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Anti-inflammatory effect of 1α,25-dihydroxyvitamin D_3 in human coronary arterial endothelial cells: Implication for the treatment of Kawasaki disease

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ABSTRACT

Kawasaki disease (KD) is an acute febrile vasculitis in childhood that is associated with inflammatory cytokines, in which the vascular inflammation results in damage to the coronary arteries. The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 {1α,25-$(OH)_2D_3$} exhibits anti-inflammatory activities. In this study, we determined the mRNA and protein expression of the vitamin D receptor in human coronary arterial endothelial cells (HCAEC) by RT-PCR and Western blotting, respectively. We examined whether or not 1α,25-$(OH)_2D_3$ inhibits the tumor necrosis factor-α (TNF-α)-induced activation of nuclear transcription factor-κB (NF-κB), which is essential for the expression of proinflammatory cytokines in HCAEC, by ELISA. In addition, we determined the inhibitory effect of 1α,25-$(OH)_2D_3$ on E-selectin expression induced by TNF-α in HCAEC by flow cytometry. RT-PCR revealed mRNA for the vitamin D receptor in HCAEC. Western blotting demonstrated vitamin D receptor protein in HCAEC. ELISA showed that pretreatment with 1α,25-$(OH)_2D_3$ significantly inhibited the TNF-α-induced NF-κB activation in HCAEC. Moreover, flow cytometry revealed that pretreatment with 1α,25-$(OH)_2D_3$ significantly inhibited the TNF-α-induced expression of E-selectin on HCAEC. Our results suggest that adjunctive 1α,25-$(OH)_2D_3$ may modulate the inflammatory response during KD vasculitis.

1. Introduction

Kawasaki disease (KD) is an acute illness of early childhood that is characterized by prolonged fever, diffuse mucosal inflammation, indurative edema of the hands and feet, polymorphous skin rash and non-suppurative lymphadenopathy [1]. The histopathological findings in KD comprise panvasculitis with endothelial necrosis, and infiltration of mononuclear cells into small and medium-sized blood vessels [2]. Coronary arterial lesion is the most important complication of KD, which may cause significant coronary stenosis resulting in ischemic heart disease [3]. Our previous studies revealed that tumor necrosis factor-α (TNF-α) activity and activation of nuclear factor-κB (NF-κB), a transcription factor that is essential for the expression of proinflammatory cytokines, play important roles in the pathogenesis of KD [4–8]. In addition, arterial endothelial cells are activated in acute KD because the levels of various soluble forms of adhesion molecules are elevated in the peripheral blood during the acute stage [9,10].

The active form of vitamin D, 1α,25-dihydroxyvitamin D_3 {1α,25-$(OH)_2D_3$} or calcitriol is known to be associated with calcium and phosphorus homeostasis and maintenance of skeletal architecture [11]. Recently, several studies have reported that 1α,25-$(OH)_2D_3$ exhibits anti-inflammatory and immunomodulatory effects [11,12]. TNF-α expression and NF-κB activation in macrophages, and TNF-α-induced expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in human umbilical vein endothelial cells are inhibited by 1α,25-$(OH)_2D_3$ [12–17]. These reports suggest that 1α,25-$(OH)_2D_3$ could be effective for acute KD. In addition, a proportion of KD patients are resistant to high-dose intravenous immunoglobulin (IVIG) therapy as a standard therapy for KD [18,19]. We hypothesize that adjunctive 1α,25-$(OH)_2D_3$ may modulate KD vasculitis. In this study, we examined the expression of vitamin D receptors in human coronary arterial endothelial cells (HCAEC), and determined whether or not 1α,25-$(OH)_2D_3$ inhibits NF-κB activation and E-selectin expression induced by TNF-α in HCAEC.

2. Materials and methods

2.1. Cell culture and stimulation conditions

HCAEC were obtained from Lonza (Walkersville, MD, USA) and maintained at 37 °C under humidified 5% CO_2 in a stationary culture. HCAEC were grown using the EGM-2 BulletKit (Lonza). Jurkat cells (a human T lymphocytic leukemia cell line), THP-1 cells (a human

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monocytic leukemia cell line), and Cos-1 cells (an African green monkey kidney fibroblast line) were obtained from the American Type Culture Collection and maintained at 37 °C under a humidified 5% CO_2 atmosphere as stationary cultures. The cells were grown in RPMI 1640 medium containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

HCAEC were exposed to 2 ng/ml TNF-α (R&D Systems, Minneapolis, MN, USA) for the indicated times after 30 min pretreatment with 10^{-7}, 10^{-8} and 10^{-9} M 1α,25-$(OH)_2D_3$ (Wako Junyaku Co., Osaka, Japan).

2.2. RNA isolation and RT-PCR

Total RNA was prepared from each cell type using TRIzol reagent (Invitrogen, Leek, The Netherlands). RT-PCR was performed with Gene Amp and an oligo-dT primer (Applied Biosystems, Foster City, CA, USA) for RT, and Taq polymerase (Roche Diagnostics GmbH, Mannheim, Germany) for PCR. The primers used were: (1) vitamin D receptor: forward, 5′-ATGCCATCTGCATCGTCTC-3′, and reverse, 5′-GCACCGCACAGGCTGTCCTA-3′; (2) GAPDH: forward, 5′-ACCACAGTCCATGCCATCAC-3′, and reverse, 5′-TCCACCACCCTGTTGCTGTA-3′. Quantitation of the bands was performed using a Kodak Digital Science 1D (Eastman Kodak Company, New Haven, CT, USA).

2.3. Western blot analysis

Whole cell lysates were obtained by incubation of cell samples in ice-cold lysis buffer (1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride) containing protease inhibitors (1 μM leupeptin and 1 μM pepstatin) and centrifugation to remove debris (12,000 × *g* for 10 min at 4 °C). The protein concentrations of the samples were determined using Bio-Rad (Hercules, CA, USA) protein concentration reagent. Samples containing 20 μg of protein were separated in denaturing 10% polyacrylamide gels and then transferred to polyvinylidene difluoride membranes. After three washes in TBST (40 mM Tris–HCl, pH 7.6, 300 mM NaCl and 0.5% Tween 20), the membranes were incubated with 1:100 dilution of mouse monoclonal anti-vitamin D receptor antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TBST containing 5% nonfat dry milk at 4 °C for 24 h. After three additional washes in TBST, the membranes were incubated with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Immunoreactive proteins were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) and analyzed by radiography.

2.4. Nuclear extracts and determination of NF-κB activation

Nuclear extracts were harvested from HCAEC using a Nuclear Extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. The protein concentrations of the nuclear extracts were determined using Coomassie Plus Protein Assay Reagent (PIERCE, Rockford, IL, USA). The levels of NF-κB were determined using an activated NF-κB ELISA kit (Active Motif). An oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) was adsorbed to polystyrene microwells. The activated NF-κB contained in nuclear extracts bound specifically to this oligonucleotide, and the NF-κB complex bound to the oligonucleotide could then be detected using an anti-NF-κB p65 antibody. Following this, a secondary antibody conjugated to horseradish peroxidase was added. The absorbance was measured at 450 nm by spectrophotometry.

2.5. Determination of E-selectin (CD62E) expression

HCAEC were exposed to 2 ng/ml TNF-α with or without pretreatment with 1α,25-$(OH)_2D_3$ for 30 min. The cells were collected 4 h after the addition of TNF-α, and the expression of E-selectin was determined by flow cytometric analysis. The cells were then labeled with a PE-conjugated anti-CD62E antibody (Ancell Co., Bayport, MN, USA). Immunofluorescence staining was analyzed with a FACScan flow cytometer equipped with CellQuest software (Becton-Dickinson Biosciences, San Diego, CA, USA). Five thousand cells were analyzed in the flow cytometric studies.

2.6. Statistical analysis

The data are presented as the mean ± S.D. Statistical analysis was performed with the *t*-test, with a *p*-value of less than 0.05 being considered as significant. Analyses and calculations were performed using SPSS-12.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Expression of vitamin D receptor in HCAEC

We investigated the presence of vitamin D receptor mRNA in HCAEC. THP-1, Jurkat, HCAEC, and Cos-1 cells showed expression of vitamin D receptor mRNA by RT-PCR (Fig. 1). We then investigated the presence of the vitamin D receptor protein in HCAEC. Western blotting analysis of THP-1, Jurkat, HCAEC, and Cos-1 cells revealed expression of the vitamin D receptor (50 kDa) (Fig. 2). There were no bands in control experiments with secondary antibodies alone (data not shown).

3.2. Inhibitory effect of 1α,25-$(OH)_2D_3$ on NF-κB activation induced by TNF-α

We investigated whether or not 1α,25-$(OH)_2D_3$ could inhibit NF-κB activation induced by TNF-α in HCAEC. The inhibitory effects of 1α,25-$(OH)_2D_3$ on NF-κB activation induced by TNF-α in HCAEC

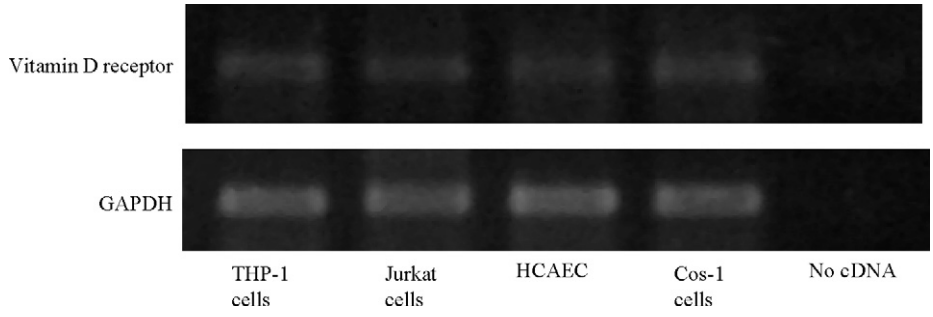

Fig. 1. Expression of vitamin D receptor mRNA measured by RT-PCR in THP-1, Jurkat, HCAEC, and Cos-1 cells. Expression of vitamin D receptor mRNA was observed in THP-1, Jurkat, HCAEC, and Cos-1 cells. Representative data are shown. Similar results were obtained in four independent experiments.

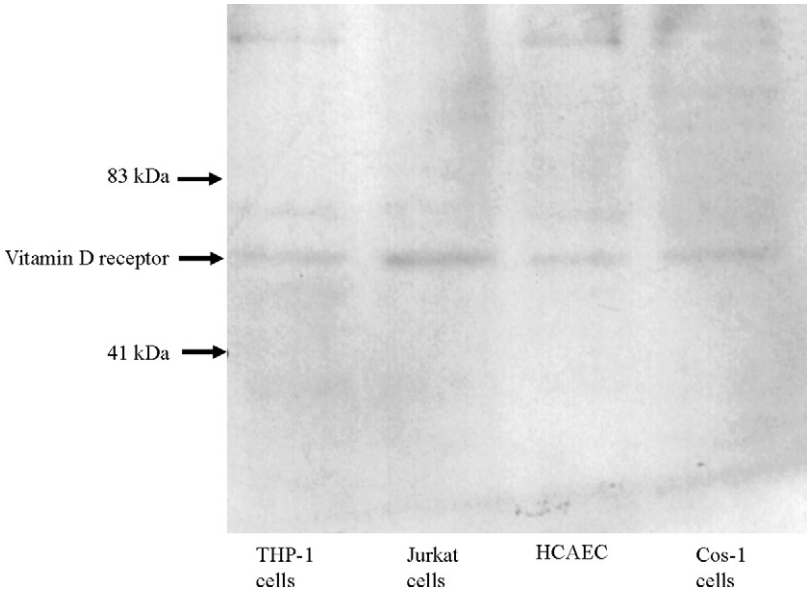

Fig. 2. Expression of vitamin D receptor measured by Western blot analysis in THP-1, Jurkat, HCAEC, and Cos-1 cells. Vitamin D receptors were expressed in THP-1, Jurkat, HCAEC, and Cos-1 cells. Representative data are shown. Similar results were obtained in four independent experiments.

are shown in Fig. 3. NF-κB activation was significantly induced by stimulation with TNF-α for 30 min ($p < 0.001$), which was significantly inhibited by pretreatment with 10^{-7}, 10^{-8} and 10^{-9} M 1α,25-$(OH)_2D_3$ ($p < 0.01$, $p < 0.01$ and $p < 0.05$, respectively). There were no significant differences in the inhibitory potency for NF-κB activation by 10^{-7}, 10^{-8} or 10^{-9} M 1α,25-$(OH)_2D_3$. NF-κB activation was not affected by 1α,25-$(OH)_2D_3$ in the absence of TNF-α.

3.3. Inhibitory effect of 1α,25-$(OH)_2D_3$ on E-selectin expression induced by TNF-α

We investigated whether or not 1α,25-$(OH)_2D_3$ could inhibit E-selectin expression induced by TNF-α in HCAEC. Flow cytometric analysis revealed that TNF-α significantly increased E-selectin expression in HCAEC ($p < 0.001$), and that pretreatment with 10^{-7}, 10^{-8} and 10^{-9} M 1α,25-$(OH)_2D_3$ significantly inhibited the TNF-α-induced expression of E-selectin, as shown in Fig. 4 ($p < 0.001$, $p < 0.001$ and $p < 0.01$, respectively). The inhibitory effects of 10^{-7} and 10^{-8} M 1α,25-$(OH)_2D_3$ on E-selectin expression induced by TNF-α were significantly greater than that of 10^{-9} M 1α,25-$(OH)_2D_3$ (both $p < 0.05$).

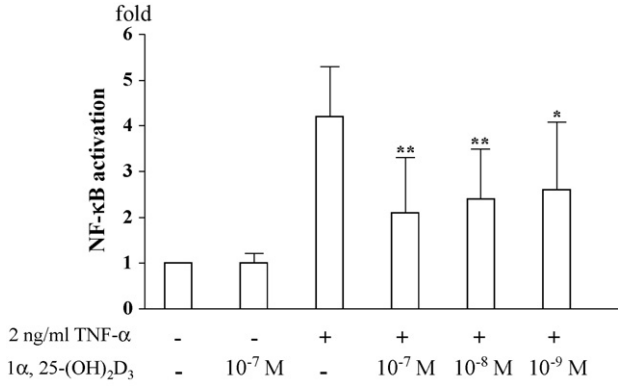

Fig. 3. Inhibitory effect of 1α,25-$(OH)_2D_3$ on the activation of NF-κB induced by TNF-α in HCAEC. NF-κB activation in HCAEC was measured by ELISA. HCAEC were pretreated with 10^{-7}, 10^{-8} and 10^{-9} M 1α,25-$(OH)_2D_3$ for 30 min prior to stimulation with TNF-α for 30 min. The data ($n = 8$) are expressed as the ratio of stimulated cells to cells cultured in medium alone, and are presented as the mean ± S.D. $^{**}p < 0.01$ and $^{*}p < 0.05$, compared to cells treated with TNF-α.

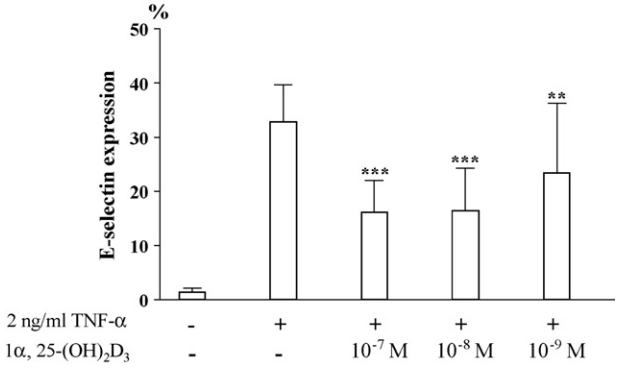

Fig. 4. The inhibitory effect of 1α,25-$(OH)_2D_3$ on E-selectin expression induced by TNF-α in HCAEC. The expression of E-selectin in HCAEC was measured by flow cytometry. HCAEC were pretreated with 10^{-7}, 10^{-8} and 10^{-9} M 1α,25-$(OH)_2D_3$ for 30 min prior to stimulation with TNF-α for 4 h. Data ($n = 10$) are presented as the mean ± S.D. $^{***}p < 0.001$ and $^{**}p < 0.01$, compared to cells treated with TNF-α.

4. Discussion

We previously demonstrated that NF-κB was markedly activated in peripheral blood monocytes/macrophages and T cells from children with acute KD [7]. Levels of soluble E-selectin are elevated in serum during acute KD [10]. E-selectin is an endothelial-specific surface adhesion molecule that is primarily found in its soluble form after endothelial cell activation and leukocyte–endothelial cell interaction [20,21]. In addition, serum TNF-α levels are elevated in KD and KD patients with high levels of soluble TNF receptor in the serum appear to be susceptible to coronary artery lesions [4–6,9]. In consideration of these previous reports, TNF-α, induced primarily by NF-κB activation, likely causes systemic vasculitis with high-E-selectin expression associated with NF-κB activation in vascular endothelial cells during acute KD.

It has been reported that 1α,25-$(OH)_2D_3$ exhibits anti-inflammatory and immunomodulatory effects. The expression of interferon-γ and interleukin-12 (IL-12) in T cells, differentiation,

maturation, activation, and survival of dendritic cells, and TNF-α expression and NF-κB activation in macrophages were all inhibited by 1α,25-$(OH)_2D_3$ [13–16]. Moreover, activation of NF-κB and release of IL-6, IL-8 and regulated upon activation normal T cell exposed and secreted (RANTES), which are induced by lipopolysaccharide in human microvascular endothelial cells, and the expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1, which are induced by TNF-α in human umbilical vein endothelial cells, were all inhibited by 1α,25-$(OH)_2D_3$ [12,17]. However, there have been no reports on the action of 1α,25-$(OH)_2D_3$ in HCAEC. In our present study, RT-PCR and Western blot analysis revealed expression of vitamin D receptor mRNA and protein, respectively, in HCAEC. Previous studies reported that Jurkat and THP-1 cells had vitamin D receptor [22,23]. Therefore, we used both cells as positive controls for the vitamin D receptor. Cos-1 cells were used as negative controls. However, they were found to express the vitamin D receptor. It was considered that the vitamin D receptor of Cos-1 cells reacted to the human primers and antibody because the sequence of monkey vitamin D receptor exhibits 91% homology with the human form [24]. This is the first report to demonstrate that HCAEC express the vitamin D receptor. Moreover, pretreatment with 1α,25-$(OH)_2D_3$ significantly inhibited the TNF-α-induced activation of NF-κB and expression of E-selectin in HCAEC. We used TNF-α to stimulate the cells because TNF-α is an important factor in the pathogenesis of KD vasculitis. The concentrations of 10^{-9} to 10^{-7} M 1α,25-$(OH)_2D_3$ used in our study are consistent with plasma levels obtained in normal humans after administration of a normal dose [25,26].

More recently, it has been reported that vitamin D may be used as a therapeutic option to treat inflammatory diseases, including Behçet's disease, inflammatory bowel disease, multiple sclerosis, experimental sepsis and inflammatory polyarthritis [27–33]. IVIG therapy has been reported to be effective for reducing the incidence of coronary artery lesions in KD patients [34–36]. However, at least 10% of KD patients fail to exhibit defervescence with IVIG therapy [18,19]. Some reports have recommended alternative therapy with steroids as anti-inflammatory therapy for KD patients [37–39]. Our present results suggest that adjunctive 1α,25-$(OH)_2D_3$, which has been widely used in children and has a high-safety index, may modulate the inflammatory response during KD vasculitis.

In conclusion, HCAEC express the vitamin D receptor and 1α,25-$(OH)_2D_3$ inhibited the TNF-α-induced activation of NF-κB and expression of E-selectin in HCAEC.

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