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The Effects of Vitamin K1 and Vitamin K2 on the Proliferation, Cytokine Production and Regulatory T Cell-Frequency in Peripheral Blood Mononuclear Cells of Pediatric Atopic Dermatitis Patients.

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ABSTRACT

We estimated the pharmacological efficacy of vitamin K₁ (VK₁) and VK₂ on the mitogen-activated peripheral-blood mononuclear cells (PBMCs) of pediatric atopic dermatitis (AD) patients. VK₂ suppressed the *in vitro* proliferation of T cell-mitogen-activated PBMCs of AD patients. In contrast, VK₁ had little effect on the PBMC proliferation. The IL-2 production from the activated PBMCs of AD patients significantly increased ($P<0.05$), while the production significantly decreased by 100 μ M VK₂ ($P<0.01$). In addition, 100 μ M VK₂ reduced the percentage of CD4⁺ and CD4⁺CD25⁺ cells in PBMCs. These results suggest that VK₂ can modulate T cell function in PBMCs of AD patients.

BACKGROUND

Atopic dermatitis (AD) is a chronic inflammatory skin disease with pruritus that predominantly occurs in early childhood [1-4]. Immunological aberrations are suggested to be the main inducers of AD [5]. However, side effects of immunosuppressants are reported in 70% of patients [6]. Selecting suitable immunosuppressive agents for young AD patients is a dilemma for pediatricians. We previously confirmed that VK₃ and VK₅ significantly suppress the T cell mitogen-activated proliferation of peripheral blood mononuclear cells (PBMCs) at concentrations of 10-100 μ M *in vitro* [7]. VK₁ and VK₂, which are used as coagulant and anti-osteoporosis drugs, respectively, share the same naphthoquinone skeleton as VK₃. Thus, VK₁ and VK₂ are suggested to have immunosuppressive efficacy with fewer side effects.

QUESTIONS ADDRESSED,

We evaluated the immunosuppressive effects of VK₁ and VK₂ using PBMCs of AD patients. The effects of VK on cytokine production and CD4⁺CD25⁺Foxp3⁺ cell ratio in concanavalin A-activated PBMCs of AD patients were also investigated.

EXPERIMENTAL DESIGN

The effects of VK₁ and VK₂ on concanavalin A-stimulated PBMCs obtained from 12 AD patients (mean age: 7.7 years) were evaluated *in vitro* by a (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [8]. The effects of VK₁ and VK₂ on the production of Th1/Th2/Th17 cytokines produced from the activated

PBMCs were examined by bead-array procedures. The percentages of

CD4⁺CD25⁺Foxp3⁺ cells as Treg cells in the lymphocyte cell fraction were estimated after staining with specific antibodies followed by flow-cytometry.

RESULTS

Even at its highest concentration, VK₁ showed little effect on the concanavalin A-activated proliferation of PBMCs derived from pediatric AD patients (**Figure 1**). In contrast, 100 μ M VK₂ showed significant inhibitory effects on the proliferation of PBMCs derived from pediatric AD patients ($P<0.05$).

The Th1/Th2/Th17 cytokine production from the concanavalin A-activated PBMCs of AD patients was unaffected by VK₁ at any concentration (**Figure 2**). The production of IL-2 from the concanavalin A-activated PBMCs of AD patients significantly increased ($P<0.05$) and the production of IL-10 significantly decreased by 100 μ M VK₂ ($P<0.01$) (**Figure 2**). VK₂ also appeared to decrease the IL-17 production and increase the IL-4 production from the activated PBMCs of pediatric AD patients, but the changes were not statistically significant.

The percentage of CD4⁺ cells significantly decreased by the treatment with 10 and 100 μ M VK₂ ($P<0.01$). 100 μ M VK₂ also reduced the percentage of CD25⁺ lymphocytes ($P<0.001$). At concentrations of 0.1-100 μ M, VK₂ tended to reduce the percentage of CD4⁺CD25⁺Foxp3⁺Treg in lymphocytes and CD4⁺ cells; however, these changes were not statistically significant (**Figure S1**).

DISCUSSION

VK₂ inhibited the mitogen-activated proliferation of PBMCs of pediatric AD patients in a dose-dependent manner. However, the MTT assay results showed that VK₁, which shares the same naphthoquinone skeleton as VK₂, had no inhibitory effect on the proliferation of mitogen-activated PBMCs in pediatric AD patients *in vitro* (**Figure 1**). Given the previously reported immunosuppressive efficacy of VK₃ and VK₅ [7], naphthoquinone skeleton is suggested to be a suitable candidate chemical unit for developing new immunosuppressive agents with fewer adverse effects, especially for the treatment of pediatric AD patients.

CD4⁺ cells can be induced to differentiate into Treg cells and Th17 phenotypes according to the local cytokine milieu [9]. Treg cells form a substantial component of the autoimmune counterbalance [10]. VK₂ at 100 μ M significantly reduced the percentages of CD4⁺ cells and CD4⁺CD25⁺ cells in peripheral lymphocytes of pediatric AD patients. The percentage of Treg cells in both CD4⁺ cells and lymphocytes tended to decrease by the VK₂ treatment (**Figure S1**). The production of cytokine IL-10 significantly inhibited by 100 μ M VK₂, and the relationship between the IL-10 concentration in the PBMC culture medium and the Treg cell ratios showed a statically significant positive correlation (**Figure S2**). These observations seem to verify the hypotheses of other researchers [11, 12]. We therefore suggest that the immunomodulation by VK₂ is independent of the Treg frequency in PBMCs and the IL-10 levels.

Although the production of most cytokines from PBMCs tended to decrease with VK₂ treatment, IL-2 and IL-4 showed the opposite tendency. Several lines of evidence disclosed that the unbalanced generation of Th2/Th1-like cells occurs in atopic disorders [13-15]. We calculated the ratio of IL-4/IL-2 and IL-4/IFN- γ before and after treatment by 100 μ M VK₂, and showed that the IL-4/IL-2 ratio decreased from 3.4 to 0.7, whereas the IL-4/IFN- γ ratio increased from 0.001 to 0.025. The results suggest that VK₂ could change the balance of Th1 and Th2 in AD patients.

IL-17 may have a high clinical impact on the pathogenesis, exacerbation and maintenance of AD, although its identity and functions remain unclear. We found in this study that the IL-17 production from PBMCs decreased, while the IL-4/IL-17 ratio increased by VK₂ treatment.

Gas6 is a plasma vitamin K-dependent protein that plays an important immunoregulatory role in various cells by interacting with the three receptors of TAM (Tyro3, Axl, and MerTK) [16-19]. However, lymphocytes do not express TAM receptors [20]; thus the GAS6 receptors do not seem to be related to the main mechanism underlying the effects of VK₂. Our present data suggest that VK₂ can effectively suppress activated T lymphocytes in the PBMCs of AD patients. Neither cytokines nor regulatory T cells appeared to be involved in the primary PBMC-suppressive mechanism of VK₂.

Acknowledgements and a list of individual author contributions

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Kehan Meng performed the research and wrote the paper. Kentaro Sugiyama and Toshihiko

Hirano designed the research study. Wencheng Xu analyzed the data. Sachiko Tanaka

provided the research methods. Taro Miura, Shunsuke Suzuki, Masako Chiyotanda and

Hisashi Kawashima contributed to the clinical study.

Conflict of interest statement

The authors declare no conflicts of interest.

Key words: Vitamin K1, Vitamin K2, Peripheral Blood Mononuclear cells, Atopic

Dermatitis Immunosuppressive efficacy

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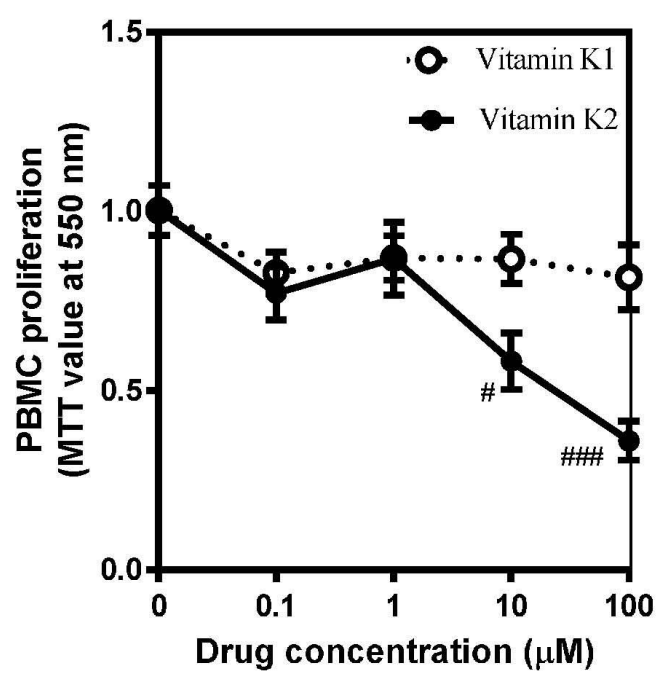


Figure 1

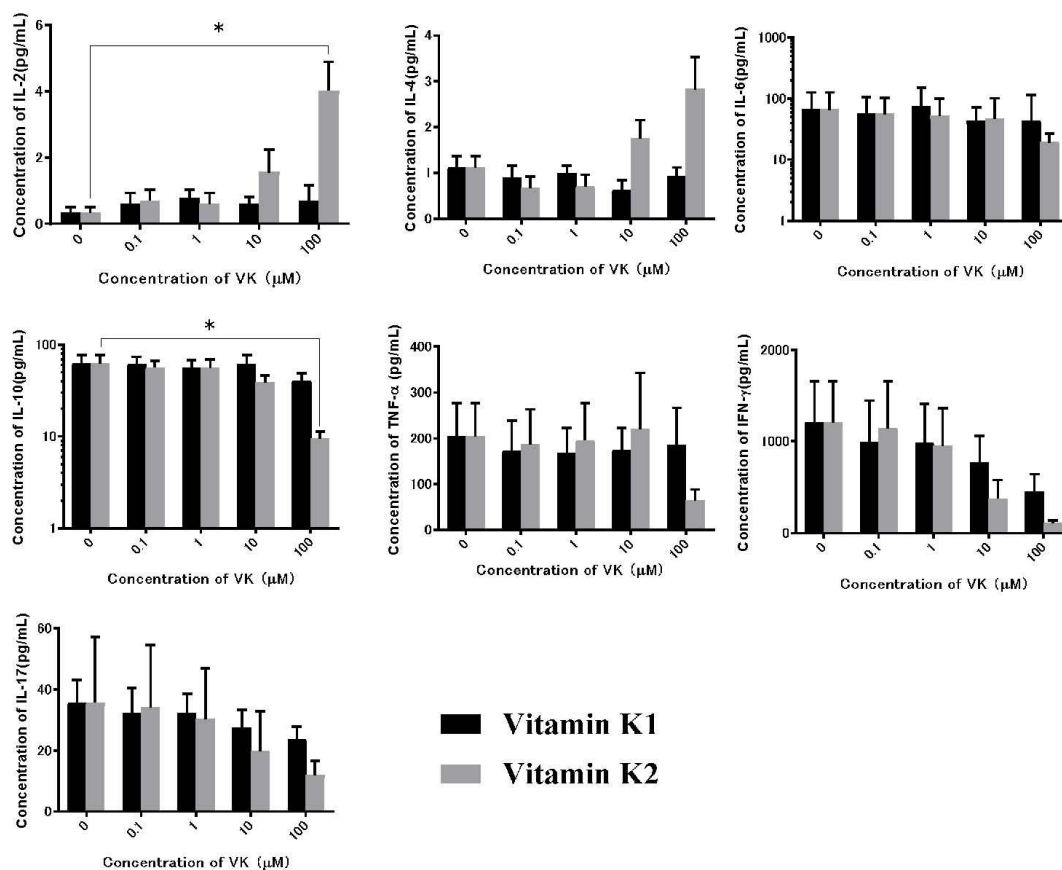


Figure 2

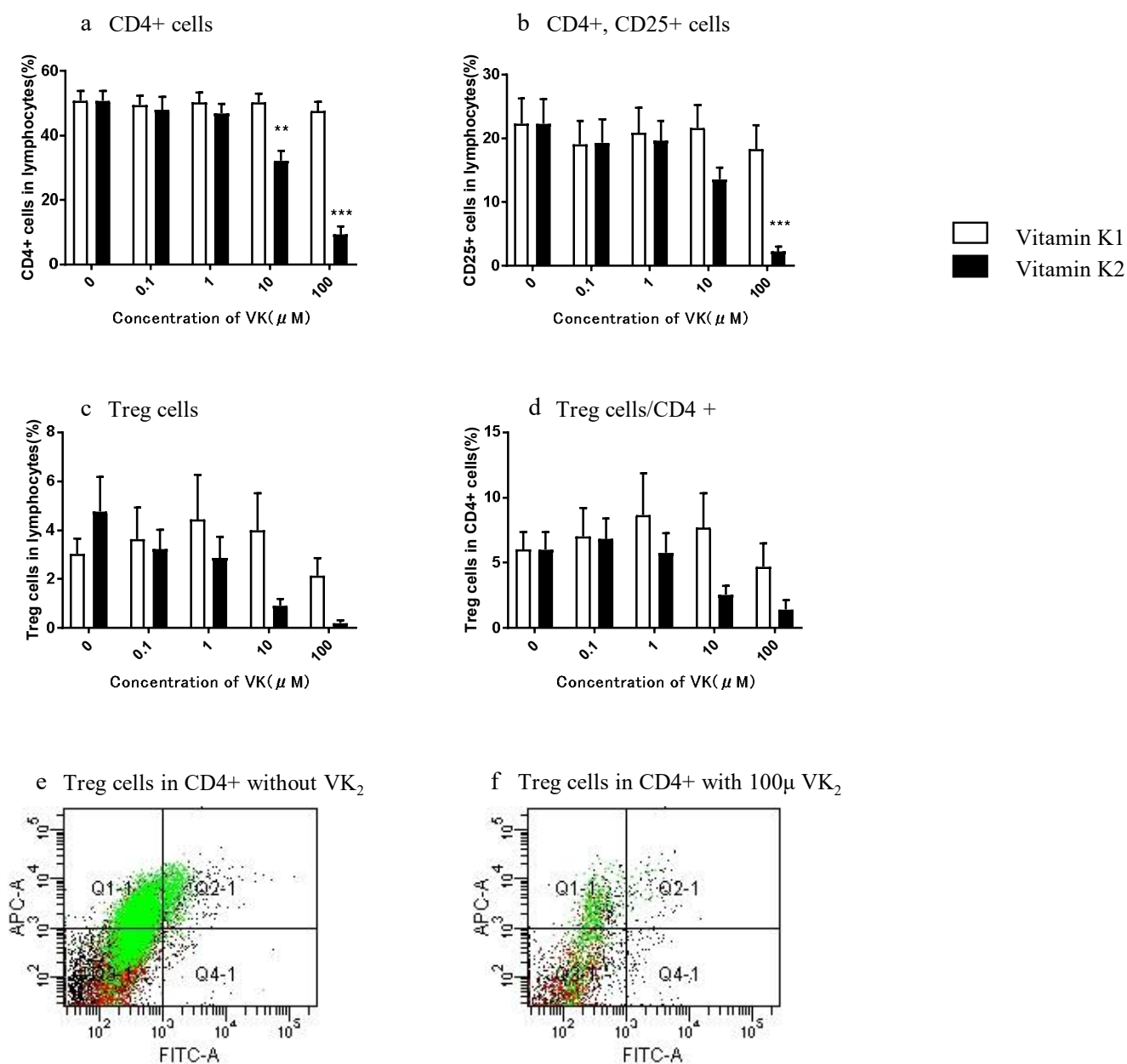


Figure S1

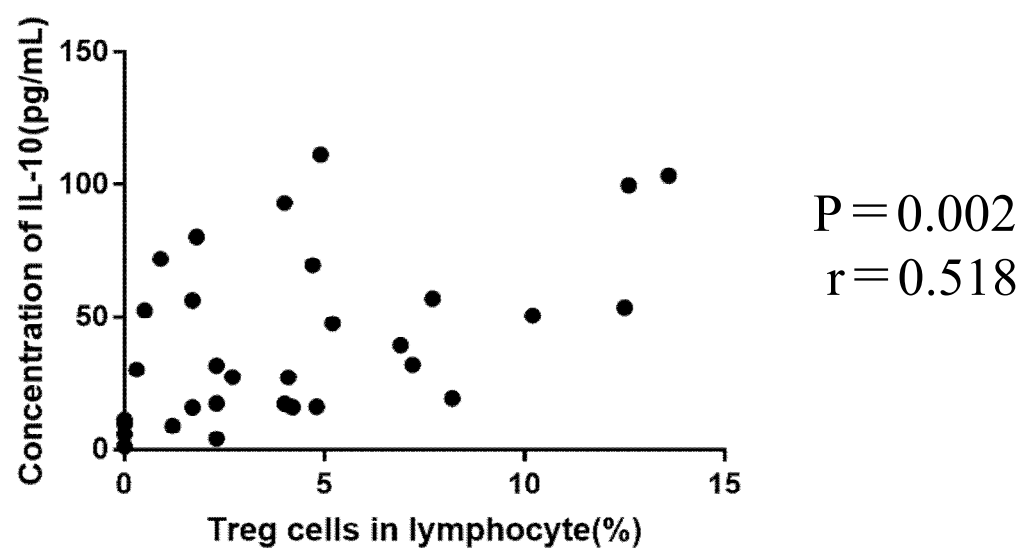


Figure S2

Figure Legends List

Figure 1. Effects of vitamin K (VK) 1 and VK₂ on the proliferation of PBMCs obtained from pediatric AD patients, as determined by MTT assay. The data were expressed as the mean $\pm$ SEM, and analyzed by Dunnett's multiple comparison test. # $P < 0.05$, ### $P < 0.001$ compared to each control groups (without drug), respectively.

Figure 2. Effects of VK₁ and VK₂ on cytokine concentrations in the supernatant of T-cell mitogen-activated PBMCs from pediatric AD patients. PBMCs from pediatric AD patients were stimulated with concanavalin A for 96 h in the absence (0 μ M) or presence (0.1-100 μ M) of VK. The production in culture supernatant was analyzed by Dunnett's test for the concentrations of IFN- γ , TNF α , IL-2, IL-4, IL-6, IL-10 and IL-17 (n=8) using a beads-array procedure followed by flow cytometry. * $P < 0.05$ and ** $P < 0.001$.

Figure S1. The percentages of CD4+CD25+Foxp3+ (Treg) cells in concanavalin A-stimulated PBMCs of AD patients cultured in the presence or absence of VK₁ (white bars) and VK₂ (black bars) for 96 h (n=8). (a) CD4+ cells in lymphocytes, (b) CD25+ cells in lymphocytes (c) CD4+CD25+Foxp3+ (Treg) cells in lymphocytes and (d) CD4+CD25+Foxp3+ (Treg) cells in CD4+ cells were analyzed by Dunnett's test. ** $P < 0.01$, *** $P < 0.001$ compared to each control (VK=0 μ M). (e, f) Typical dot-plot data of CD4+CD25+Foxp3+ (Treg) cells in CD4+ cells after treatment without (e) or with (f) 100 μ M VK₂.

Figure S2. The relationship between the concentrations of IL-10 in the PBMC culture medium and the CD4+CD25+Foxp3+ cell ratios in lymphocytes stimulated by concanavalin A in the presence of several concentrations (0.1-100 μ M) of VK₂. A statistically significant positive correlation was observed by Spearman's test between the two indices ($r=0.563$, $p=0.0019$).