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The effect of vitamin D on GATA3 gene expression in peripheral blood mononuclear cells in allergic asthma

Abstract

Introduction: Asthma is becoming a major health problem in many countries. Immune responses in allergic asthma, as the most prevalent asthmatic phenotype, are mediated mostly by a subtype of T lymphocytes referred to as the effector lineage of Type 2 Th cells (Th2). The development of Th2 cells is mainly governed by a zinc finger transcription factor, i.e., GATA-binding protein 3 (GATA3). Allergic asthma is a complex disease, and vitamin D deficiency has been named as a non-genetic risk factor for its development. Vitamin D, a steroid hormone belonging to the family of nuclear receptors, has shown significant immunosuppressive effects in previous studies.

Material and methods: In this study, given its immunomodulatory properties, we aimed to investigate the effects of different concentrations of vitamin D on GATA3 gene expression in peripheral blood mononuclear cells (PBMCs), including Th2 cells, and compare GATA3 expression levels between PBMCs taken from allergic asthmatic patients and healthy controls.

Results: The total sample size was 40 and the quantitative real-time PCR (qPCR) procedure was applied to assess the mRNA expression levels of GATA3 in different groups. Collectively, our results demonstrated that the expression of GATA3 in PBMCs taken from patients with allergic asthma is lower than in that from healthy controls. In addition, in the control group, cells co-cultured with vitamin D had a significantly increased GATA3 expression. However, in the patient group, such an increase was only observed in cells treated with 10^{-7}M-vitamin D. By contrast, incubation with vitamin D at the concentration of 10^{-6} M slightly decreased the expression of GATA3 among patients.

Conclusion: In summary, it is likely that vitamin D should regulate GATA3 gene expression in the PBMCs in a dose-dependent manner. The impacts of this steroid hormone can also differ between the status of health and allergic asthma in either extent or direction.

Key words: allergic asthma, immune response, GATA3, Th2, vitamin D

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Introduction

Asthma is currently becoming a major health problem in many countries, from Australia to Brazil. At the present time, more than 330 people of all age groups are suffering from asthma globally. As per the Global Initiatives of Asthma, asthma is defined as a chronic disease of the respiratory system that is heterogeneous in nature and often bears the hallmark of inflammation. Currently, it is widely accepted that asthma phenotypes can be classified based on a number of recognizable characteristics in terms of association with allergy, underlying molecular mechanisms, the age of onset, and so on [1, 2]. The most prevalent asthmatic phenotype is allergic asthma. Almost

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half of all asthmatic patients are given a diagnosis of allergic asthma [3]. This subtype is generally accompanied by a personal and/or familial background of allergic conditions such as hay fever and atopic dermatitis [1, 2]. Although the fundamental cause of asthma is yet unknown, research has demonstrated that both genetic predisposition and environmental factors might have an effect on either the development or severity of asthma, and even on developing resistance to the disease. Air pollution, unhealthy diet, antibiotic exposure, and vitamin D deficiency are among the non-genetic risk factors of asthma. All of these factors may contribute to asthma through complex interactions with the genome. In the case of vitamin D, it has been demonstrated that it can modulate some aspects of the inflammatory process through the regulation of the expression of various relevant genes, including a number of inflammatory cytokines [4, 5]. From a molecular standpoint, pathways inducing airway inflammation can precipitate asthma. In the present study, we focused on allergic asthma due to the importance of this subtype as the most common phenotype of asthma. Allergic asthma pathogenesis is mostly mediated by a subtype of T lymphocytes referred to as T helper (Th) cells [6]. Following the allergen challenge, naive $CD4^+$ T helper (Th) cells activate and subsequently differentiate mostly into the effector lineage of Type 2 Th cells (Th2) [7]. Inducement of differentiation into Th2 cells is governed by the GATA-binding protein 3 (GATA3) [8], a transcription factor which belongs to the GATA family and regulates the transcription of target genes by binding to the (T/A) GATA (A/G) sequence on DNA [9]. Upregulation of the GATA3 gene within the $CD4^+$ T-cells causes a major change in the cell genetic programming, promotes Th2 polarization, and simultaneously inhibits Th1 cell lineage differentiation [10, 11]. GATA3 exerts these effects through activating the expression of Th2 signature cytokines (Il4, Il5, Il13) and suppressing the expression of interleukin-12 receptor 2 (Il12R 2) and signal transducer and activator of transcription 4 (STAT4) genes, respectively [12]. Vitamin D has been also shown to alter the balance between Th1 and Th2 cells in favor of Th2 development and thus may play a role in determining the endpoint immune response [13]. However, to date, the biochemical mechanisms for Th2 cell enhancement induced by vitamin D are not entirely clear. A few studies have suggested that GATA3 can act as an important mediator in vitamin D-induced Th2 development, whereas the data reported on the effects of vitamin D on GATA3 gene expression are controversial [14–16]. Therefore, given the established immunomodulatory properties of vitamin D as well as the importance of stringent regulation of GATA3 gene expression in the development of Th2-mediated immune reactions in allergic asthma, the present study aimed to examine the possible effects of vitamin D on GATA3 gene expression levels in peripheral blood mononuclear cells (PBMCs) as an indicator of circulating Th cells and to compare GATA3 gene expression between PBMCs taken from allergic asthmatic patients and healthy controls. The data can provide new insights into the effects of vitamin D on regulatory pathways involved in the inappropriate immune response in allergic diseases and asthma.

Material an methods

Participants

This study was approved by the Research Ethics Committee of Jahrom University of Medical Sciences (Jahrom, Iran), and all the participants signed the consent form approved by the Ethics Committee of said university. In this study, 20 patients treated for allergic asthma in Motahari Hospital, Jahrom University of Medical Sciences, Iran, between 2014 and 2015, were enrolled. Cases eligible for participation were men and women aged > 18 years. Patients with other chronic disorders such as cancer and cardiovascular diseases were excluded from the study. Controls were 20 individuals who visited Motahari Hospital for annual check-ups and were not diagnosed with allergic asthma until the same age as that of the patient group.

Separating PBMCs and cell culture

Peripheral blood mononuclear cells (PBMCs) were separated using Ficoll and gradient of concentration methods. Briefly, 10 mL of peripheral blood with the anticoagulant was collected. The blood was diluted as PBS and poured onto the Ficoll as separated by two layers, and subsequently centrifuged for 20 to 25 minutes at 2500 rpm.

Cell culture

The isolated monoclonal cells were washed with PBS, and the cell suspension was suppressed in an enriched RPMI medium. In all the steps, RPMI-1640 (Merck KGaA, Darmstadt, Germany) was utilized while adding 2mM L-glutamine (Merck KGaA, Darmstadt, Germany), 100 U/mL penicillin (Merck KGaA, Darmstadt, Germany), 100 mg/mL streptomycin (Merck KGaA,

Darmstadt, Germany), and 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the treatment was performed in three groups of 10^{-7} M vitamin D, 10^{-6} M vitamin D (Merck KGaA, Darmstadt, Germany), 50μM dexamethasone (as a therapeutic intervention currently in use), while a control group that did not receive any treatment was considered as the calibrator for all samples of patients and control. The concentrations of vitamin D used in cell cultures were not cytotoxic and were driven from previous in vitro studies [17]. After 24 hours of culture, PBMCS was isolated from their culture media and their lysis using TRIZOL was performed in order to extract total-RNA, which was measured using the Bio photometer (Eppendorf, Hamburg, Germany). To this end, RNA was diluted with distilled water (1/100) and absorbance was read at 260, 280, and 320 nm wavelengths. The amount of RNA was calculated using the following formula:

$$\text{Total RNA} = (\text{OD } 260 - \text{OD } 280) \times 40 \times 100$$

Next, 5μg of RNA was converted into cDNA using the Revert Aid First cDNA synthesis kit (# K1632, Fermentas, Thermo Fisher Scientific, Waltham, MA, USA) and random hexamer primers according to the manufacturer's instruction. The DNasel enzyme was utilized to prepare the CDNA to prevent contamination with genomic DNA.

Quantitative real-time PCR (qPCR) procedure

In this procedure, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Two primer sequences for the GATA3 gene were designed by the Primer3 web service. The following primer sequences were employed for the GATA3 gene: forward (5′-AAGCCTAAACGCGATGGATA-3′) and reverse (5′-AGTGGTTGGAACACAGACACC-3′). The sequences of specific primers for GAPDH were as follows: forward (5′-ATTATTCTCTGATTTGGTCGTAT-3′) and reverse (5′-CTCCTGGAAGATGGTGAT-3′). Bioneer Corporation (Daejeon, South Korea) synthesized custom primers. The gene expression levels of GATA3 transcription factor and GAPDH were measured by qPCR performed using a Step One Plus Real-Time PCR Thermo cycler system (Applied Biosystems, Thermo Fisher Scientific, Foster City, USA). PCR amplification was performed at a final volume of 20 μl. The reaction mixture contained 1X DNA polymerase, with the final $MgCl_2$ concentration of 1.5 mM (Bioneer, Daejeon, South Korea) and KCl concentration of 1 mM (Bioneer, Daejeon, South Korea), 250 ng of cDNA, 1X from each primer, and 2X SYBR Green PCR master mix (Applied Biosystems, Thermo Fisher Scientific, Foster City, USA). The thermal cycler was set to 10 min of initial denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The final extension was performed at 72°C for 10 min. In the final step of real-time PCR, melting curves were incorporated from 60 to 90°C rising by 0.3 degrees. Samples were run in triplicate, and the fold difference in gene expression in samples was calculated by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The data were reported as mean ± S.D. In order to compare gene expression levels among different groups, analysis of variance (nonparametric Kruskal-Wallis and Mann-Whitney U tests) was applied using Graph Pad PRISM software (Version 6, Graph Pad Software, CA). The two-tailed significance level of <0.05 was considered in all statistical analyses.

Results

The total sample size was 40, including 20 patients and 20 healthy controls. The average age in the patient and control group was 32.58 ± 3.22 and 30.57 ± 2.88, respectively. Both groups were composed mainly of males, and there were no statistically significant differences between case and control groups in terms of age and sex. As shown in Figure 1, the analysis of data resulting from qPCR experiments showed that the ex-

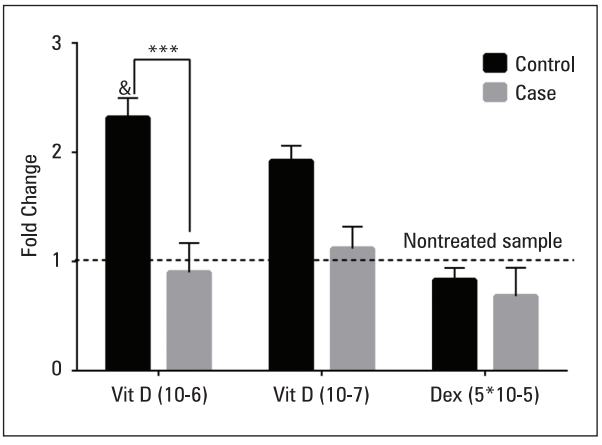

Figure 1. Effect of vitamin D and Dexamethasone on mRNA expression of GATA3 gene. ***P-value < 0.001, &P-value < 0.01 in comparison to non-treated sample

pression of GATA3 in PBMCs taken from patients with allergic asthma is lower than in those from healthy controls in all treatment groups. The difference between cases and controls reached the statistically significant threshold in the cells cultured with 10^{-6} M concentration of vitamin D (0.89 ± 0.27 *vs.* 2.32 ± 0.18, P-value < 0.01), while the difference was not significant in the cell cultures treated with either 10^{-7}M-vitamin D or dexamethasone (1.12 ± 1.2 *vs.* 1.92 ± 0.14 and 0.68 ± 0.26 *vs.* 0.83 ± 0.11, respectively). In both case and control groups, dexamethasone slightly reduced the expression of GATA3 compared to the untreated samples. In the cell cultures from healthy individuals, treatment with vitamin D increased the expression levels of the GATA3 gene in comparison to the untreated samples. This increase was only significant in the presence of 10^{-6}M-vitamin D (*P*-value<0.05). Indeed, a higher concentration of vitamin D resulted in a higher expression of GATA3 among controls. Nevertheless, results were different in the PBMCs from asthmatic patients where such an increase was observed only in the cells treated with a lower concentration of vitamin D, i.e., 10^{-7}M. By contrast, incubation with vitamin D at the concentration of 10^{-6} M slightly decreased the expression of GATA3 among patients.

Discussion

Based on our data, PBMCs from healthy individuals expressed greater GATA3 than did those coming from subjects with allergic asthma. Furthermore, the 24-hour incubation of PBMCs with 10^{-7} M-vitamin D increased the expression of GATA3 in both case and control groups. In the control group, vitamin D concentrations at 10^{-6} M were also shown to have a significant positive effect on GATA3 expression (*P*< 0.05). Nevertheless, in the patient group, unlike the control group, the addition of 10^{-6} M-vitamin D provided a weak orientation toward decreasing the expression of GATA3. Apparently, 10^{-6} M-vitamin D exerted statistically significant opposite effects on case and control groups (P < 0.001). We observed lower expression of GATA3 in PBMCs from subjects with allergic asthma. A possible reason of that observation might be the fact that blood is not necessarily reflective of the lung parenchyma as the main involved organ in allergic asthma. Simply, this probably means that a substantial number of Th2 cells migrate from circulating blood to the lungs during allergic asthma. In fact, such a migration also occurs during tuberculosis infection, where the frequency of mucosal-associated invariant T cells (MAIT) dramatically drops in the peripheral blood [18]. Vitamin D is a steroid pre-hormone belonging to the family of nuclear receptors [19]. The active form of vitamin D, $1,25(OH)_2D_3$, exerts its effects through binding to the vitamin D receptor (VDR) in the cytoplasm. The complex of vitamin D/VDR then forms a heterodimer with retinoid X receptor (RXR) and, after translocation to the nucleus, binds to vitamin D response elements (VDRE) on the genome and regulates the transcription of target genes either positively or negatively [20]. It has been well established that the GATA3 gene is upregulated in asthmatic lungs [21–23]. As mentioned earlier, multiple studies have demonstrated the suppression of Th1-mediated immune reactions in response to vitamin D [24, 25]. One suggested mechanism for the inhibition of Th1 immune response by vitamin D is altering the balance between Th1 and Th2 cells in favor of Th2 development [26, 27]. Indeed, results from some previous studies indicated that vitamin D induces the significant augmentation of GATA3 as the specific transcription factor of Th2 cells [28, 29], while other research groups reported the minimal impact of vitamin D in this regard [15, 16]. Collectively, these findings have shown the potential of vitamin D to fortify Th2 development in vitro. Partially consistent with the published data, our results imply that vitamin D can alter GATA3 gene expression in the PBMCs, including Th2 cells, in a dose-dependent manner. The impacts of this steroid hormone were also different between the status of health and allergic asthma in either extent or direction. A possible explanation is that the mRNA expression of GATA3 may be context-specific, that is, influenced by the presence of other cell-cell interactions or cytokines. Our results demonstrated that 10^{-6}M-vitamin D caused a slight reduction in GATA3 expression in asthmatic patients. In line with our data, some animal studies on an experimental model of allergic asthma in mice reported the beneficial effects of treatment with vitamin D on the airway eosinophilia and Th2 responses [30, 31]. However, these results were not confirmed using a different strain of mice [32]. Also, it seems that GATA3 mRNA expression can be genotype-dependent as well. The finding that GATA3 had an about two-fold greater amount in vitamin D-stimulated cultures versus untreated ones from healthy subjects points out that vitamin D is likely to play an important role in the regulatory pathways of GATA3 gene. However, vitamin D supplementation failed to

confirm such a significant effect on GATA3 expression in healthy females [14]. In fact, in vitro and in vivo studies are not comparable, since it is mostly challenging to determine the localized concentration of vitamin D in vivo. Cells such as macrophages and dendritic cells can produce vitamin D which can make regulatory changes in target genes in neighboring cells [13]. The role of vitamin D in allergic asthma is also supported by some evidence; the serum concentrations of vitamin D are associated with the incidence of asthma [33]. Further strengthening the role of vitamin D are studies demonstrating that vitamin D receptors are present in a wide range of cells involved in asthma [20] and VDR expression is considerably lower in subjects with asthma compared to healthy individuals [34]. Finally, allergic asthma was not developed in VDR deficient mice following ovalbumin (OVA) with aluminum hydroxide sensitization [32]. Topilski et.al in 2004 [31] suggested that vitamin D can reduce integrin-mediated T lymphocyte homing, thereby diminishing Th2 responses in vivo. Vitamin D exerts its effects through attachment to VDRE. Thus, we investigated more to find out which human genes containing VDRE sequence and might play a role in allergic asthma and its associated immune response [35, 36]. To this aim, we used NCBI Basic Local Alignment Search Tool (BLAST) to determine the human genes containing VDRE sequences; then, through ontology analysis, we investigated the functional relationship of target genes to GATA3 and T cell development in order to speculate about other possible involved mechanisms. Based on the results, the VDRE sequence, GGGTGA ACG GGGGCA, exists in two genes. This sequence was present in the transcription factor B cell lymphoma 11b gene (BCL11B) and the NEDD4 like E3 ubiquitin protein ligase gene (NEDD4L) in 93% and 100% similarity, respectively. Bcl11b, have been recently shown to function as an essential factor contributing to the regulation of Gata3 in mice [7], while the second protein regulates the expansion of CD4+ effector T cells [37]. Lorentsen et al. [7] reported that Bcl11b is capable of binding to intronic and downstream-noncoding regulatory elements of Gata3 to sustain its expression. In addition, Bcl11b deficient mice were unable to develop increased Th2 responses during allergic asthma. Therefore, it is likely that vitamin D should affect GATA3 expression through acting on BCL11B in the human genome. Furthermore, the active form of Nedd4, the mouse ortholog for NEDD4L, reduces the number of CD4+ T cells through degradation of Janus kinase 1 (Jak1) [37]. JAK1 as a component of Janus kinase 1/signal transducer, and the activator of transcription 6 (JAK1/STAT6) pathway induces the expression of GATA3 [38]. Accordingly, it is likely that vitamin D should indirectly affect the expression of GATA3 by acting on GATA3 regulatory genes harboring VDRE sequences. Due to the importance of the precise regulation of major transcription factors such as GATA3, even small regulatory changes may have far-reaching consequences for the modulation of immune responses. Thus, vitamin D deserves further studies in order to elucidate its effects on GATA3 regulatory pathways and Th2-mediated immune responses in both healthy and disease status in vivo.

The present study had some limitations. The most important limitation was that we used a mixed population of cells to examine GATA3 expression in different groups, e.g., asthma *vs.* control group. Therefore, it was difficult to interpret the effects of vitamin D on Th2 cells alone. This field is moving towards purified populations e.g. Th2 or type 2 innate lymphoid cells (ILC2) and, more recently, single-cell transcriptomic to pin down these relationships [21].

Conclusion

In conclusion, our results revealed that vitamin D affects GATA3 gene expression, as the specific transcription factor in Th2 development, in the PBMCs in a dose-dependent manner. The effects of vitamin D differed between the status of health and allergic asthma. Future studies are required to clearly understand the impact of this steroid hormone on the regulation of the GATA3 gene as well as the relevant mechanisms of action. Apparently, vitamin D has the potential to be considered as an effective treatment for altering the GATA3 gene expression levels and thus diminishing immune responses mediated mostly by Th2 cells.

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Ethics approval and Consent to participate

All procedures performed in studies involving human participants were in accordance

with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All the participants signed the consent form approved by the Ethics Committee of the mentioned university.

Conflict of interests

None.

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