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알레르기비염 마우스 모델에서 비강 내 국소 투여한 1, 25-dihydroxyvitamin D3 의 항알레르기 효과에 대한 연구

Anti-allergic effect of intranasal 1, 25-dihydroxyvitamin D3 treatment in allergic rhinitis mouse model

2018 년 2 월

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Abstract

Introduction

The role of Vitamin D as a potential immune-modulator has been recently elucidated. However, the role of vitamin D in the pathogenesis of allergic rhinitis is unclear and its anti-allergic effect has not been established yet, especially in the mouse model. The aim of this study is to evaluate the anti-allergic effect of topically applied Vitamin D in the allergic rhinitis mouse model.

Methods

BALB/c mice were intraperitoneally sensitized with ovalbumin (OVA) and alum, and they were intranasally challenged with OVA. Intranasal 1, 25-dihydroxyvitamin D3 (0.02μg) was given to treatment group and solvent was given intranasally to sham treatment group. Allergic symptom scores,

eosinophil infiltration, cytokine mRNA levels (IL-4, IL-5, IL-10, IL-13, IFN-γ) in the nasal tissue, serum total and OVA-specific IgE, IgG1, and IgG2a were analyzed and compared with negative and positive controls. Cervical lymph nodes were harvested for flow cytometry analysis and cell proliferation assay.

Results

In the treatment group, allergic symptom scores, eosinophil infiltration, and the mRNA levels of IL-4 and IL-13 in the nasal tissue were significantly reduced compared to positive control. IL-5 mRNA level, serum total IgE, and OVA-specific IgE and IgG1 levels showed a tendency to decrease in the treatment group, but did not reach to a significant level. In the cervical lymph nodes, $CD11c^{+}$, $MHCII^{high}$, $CD86^{+}$ activated dendritic cells were significantly reduced in the treatment group. There was a significant decrease of IL-4 secretion in the lymphocyte culture from cervical lymph node.

Conclusion

We have confirmed the anti-allergic effect of 1, 25-dihydroxyvitamin D3 when intranasal topically applied. Also 1, 25-dihydroxyvitamin D3 decreased dendritic cell activation and Th2 mediated inflammation in the cervical lymphnode. Topical application of 1, 25-dihydroxyvitamin D3 may be a future therapeutic agent in allergic diseases.

Keywords: Vitamin D, allergic rhinitis, dendritic cell, anti-allergic effect

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LIST OF ABBREVIATIONS

AR : Allergic rhinitis

OVA : Ovalbumin

H&E: hematoxylin & eosin

Alum : aluminum hydroxide

PCR : polymerase chain reaction

DC : dendritic cell

MHC : major histocompatibility complex

Introduction

Allergic rhinitis (AR) is an inflammatory, IgE mediated, disease characterized by allergic symptoms such as nasal congestion, rhinorrhea, sneezing, and /or nasal itching after exposure to causative allergen. Although exact pathogenesis remains unclear, it is known that Th2 driven immune response to allergen is a center of the disease pathogenesis, and this is thought to be resulted from surrounding signals provided by mucosal epithelial and dendritic cells[1].

Vitamin D, originally known to be key regulator in calcium homeostasis, recently had been discovered as a potent immune modulator with a broad spectrum of effects. The serum concentrations of vitamin D have been shown to be lower in patients with autoimmune diseases such as multiple sclerosis[2]. Also deficiency in serum level of vitamin D may contribute to the

development of Th2 skewed respiratory disease such as asthma, allergic rhinitis or chronic rhinosinusitis with nasal polyps[3],[4, 5]. However one study demonstrated low vitamin D levels associated with atopic dermatitis rather but not allergic rhinitis, asthma or IgE sensitization [6] Although previous epidemiological studies are somewhat controversy, studies implicate at least there are association with Th2 mediated allergic disease.

The action of vitamin D is mediated through vitamin D receptor(VDR) located in the cytoplasm, and it is known that virtually all the immune cells are known to express VDR [7, 8]. Previous studies have shown that T cell, B cell, monocytes and macrophages are influenced by the vitamin D[9]. Vitamin D also regulates the activity DC which is a key immune modulatory cell. Vitamin D blocks differentiation and maturation of DC from monocyte and down regulates the expression of costimulatory molecules including CD80,

CD 83, and CD86 [10, 11]. Down regulation of these costimulatory molecules reduce activation of T cell and thus leading to immune tolerance[12].

Normally, in healthy tissue, dendritic cells (DC) reside as an immature state, i.e. it has a highly active endocytic capability for uptaking a foreign antigen and yet have not acquired the fully active ability for priming naïve T cells[13]. When certain antigens are exposed and interacts with epithelium, variety of cytokines such as IL-33, thymic stromal lymphoprotein (TSLP), and other members of IL-1 family are released[14-16]. Under theses stimulation, maturation of DC occurs and the major events in DC maturation are the up-regulation of major histocompatibility complex (MHC) and costimulatory molecules on their surface[12]. Upon activation, migratory capacity increases with up-regulation of homing receptors such as C-C chemokine receptor type 7(CCR7)[17] which interacts with chemokine (C-C motif) ligand 19 (CCL19)

and CCL21 expressed on lymphatics, that migration to regional lymph node (LN) is accelerated. These migratory DCs then enter T cell zone of secondary lymphoid organs to stimulate and differentiate naïve T cells[18].

Even though vitamin D is known to regulate the tolerogenicity of dendritic cell, most of them were from in-vitro studies. Also the exact role of vitamin D in allergic rhinitis seems to be unclear. So far there had been limited publications regarding its' therapeutic effect in allergic disease. In this study, we have measured 1) the anti-allergic effect of topically applied 1, 25-dihydroxyvitamin D3, a biologically activated form of vitamin D, in the allergic rhinitis mouse model, and 2) the effect of 1, 25-dihydroxyvitamin D3 on dendritic cell activation.

Materials and Methods

Animals

Four-week-old female BALB/c mice were used as the experimental animals (YoungBio, Seongnam, Korea). Each mouse weighed 18-22 g and was maintained under specific pathogen-free conditions. All animal experiments in the present study followed the guidelines and ethics of the Institutional Animal Care and Use Committee of the Biomedical Research Institute of Seoul National University Hospitalospital.

Induction of the murine model of AR and treatment

Mice were divided into 4 groups as follows: Group 1 (n=4) as a negative control group, Group 2 (n=6) as a positive control group, Group 3 (n=6) as a 1, 25-dihydroxyvitamin D3 treatment group, and Group 4 (n=4) as a solvent, sham treatment group. 1, 25-dihydroxyvitamin D3 (Bonky®), and solvent was acquired from YuYu Pharma, Inc (Seoul,Korea). The main ingranients for the

solvent were polysorbate 20 (4.0mg), NaCl(1.5mg), sodium L-ascorbate (10.0mg), sisociumn edetate (1.11mg), anhydrous (7.5mg), monobasic sodium hydrogen phosphate monohydrate(1.84mg) per 1mL. Allergen sensitization, challenge for development of the AR murine model, and treatment are summarized in Fig. 1. Briefly, the mice were sensitized by intraperitoneal injection with 25 μg of OVA (grade V; Sigma, St. Louis, MO, USA) and 2 mg of aluminum hydroxide (alum) on days 0, 7, and 14. The mice were then subjected to an intranasal challenge with 100 μg of OVA on 7 consecutive days from days 21 to day 27. The negative control mice were intraperitoneally injected and intranasally challenged with phosphate-buffered saline (PBS) instead of OVA on the same schedule. 1, 25-dihydroxyvitamin D3 (20μL of Bonky® containing 0.02μg per mouse) and solvent(20μL per mouse) were

given by intranasal instillation on days 21 to 27 (3 hours before intranasal OVA challenge) to mice in groups 3 and 4, respectively.

Symptom scores

On day 27, after intranasal allergen provocation with 100 μg of OVA, the frequencies of sneezing and nasal rubbing behaviors were recorded during a 15-minute period to evaluate early allergic responses by blinded observers.

Tissue preperation

In this study, experiments had been repeated twice (40 mouse in total). In both experiments, symptom scores had been checked and the mice were then killed 24 hours after the last OVA challenge. Cervical lymph nodes(LNs) (primarily submandibular, 2 to 3 nodes at each side) were harvested from each mouse and were phyisically dissociated. In the first experiment, the nasal tissues were obtained and were further homogenized for each mouse to

measure cytokine expression. Also serum fluid from each mouse were obtained for further analysis. Cells from the cervical LNs underwent for flow cytometry analysis. In the second experiment, nasal tissuse in each mouse was fixed in formaldehyde solution for histologic analysis. Cells from the cervical LNs were cultured to measure the cytokine from the supernatant in the presence of OVA.

Real-time polymerase chain reaction (PCR) for cytokines in the nasal tissue

After sacrifice, head of each mouse was removed. After exposing the nasal cavity, the nasal mucosa in each mouse was carefully taken out by using a curette. Total RNA was isolated from the nasal mucosa using the TriZol reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized using -cDNA synthesis kit (Gendepot). For the analysis of

interleukin (IL)-4 (Mm 00445258_g1), IL-5 (Mm 01290072_g1), 1), interferon gamma (IFN-γ) (Mm 99999071_m1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Mm 03302249_g1), predeveloped assay reagent (PDAR) kits of primers and probes were purchased from Applied Biosystems (Foster City, CA, USA). Amplification of IL-4, IL-5, IL-10, IL-13, IFN-γ, Foxp3 and GAPDH cDNA was performed in MicroAmp optical 96-well reaction plates (Applied Biosystems). The reaction was performed using a QuantStudio™ 3 Real-Time PCR System (Applied Biosystems). The average transcript levels of genes were then normalized to GAPDH expressed as $2^{-\Delta Ct}$ values.

Evaluation of histologic findings in the nasal tissue.

For the evaluation of nasal histology, the heads of mice in each group were fixed with 10% formaldehyde solution. The nasal tissues were decalcified with

ethylenediaminetetracetic acid (EDTA) solution, embedded in paraffin, sectioned coronally into 4-μm slices, and stained with hematoxylin and eosin (H&E) for the visualization of eosinophils. Under a light microscope (×400 magnification), infiltrating eosinophils were counted in 4 fields of the nasal septal mucosa by a single-blinded observer. Eosinophils were morphologically defined by the presence of eosinophilic granules that were stained by H&E and the presence of a two-lobed nucleus[19].

Determination of serum levels of total and OVA-specific immunoglobulins (Igs)

Serum samples from each mouse were obtained at the time of sacrifice. Serum levels of total IgE and OVA-specific IgE, IgG1, and IgG2a were measured by ELISA. For the analysis of total IgE, 96 well flat-bottom plates were coated overnight with anti-mouse IgE monoclonal antibody (mAb) (BD PharMingen,

San Jose, CA, USA) at 4°C. The plate was washed with PBST (PBS containing 0.05% Tween-20) 3 times and nonspecific antigen-antibody reactions were blocked with 300 μL of 3% bovine serum albumin (BSA) per well for 1 hour at room temperature. Serum samples were added to the 96-well plates along with purified mouse IgE isotype (BD PharMingen) used as a standard, and the plates were incubated for 3 hours at 4°C. For the analysis of OVA-specific IgE, 96-well flat-bottom plates were coated with OVA 100 μg/mL in coating buffer (0.05M carbonate-bicarbonate) overnight at 4°C. The plates were washed 3 times with PBST, and blocked with 3% BSA in PBS for 1 hour at 37°C. Serum samples were added to OVA-coated plates along with serial dilutions of a high-titer OVA IgE standard and incubated for 2 hours at 37°C. After washing, 100 μL of biotin-conjugated rat anti-mouse IgE mAb (BD PharMingen, San Jose, CA, USA) was added to each well and incubated for 1 hour at 37°C. For

the analysis of OVA-specific IgG1 and IgG2a, 96-well plates were coated with 100 μg/mL OVA in coating buffer overnight at 4°C. Serially diluted serum samples were incubated with biotinylated rat anti-mouse IgG1 and IgG2a (BD PharMingen), respectively. After washing three times, the plates were then incubated with 100 μL of horseradish peroxidase (HRP)-conjugated secondary Ab (BD PharMingen) for 30 minutes at 37°C. The reactions were developed using 3, 3′, 5, 5′-tetramethylbenzidine (TMB) (Moss Inc., Belfast, ME, USA) and terminated by adding 1N HCL. OD was measured in a microplate reader at 450 nm.

Flow cytometric analysis of cervical LNs

For analysis, harvested cells from each mouse were distributed into two sets and were suspended in 50μL of cold PBS containing 2% FCS. Cells were stained with V450 conjugated anti-CD45 (BD), BB515 conjugated anti-MHC

II (BD) , phycoerythrin (PE) conjugated anti-CD11c (BD), and APC conjugated anti-CD 86 (BD) for detection of activated dendritic cells, and with PerCP conjugated anti-CD3 (BD), FITC conjugated anti-CD4 (eBiosience) , APC conjugated anti-CD25, and PE conjugated anti-Foxp3 for detection of T regulatory cells(Treg) . The cells were analyzed by Flow cytometry (LSR II, BD Biosciences). $CD11c^{+}MHC\text{-}II^{high}$ cells were considered as migratory DCs[20] (Fig. 2).

Measurement of cytokines in the LN cell culture

Cervival LN single-cell suspensions from each mouse were plated in 24-well cell culture plates at a final concentration of 5×10^{6} cells/well using RPMI 1640 containing 10% fetal bovine serum (FBS) supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin (Gibco, Grand Island, NY, USA). The cells were incubated in a CO_2 incubator at 37°C for 72 hours and

stimulated with OVA for 72 hours. The culture supernatant was collected and stored at -70°C until cytokines were measured. Cytokine levels in the culture supernatant were assayed using a DuoSet ELISA kit (R&D Systems), according to the manufacturer's protocol. After measuring the optical density (OD) at 450 nm, the concentrations of IL-4, IL-5, IL-10, and IL-13 were determined by interpolation from a standard curve, and all data are expressed in pg/mL.

Statistical analysis

The data are presented as means±standard error mean (SEM). A Mann-Whitney U test was used to compare results between negative and positive controls, and treatment groups and positive control. A P value of <0.05 was considered statistically significant. Statistical analysis was performed using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA)

Result

Intranasal 1, 25-dihydroxyvitamin D3 treatment alleviates allergic symptom

Fig. 3 shows symptom scores for each group after nasal challange with OVA. In mice of group 2 (positive control), the mean number of sneezing and rubbing nose was significantly higher than those of group 1 (negative control) ($P<0.001$, $P=0.001$, respectively). Mice of group 3 (treatment group) showed significatly reduced number of sneezing and nose rubbing compared to those of group 2 ($P<0.001$, both) and 4 ($P<0.001$, $P=0.001$ respectively). There was no signficiant difference in the mean number of sneeizng and nose rubbing between group 2 and 4 ($P=0.193$, $P=0.754$, respectively). .

Histologic findings and eosinophil infiltration in nasal mucosa indicates 1, 25-dihydroxyvitamin D3 treament significalty decreased infiltration of tissue eosinophil

Fig. 4 shows eosinophil infiltration in the nasal mucosa for each group. The mice of group 2 had significantly higher number of eosinophil infiltration per high power field than the those of group 1 (P=0.004). The mean number of eosinophils infiltrating the nasal mucosa per high-powerfiled field were significantly lower in group 3, compared to group 2 (P=0.002) and 4 (P=0.004) . However there was not much difference between group 2 and 4 (P>0.999)

Decrased Th2 cytokine expression in the nasal tissue after topical 1, 25-dihydroxyvitamin D3 treatment

Fig. 5 shows cytokine expression in the nasal tissue. In group 2, compared to group 1, there was a signifiant increased level of mRNA expression of IL-4 (P=0.010), IL-5 (P=0.010), IL-10 (P=0.010), IL-13 (P=0.010) and IFN-γ(P=0.019) in the nasal tissue. IL-4 and IL-13 mRNA expression in group 3 was significantly decreased compared to those with group 2 (P=0.010, P=0.038, respectively), and group 4 (P=0.029, both). Expression of IL-5 in group 3 tended to follow the same pattern however, the result did not reach to a significant level (P=0.240, P>0.999, compared to group 2 and 4 respectively). Other mRNA expression including IFN-γ and IL-10 in group 3 did not show a significant difference compared to group 2 (P=0.132, both), and 4 (P=0.257, P=0.352, respectively). Between group 2 and 4, there was not much difference in mRNA expression of IL-4 (P=0.914), IL-5 (P=0.476), IL-10 (P=0.762), IL-13 (P=0.476), and IFN-γ (P=0.610).

Down-regulation of CD86 expression in cervical LN DCs after topical 1, 25-dihydroxyvitamin D3 treatment

The expression of CD86 among $CD11c^{+}$, MHC^{high} cells from cervical LNs were examined. Group 2 showed significalty increased expression of CD 86 among CD11c+, MHC^{high} cells compared to group 1 (p=0.038). Despite the number of CD 86+ cells among CD11c+MHC^{hi} cells was not significantly different among group 2,3 and 4 (data not shown), mean fluorescence intensity showed that the expression of CD86 was significantly higher in group 3 compared to group 2 (P =0.041) and 4 (P=0.010). When compared to group 1, there was no significant difference of CD86 expression on group 3 (P=0.069). Between group 2 and 4, expression of CD86 did not show a significant difference (P>0.999). The number of $CD4^{+}CD25^{+}$ $Foxp3^{+}$ was tended to be higher in group 3 compared to group 2 and 4, however, did not reach to a

significant level (Fig. 6). There was also no significant difference in mean fluorescence intensity of Foxp3 expressed by CD4+CD25+cells with most of the cells (≥90%) expressing Foxp3 (data not shown).

Decreased IL-4 secretion in the lymphocyte culture from cervical LN after topical 1, 25-dihydroxyvitamin D3 treatment

To investigate wether the decreased CD 86 expresison in the cervical LN DC actually down regulate T cell activity, we have measured the of cytokines from the culture supernantants (Fig. 7). In group 2, levels of IL-4 (p=0.016), IL-5 (p=0.008), IL-10 (p=0.008), and IL-13 (p=0.008) were significanlty higher than those from group 1. In group 3, IL-4 was significantly decreased compared to group 2 (p=0.029) and 4 (p=0.029). Levels of IL-5, IL-13 showed similar pattern however the difference was not significant when compared to

group 2 (p=0.548) and group 4 (p=0.905). IL-10 levels were not significantly different among group 2, 3 and 4.

Serum total IgE and OVA-specific Igs

In group 2, serum total IgE and OVA-specific IgE, IgG1, IgG2a were significantly higher when compared to group 1 (p=0.010). There were no significant difference among group 2, 3, and 4. Despite the lack of significance, the mean level of total level of IgE, OVA-specific IgE, OVA-specific IgG1 which represent Th2-related Ig, tended to be lowered in group 3 compared to group 2 and 4, meanwhile, the level of OVA-specific IgG2a, which represent Th1-related Ig tended to be higher in group 3 compared to group 2 and group 4

Discussion

In this study, we obeserved that intranasally instilled 1, 25-dihydroxyvitamin D3 decreased symptom score, tissue eosinophil infiltration, and decreased Th2 cytokine expression in the nasal tissue. Also 1, 25-dihydroxyvitamin D3 treatment downregulated the activation of DC in the cervical LN. The decreased IL-4 secretion in the cervical LN in the proliferation assay indicated the decreased activated OVA spefic Th2 cell population in the cervical LN. Thus from these observations we assumed that 1, 25-dihydroxyvitamin D3 have antiallergic effect which may partialy resulted from the decreased DC activation leading to less Th2 polarization in the cervical LN.

Cervical LNs are the regional LN of the upper airway, and the nasal sensitization with antigen induced activated DC migrates to this regional LN[21]. Therefore in order to identify mature or immature tolerogenic DC and it's interaction with naïve T cells, we have analyzed cervical LN.

Several distinct signals are required for naïve CD4 T cells to be activated and this includes 1) MHC II in complex with peptide processed from antigens and 2) a co-stimulatory factor expressed over DC. With the absence of costimulatory signal, T cells become anergic[22].

Among the co-stimulatory molecules, CD 80 and CD 86 expression on DC may be the most important pathway in T cell activation. The expression of these molecules are quickly mediated by a variety of inflammatory cytokines or pathogen derived mediators that these molecules act as early costimulatory molecules [23]. In the murine system, CD86 is the main activation marker of bone-marrow derived DC, strongly up-regulated after maturation, while CD 80 expression is less pronounced [24]

Previous studies have demonstrated that CD80 and CD 86 levels are elevated in patients with asthma [25, 26]and allergic rhinitis[27, 28] , and

blockage of this molecules (either by knockdown or by blocking antibody) had been proved to be effective with less tissue eosinophil infiltration, less production of Th2 cytokine [29, 30]

1, 25-dihydroxyvitamin D3 has long been considered to have immune modulator effect through its' ability to induce less T cell activation and proliferation but rather enhanced Treg induction.[31-33] Densities of co-stimulatory molecues CD80 and CD86 of DC are decreased in the presence of 1, 25-dihydroxyvitamin D3[10, 11]. Thus it seems that this immumomodulatory effect of 1, 25-dihydroxyvitamin D3 enables a potential therapeutic agent for disease with inappropraite or overwhelming inflammtion.

In this study, despite the significant reduction of nasal tissue IL-4, IL13 expression in intranasal 1, 25-dihydroxyvitamin D3 treated mouse compared to positive control or sham treatment group, there was no significant reduction

expression of other Th2 cytokines in nasal tissue (IL-5) and cervical LNs after prolfertaion assay (IL-5, IL-13). The initiail production of IL-4 from the naïve T cell by T cell receptor signaling pathway is important in Th2 response since it's main role is to polarize and maintenance of Th2 cells. IL-4 upregulates the expression of GATA-binding protein 3 (GATA3), known as a master transcription factor for Th2 cell produing IL-4, IL-5 and IL-13. Also IL-4 is known to reglulate B cell class switching to IgE [34]. 1, 25-dihydroxyvitamin D3 through its action by suppressing dendritic cell activation may eventually supress IL-4 production from the naïve T cell thus further leading to supressing Th2 inflammtion. However, production of other Th2 cytokinse such as IL-5, is also known to be produced via IL-4 independent pathyway[35]. Thus even in the significant decreased production of IL-4 in our study, other Th2 cytokine such as IL-5 can be produced resulting insignificant decrease in

the presence of 1, 25-dihydroxyvitamin D3 . In our study, despite the insignificant change in IL-5 in the nasal tissue, tissue eosinophilia had been dramatically decreased in the control group and 1, 25-dihydroxyvitamin D3 treatment group compared to positive group and sham treatment group. IL-5 is a key regulator for eosinophil proliferation in the bone marrow, and amplifies tissue recruitment of eosinophil in response to locally derived chamotactic signals. However, IL-5 does not play an obligatory role in the homing eosinophil to the local tissue. Thus it seems that primary role for IL-5 is the promotion of peripheral eosinophilia in response to allergic stimulation. In this regard, our result also implicates that reduced tissue eosinophilia in the 1, 25-dihydroxyvitamin D3 treatmed mouse may rather be associated with decreased IL-4 axis[27]. Also this may be due to anti-allergic effect of 1, 25-dihydroxyvitamin D3 such as by directly down regulating and stabilizing the

mast cell which may further decrease the release of chemokines crucial for eosinophil recruitment[36, 37]. Also there had been report about the role of 1, 25-dihydroxyvitamin D3 regulating crosstalk between NK cells and eosinophils via IL-15/IL-8 axis and therefore further decreasing tissue eosinophilia[38].

Also despite the fact that IL-4 had been significatnly reduced in the nasal tissue and the culture superntnat from the cervical LN, there was not so much difference OVA specific immunoglobulins including IgE, IgG1, IgG2a among groups. Despite decreased IL-4 production may have led to decreased B cell's class switching to IgE, sensitization of OVA with alum by injecting intraperitoneally before intranasal treatment of 1, 25-dihydroxyvitamin D3 may have resulted the pruduction of OVA specific IgE. OVA-specific IgG1 has been used as a Th2 marker and is correlated with OVA-specific IgE levels[39].

Despite the lack of significance, both OVA specific IgE and IgG1 were lower in intranasal 1, 25-dihydroxyvitamin D3 treatment group and this may further support decreased Th2 inflammtion.

The purpose of intranasal instialtion of 1, 25-dihydroxyvitamin D3 in this study was to increase the local concentration. Before this study began, we have performed preliminary studies to decide the optimal timing, route of administration and treatment dosage. First we have compared the effecacy of treatment according to the treament period. Administration of 1, 25-dihydroxyvitamin D3 had been performed subcutaneously with an amount of 0.1 μg during the sensitization preiod at day 1, day 7 and day 14 at 3 hours before intraperitoneal OVA injection. Subcutaneous injection of 0.1 μg 1, 25-dihydroxyvitamin D3 during the allergen challenge had been also performed to another group of mouse 3 hours before intranasal OVA challenge at day 21, 23,

25, 17 and were compared to the group of mouse treated during sensitization period. Total IgE, and OVA speficic IgE was more decreased in the mouse treated during sensitiztion period while as tissue cytokine expression including IL-4, and IL-5 were lowest in the mouse treated during allergen challaning period (Fig. 9).

Second, we have compared the efficay of treatment by compring the route of adminstration during the allergen challaning period. For this we have subgrouped mouse as subcutanous injected group, and intranasally applied group. Treatment groups were subcategorized by the treatment dose of 0.1 μg, and 0.001 μg of 1, 25-dihydroxyvitamin D3. Cytokine expression including IL-4 and IL-5 had been measured by quantitative real-time PCR and was found out that intranasally treated groups tended to be more effective (Fig. 10).

Finally we have determined the dose for intratnasal application by comparing treatment dose of 0.02 μg and 0.0002 μg during allergen challenging period. Cytokine expression including IL-4 and IL-5 had been the lowest in the mouse group treated with 0.02 μg (Fig. 11). To summrize our preliminary studies, we have found out that treating during the allergen challenging period, at the dose of 0.02 μg per mouse and intranasally is the optimal treatment.

It is known that the serum half life of vitamin D is about 4 to 6 h. 24-hydroxylase (CPY24A) which is the major protein that metabolize 1, 25-dihydroxyvitamin D3 is usually expressed in the kidney or bladder tissue (http://www.proteinatlas.org/ENSG00000019186-CYP24A1/tissue) thus we tought that systemic injection of vitamin D may not reach to a locally effective level. Also higher concentration can be applied whan applied intranasally with less systemic side effect. The study has shown the

antiallergic effect of intranasally instillated 1, 25-dihydroxyvitamin D3 and this implicates that it could be one of the possible future therapeutic agents alone or in combination with intranasal corticosteroid since it may have syndergistic effect when combined with corticosteroid[40]. It can be also be applied in combination with antigen specific immunotherapy[41].

The underlying down stream mechanism of 1, 25-dihydroxyvitamin D3 on inducing tolererogenic phenotype of dendritic cell remains unclear, however, one of the downstraim effect of 1, 25-dihydroxyvitamin D3 is a metabolic imprinting of on of oxidative phosphoryation. Toll like receptor activation induces a metabolic transition in DCs from oxidative phosphorylation to aerobic glycolysis which is essential for DC activation[42]. However, in the presence of 1, 25-dihydroxyvitamin D3 transcriptional programme is started that engages oxidative phosphorylation[43, 44]. By sustaining oxidative

phosphorylation as a mode of glucose breakdown, the metabolic pattern used by quiescent cells, 1, 25-dihydroxyvitamin D3 may support or favour immune quiescence and tolerance[45]. Another study also demostrated activation of PI3K-AkT-mTOD pathyway by the 1, 25-dihydroxyvitamin D3 for the development of tolerogenic DC and thus the tolerogenic effect may arise from its impact on the PI3K pathway, which can control essential transcription factors[46]

The limitation of this study is that the direct effect of 1, 25-dihydroxyvitamin D3 on dendritic cell had not been proven. The anti-allergic effect may also have resulted from other type of inflammtory cells, such as mast cells, lymphocytes or macrophages, since all of these cells are known to possess and express VDR[47], and with decreased allerigc inflammtion, maturation of DC seems may also have been decreased. Therefore further studies are required.

To summerize, 1, 25-dihydroxyvitamin D3 when treated intranasally alleviates allergic rhinitis in murine model. One of the possible mehanism is via down regulation of DC activation leading to decreased IL-4 production from the T cells.

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Figures

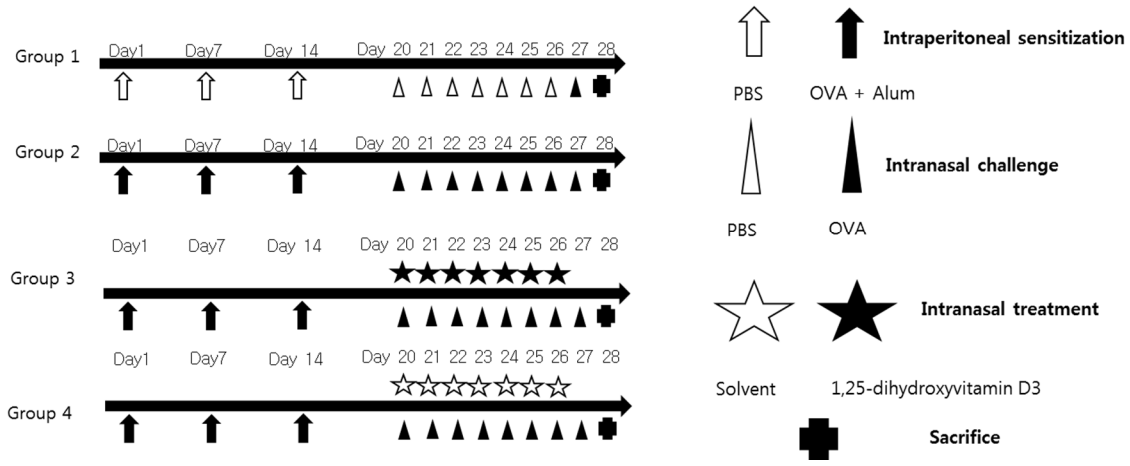


Figure 1. Experimental protocol. BALB/c mice were sensitized with ovalbumin (OVA) and 1 mg of aluminum hydroxide gel (Alum) on days 0, 7, and 14 (general sensitization). All groups except for group 1 received intranasal OVA from day 21 to day 27. In addition to sensitization and challenge, 1, 25-dihydroxyvitamin D3 (0.02μg) and solvent were given by intranasal instillation on days 21 to 27 (6 hours before intranasal OVA challenge) to mice in groups 3 and 4, respectively

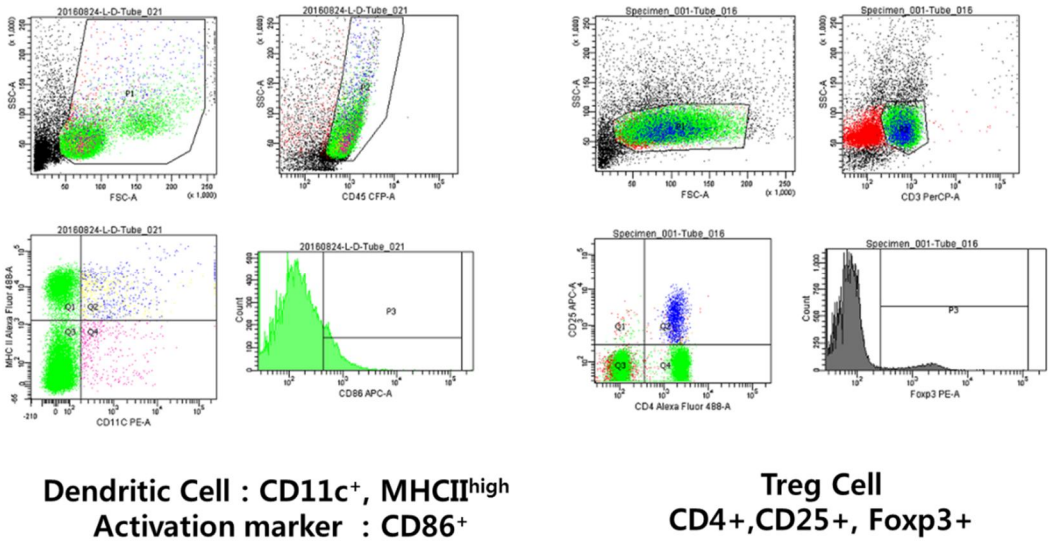


Figure 2. $CD11c^{+}MHC\text{-}II^{high}$ cells were considered as migratory dendritic cells (DC). And among them, $CD86^{+}$ cells were considered as activated DCs. CD4+,CD25+, Foxp3+ cells were considered as T regulatory cells

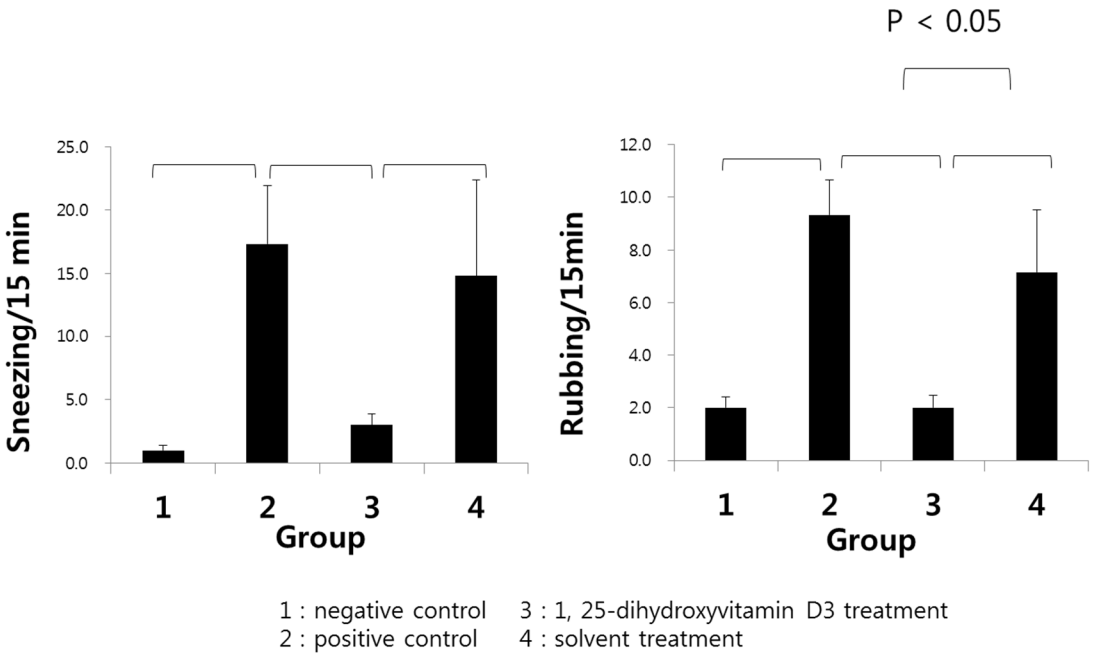


Figure 3. Symptom scores. Intranasal treatment of 1, 25-dihydroxyvitamin D3 (Group 3) suppressed allergic symptoms. Sneezing symptom score (left) and nasal rubbing symptom score (right) of each group. Data are expressed as mean±standard error mean (SEM)

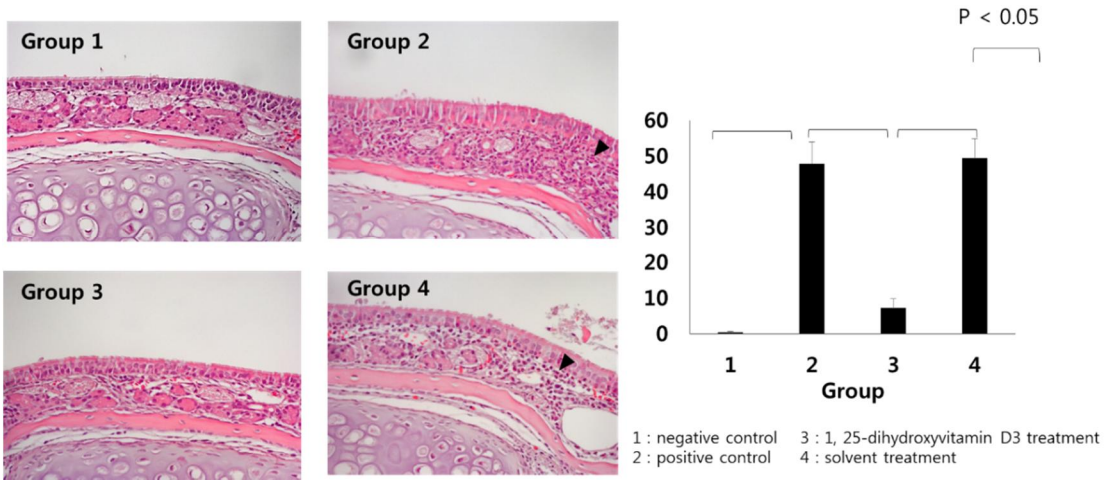


Figure 4. Local treatment of Intranasal treatment of 1, 25-dihydroxyvitamin D3 (Group 3) suppressed eosinophil infiltration in the nasal mucosa. Hematoxylin and eosin staining (×400 magnification) of each group. Eosinophils were morphologically defined by the presence of eosinophilic granules that were stained by H&E and the presence of a two-lobed nucleus (arrow head). Graph on the right denotes that tissue infiltrated eosinophils were significantly decreased in group 3 compared to group 2 and 4. Data are expressed as mean±standard error mean (SEM)

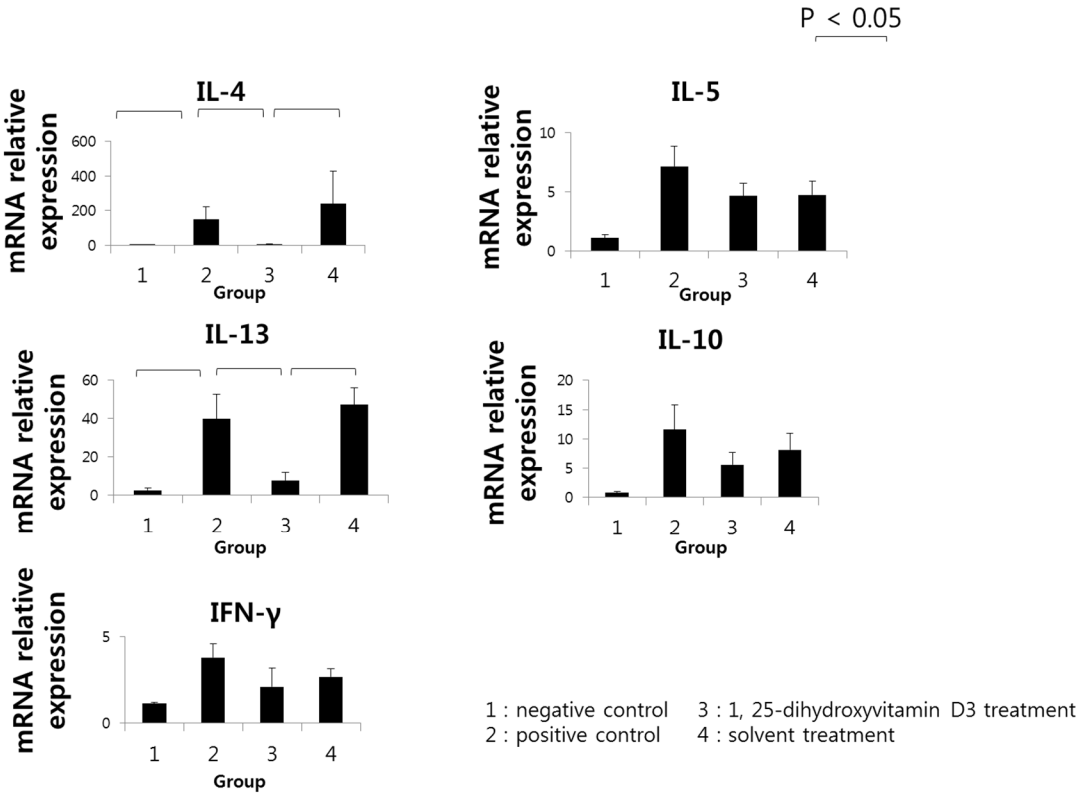


Figure 5. IL-4 and IL-13 mRNA expression in group 3 was significantly decreased compared to those with group 2 (P=0.010, P=0.038, respectively), and group 4 (P=0.029, both). Expression of IL-5 in group 3 tended to follow the same pattern however, the result did not reach to a significant level (P=0.240, P>0.999, compared to group 2 and 4 respectively). Data are expressed as mean±standard error mean (SEM)

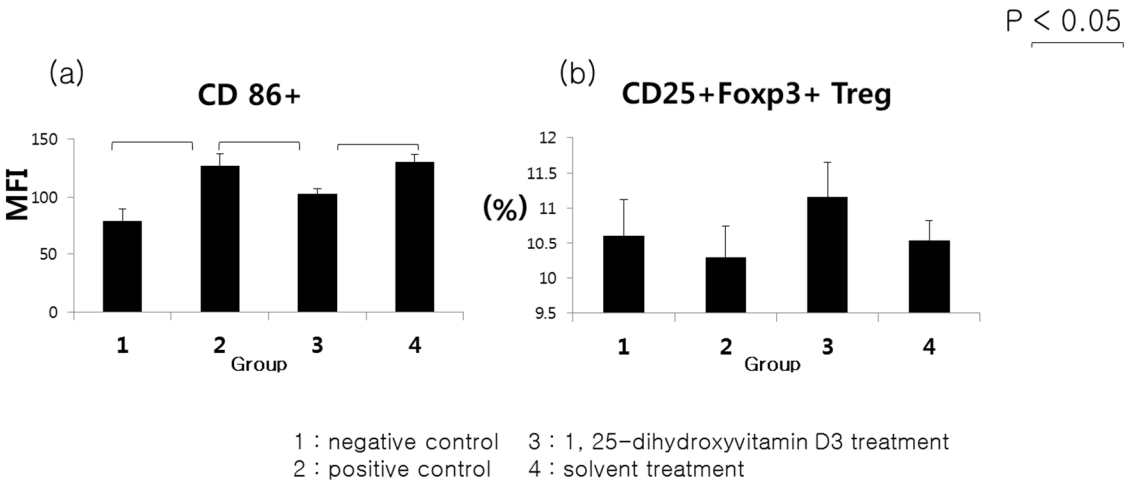


Figure 6. (a) Decreased expression of CD86 on CD11c+MHC^{high} cells in the cervical lymph nodes from the mouse treated with intranasal 1, 25-dihydroxyvitamin D3. Mean fluorescence intensity(MFI) showed that the expression of CD86 was significantly higher in group 3 compared to group 2 and 4. When compared to group 1, there was no significant difference of CD86 expression on group 3. (b) Number of CD25+/Foxp3+(%) among CD3+, CD4+ cells. There was no significant difference in the number of CD25+Foxp3+cells among CD3+CD4+ cells. Data are expressed as mean±standard error mean (SEM)

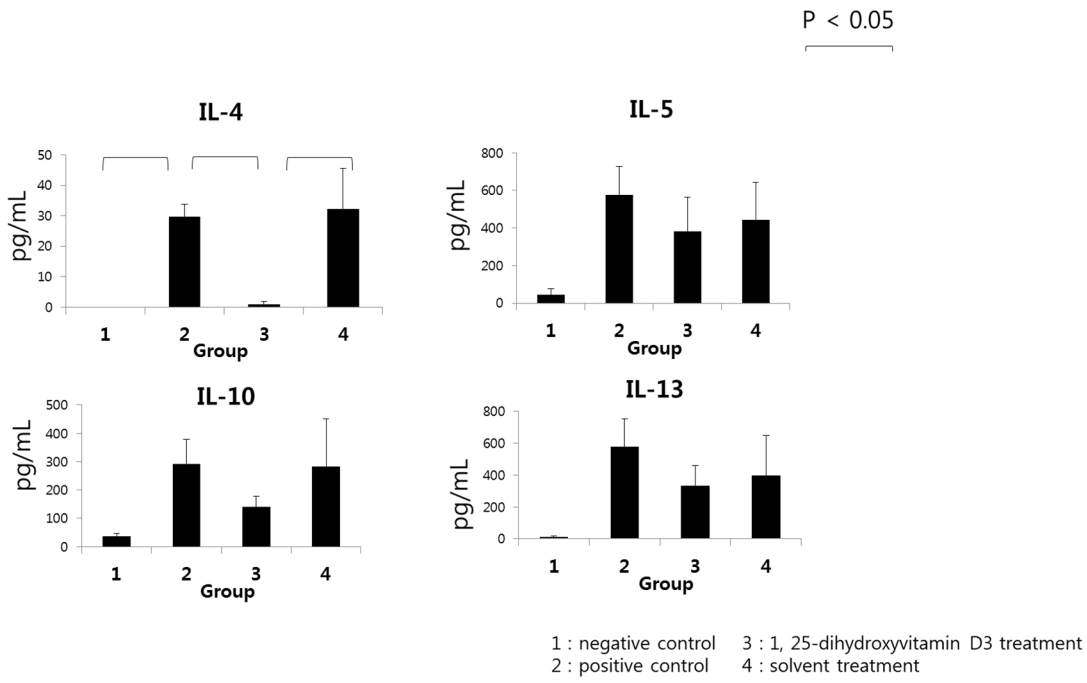


Figure 7. In group 3, IL-4 was significantly decreased compared to group 2 (p=0.029) and 4 (p=0.029). Levels of IL-5, IL-13 showed similar pattern however the difference was not significant when compared to group 2 (p=0.548) and group 4 (p=0.905). IL-10 levels were not significantly different among group 2, 3 and 4. Data are expressed as mean±standard error mean (SEM)

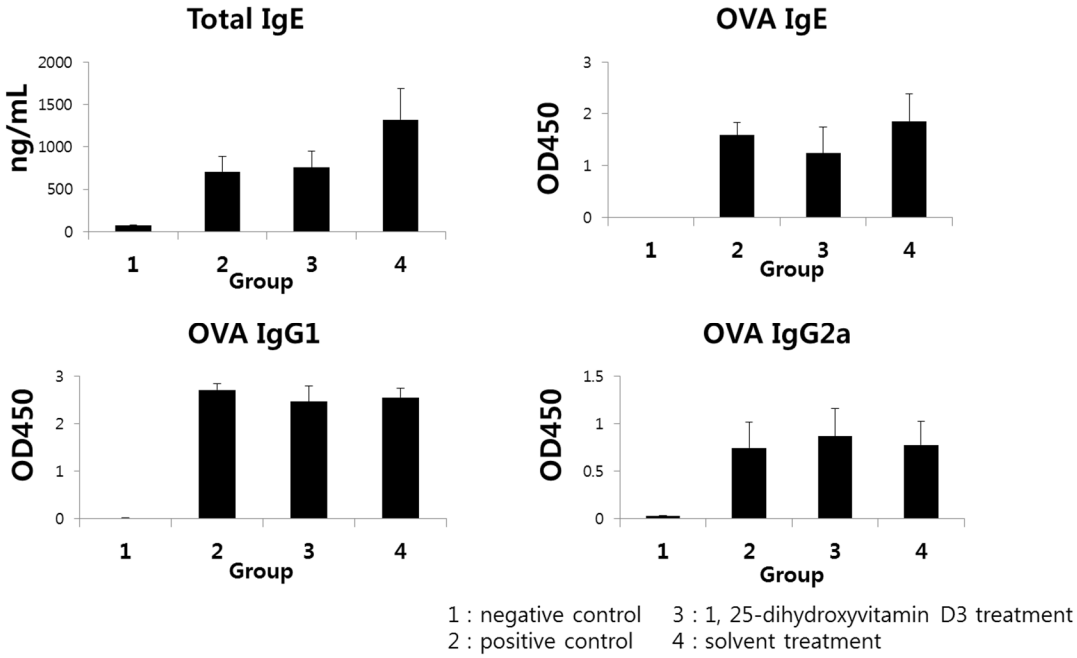


Figure 8. There were no significant difference in the level of serum total IgE, OVA-spedicific IgE, IgG1, or IgG2a among group 2, 3, and 4. Despite the lack of significance, the mean level of total level of IgE, OVA-specific IgE, OVA-specific IgG1 tended to be lowered in group 3 compared to group 2 and 4, meanwhile, the level of OVA-specific IgG2a tended to be higher in group 3 compared to group 2 and group 4. Data are expressed as mean±standard error mean (SEM)

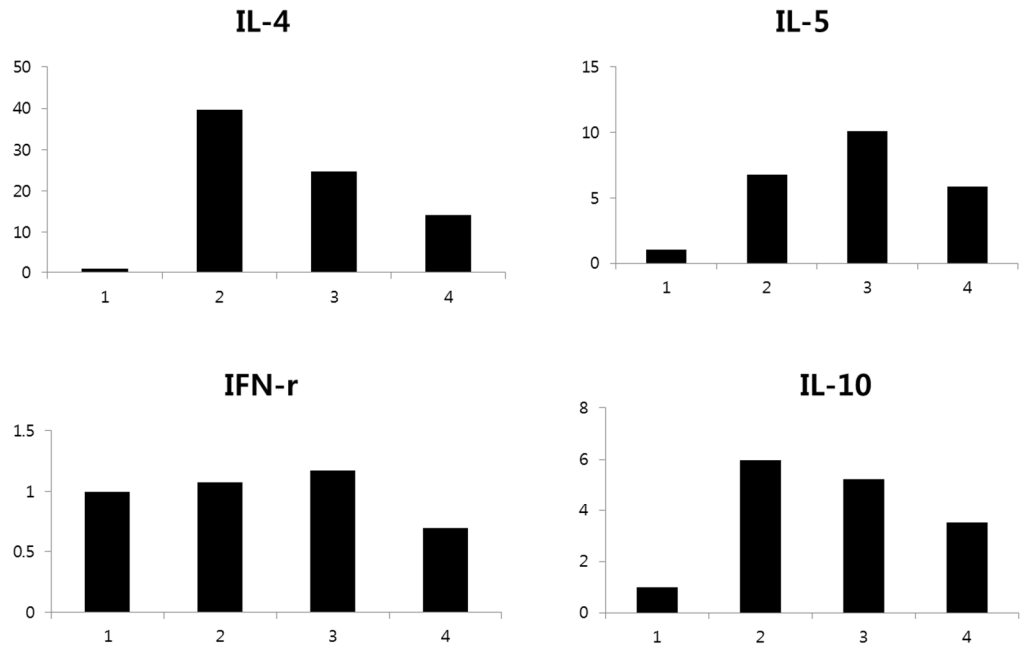
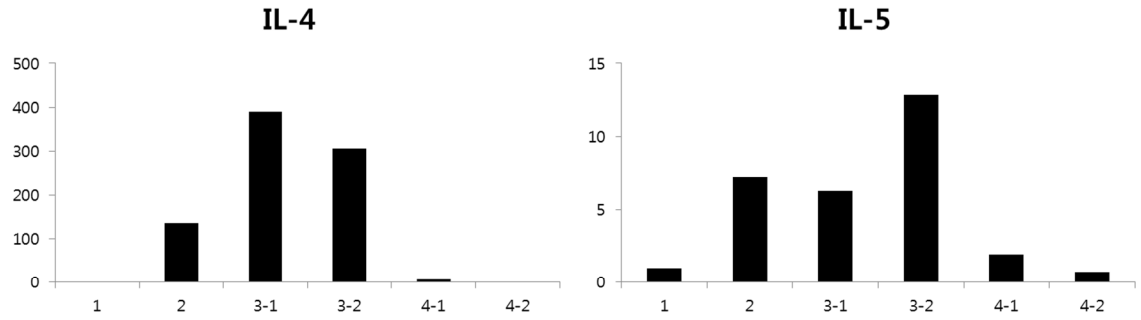


Figure 9. Relative mRNA expression in the nasal tissue. In the first preliminary study, 0.1μg 1, 25-dihydroxyvitamin D3 was injected into mouse during sensitization period (group 3) and challenging period (group 4). mRNA expression of IL-4, and IL-5 tended to be higher in mouse treated during challenging period (group 4).



1 : Negative control
2 : Positive control
3-1 : 1,25-Dihydroxyvitamin D3 (calcitriol) 0.1 μg, subcutaneous
3-2 : 1,25-Dihydroxyvitamin D3 (calcitriol) 0.001 μg subcutaneous
4-1 : 1,25-Dihydroxyvitamin D3 (calcitriol) 0.1 μg Intranasal
4-2 : 1,25-Dihydroxyvitamin D3 (calcitriol) 0.001 μg Intranasal

Figure 10. Relative mRNA expression in the nasal tissue. In the preliminary second study, we have subgrouped mouse as subcutanous injected group, and intranasally applied group. Treatment groups were subcategorized by the treatment dose of 0.1 μg, and 0.001 μg of 1, 25-dihydroxyvitamin D3. Cytokine expression including IL-4 and IL-5 had been measured by quantitative real-time PCR and was found out that intranasally treated groups tended to be more effective.

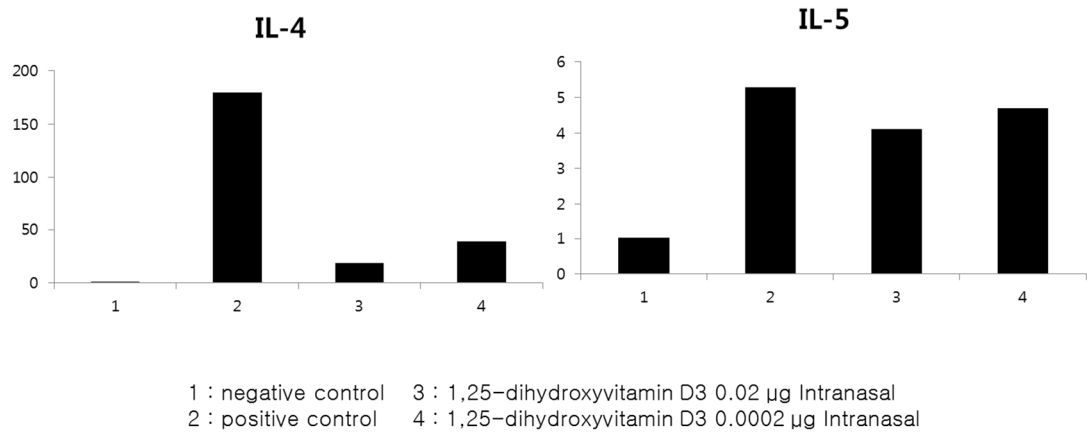


Figure 11. Relative mRNA expression in the nasal tissue. We have determined the dose for intratnasal application by comparing treatment dose of 0.02 μg and 0.0002 μg during allergen challenging period. Cytokine expression including IL-4 and IL-5 had been the lowest in the mouse group treated with 0.02 μg

국문초록

서론

최근 들어서 비타민 D의 면역중재자의 역할이 많이 알려지고 있으며, 특히 비타민 D는 알레르기성 질환에서 Th2 반응을 개시하는 것으로 알려진 수지상세포를 조절하는 것으로 알려져 있다. 하지만 알레르기 비염에서의 비타민 D의 역할은 아직까지 잘 알려지지 않았고, 특히 마우스 모델에서의 역할은 아직까지 보고된 적이 없었다.

방법

Ovalbumin (OVA) 에 감작된 마우스를 제작하기 위하여 BALB/C 마우스의 복강에 OVA 및 alum을 복강 내 주입하였고, 이 후 OVA을 비강 내로 투여 하였다. 치료 군에는 1, 25-dihydroxyvitamin D3 0.02μg 을 비강 내로 투여 하였고, 위약 치료 군에는 용매제를 투여하였다. 비염 증상 점수, 비강 내 조직의 호산구 침윤, 조직 내 싸이토카인 (IL-4, IL-5, IL-10, IL-13, IFN- γ)의 mRNA 발현량과, 그리고 혈청 total IgE, OVA 특이 IgE, IgG1, IgG2a 농도를 측청하여, 양성 대조군과 음성대조군과 비교하였다. 또한 경부 림프절에서 조직을 채취하여 유세포분석 및 세포 증식 분석을 시행하였다.

결과

치료 군에서는 양성 대조군에 비해 비염 증상, 비강 조직 내 호산구의 침윤, 그리고 비강 조직 내 IL-4, IL-13 mRNA의 발현이

유의하게 감소하였다. IL-5, 혈청 내 total IgE, OVA 특이 IgE, IgG1의 농도는 양성 대조군에 비해 감소하는 경향을 보였지만 유의성을 보이지는 않았다. 경부 림프절에서는 치료 군에서 $CD11c^{+}$, $MHCII^{high}$, $CD86^{+}$의 활성화된 수지상세포가 양성 대조군에 비해 유의하게 줄어든 것을 볼 수 있었고, 세포 증식 분석 결과 치료 군에서 유의하게 IL-4의 분비가 줄어든 것을 볼 수 있었다.

결론

이번 실험에서는 알레르기 비염 마우스 모델의 비강 내 투여된 1, 25-dihydroxyvitamin D3의 항알레르기 효과를 확인하였다. 또한 1, 25-dihydroxyvitamin D3는 경부 림프절에서 수지상세포의 활성화와 이로 인한 Th2 염증 반응을 감소 시킴을 확인하였다. 즉, 이번 실험에서 비강 내 국소 투여된, 25-dihydroxyvitamin D3의 향후 비염 치료제로서의 가능성을 확인하였다.

주요어: 비타민 D, 알레르기성 비염, 수지상세포, 항알레르기 효과

학번 : 2012-23625