Evaluation of the ratio of T helper 17 and T regulatory cells in patients with chronic idiopathic urticaria

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Abstract

Chronic idiopathic urticaria (CIU) is a common skin disorder characterized by the emergence of hives for at least 6 weeks without any known etiologic agents. The T helper 17 (Th17) and regulatory T-cells (Treg) balance plays a critical role in both suppressing immune response and maintaining immunological homeostasis. In this study, the gene expression of RORγt and FOXP3 were evaluated to exam the potential immunological roles of Th17 and Treg in CIU patients. In a cross sectional study twenty CIU patients and twenty healthy individuals were evaluated for RORγt and FOXP3 genes expression. Peripheral blood mononuclear cells (PBMCs) were isolated and stimulated by phytohemmaglutinin. Real-time PCR, two standard curves TaqMan method was applied to quantify gene expression. The mean age of patients and controls was 30.5±2 and 30.2±1.6 years, mean duration of disease: 17.3±5 months. FOXP3 gene expression significantly increased in activated PBMCs of CIU patients (2.28 ± 0.6) compared to controls (0.24±0.1) (p=0.05). Non-activated PBMCs demonstrated remarkable increase in gene expression but it was not significant (p=0.054). Patients and healthy individuals did not show significant alteration in RORγt gene expression. Meanwhile, the ratio of RORγt/FOXP3 in patients was significantly lower than controls (p<0.05). The high expression of FOXP3 in patients without any significant changes in RORγt might indicate the presence of an independent inflammatory pathway such as neurogenic inflammation, which induces Treg cells and mediates inflammation through the degranulation of mast cells.

Key words: Urticaria, T lymphocytes, Forkhead transcription factors, Inflammation.

Introduction

Chronic urticaria (CU) is usually described as the persistence of wheal and flare for at least 6 weeks. When the other causes of disease including drugs, foods, aeroallergens, vasculitis, and/or physical factors are excluded; it is entitled "chronic spontaneous urticaria" (CSU). CSU is divided into two groups: the autoimmune (45%) and chronic idiopathic urticaria [(CIU) (55%)]. In the autoimmune form, the auto-antibody such as anti-α-subunit of FCεR antibody or anti-IgE is present while in the CIU form, autoantibody is absent (1). CIU affects approximately 0.1 percent of the population (2) and has a great impact on life quality, satisfaction, and performance (3). It has been shown that the health status scores for these patients are comparable to those suffering from coronary artery disease (4). Furthermore, psychological conditions such as depression and anxiety are prevalent among CIU patients (5).

T helper 17 (Th17) and forkhead box P3 positive (FOXP3+) regulatory T cells (Treg) are two important T-cell subsets (6, 7). The most important function of Th17 is secretion of interleukin17 (IL-17) (8). IL-17 production is dependent on retinoid orphan receptor C (RORγt), the main master regulator transcription factor for the conversion of the naïve T-cell into Th17 (9). In addition to this primary secretion role, Th17 also plays a positive role against harmful microorganisms, and a negative role in promoting some disorders such as autoimmunity, allergic reactions, and inflammation (10-12).

Treg cells play a fundamental regulatory and inhibitory role on immune cells such as B cells, CD4+ and CD8+ T cells, monocytes and dendritic cells (DCs)(13). The regulatory effects of Treg cells are mediated through IL-10 and TGF-β production (14). The FOXP3 transcription factor gene is essential for the induction of inhibitory function (15). Interestingly, recent studies have shown that Th17 and Treg have controversial affects on immune reactions (16). TGF-β and IL-6 presence promotes Th17 development, while in the presence of only TGF-β, naïve T-cells shift towards Treg (17). Evaluation of the Th17/Treg ratio has significantly expanded our knowledge about the pathogenesis of many disorders such as rheumatoid arthritis (18), graft versus host disease (19) and coronary artery disease (20).

Considering the importance of Treg and Th17 in immune responses, in this study the gene expression of RORγt and FOXP3 were evaluated in CIU patients and healthy individuals.

Materials, Patients and Methods

Twenty CIU patients (male: 6, female: 14) and twenty healthy people (male: 6, female: 14) participated from the Allergy clinic of Qaem hospital (Mashhad, Iran) in this study. The patients were selected as CIU if they had recurrent wheals occurring at least three times per week for more than six weeks without any particular cause, as we previously reported (21). Patients with lesions which had lasted more than 24 hours were excluded. The patients with IgE-mediated urticaria or with any other known cause such as urticarial vasculitis, physical urticaria, autoinflammatory diseases and food allergy were also excluded from the study. Standard laboratory work-ups included: complete blood cell count, stool exam, urinalysis, complement evaluation, function of thyroid hormones and anti-thyroid antibodies, anti-nuclear antibodies, anti-H. pylori and total serum IgE. Patients and controls gave written informed consent and the study design was approved by the Ethics committee of Mashhad University of Medical Sciences (number 91641).

Autologous Serum Skin Test (ASST)

None of the patients participating in the study had taken an oral corticosteroid or other immunosuppressive agents before the test. The patients did not use antihistamine for the 3 days prior to the test. The ASST was performed according to the Grattan protocol (22). Briefly 0.05 ml of fresh autologous serum and normal saline (as control) were injected separately and intradermally into the volar surface of the forearm and evaluated 30 minutes later. The test was considered as positive if the difference of wheal diameters between serum and controls was more than 1.5 mm.

PBMCs isolation and stimulation

Up to 4 ml of venous blood was taken from each participant. PBMCs were then isolated by a Ficoll-Hypaque (Sigma, UK) density centrifugation. A total of 1.5×10^6 cells/well were cultured in RPMI-1640 (Gibco-Bio-Cult, Glasgow, Scotland) supplemented with 10% fetal bovine serum (FBS) and stimulated by phytohemmaglutinin (PHA) (2μg/ml) (Sigma Chemical, USA) for 48 hours at 37 ^{0}C in a 5% CO_2 atmosphere. The cells were collected and Tripure (Roche) was added to extract RNA.

RNA Extraction, cDNA Synthesis, and Gene Expression

RNA extraction was performed using Tripure (Roche) according to the standard protocol. cDNA was produced using a RevertAidTM H Minus First Strand cDNA Synthesis Kit (Fermentas, Germany). FOXP3 and RORγt gene expressions were measured using Real-time PCR. Primers and probes were designed using Beacon Designer 7 software (Premier Biosoft International, USA). Real-time PCR was performed in Taqman method for FOXP3 and RORγt PCR kit. The sequence of primers and probes of respective genes are shown in Table 1. β_2 –microglobulin, which express in all nucleated cells, was used as an endogenous control. The Real-time PCR was performed on a Rotor-Gene6000 Cycler (Corbet, Hilden, Germany). Real-time PCR was performed according to the Taqman method in a 10 μl volume using 4 μg total cDNA, 5 μl PrimeScript RT Master Mix (Takara Corporation), 0.4 μl forward and reverse primers and also 0.2 μl probe. All reactions were performed in duplicate. After adjustment of the respective concentrations of primers, probes, and Mg2+, cycling protocols were finally implemented as

follows: 40-cycle amplification program consisting of 10 s at 95 °C and 40 s at 60 °C. Gene expression level for each gene was calculated using the standard curve method. Target efficiency (FOXP3, RORγt) and reference genes were approximately equal.

Statistical Analysis

The Statistical Package for the Social Sciences, version 16 (SPSS 16.0, WinWrap Basic, Polar Engineering and Consulting, Nikiski, AK, USA), was used to conduct statistical analysis. Kolmogrov–Smirnov (K-S) Test and Mann-Whitney U Test were used to compare the gene expressions between the CIU and control groups. The significance level of this test was estimated at less than 0.05, with a confidence interval of 95%.

Results

Totally, 40 subjects completed the study (20 CIU patients, and 20 healthy cases). The mean age of patients and controls was 30.5±2 and 30.2±1.6 years, respectively. The mean duration of disease for the CIU group was 17.3±5 months.

RORγt and FOXP3 expression before PHA stimulation

The mean of RORγt mRNA expression in the lymphocytes of the healthy group showed an expression index (ei) of 0.065±0.01. In the CIU patients, the mean RORγt mRNA expression was 0.17±0.1 ei. No significant difference in RORγt gene expression was found between the CIU and control groups (p>0.05).

The mean FOXP3 mRNA expression in the lymphocytes of the CIU patients was higher (0.023±0.01) than control groups (0.0003±0.0001 ei), however the result did not reach significant levels (p=0. 054).

RORγt and FOXP3 expression after PHA stimulation

The mean of RORγt mRNA expression in the lymphocytes of the healthy group and patients was 1.14±0.2 ei and 1.17±0.3 ei, respectively. No significant difference in RORγt gene expression was found between the CIU and control groups (p>0.05).

The mean FOXP3 mRNA expression in the lymphocytes of the CIU patients was significantly higher than healthy (p<0.05) (Table 2).

The ratio of RORγt to FOX3 in non-activated PBMCs of CIU patients and controls was 387±377 and 147±116 respectively and no significance (p>0.05) was observed between two groups.

The ratio of RORγt to FOX3 in activated PBMCs of CIU patients and controls was 0.4±0.1 and 3030±2477 (p<0.001), respectively which was significant.

Table 1: Primers and probes sequences of FOXP3 and RORγt genes

Gene	Primer	Probe
FOXP3*	Forward:5-ACTACTTCAATTTCCACAACACGC-3 Reverse: 5-GAG TGT CCG CTG CTT CTC TG-3	Fam-TCACCTACGCCACGTTCATCCGCT-BHQ1
RORγt**	Forward: 5-GCT AGG TGC AGA GCT TCAGG-3 Reverse: 5- TGTTCTCATGACTGAGCCTTGG-3	Fam-CCTTGGCTCCCTGTCCTTCTCAGCA-BHQ1
B2-microglobulin	Forward: 5- CGGAAGGAACCATCTCACTGTG-3 Reverse: 5-AGAAATCAGGAAGGCTGCCAAG-3	Fam-ATGGTTCACACGGCAGGCATACTCATCT-BHQ1

* Forkhead box P3 positive, ** Retinoid orphan receptor C

Table 2: Expression of FOxp3 and RORγt expression in patients with CIU and healthy controls

Groups	Before stimulation			After stimulation		
	RORγt	FOXP3	RORγt FOXP3 ratio	RORγt	FOXP3	RORγt FOXP3 ratio
Healthy group	0.065 ±0.01	0.0003±0.0001	147±116	1.14 ±0.2	0.24±0.1	3030±2477
Patient group	0.17 ±0.1	0.023 ± 0.01	387±377	1.17 ±0.	2.28 ± 0.6	0.4±0.1
Significance (*p*-value)	*p*=0.38	*p*=0.054	*P*=0.2	*p*=0.9	*p*=0.003	*P*<0.001

Discussion

In this study, we examined the expression of FOXP3 and RORγt and also the ratio of these factors in CIU patients and healthy individuals. The increase in FOXP3 gene expression and subsequently decrease in Th17/Treg ratio in activated PBMC of CIU patients were significant compared to healthy individuals.

The result of this study does not support the hypothesis of a reciprocal relationship between Th17 and Treg cells reported in the previous studies (18-20), but it could be justified with another important mechanism, neurogenic inflammation (23), in which the number of Treg cells is increased (24). Neuron fibers and mast cells are within a close proximity to the skin, therefore many factors such as stresses, hot weather, physical factors, and histamine, affect neuron fibers and trigger the secretion of the chemical mediators, neuropeptides. Several neuropeptides are present in the skin, of which the most important are calcitonin gene-related peptides (CGRP), substance p (SP), and vasoactive intestinal peptides (VIP) (25). These mediators induce NI in two main pathways (26): directly binding to micro vascular or mast cell receptors, the latter stimulates the release of histamine. It has been shown that CGRP and VIP also have regulatory functions in NI (27-29). Smith et al. showed that the effects of VIP are more severe than the other wheal inducers in CIU patients (30). Furthermore, VIP has an important immunomodulatory function in this kind of inflammation (31-33), especially after the acute inflammation phase that induces FOXP3 expression (34, 35). It is consistent with our finding in which FOXP3 expression is increased after activating PBMCs in CIU patients.

The Th17 plasticity has been demonstrated in several studies (36-38); it is probable that in CIU patients, VIP secretion induces Treg and therefore decreases the ratio of Th17 to Treg. The serum level of SP in CIU patients is controversial (39, 40), and to our knowledge there is no study regarding the role of VIP in patients with CIU. Further studies are needed to clarify the role of VIP and other neuropeptides in CIU.

Taken together, our results showed that FOXp3 expression is increased in CIU patients and therefore the ratio of Th17 to Treg is decreased. It seems that the production of neuropeptides factors such as PS, VIP, and CGRP might be involved in this issue. Thus further studies should be taken into account to determine the relation between these mediators and Treg cells in patients with CIU.

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