Evaluating the Serum Levels of CCL17, CCL22, and CCL28 Chemokines and the Gene Expression of α4β1 and α4β7 Integrins in Patients With Allergic Rhinitis

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Received: 04 Oct. 2022; Accepted: 28 Jul. 2023

Abstract- Allergic rhinitis (AR) is a chronic inflammatory disease involving the nasal mucosa. Leukocytes recruitment to the inflammation sites is controlled by chemokines, cytokines, and adhesion molecules. Retinoic acid (RA), a vitamin A metabolite, plays an essential role in mucosal immunity and the production of inflammatory cytokines and chemokines. This study intended to evaluate the serum levels of RA, CCL17, CCL22, CCL28, and the mRNA expression levels of α4, β1, and β7 integrins in AR patients compared to healthy subjects. Peripheral blood was collected from 37 patients with AR and 30 age- and gender-matched healthy individuals. Serum levels of RA, CCL17, CCL22, and CCL28 were measured by the enzyme-linked immunosorbent assay (ELISA) technique, and the mRNA expression levels for α4, β1, and β7 integrins were assessed using the quantitative real-time PCR method. Our results showed that the serum levels of CCL22 and CCL28 chemokines are significantly higher in the AR group compared to the healthy controls (P<0.01). However, the gene expression of the β1 integrin in the AR group was significantly lower than that of the control group (P<0.001). Besides, there was a positive association between serum RA and CCL17 levels in patients (P<0.0001, r=0.6). In conclusion, increased serum levels of CCL22 and CCL28 chemokines, as well as decreased gene expression of β1 integrin in AR patients, may contribute to the pathogenesis and/or exacerbation of AR.

Acta Med Iran 2023;61(8):459-467.

Keywords: Allergic rhinitis; Retinoic acid; Chemokine (C-C Motif) ligand 17 (CCL17); α4β1; α4β7

Introduction

Allergic rhinitis (AR) is defined as a chronic immunoglobulin E (IgE)-mediated inflammatory disease of the nasal mucous membranes (1). During past decades, the prevalence of AR has considerably increased worldwide. Although the exact etiology of this disease is yet to be understood, changes in lifestyle and diet are proposed as the main factors (2,3).

Vitamin A and its active metabolite, retinoic acid (RA), are necessary for the regulation, expansion, and function of the immune system (4,5). Studies have shown that naïve T-cells differentiation into T helper 1 (Th1) and Th2 cells is regulated by RA (6). However, the results obtained from different studies showed contradictions. Some studies have shown that high

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consumption of all-trans retinoic acid (ATRA) may increase IgE levels in serum, develop Th2 responses, and inhibit the synthesis of interleukin (IL)-12 (7). In addition, another study showed that blocking retinoid X receptor (RXR) subunits enhanced the production of IL-12 by activation of monocytes and inhibition of Th2 responses (8). In another study using mice models, it has been shown that the effect of RA on the allergy is dose-dependent such that at low doses, it directly increases the allergic responses. However, RA at high doses may suppress eosinophil migration and its functional effects on the airways (9). Interestingly, in the lung mucosa, CD103+ dendritic cells (DCs) can produce RA (10).

Trafficking of activated T cells into inflammatory sites is affected by multiple factors, in particular, chemokines and adhesion molecules (11,12). Chemokines play a crucial role in the pathogenesis of allergic diseases by mediating leukocyte influx into the lungs (13). Many chemokines, including chemokine (C-C motif) ligand 17 (CCL17)/thymus- and activation-regulated chemokine (TARC), and CCL22/macrophage-derived chemokine (MDC), are essential for the advent of allergic airway diseases (14). Human "myeloid DC" produces CCL17 and CCL22 in response to toll-like receptor (TLR) ligand and cytokine stimulation (15). Also, there is evidence that points toward CCL28's important role in allergy disease pathogenesis. First, high levels of CCL28 have been recognized in the sputum and airways of humans with asthma and allergy disease (16). Second, CCL28 receptors (i.e. CCR3 and CCR10) are expressed on eosinophils, Th2 cells, regulatory T (Treg) cells, and other cell types related to allergy disease (17). Adhesion molecules also play a vital role in attracting leukocytes to the sites of inflammation (18-20). Integrins are heterodimeric cell surface-expressed adhesion molecules composed of noncovalently linked α and β subunits (21). On T cells, the α4 integrin subunit associates with either the β1 subunit, to form α4β1 integrin, or the β7 subunit, to form α4β7 integrin (22). Human memory CD4+ T cells express high levels of either α4β1 or α4β7 integrin (22-24). This high reciprocal expression of either α4β1 or α4β7 promotes altered trafficking properties based on the site-specific expression of the α4β1 ligand, vascular cell adhesion molecule-1 (VCAM-1), and the α4β7 ligand, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) (25). α4β7 integrin is expressed on a subset of eosinophils, T, B, and natural killer (NK) cells (26-28). This integrin plays an essential role in physiological conditions by lymphocytes migration to the peyer's patches and lymph nodes in the intestine. α4β1 integrin is expressed at high levels on all circulating leukocytes except neutrophils and has a significant role in lymphocytes homing in inflammatory conditions (26,29).

Considering the crucial roles of RA, inflammatory chemokines, and integrins in allergic rhinitis pathogenesis, the present study aims to assess the association between the serum levels of RA, CCL17, CCL22, and CCL28 chemokines, and also gene expression levels of α4, β1, and β7 integrins in the peripheral blood cells of allergic rhinitis patients compared to healthy subjects.

Materials and Methods

Studied subjects

A total of 37 patients with allergic rhinitis (age: 35.18±1.74 years (mean±SEM), sex: 24/13 (F/M)) and 30 age/sex-matched healthy controls (age: 34.96±2.33 years (mean±SEM), sex: 20/10 (F/M)) were included in this study. The height and weight of the participants were accurately measured, and then their body mass index (BMI) was calculated accordingly. The allergist physician made the diagnosis of AR according to the presence of typical clinical symptoms (including airway obstruction, sneezing, rhinorrhea, itchy nose, and itchy and watery eyes), and the control group was selected from healthy individuals without any chronic inflammatory disease. The demographic information and clinical features of patients and controls, as well as the frequency of rhinitis symptoms which was assessed by using the Rhinitis Quality of Life Questionnaire (RQLQ), have been shown in Table 1. Written informed consent was also obtained from all subjects. This study was performed with approval from the Ethics committee of the Kermanshah University of Medical Sciences (Ethical code: IR.KUMS.REC.1396.449).

Sample collection

Peripheral blood (10 ml) was obtained from all patients and healthy subjects. 7.5 ml of the blood sample was collected into a clot tube and was centrifuged at 3,000 rpm for 15 min to separate serum for measuring the serum levels of retinoic acid, CCL17, CCL22, and CCL28 chemokines. 2.5 ml of the remained blood was collected into a tube containing the ethylenediamine tetra acetic acid (EDTA) anticoagulant for the measurement of α4, β1, and β7 integrins gene expression levels. The serum samples were stored at -70° C until the performance of experiments by enzyme-linked immunosorbent assay (ELISA) procedure.

Measurement of serum CCL17, CCL22, CCL28, and retinoic acid levels

Serum levels of CCL17, CCL22, CCL28, and retinoic acid were measured using ELISA according to the manufacturer's instructions (Eastbiopharm, China).

α4, β1, and β7 gene expression levels

Total RNA extraction was carried out according to the manufacturer's protocol (Favorgen Biotech Corp., Taiwan), and the extracted RNA was then kept at -70° C. PCR was performed to synthesize cDNA using 10 µL total RNA and random hexamers according to the manufacturer's protocol (Favorgen Biotech Corp., Taiwan). The reactions were performed in a Thermocycler (Bio RAD Thermal Cycler C1000 Touch system) using the following steps: 5 min at 70° C, 60 min at 37° C, 5 min at 70° C, and a final hold at 4° C. The synthesized cDNA was stored at -20° C until analysis. To measure the *g*ene expression levels of α4β1 and α4β7, the synthesized cDNA was amplified using real-time PCR (light cycler 96, Roche Inc., CA, U.S.A.) and SYBR Green master mix (Favorgen Biotech Corp., Taiwan), according to the manufacturer's recommendations. Primers were designed using Beacon Designer™ Software (Primer Biosoft, USA). The oligonucleotide primer sequences were used as follows (listed as the forward primer and reverse primer, respectively): α4 integrin: 5´-CGAACCGATGGCTCCTAGTGG-3´, 5´-CTTTCCGATCCTGCATCTGTAAATCG-3´: β1 integrin: 5´-GCAAAGGAACAGCAGAGAAGCTC-3´, 5´-TGGCTCCCCTGATCTTAATCGC-3´: β7 integrin: 5´-GCATCCTCTGCGGAGGCTTT-3´, 5´-TGCATTTGCAGCGTCCATGC-3´; GAPDH: 5´-GACCCCTTCATTGACCTCAAC-3´, 5´-GATCTCGCTCCTGGAAGATG-3´. GAPDH was used as the internal control, and relative mRNA expression levels were calculated using the Pfaffl method ($R=(E_{target})^{\Delta Ct\ target\ (control-sample)} / (E_{Ref})^{\Delta Ct\ Ref\ (control-sample)}$) (30).

Statistical analysis

All statistical analyses, calculations, and graph drawings were performed using SPSS software version 23 (SPSS, Chicago, IL, USA) and GraphPad Prism version 8 (GraphPad, Software, La Jolla, California, USA). At first, the data normality was determined by the 1-sample Kolmogorov-Smirnov (K-S) test. Afterward, the Mann-Whitney test was used for the analysis of nonparametric data. Spearman rank-order correlation coefficient analysis was also applied to assess the correlations between variables. A *P* of less than 0.05 was considered to be statistically significant. In all statistical analyses, data are expressed as the Mean±standard (Std) Error of the Mean (SEM).

Table 1. The demographic information and clinical characteristics of the studied participants

Variables	Allergic rhinitis group (N=37)	Control group (N=30)	*P*
Sex (Female/Male, %)	64.86/35.14	66.67/33.33	0.29
Average age, years	35.18 ± 1.74	34.96 ± 2.33	0.84
The average period of the disease	Six years	-	-
History of asthma	-	-	-
Smoking (N)	8	-	-
Body mass index (BMI)	25.47 ± 0.71	24.22 ± 0.78	0.84
Total IgE, IU/ml	85.32 ± 15.93	53.08 ± 10.50	0.34
Peripheral blood eosinophils count (%)	5 ± 0.49	1.29 ± 0.27	<0.0001
The frequency of rhinitis symptoms			
Nasal obstruction (%)	91.7	-	
Nasal rhinorrhea (%)	94.4	-	
Sneezing (%)	91.7	-	
Nasal itching (%)	86.1	-	
Watery eyes (%)	70	-	
Eye itching (%)	66.7	-	

Data are expressed as the Mean ± SEM

Results

Serum retinoic acid, CCL17, CCL22, and CCL28 levels

The serum analysis showed that the mean serum level of retinoic acid in the AR group (1.7 ng/ml) was lower than that of the control group (2.2 ng/ml); however, this difference was not statistically significant. The mean serum CCL17 level in the AR and the control groups was 28.0 and 32.62 ng/l, respectively. Besides, serum analysis revealed that patients with AR had a significantly higher level of CCL22 (*P*=0.005) and

CCL28 (P=0.001) compared to the control group. The mean serum CCL22 level in the AR and the control groups was 39.8 and 26.8 ng/ml, and the mean serum CCL28 level was 37.3 and 23.03 ng/ml, respectively. Data are shown in Figure 1.

Expression levels of α4, β1, and β7 integrins

Our data analyses showed that the relative expression levels of the α4 and β7 integrin genes were not significantly different between AR and the control groups, but the relative expression of the β1 integrin gene in the AR group was significantly lower than that of the control group (P<0.001). Data are shown in Figure 2.

Correlation between serum retinoic acid and CCL17, CCL22, and CCL28 levels

The independent effects of RA on serum CCL17, CCL22, and CCL28 levels in patients were assessed by Spearman rank-order correlation coefficient analysis. In the patient group, RA was positively correlated with CCL17 levels (r=0.6, P<0.0001) (Figure 3). However, there was no statistically significant relationship between serum RA and CCL28 (r=0.3, P=0.07) and CCL22 (r=-0.2, P=0.1) levels.

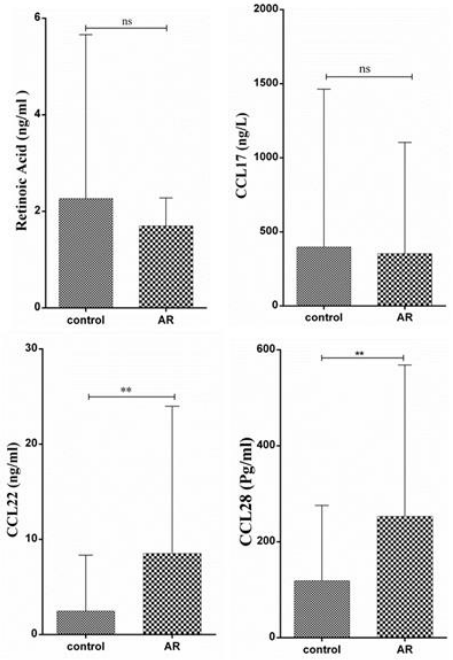

Figure 1. Serum levels of RA, CCL17, CCL22, and CCL28 in the studied groups. The serum concentrations of retinoic acid and CCL17 were reduced in the AR patients compared to the controls; nonetheless, these differences were not statistically significant. Besides, the serum CCL22 and CCL28 concentrations in the AR patients were meaningfully greater than controls (P<0.01). AR, allergic rhinitis; RA, retinoic acid; ** P<0.01 and ns: not statistically significant

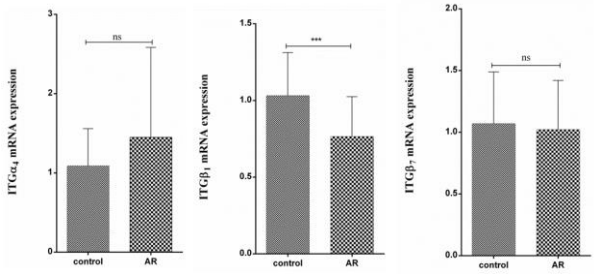

Figure 2. mRNA expression levels of α4, β1, and β7 integrins in the studied groups. There was no meaningful difference in the mRNA expression levels of α4 and β7 integrins between AR patients and controls. However, the mRNA expression level of β1 integrin was significantly diminished in the AR patients relative to the control group (P<0.001). AR, allergic rhinitis; ITG, Integrin; *** P<0.001 and ns: not statistically significant

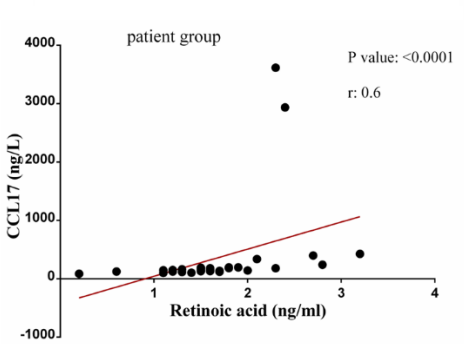

Figure 3. Correlation between serum retinoic acid and CCL17 levels in the patient group. There was a positive association between serum retinoic acid and CCL17 levels in the patient group (P<0.0001, r=0.6)

Discussion

In this study, retinoic acid, CCL17, CCL22, and CCL28 serum levels, as well as gene expression levels of α4, β1, and β7 integrins in the peripheral blood of patients with allergic rhinitis and healthy subjects, were evaluated.

Allergic rhinitis is a common inflammatory disease engaging the nasal mucosa and upper airways (31). The most prominent characteristic of inflammation is the accumulation of leukocytes at the site of inflammation. Leukocytes migration into the site of inflammation is a complex process influenced by several factors, like chemokines, cytokines, and adhesion molecules (32). CCL17 and CCL22 chemokines have a prominent role in allergic reactions by attracting Th2 cells via binding to CCR4 on the surface of the lymphocytes (33,34). Evidence indicated that the nasal epithelium produces CCL17, which has an essential role in respiratory allergic diseases, including asthma and rhinitis (35). Furthermore, a previous study showed elevated CCL17 and CCL22 levels in supernatants of bronchial mucosa biopsy cultures of patients with asthma after the allergen challenge (36). Another study in 2008 also showed a significantly high expression level of CCL22, but not CCL17, in colorectal cancerous tissues, compared to paired normal tissue from colorectal cancer patients (37). Our results indicated a significant increase in CCL22 serum levels in the patient group; however, there was no significant difference in CCL17 serum levels between allergic rhinitis patients and the control group. This might be explained by the local secretion of CCL17 by the nasal mucosa epithelium which may not be sufficient to increase chemokine levels systematically in the bloodstream (35).

CCL28 chemokine is expressed in the airway's epithelial mucosa with a vital role in the development of asthma and allergies (16,17,38-40). Previous investigations showed that the anti-CCL28 and inhibition of CCL28 could reduce the eosinophilic infiltration into the inflammation site (16,41,42). Chemokine-specific receptors of CCL28 are CCR3 and CCR10. CCR3 is expressed on the surface of eosinophil and Th2 cells, and CCR10 is expressed by airways epithelial and mucous membranes (17,43,44). In the present study, the CCL28 serum level in AR patients showed a significant increase when compared to the controls. Some studies have confirmed the increased CCL28 and its mRNA expression in the airway mucous membranes of allergen-susceptible patients. Also, there is evidence demonstrating communication between the CCL28 and chronic asthma pathogenesis (16,17). Prior studies have also reported a high CCL28 expression in mucous membranes of animal models suffering from allergic rhinitis (45). The interaction between CCL28 and CCR3/CCR10 is likely to result in the absorption of memory CD4+ T cells into the nasal mucosa (46). In homeostasis conditions, the nasal mucosa contains a low number of Th2 cells, but in inflammatory conditions, CCL28 increases the migration of CD4+ Th2 cells to the nasal mucosa (17,44). Indeed, after allergens exposure, the level of this chemokine is increased in the nasal mucosa. Studies have shown that pro-inflammatory cytokines such as IL-1β and TNF-α could induce the expression of CCL28 in airway epithelial of animal models suffering allergic rhinitis (40). Besides, our results showed increased eosinophil counts in AR patients compared to healthy subjects, and in vitro studies demonstrated an essential role of CCL28 in eosinophil migration to the inflammation site (44).

Vitamin A and its metabolite, retinoic acid (RA), have a critical role in mucosal immunity (47,48). In this study, we found a reduced serum level of RA in AR patients than in controls, although this reduction was not

statistically significant. Besides, there was a positive relationship between serum levels of CCL17 and RA in the AR patients. Studies reported that CD103+ DCs in the lung mucosa play a significant role in stimulating responses to respiratory allergens (10). Also, some studies showed that these DCs could affect RA production (49-51). The high expression of IL-4 and IL-13 cytokines is one of the characteristics of Th2 cells. Some studies have confirmed the ATRA's role in enhancing the expression of IL-4 and IL-13. Some previous researches also proved that ATRA nutrition (feeding) in animal models increased the expression of IL-4 and IL-13 in the lung and liver (52,53). Moreover, some studies have confirmed the IL-4 and IL-13 effects on the increase of CCL17 and CCL22 chemokines in the human lung (54,55). Another animal model study showed that the RA effect on allergy is dose-dependent, which directly increases the allergic responses at low doses; however, RA in high doses stimulates eosinophilic suppression and its functional effects in the airways (9).

Integrins also play a vital role in attracting leukocytes to the inflammation site (56). Each integrin molecule is composed of two subunits α and β, which are connected by a non-covalent bond. The α4 subunit is a subunit shared between two integrins α4β1 and α4β7 (32,57,58). In this study, there was a significant decrease in the β1 integrin subunit expression in AR patients compared to the controls; however, there was no significant difference between the α4 and β7 subunits in AR patients and controls. Some previous studies reported the increased expression of α4β1 integrin, but not α4β7 integrin, in the peripheral blood of animal models (59). α4β1 is an essential mediator in eosinophils migration (60). The interaction between adhesion molecules on the eosinophil surface and their receptors on the epithelial and vascular endothelium surface plays an important role in cell migration to the inflammation site (61). Increased VCAM-1 expression by inflammatory mediators, like IL-4 and IL-13, causes the lymphocytes and eosinophils to migrate from blood to the mucous membranes of asthmatic patients, while these cytokines do not affect the expression of other adhesion molecules, like ICAM and E-selection (61-64). Although studies have confirmed the increased β1 integrin expression in allergies and inflammation, we report a reduction in the β1 integrin expression in this study. Given the low expression of α4β1 integrin on the naïve lymphocyte surface, as well as the migration of effector lymphocytes from peripheral blood to the site of inflammation and the lung mucosa, it may be possible to justify the decrease in expression of this integrin in peripheral blood (65).

In the present study, there was a non-significant difference in RA serum levels between the AR and control groups; however, a positive relationship between serum CCL17 and RA levels was observed, which might show the role of RA in allergic rhinitis pathogenesis. Indeed, the insignificance of RA serum levels can be due to the low detection ability of chemokines and RA kits. Besides, we found a significant increase in CCL22 and CCL28 serum levels in AR patients. However, the CCL17 chemokine serum level was not significantly different in the patient's group compared to the control group. This may be because the local secretion of CCL17 by the nasal mucosa epithelium was not sufficient to increase chemokine levels systematically in the bloodstream. Also, contrary to our anticipation, the β1 integrin gene expression in peripheral blood cells of allergic rhinitis patients showed a significant decrease compared to the control group. This decrease of β1 gene expression in peripheral blood cells can be due to the migration of lymphocytes expressing this integrin to the lung mucosa and upper respiratory tract. In conclusion, increased serum levels of CCL22 and CCL28 chemokines, as well as reduced gene expression of β1 integrin in AR patients, may be involved in the pathogenesis and/or intensification of AR. However, further studies with larger sample sizes are required to confirm our findings and to better understand the underlying molecular mechanisms of the involvement of RA, CCL22, and CCL28 chemokines, as well as β1 integrin, in AR pathogenesis.

Acknowledgments

The authors appreciate all participating patients and individuals. Besides, the authors would like to thank Dr. Misagh Rajabinejad for his precious assistance in editing this article. Financial support from the Kermanshah University of Medical Sciences (KUMS) is also highly appreciated.

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