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High-dose of vitamin D supplement is associated with reduced susceptibility of monocyte-derived macrophages to dengue virus infection and pro-inflammatory cytokine production: An exploratory study

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ABSTRACT

Background: Dengue, one the most important public health problems in tropical and subtropical areas, is the most important mosquito-borne viral infection in humans. In the absence of effective treatment and vaccine against dengue, the active form of vitamin D could play a central role in protection against dengue virus (DENV), the causal agent of dengue. Recently we reported that monocyte-derived macrophages (MDMs) differentiated in the presence of vitamin D, in addition to expressing lower levels of mannose receptor, are less susceptible to DENV infection and produce low levels of pro-inflammatory cytokines, compared to MDMs differentiated in the absence of vitamin D.

Objective: The aim of this study was to determine that oral vitamin D supplementation exerts an effect on DENV susceptibility and pro-inflammatory cytokine production in MDMs.

Methods: Healthy individuals were supplemented with 1000 or 4000 international units (IU)/day of vitamin D during 10 days. Before and after vitamin D supplementation, a peripheral blood (PB) sample was taken and the monocytes recovered were used to obtain MDMs and were challenged with DENV-2. Furthermore, the expression of genes encoding vitamin D receptor (VDR), CYP24A1 and CAMP were analyzed using real-time quantitative PCR.

Results: The data indicate that macrophages differentiated from monocytes obtained from healthy donors who received higher doses of vitamin D (4000 IU/day), exhibited higher resistance to DENV-2 infection and produced a significant decrease of pro-inflammatory cytokines and high production of interleukin-10 (IL-10). Furthermore, a significant decrease in intracellular toll-like receptor (TLR) and CAMP mRNA was observed.

Conclusion: A supplement of 4000 IU/day of vitamin D may represent an adequate dose to control dengue progression and DENV replication. Although the results of our study suggest that the vitamin D status can influence the immune response, further studies are needed to determine the feasibility of vitamin D as anti-DENV agent and immune modulator.

1. Introduction

According to the World Health Organization (WHO), the incidence of dengue has increased dramatically in the last three decades and has expanded to new geographical areas, becoming an important problem in public health worldwide [1]. In humans, infection with dengue virus (DENV) results in a wide spectrum of outcomes leading to diverse clinical manifestations ranging from asymptomatic infection or mild undifferentiated fever, to one of the following clinical manifestations, dengue with or without warning signs and severe dengue [2]. Although the pathology of DENV is not completely understood, one of the hallmark manifestations in dengue patients includes capillary leak syndrome caused by endothelial dysfunction associated with an alteration in the balance of secreted pro-inflammatory cytokines [3]. Diverse studies have reported that DENV-infected monocytes and macrophages release soluble mediators, including interleukin (IL)-1β, and IL-6 that exert prominent effects on the function and properties of endothelial cells [4], suggesting that these cells play a role in DENV pathogenesis. Toll-like receptors (TLRs), one of the most important categories of pattern recognition receptors (PRRs), recognize viral particles or viral components and subsequently initiate signaling for pro-inflammatory cytokine production [5]. Interestingly, the DENV NS1 protein induces cytokine secretion through TLR4 [6], and contributes to vascular leak in dengue patients [7]. Furthermore, DENV up-regulates

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the expression of TLR3 and TLR8 in HepG2 cells and increase the expression interferon-beta and pro-inflammatory cytokines [8]. Taken together, these results show that DENV infection induces TLR activation and consequently that the interaction of DENV-TLRs can result in dengue disease progression.

It is known that 1,25-dihydroxyvitamin D [1,25(OH)2D3], the active metabolite of vitamin D, acts at multiples levels, including cell proliferation and cell differentiation, and has potent immunomodulatory activities [9]. However, the inactive form of vitamin D (25-hydroxyvitamin D) is hydroxylated to its active form by 25(OH) D-1alfa-hydroxylase (CYP27B1) [10]. The active form of vitamin D then binds to the intracellular vitamin D receptor (VDR) and induces the expression of 1,25(OH)2D- 24-hydroxylase (24-OHase; CYP24A1) which initiates the degradation of the physiologically active form of vitamin D3 [11]. The human antimicrobial peptide LL-37, which is produced as part of the innate immunity, is induced by 1,25(OH)2D3 macrophages [12],and has received considerable attention due to its antimicrobial activity against diverse pathogens [13]. However, our understanding of the impact of vitamin D on the immune response, including cytokine production by regulating the innate immune receptors is more limited. To date it is well known that vitamin D metabolites down-regulate pro-inflammatory cytokine production, but their impact on virus or viral PRR-stimulated response is very limited. Some studies reported that patients with Hepatitis C virus (HCV) and with vitamin D deficiency present severe manifestations such as hepatic cirrhosis, chronic liver disease and severe hepatic fibrosis [14,15]. Likewise, it was reported that vitamin D supplementation increases the probability of achieving a sustained viral response following antiviral treatment, suggesting a relationship between vitamin D and HCV infection [16]. Epidemiological studies have also provided evidence that vitamin D deficiency could be associated with a risk of viral infections including influenza virus (IV), respiratory tract infections and human immunodeficiency virus 1 (HIV-1) [17]. Other reports strongly point towards vitamin D as having significant beneficial effects in the prevention of infectious diseases [18]. This is highly interesting, since vitamin D is one of the most cost-effective supplements used to improve overall human health [19,20]. Puerta-Guardo et al., (2012) demonstrated that vitamin D treatment resulted both in a significant decrease in the percentage of U937- and Huh-7-cells infected with DENV and a reduction in the production of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 [21]. Moreover, an association between the polymorphism of the vitamin D receptor (VDR) gene and the risk of symptomatic dengue requiring hospitalization was reported [22]. While these results show an association between vitamin D and clinical outcomes of DENV infection including decreased levels of pro-inflammatory cytokines, the importance of vitamin D supplementation in the context of dengue patients is unknown.

Although vitamin D has been assigned functions in monocytes and macrophages [23], the macrophage cell type resulting in response to vitamin D treatment has not been sufficiently characterized. It has been established that the differentiation state of monocytes and macrophages is crucial in determining the susceptibility and productive infection by different viruses [24], but the influence of these differentiations on DENV infection has been sparsely examined. We observed that monocyte-derived macrophages (MDMs) differentiated in the presence of vitamin D, in addition to expressing lower levels of mannose receptor, are less susceptible to DENV infection and produce low levels of pro-inflammatory cytokines compared with MDMs differentiated in the absence of vitamin D (submitted manuscript). Therefore, based on these results the aim of the present study was to explore if oral vitamin D supplementation exerts an effect on DENV susceptibility and pro-inflammatory cytokine production in MDMs. To accomplish this, the healthy individuals received 1000 or 4000 international units (IU)/day of vitamin D supplement during 10 days. Then, from each individual, before and after the vitamin D supplement, a PB sample was taken and the monocytes isolated. They were used to obtain MDMs in the presence of autologous serum, and were then challenged with DENV-2. The data indicated that macrophages differentiated from monocytes obtained from healthy donors who received higher doses of vitamin D (4000 IU/day), exhibited higher resistance to DENV-2 infection and produced a significant decrease of pro-inflammatory cytokines and high production of IL-10. Furthermore, a significant decrease of intracellular TLR mRNA was observed. Although the results of our study suggest that the status of vitamin D can influence the immune response and DENV resistance/susceptibility to infection, further studies are required to determine the feasibility of vitamin D as anti-DENV agent and immune modulator.

2. Materials and methods

2.1. Ethics statement

The protocols for patient enrollment and sample collection were approved by the Committee of Bioethics Research of the Sede de Investigación Universitaria, Universidad de Antioquia (Medellín, Colombia) and an individual's inclusion was preceded by a signed informed consent form, according to the principles expressed in the Declaration of Helsinki.

2.2. Study subjects

Since two oral daily doses (4000 and 7000 IU) of cholecalciferol (D_3) over a 12-week period was reported safe and effective [25], our design was to enroll 2 groups, both with 10 healthy volunteer subjects. Each group was randomized to 1000 or 4000 IU vitamin D (cholecalciferol)/day, during 10 days. The 1000 IU/day group (1000VitD) received one gelatin capsule and the 4000 IU/day group (4000VitD) received two 2000 IU vitamin D gelatin capsules (Farma D, Colombia) per day. The 20 healthy individuals (HIs) from Medellin voluntarily agreed to participate in this study and signed a written informed consent before participation. Furthermore, HIs were negative for the DENV NS1 antigen and DENV IgM/IgG antibodies according to the Dengue NS1 Antigen kit (Bio-Rad Laboratories, Marnes La Coquette, France) and had not been vaccinated against yellow fever virus. The inclusion criteria included individuals between 18 and 50 years of age, and for each individual the C-reactive protein (CRP) concentration was quantified to rule out any active infectious process. The HIs declared that they were nonsmokers and were not taking any medication. The exclusion criteria included any clinical presentation associated with infectious disease and pregnancy. Subjects taking supplements that contained vitamin D or calcium supplements were excluded of the study.

2.3. Sample collection

After removing 50 ml (VD0) of PB collected with a syringe and 8 ml of PB in red top tube for clotted blood that was used to obtain autologous serum, the participants who agreed to take the supplement were assembled in the 1000VitD and 4000VitD groups. Immediately after the first blood sample was removed, the participants started taking the daily doses for 10 days. Not incidents were reported to us by the participants during the course of the experiment. The second 50 ml of PB collected was taken on day 10 (VD10).

2.4. Monocyte purification and monocyte-derived macrophage differentiation

The PB samples, VD0 and VD10, from each subject were mixed with EDTA 2% v/v and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Histopaque (Sigma-Aldrich, St. Louis, USA) gradient at 650 g during 30 min as described [26]. Platelet depletion was performed by washing with PBS (Sigma-Aldrich, St. Louis, USA) three times at 250 g during 10 min. The percentage of CD14 + cells (monocytes) was then determined by staining 1′000.000 of PBMCs with 1 μl of