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Research Article

Evaluation of Indoleamine 2, 3- Dioxygenase Gene Expression and Activation in Chronic Spontaneous Urticaria

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ABSTRACT

Chronic spontaneous urticaria (CSU) is a common skin disorder characterized by the emergence of hives for at least six weeks without any known etiologic agent. Indoleamine 2,3- dioxygenase (IDO) which catalyzes tryptophan (Trp) to kynorenin (KYN) is an immunomedulatory enzyme and complicated in immunological diseases. In this study, Trp, KYN and IDO gene expression in CSU patients were analyzed. We studied 20 CSU patients (mean age: 28±6 years, mean duration: 27±4 months) and 20 healthy individuals (mean age: 28±9 years). Peripheral blood mononuclear cells (PBMCs) were isolated from both patients and healthy control and stimulated by phytohemmaglutinin (PHA). Real-time PCR was applied to quantify IDO gene expression and its activity was estimated by KYN/Trp ratio in supernatant of PBMCs by HPLC. Our study results showed that the gene expression of IDO was higher in CSU patients (0.33±0.27) compare to healthy individuals (0.31±0.6, p=0.02). Amazing, the activity of IDO (KYN/Trp) was decreased in CSU patients (322.5±432.3) contrast of healthy ones (685.9±531.7, p=0.02). Previous studies documented the impaired of IDO gene expression in CSU patients, however in the present study we observed a decrease activity of IDO in CSU patients which might suggest the function of this factor is impaired in CSU patients.

Key words: Chronic spontaneous urticaria**,** Indoleamine 2,3- dioxygenase, Tryptophan, Kynorenin.

INTRODUCTION

Chronic spontaneous urticaria (CSU), formerly named chronic idiopathic urticaria (CIU) is defined as persistent symptoms of wheal and flare for 6 week or more without an obvious stimulus (1) and affect up to %1 of population (2). There is a clear association of a subpopulation of chronic urticaria (40%-45%) with autoimmunity (3), the non-autoimmune remaining 55%-60% of patients up to now is considered to be idiopathic and all these are entitled "spontaneous"(1). CSU has significant detrimental effects on patient's health-related quality of life (4). Many studies have shown high prevalence of psychosocial factors in these patients (5). Pathogenesis of CSU is not clear, however disturbances in some components such as mast cells and basophils are proposed (6). Hence investigation for immunomodulating agents in CSU is helpful. Indoleamne 2,3-dioxygenase

(IDO) is a rate-limiting enzyme which degrades tryptophan to kynorenin (7). Several studies have shown its important role in T cell immunomodulation and tolerance (8-11). IDO has a major role in inducing tumors by immune suppression in different cancers(8, 12). Furthermore its immunosuppression role has demonstrated in various situations such as: feto-maternal tolerance (13), allergic rhinitis (14), asthma (15), atopic dermatitis (16) and transplantation (17). Therefore, IDO enzyme activity condition maybe affected in CSU patients so identifying the possible relationship between this condition and CSU may hopefully help to establish an appropriate treatment. The present study, then, aimed to evaluate the IDO enzyme activity in CSU patients. In addition, IDO gene expression was concurrently checked to be compared with its activation.

MATERIALS AND METHODS

Population

Twenty CSU patients (male: 6, female: 14) and twenty healthy people (male: 6, female: 14) participated from allergy clinic of Qaem hospital in this study. The patients were selected as CSU if they had a recurrent wheals occurring at least three times per week for more than six weeks without any particular cause as we previously reported. 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 were including: 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 of ethics committee of Mashhad University of Medical Sciences (number 91641).

Autologous Serum Skin Test (ASST)

ASST differentiates autoimmune and non-autoimmune urticaria. 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. 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 1.5mm.

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 PHA (2µg/ml) (Sigma Chemical, USA) for 48 hours at 37 C in a 5% CO_2 atmosphere. The cells were collected and Tripure (Roche) was added to extract RNA.

Preparation of samples for high performance liquid chromatography (HPLC)

Samples were Deproteinized by adding 10 µl of 30% perchloric acid to 100 µl plasma. After resting for 10 min the samples were mixed for 1 min and centrifuged at room temperature at 13,000 rps for 10 min (Minispin, Eppendorf). Thereafter, 20 µl of the supernatant was injected into the HPLC system. Standard stock solutions of Trp (100 ppm) and Kyn (4ppm) were prepared by dissolving 10 mg of Trp and 0.4 mg of Kyn in 100 ml of 15 mmol/l sodium acetate–acetic acid (pH 5.5) and were stable for at least 3 months at − 20 °C. The mixture working solutions were prepared by diluting to appropriate concentration with 15 mmol/l sodium acetate–acetic acid (pH 5.5).

Chromatography

The HPLC equipment was waters (600E pump). The analytical column (150 mm × 3.9 mm; Nova-

pakl® C-18) were obtained from waters. The mobile phase consisted of 15 mmol/l sodium acetate buffer solution and 6% (v/v) acetonitrile. The pH of the buffer solution was adjusted to 5.5 with acetic acid. Freshly prepared buffer solution was filtered through 0.45 µm Millipore membrane. The chromatographic separation was performed at 25 °C. The rate of the mobile phase was 0.8 ml/min. wavelength detection (2487detector) setting at 360 nm for Kyn and at 302 nm for Trp. The linear range for Kyn was (80-400 ppb) and for Trp (2-10 ppm) respectively.

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). IDO gene expression was measured using Real-time PCR. Primers and probes were designed using Beacon Designer 7 software (Premier Biosoft International, USA). The sequence of primers and probes of respected 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 µl 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 Mg^{2+}, cycling protocols was 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 (IDO) and reference genes were approximately equal.

Table 1: Primers and probes sequences of IDO and reference genes

Genes	Primers	Probes
IDO*	Forward: 5-GCCAAGGTCATCCAACTACT-3 Reverse: 3- GCCTGCTTCACCACCTTCTGATG-5	Fam- AAGCTGCTCGAGATTTCCACCAATAGA -BHQ1
B2-microglobulin	Forward: 5- CGGAAGGAACCATCTCACTGTG-3 Reverse: 5-AGAAATCAGGAAGGCTGCCAAG-3	Fam-ATGGTTCACACGGCAGGCATACTCATCT-BHQ1

* Indoleamine 2, 3 di-oxygenase

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 CSU and control groups. The significance level of this test was estimated at less than 0.05, with a confidence interval of 95%.

RESULTS AND DISCUSSION:

Totally, 40 subjects completed the study (20 CSU patients, and 20 healthy cases). The mean age of patients and controls was 28±6 and 28±9 years, respectively. The mean duration of disease for the CSU group was 27±4 months.

IDO expression and IDO activity (KYN/Trp) before PHA stimulation

The mean of IDO mRNA expression in the lymphocytes of the healthy group showed an expression index (ei) of 0.01(SD=0.02). In the CSU patients, the mean IDO mRNA expression was 0.02(SD=0.03) ei. No significant difference in IDO gene expression was found between the CSU and control groups (p>0.05).

The mean of IDO activity (KYN/Trp) in the serum of control groups was 41.5 (SD=16.4) ei, compare to patients group 44.3(SD=16.3), which was not significant (p>0.05).

IDO expression and IDO activity (KYN/Trp) after PHA stimulation

The mean of IDO mRNA expression in the lymphocytes of the healthy group and patients was 0.31(SD=0.6) and 0.33 (SD=0.27) respectively. The results showed that the gene expression of IDO was significantly higher in CSU patients (p=0.02).

The mean of IDO activity (KYN/Trp) in the supernatant of PBMCs of the CSU patients was significantly lower (322.5) than healthy group (685.9) (*p=0.02*) (Table 2).

Table 2: IDO expression and activation before and after stimulation by PHA

	IDO expression Mean (SD)			**IDO activation Mean (SD)**		
	before stimulation	after stimulation	Percentage change	before stimulation	after stimulation	Percentage change
Healthy group	0.01(0.02)	0.31(0.6)	53700.9	41.5(16.4)	685.9 (531.7)	1700.7
Patients group	0.02(0.03)	0.33(0.27)	700.0	44.3(16.3)	322.5 (432.3)	9600.2
***p*-value**	0.24	0.02		0.18	0.02	

The results of this study showed that the gene expression of IDO was higher in CSU patients compared to healthy individuals (p=0.02); however, we observed a significant decreased activity of IDO in CSU patients (*p=0.02*).

In the situation of low activity of IDO (KYN/TRP) and following high level of immune response(18), more inflammation will be constructed, which was compatible to the symptoms of the patients. Although another study has reported impaired IDO gene expression in chronic idiopathic urticaria, they did not differentiate CSU from chronic idiopathic urticaria and IDO activation was not evaluated(19).

Many studies have shown high IDO gene expression in malignant condition(12), while high enzyme activation has not been scored simultaneously. Moreover, FCεRI with an essential role in atopic conditions promotes the tryptophan catabolism and is followed by regulating T cell responses. Albeit as mentioned in the methods section, the patients were non-autoimmune chronic urticaria using ASST. Likewise in non-symptomatic allergic rhinitis high IDO gene expression has illustrated meanwhile enzyme activation has not evaluated (20). Although an increase in IDO enzyme is expected to increase the tryptophan catabolism (increase of IDO activity) and to hamper the immune response or consequent inflammation, in this study, IDO gene expression and activity did not simultaneously increase. Nevertheless, other researchers have identified genetic variants in IDO some of which are non-functional (21). In addition, impaired functional variants may produce impaired folding isoenzyme that may be degraded by proteasome in cytoplasm (22). Therefore, the authors recommend further future studies on IDO gene polymorphism and isoenzyme structure to distinguish probable enzyme disturbances.

CONCLUSIONS

There are increasing evidences that IDO plays an essential role in inflammatory situations. Although no clear data was found about IDO and chronic urticaria, the authors propose more studies about its function in chronic urticaria. Distinguishing of

probable enzyme disturbances maybe help in future proposed treatments.

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CONFLICT OF INTEREST:
The authors declare that they are no conflict of interest regarding this manuscript.

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