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1,25-Dihydroxyvitamin D3 induces splenocyte apoptosis and enhances BALB/c mice sensitivity to toxoplasmosis

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Abstract

The hormonal form of Vitamin D, 1,25-dihydroxyvitamin D3, is well known for its immunosuppressive, anti-proliferative and pro-apoptotic activities. In the present work, we studied the effect of 1,25-dihydroxyvitamin D3 on *Toxoplasma gondii*-infected mice. We observed that 1,25-dihydroxyvitamin D3 reduces the survival rate of infected mice by up to 37% at day 10 post-infection compared to untreated infected mice ($P < 0.0001$). IFN-γ and IL-12p40 levels were significantly reduced by 1,25-dihydroxyvitamin D3 in infected mice sera indicating an inhibition of Th-1-type cytokines. CD_4+ T lymphocyte and splenocyte counts were also reduced following 1,25-dihydroxyvitamin D3 treatment and a marked induction of apoptosis, accompanied with down-regulation of the anti-apoptotic proteins Bcl-2 and Bcl-X_L, was observed. The above results indicate that 1,25-dihydroxyvitamin D3 induces splenocyte apoptosis and enhances host susceptibility to toxoplasmosis.

Keywords: Apoptosis; 1,25-Dihydroxyvitamin D3; *Toxoplasma gondii*; Th-1 response

1. Introduction

In addition to its role as a calcium-regulating hormone, the active Vitamin D metabolite, 1,25-dihydroxyvitamin D3, exerts immunosuppressive [1] and pro-apoptotic [2] properties. The discovery of Vitamin D receptors (VDR) in antigen-presenting cells, such as dendritic cells [3], macrophages and activated lymphocytes [4], has led to determination of the role of 1,25-dihydroxyvitamin D3 in immunosuppression [5]. Moreover, it has been reported that 1,25-dihydroxyvitamin D3 inhibits IFN-γ production, while it has little effect on IL-4 [6]. In addition, 1,25-dihydroxyvitamin D3 reduces class II antigen expression on monocytes without significantly reducing class I antigen expression [7]. An inhibitory effect of 1,25-dihydroxyvitamin D3 on the immune system by targeting Th-1 lymphocytes has also been suggested [1].

The coccidian intracellular protozoan, *Toxoplasma gondii*, which belong to the apicocomplexan phylum, is a widespread opportunistic parasite of humans and animals. Primary acquired infection of *T. gondii* in immunocompetent individuals is usually asymptomatic due to an effective immune response [8]. In contrast, altered immune functions in immunocompromised individuals may induce a resurgence of toxoplasmosis due to disruption of the cyst form of the parasite. This phenomenon occurs especially in AIDS patients, who frequently develop toxoplasmic encephalitis [9]. The acute phase of the infection is marked by an elevated level of Th-1-characterized cytokines, such as IFN-γ and IL-12, as well as of other pro-inflammatory cytokines [10]. Indeed, a highly polarized Th-1

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cytokine expression pattern plays a crucial role in controlling replication of *T. gondii* tachyzoïtes in early host infection [8].

Recently, a great success has been made in developing 1,25-dihydroxyvitamin D3 analogues with high potency and less hypercalcemic effect as a potential therapeutic agent for treatment of certain autoimmune diseases and tumors [11]. However, the inhibition of type-1 response by 1,25-dihydroxyvitamin D3 treatment on a host infected with opportunistic infectious agents, such as *T. gondii* are not yet elucidated. In the present study, we hypothesized that 1,25-dihydroxyvitamin D3 may be associated with detrimental effects on hosts infected with *T. gondii*.

2. Materials and methods

2.1. Infection and 1,25-dihydroxyvitamin D3 treatment of mice

Six- to 8-week-old female BALB/c mice purchased from the Centre d'Elevage R. Janvier (Le-Genest-St-Isle, France) were housed under pathogen-free conditions and received water and food ad libitum. The food consisted of a standard laboratory diet (SAFE, Villemoisson-sur-Orge, France) containing 0.87% calcium and 800 IU/kg of 1,25-dihydroxyvitamin D3. The mice were infected intraperitoneally (i.p.) with 20 cysts of the *T. gondii* avirulent strain, ME49 (type II), obtained from brains of Swiss-Webster mice infected i.p. 3–4 months earlier as described previously [12].

1,25-Dihydroxyvitamin D3 (Sigma–Aldrich Chimie, Lyon, France) was administered (i.p.) to each mouse at a dose of 0.5 μg/kg/2 days. Treatment begun 3 days before infection (two doses) and continued over a period of 13 days (seven doses). In preliminary experiments, we assessed the 1,25-dihydroxyvitamin D3 dosage to avoid an hypercalcemic effect. Total calcium levels in mice sera samples were determined by spectrophotometry (ADVIA 1650 Chemistry System, Bayer, Puteaux, France). Data analysis indicated that calcium levels were less than 2.6 mM in 1,25-dihydroxyvitamin D3-treated mice. Four experimental groups of 10 mice each were maintained throughout each experiment. In order to investigate the toxic effect of 1,25-dihydroxyvitamin D3 on the parasite, we pre-incubated *T. gondii* with increased concentrations of 1,25-dihydroxyvitamin D3 for 3 h at 37 °C. Analysis by trypan blue cell death assay indicated that no toxic effect introduced by 1,25-dihydroxyvitamin D3 for *T. gondii* (data not shown).

2.2. Biological and histological analysis

Blood samples were collected from the orbital vein of the eye under sedation. Serum levels of IFN-γ and IL-12p40 were measured at day 7 post-infection by ELISA according to the manufacturer's instructions (OptEIA kit, PharMingen, San Diego, CA, USA). The threshold value for delectability of IFN-γ and IL-12p40 was 31 pg/ml. At day 10 post-infection, 4 mice from each group were euthanized by isoflurane inhalation (Abbott, Rungis, France). The brain, lung, liver, spleen, heart, small intestine and large intestine were removed and immediately fixed in 4% paraformaldehyde. Two to 5 μm thick paraffin sections of each organ were stained with hematoxylin/eosin to evaluate inflammatory lesions or used for immunohistochemistry to detect *T. gondii* parasites and antigen in tissues by using a peroxidase–anti-peroxidase technique with diaminobenzidine as the chromogen [13].

2.3. Cell culture and flow cytometry analysis

Spleens were harvested from infected mice at day 7 post-infection and disrupted in endotoxin-free RPMI 1640 with 10% FCS, penicillin (100 U/ml)–streptomycin (100 U/ml) and amphotericin B (25 ng/ml). All the reagents were purchased from Invitrogen (Cergy Pontoise, France). The erythrocytes were lysed with 0.83% NH_4Cl in 0.01 M Tris–HCl (pH 7.2). The cells were washed twice with medium and counted under light microscopy. Spleen T-lymphocyte subsets (CD_4+/CD_3+, CD_8+/CD_3+) were analyzed by flow cytometry using mAbs bound to FITC or PE (Immunotech, Marseille, France). Cell cycle analysis was performed by measuring DNA binding to propidium iodide (PI) after 1 h of incubation of 1×10^6 spleen cells with PI using a commercial kit (DNAcon3, Dako S.A., Trappes, France), followed by flow cytometry analysis. The data were analyzed using Win cycle software (Phoenix Flow Systems, San Diego, CA). Analysis of apoptosis by annexin-V labeling was performed using a total of 1×10^6 spleen cells, which were washed in PBS and resuspended in 100 μl of binding buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$). A volume of 5 μl of annexin-V and 2.5 μl of PI was added to the cells, followed by incubation for 10 min. The final volume was then adjusted by adding 400 μl of binding buffer and analyzed by flow cytometry. Annexin-V data were obtained using a Beckman Coulter flow cytometer (Cytomics FC 500, Miami, Florida) and analyzed with RXP software.

2.4. Analysis of pro-apototic and anti-apoptotic protein expressions

Crude cell lysates from spleen cells were prepared by harvesting cells in PBS followed by sonication. For immunoblotting, total proteins from cell lysates were loaded for one-dimensional electrophoresis on an SDS 8% polyacrylamide gel. Proteins were blotted onto a nitrocellulose membrane, blocked with 10% blocking reagent (Roche Diagnostics, Mannheim, Germany) and incubated for 1 h at room temperature with anti-ICBP90 (0.1 μg/ml); engineered in our laboratory as described previously [14], anti-Bcl-2 or anti-Bcl-X_L mAbs (Santa Cruz Biotechnology, Le Perray en Yvelines, France) diluted to 1:100. Alkaline phosphatase conjugated sheep anti-mouse Ig (Fab fragments, Roche Diagnostics) was used at a dilution of 1:2500. Actin detection