of $1,25(OH)_2D_3$ have been suggested to be mediated by VDR. When normal MDM were treated overnight with $1,25(OH)_2D_3$ or LPS, they apparently augmented the capacity for O_2^- release stimulated by PMA. MDM from patients with VDDR II were similarly activated after treatment overnight with $1,25(OH)_2D_3$ or LPS, thus demonstrating that MDM from patients with VDDR II exhibit normal respiratory burst activity, and suggesting that the priming of MDM for enhanced capacity of O_2 release by $1,25(OH)_2D_3$ could be induced in the absence of VDR action.

Children with vitamin D deficiency appear to have a high incidence of infection [26], and vitamin D-deficient rat macrophages showed abnormalities in phagocytic and microbicidal activities [27], suggesting that vitamin D is one of the important factors for defense against microbial infection. However, our patients with VDDR II did not show any recurrent infection throughout their lives, and MDM from these patients normally responded to $1,25(OH)_2D_3$. These findings indicate that macrophages are able to exhibit distinct non-genomic actions and/or to compensate VDR actions by other nuclear signaling pathways, which may, at least in part, maintain their functions in the absence of VDR-mediated pathways, and may explain the absence of immunodeficiency in the patients with VDDR II.

In our patients with VDDR II, severe rickets, alopecia, and muscular weakness manifesting before treatment completely improved with massive doses of vitamin D_3 except for total alopecia [5]. Furthermore, rickets did not recur after cessation of the therapy for 7–15 years of follow up, although their cells still continued to exhibit a genetic defect [6]. These clinical findings strongly suggest that the treatment with vitamin D_3 up-regulates non-genomic pathways of vitamin D_3 and/or a VDR-independent nuclear signaling bypass, i.e. via other steroid receptors including retinoid receptors and retinoid X receptors, to compensate the VDR actions. To address this question, we are currently studying the changes in vitamin D_3 responses of MDM from patients freshly diagnosed as VDDR II before and after long-term treatment with massive doses of vitamin D_3.

Differentiation of mononuclear phagocytes into osteoclasts is also stimulated by $1,25(OH)_2D_3$ via a mechanism involving osteoblastic cells [28]. However, since VDR is present in osteoblasts but not in osteoclasts [28], the present findings indicate that non-genomic actions of vitamin D_3 might be an important subject to study for understanding the bone formation and remodeling in patients with VDDR II.

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