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Master's Thesis in Biomedical Science

Role of Translationally Controlled Tumor Protein in Chronic Spontaneous Urticaria

Major in Molecular Medicine

Graduate School of Ajou University

Bastsetseg Ulambaya

Role of Translationally Controlled Tumor Protein in Chronic Spontaneous Urticaria

Hae-Sim Park, Advisor

I submit this thesis as the Master's thesis in 2019

February, 2019

Major in Molecular Medicine

Graduate School of Ajou University

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The Master's thesis thesis of Bastsetseg Ulambayar in Biomedical Science is hereby approved

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December, 24th, 2018

-ABSTRACT-

Background and objectives: Translationally controlled tumor protein (TCTP) known as histamine releasing factor (HRF) is widely expressed as housekeeping protein that has various intracellular and extracellular functions including its ability to activate mast cells. Extracellular TCTP has been found in nasal, bronchoalveolar lavage and skin blister fluids and exhibits the capacity to induce histamine release from basophils, suggesting that TCTP plays a role in allergic diseases including asthma, atopic dermatitis and urticaria. Dimerization of TCTP is critical for its cytokine-like activity, therefore, we hypothesized that the dimerized TCTP may have an important role by inducing release of histamine and other mediators, which drives the development of chronic spontaneous urticaria (CSU) symptoms.

Materials and Methods: Total 116 CSU patients and 70 healthy normal controls (NCs) were enrolled at Ajou University Medical Center, Suwon, South Korea. TCTP levels in the sera from CSU patients and NCs were measured by enzyme linked immunosorbent assay (ELISA) and compared according to other CSU-related clinical parameters. Basophil activation test (BAT) was performed by measuring CD203c expression on peripheral basophils from CSU patients. *In vitro*, β-hexosaminidase release from human mast cell line, LAD-2 mast cells was evaluated by stimulating monomeric

and dimeric TCTP. Non-reducing Western blot (WB) analysis was performed to detect dimeric TCTP from sera of CSU patients and NCs. Rapid dot-blot immunoassay was used for measuring the level of IgG to high affinity Fc epsilon receptor I alpha subunit (FcεRIα) level in sera from CSU patients and NCs.

Results: No significant differences were observed in serum levels of TCTP between CSU patients and NCs. However, the intensity of dimeric TCTP on WB was stronger in CSU patients than in NCs. Serum TCTP levels were higher in patients with severe CSU (51.1 ± 31.6 vs 44.6 ± 45.6 ng/ml, *P*=0.049) and those having IgG positivity to FcεRIα (60.4 ± 49.8 vs 42.5 ± 37.6 ng/ml, *P*=0.038). A significant positive correlation between TCTP and eosinophil cationic protein levels was also noted (Spearman's rho 0.341, *P*=0.001). Both basophil activation and mast cell degranulation were significantly increased after the stimulation with dimeric TCTP, but, not with monomic TCTP.

Conclusion: The ability of TCTP to activate basophils and mast cells is critically dependent on its dimerization status, suggesting that the inhibition of TCTP dimerization can be a therapeutic option for CSU. Association between TCTP levels and the presence of IgG to FcεRIα in CSU patients indicates that autoimmune mechanisms on CSU may be related to

dimerization of TCTP.

Keywords: Chronic spontaneous urticaria, translationally controlled tumor protein**,** basophils, mast cells, IgG autoantibody

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ABBREVIATIONS

CSU	chronic spontaneous urticaria
CU	chronic urticaria
TCTP	translationally controlled tumor protein
Ig	immunoglobulin
NCs	healthy controls
UAS	urticaria activity score
BSA	body surface area
BAT	basophil activation test
ASST	autologous serum skin test
ECP	eosinophil cationic protein
ATA	anti-thyroid autoantibody
FcεRIα	high affinity Fc epsilon receptor I alpha subunit
PBS	phosphate buffer saline
ELISA	enzyme-linked immunosorbent assay
HRP	horseradish peroxidase
FITC	fluorescein isothiocyanate
APC	allophycocyanin
HLA	human leukocyte antigen

LAD-2	laboratory of Allergic Disease 2
IQR	interquartile range
log	logarithm

I. INTRODUCTION

Chronic spontaneous urticaria (CSU) is a common skin disorder characterized by recurrent wheals and angioedema or both lasting for more than 6 weeks, with no definite extrinsic cause (Zuberbier et al., 2018). The lifetime prevalence of chronic urticaria (CU) is increasing ranging between 0.3-11.3% and hospitalization rate is increasing due to CU worldwide (Sánchez-Borges et al., 2012). The overall ratio of CU among all urticaria patients was 23.5% according to a 5-year nationwide epidemiological study in Korea (Lee et al., 2017). CSU is significantly associated with poor quality of life in patients (physiological and socioeconomic) and the majority of CSU patients requires prolonged pharmacological treatment to control their disease (Maurer et al., 2017). Mast cells and basophils play key roles in the pathogenesis of urticarial via inducing release of histamine and other vasoactive mediators or cytokines which result vasodilatation, plasma extravasation and recruitment of inflammatory cells into skin lesions (Zuberbier et al., 2018). Several mechanisms have been suggested to cause urticaria including immunoglobulin (Ig)E-mediated pathway, physical trigger inducible and autoimmunity, however, the etiology of CU is not fully understood yet.

Translationally controlled tumor protein (TCTP), known as histamine releasing factor, is a multifunctional protein widely expressed in almost all

eukaryotic organisms (MacDonald, 2012). Previous reports have shown that intracellular TCTP expression is dependent on calcium concentrations and microtubule binding protein (Gachet et al., 1999; Xu et al., 1999). In allergic disease, extracellular TCTP, which is observed in nasal, bronchoalveolar lavage and skin blister fluids could induce release of histamine from basophils (MacDonald et al., 1987). Recombinant TCTP has been found to enhance IL-4, IL-13 production and histamine release from IgE-sensitized human basophils (Schroeder et al., 1996, 1997). Also, other immune cells involved in allergic disease, such as eosinophils, B cells, bronchial epithelial cells and T cells, appear to be at least partly regulated by TCTP (Kang et al., 2001; Vonakis et al., 2003; Vonakis et al., 2008). While some studies have suggested that the role of TCTP in basophil activation is dependent on IgE, Wantke et al. demonstrated that TCTP exerts a stimulating effect via its specific receptor on the surface of human basophils and that it does not bind with IgE (Schroeder et al., 1997; Wantke et al., 1999; Kawakami et al., 2014a). Notwithstanding, a recent study reported that both serum TCTP levels and specific IgE antibody against TCTP were increased in CSU patients (Huang et al., 2017). However, the activating mechanism of TCTP on basophils or mast cells in CSU has not been fully understood. Kim et al. demonstrated dimerization of TCTP is critical for its cytokine-like activity in ovalbumin-induced airway inflammed mice and atopic

dermatitis-like NC/Nga mice *in vivo* (Kim et al., 2009; Jin et al., 2017). Based on previous findings, we aimed to investigate the role of TCTP in the pathogenesis of CSU by measuring TCTP levels in sera of CSU patients compared with healthy controls and to find whether monomeric or dimeric TCTP has different effect on basophil and mast cell activation.

II. MATERIALS AND METHODS

A. Clinical characteristics of the study subjects

In this study, we enrolled 70 normal healthy controls (NCs) and 116 CSU patients who had urticaria symptoms for at least 6 weeks (Table 1). The diagnosis of CSU in all patients were conducted in accordance with the European Academy of Allergy and Clinical Immunology/ Global Allergy and Asthma European Network/ European Dermatology Forum/ World Allergy Organization guideline (Zuberbier et al., 2018). Disease activity of CSU was evaluated by urticaria activity score (UAS), which evaluates wheal characteristics, pruritus status, and symptom duration. Total scores ranged from 0–15, with higher scores indicating higher disease activity (Ye et al., 2016).Wheals were scored according to quantity (0, none; 1, <20 wheals; 2, 20-50 wheals; 3, >50 wheals), distribution range (0, none; 1, <25% of body surface area (BSA); 2, 25-50% of BSA; 3, >50% of BSA), diameter (0, no wheals; 1, <1cm; 2, 1-3cm; and 3, >3 cm) and duration (0, no wheals; 1, <4 hours; 2, 4-12 hours; and 3, >12 hours). Pruritis was scored according to the intensity (0, no pruritus; 1, mild; 2, moderate; and 3, severe). Severe CSU patients were classified UAS evaluated as 13 and/or more. Refractory CSU was defined if

urticaria symptoms were not controlled by increasing dose of antihistamines up to 4 folds within 3 months (Trinh et al., 2016). For basophil activation test (BAT), peripheral blood was collected from 24 CSU patients for whom antihistamine medication was withdrawn for at least 5 days before collecting their blood samples. Each patient provided the informed consent.

B. Evaluation of clinical parameters of CSU patients

Autologous serum skin test (ASST) was performed and interpreted according to the guideline described previously (Zuberbier et al., 2018). Atopy status was defined if patients showed a positive result to at least one allergen on skin prick tests with common inhalant allergens (tree mixture, grass mixture, mugwort, ragweed, cat fur, dog fur, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, and *Alternaria spp*) (Bencard, Bretford, UK). Total IgE and eosinophil cationic protein (ECP) levels were measured by the ImmunoCAP system (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Anti-thyroid autoantibodies (ATA), including anti-thyroglobulin and thyroid microsomal antibodies, were detected by radioimmunoassay (BRAHMS Aktiengesellschaft, Hennigsdorf, Germany). To measure IgG to high affinity Fc epsilon receptor I alpha subunit (FcεRIα) we performed rapid dot-blot immunoassay as described previously (Lee et al., 2014). Extracellular fragments of FcεRIα protein (30 μg/ml in PBS) were dotted onto nitrocellulose

membranes and air dried. The membranes were blocked with 0.15% casein in PBS for 10 minutes and washed. After washing, membranes were incubated with diluted (1:4 casein) patient serum for 10 minutes. Bound IgG was detected using peroxidase conjugated goat anti-human IgG antibody (Zymed, CA, USA). The reaction was developed using a chemiluminescent substrate solution (Applied Biosystems, Bedford, MA, USA), and signals were analyzed by exposure to X-ray film. Quantitative analysis of the spots was conducted with a video densitometer using Kodak analyzer software. A cut-off value for IgG to FcεRIα positivity was determined as the mean value plus two standard deviations in healthy controls which determined as 200 (intensity) in this study.

C. Measurement of TCTP in serum

Serum TCTP level was measured by enzyme-linked immunosorbent assay (ELISA) (MyBioSource Ltd, San Diego, CA, USA) according to the manufacturer's instructions. 50ul of undiluted serum samples and standards were loaded into each well followed by adding 100ul of HRP-conjugate reagent and incubated for 60 minutes at 37C. Plates were washed by 1x washing buffer 4 times. Then, the plates were incubated with 100ul of chromogen A and B solution mix for 15 minutes at 37C in dark. Reaction was stopped by stop solution and optical density (OD) was read in 450nm using spectrophotometry within 15 minutes after stop solution. Both intra-assay and inter-assay

coefficients of variation (%) were less than 15%, and the sensitivity of the kit was 1 ng/ml.

D. Purification of recombinant TCTP protein

Recombinant monomeric and dimeric TCTP used for stimulation was purified in an *E. coli* system, as described previously (Kim et al., 2009). The purity of the proteins was 85%–98%. Briefly, pRSET A/TCTP or Del-N11 TCTP was transformed to *E. coli* strain BL21 (DE3)pLysS, and the cells were grown in Luria-Bertani medium containing 100 μg/ml of ampicillin and 34 μg/ml of chloramphenicol. The pre-culture medium was diluted and cultured until reaching an OD_{600} of 0.6–0.8. IPTG was added to induce recombinant protein, and then, the cell pellets were stored at -70℃ or used directly. Cell pellets were disrupted by sonication, and supernatants containing soluble proteins were purified with HisPur^{TM} Cobalt Resin (Thermo-Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Subsequently, eluted proteins were loaded onto a HiTrap Q HP column (GE Healthcare, Princeton, NJ, USA), which was equilibrated with buffer A (20 mM Tris, 1 mM EDTA, 50 mM NaCl; pH 7.4). Proteins were separated by AKTA FPLC systems (GE Healthcare, Princeton, NJ, USA) and eluted with buffer B (20 mM Tris, 1 mM EDTA, 1 M NaCl; pH 7.4) at a constant flow rate of 1 ml/min. The eluted fractions were screened by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

PAGE) and Coomassie blue staining. Samples containing monomeric or dimeric TCTP were collected, desalted with PBS, concentrated using Vivaspin 500 (Sartorius, Goettingen, Germany), and stored at -70℃ until ready for use.

E. Western blotting for detecting the dimerized TCTP in sera from CSU patients and NCs

Serum was diluted 2X Laemmli sample buffer and boiled for 5 min. After centrifugation, the supernatants were separated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (GE Healthcare, Princeton, NJ, USA). Membranes were blocked for 1 h at room temperature in 5% BSA/TBS-T. The primary antibody against TCTP (Santa Cruz Biotechnology, Dallas, TX, USA) was diluted 1:1000 with blocking buffer and incubated overnight at 4℃ with the membrane. After washing three times with TBS-T, the membranes were incubated with a goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugated secondary antibody (Bio-Rad Laboratories Inc., Hercules, CA, USA) for 1 h at room temperature. The membranes were washed again, visualized with ECL plus reagent (SurModics Inc., Eden Prairie, MN, USA), and detected by a LAS-3000 CCD camera (Fuji Film, Tokyo, Japan).

F. Basophil activation test

The surface expression of CD203c in peripheral blood basophils was measured by flow cytometry. Whole blood was collected in acid citrate dextrose tubes, and

red blood cells were lysed with red blood cell lysis buffer (0.154 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2–7.4). After washing with PBS, resuspended cells were stimulated by recombinant TCTP protein at different concentrations (0.08, 0.16, 0.32, 0.65 uM) for 2 hours at 37℃. Based on molecular weight (monomer 26.5 kDa and dimer 50 kDa), stimulating concentrations were adjusted to 2.1, 4.2, 8.5, and 17.2 ug/ml and 4, 8, 16, and 32.5 ug/ml, respectively. After incubation, the cells were washed with PBS and stained by phycoerythrin-conjugated anti-human CD203c (Beckman Coulter, Marseille, France), fluorescein isothiocyanate (FITC)-conjugated anti-human CD123 (BD PharMingen, San Jose, CA, USA), and allophycocyanin-conjugated anti-human human leukocyte antigen (HLA)-DR (BD PharMingen, San Jose, CA, USA) or isotype-matched controls on ice in the dark for 30 min. After washing once with PBS, the cells were analyzed on a FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA, USA). Basophils were identified as $CD123^+$ $HLA\text{-}DR^-$populations. The percentage of cells expressing CD203c was evaluated by BD FACSDIVA software.

G. Measurement of ß-hexosaminidase release from LAD-2 cells

Laboratory of Allergic Disease 2 (LAD-2) mast cells were kindly provided by Dr. Arnold Kirshenbaum (National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA). The release of ß-hexosaminidase from LAD-2 cells was

measured as previously described (Kuehn et al., 2010). Cells were treated by monomeric and dimeric *TCTP* (1 and 2 uM) with or without simultaneous biotinylated-IgE (100 ng/mL, BioPorto Diagnostics, Hellerup, Denmark) overnight sensitization, followed by 30 min of stimulation with streptavidin peroxidase (100 ng/mL) or calcium ionophore A23187 (1 uM) in HEPES buffer (10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM $Na_2HPO_4 \cdot 7H_2O$, 5.6 mM glucose, 1.8mM $CaCl_2 \cdot 2H_2O$, 1.3mM $MgSO_4 \cdot 7H_2O$, 0.04% bovine serum albumin (BSA) at 37℃. Total ß-hexosaminidase was obtained by lysing LAD-2 cells in 0.1% Triton X-100 in PBS. The supernatants were collected and incubated with an equal volume of p-nitrophenyl N-acetyl-β-D-glucosamide in citrate buffer (pH = 4.5, 40 mM citric acid/20 mM Na_2HPO_4*$7H_20$) for 30 min. The reactions were stopped by adding 0.4 M glycine buffer (pH=10.7), and signals were read at 405 nM in spectrophotometry. The percentage of degranulation was calculated as $100 \times (OD_{stimulated} - OD_{non\text{-}stimulated})/OD_{total\ lysate}$.

H. Statistical analysis

Statistical analyses were performed using IBM SPSS version 20 for Windows (SPSS Inc., Chicago, IL, USA). Data are presented as a mean ± standard deviation or as a median (interquartile range [IQR]) value when variables were not normally distributed. A multiple regression model was applied to determine the variables most predictive of circulating TCTP concentrations as logarithm

(log)-transformed values among those known to be associated with TCTP, including age, gender, atopy status, IgG to FcεRIα positivity, disease severity, and serum ECP level. Pearson correlation analysis identified associations among the continuous parameters. *P* values < .05 were considered indicative of statistical significance.

III. RESULTS

A. Clinical characteristics of study subjects

The clinical characteristics of study subjects are summarized in Table 1. Age and sex-matched 116 CSU patients (74.4% women) with mean age of 39.8±10.5 years and 70 NCs (74.2% women) with mean age of 39.9± 13.4 years were recruited (Table 1). Median range of urticaria duration was 4 months (IQR, 2.0-12.0 months) with 50.9% atopy rate. The median level of total IgE was 117kU/L (IQR, 55-234 kU/L) and median ECP level was 17.4 ug/L (IQR, 10.0-31.1 ug/L). The median UAS was 10 (IQR, 7-13) and 33.6% of them were classified into severe urticaria. 53.4% of CSU patients exhibited positive result in ASST and 23.3% had positive responses to ATA tests. 25.0% of CSU patients had positive IgG to FcεRIα autoantibody response. 37.1% of them had refractory urticaria who were not controlled under the treatment of second generation antihistamines with up to 4 fold increased dose.

B. Serum TCTP levels in CSU patients and NCs

Median levels of serum TCTP in CSU patients (34.4 [2.93–242.9]) were not different from those in NCs (37.5 ng/ml [1.19–195.7], Figure 1). We attempted to differentiate dimeric from monomeric TCTP in the sera from CSU patients and NCs using non-reducing Western blot analysis. In doings so, we identified separate bands of higher and lower molecular weights. The bands located in a

position of higher molecular weight were regarded as dimerized or oligomerized TCTP, which are conformationally modified. Relatively higher signal intensity for dimeric bands was noted for the sera from 3 patients with CSU, while the sera from healthy controls (NCs) showed higher signal intensity for monomeric TCTP bands (Figure 2). However, we could not quantitatively analyze dimeric and monomeric TCTP amounts.

C. Correlation of serum TCTP levels with clinical characteristics of chronic spontaneous urticaria

The median level of serum TCTP was higher in atopic CSU patients than in non-atopic patients (40.5 [25.5–65.0] vs 29.0 [12.0–61.0] ng/ml, respectively, *P* = 0.019, Figure 3A). However, no difference in TCTP levels was observed regarding to ASST positivity (38.8 [22.1–65.0] vs 30.4 [19.1–56.3] ng/ml, respectively, *P* = 0.429). Patients with positive IgG to FcεRIα autoantibody had significantly higher serum TCTP levels than patients with negative IgG to FcεRIα autoantibody (50.1 [25.4–73.7] vs 31.4 [19.5–64.1] ng/ml, respectively, *P* = 0.03, Figure 3B). Serum TCTP levels did not differ according to the presence of anti-thyroid antibody, anti-nuclear antibody, or angioedema among CSU patients. Regarding therapeutic responses to antihistamine, serum TCTP levels tended to be higher in refractory CSU patients than in patients responsive to antihistamines, although statistical significance was not observed (40.5 [22.2–77.1] vs 31.0 [19.4–56.0] ng/ml, *P* = 0.141). A significant difference in serum TCTP levels was found between severe and non-severe CSU patients, however (41.1 [28.0–70.4] vs 30.2 [19.4–54.3] ng/ml, *P* = 0.049, Figure 3C). Log-transformed serum TCTP levels were negatively correlated with age (r = -0.244; *P* = 0.008), whereas they had a positive correlation with log-transformed ECP in CSU patients (r = 0.346, *P* = 0.002). No significant correlation was found

between TCTP and total IgE levels in CSU patients (data not shown). We performed multiple regression analysis to identify predictors of serum TCTP levels in patients with CSU. Significant positive correlations were noted between log-transformed TCTP in sera from CSU patients and both severe CSU (defined as a UAS ≥13; B = 0.150, *P* = 0.043) and log-transformed ECP levels (B = 0.265, *P* = 0.002) after adjustment for confounders, including age, gender, atopy, and IgG positivity to FcεRIα (Table 2).

Table 1. Clinical characteristics of the study subjects

Characteristics	CSU (n = 116)	NC (n = 70)
Age	39.8 ± 10.5	39.9 ± 13.4
Female	86 (74.1%)	52 (74.2%)
Urticaria duration (month)	4.0 (2.0–12.0)	NA
Total IgE (kU/L)	117 (55–234)	NA
Atopy rate	59 (50.9%)	NA
ASST positivity	62 (53.4%)	NA
ATA positivity	27 (23.3%)	NA
IgG to FcεRIα positivity	29 (25.0%)	1 (1.6%)
UAS (0–15)	10 (7–13)	NA
Severe CSU	39 (33.6%)	NA
H1RA refractoriness	43 (37.1%)	NA
ECP (μg/L)	17.4 (10.0–31.1)	NA

ASST, autologous serum skin test; ATA, antithyroid antibody including anti-microsomal and anti-thyroglobulin antibodies; CSU, chronic spontaneous urticaria; ECP, eosinophil cationic protein; FcεRIα, high affinity Fc epsilon RI alpha subunit; H1RA, histamine receptor 1 antagonist; IgE, immunoglobulin E; IgG, immunoglobulin G; NC, healthy control; UAS, urticaria activity score; NA, not available.

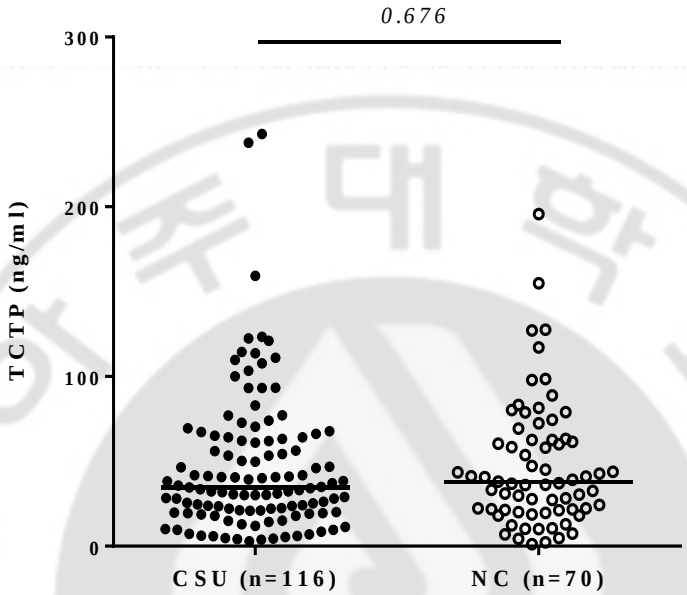

Figure 1. Comparison of serum TCTP levels between CSU patients and NCs. Serum TCTP levels were measured by ELISA. *P* value was calculated by Mann-Whitney U test. CSU, chronic spontaneous urticarial; NC, healthy control; TCTP, translationally controlled tumor protein

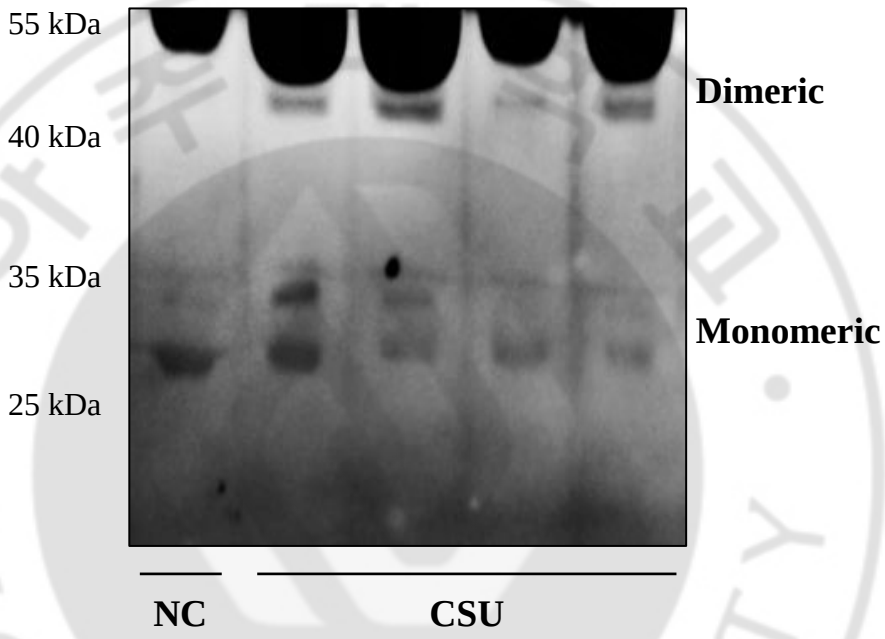

Figure 2. Detection of the dimeric TCTP in sera of the study subjects. The sera from 4 CSU patients and one NC were analyzed by non-reducing SDS-PAGE and Western blotting. Upper bands hypothesized to be dimeric TCTP with higher molecular weight (50kDa) and lower bands exhibiting monomeric form of TCTP (26-27kDa). CSU, chronic spontaneous urticarial; NC, healthy control.

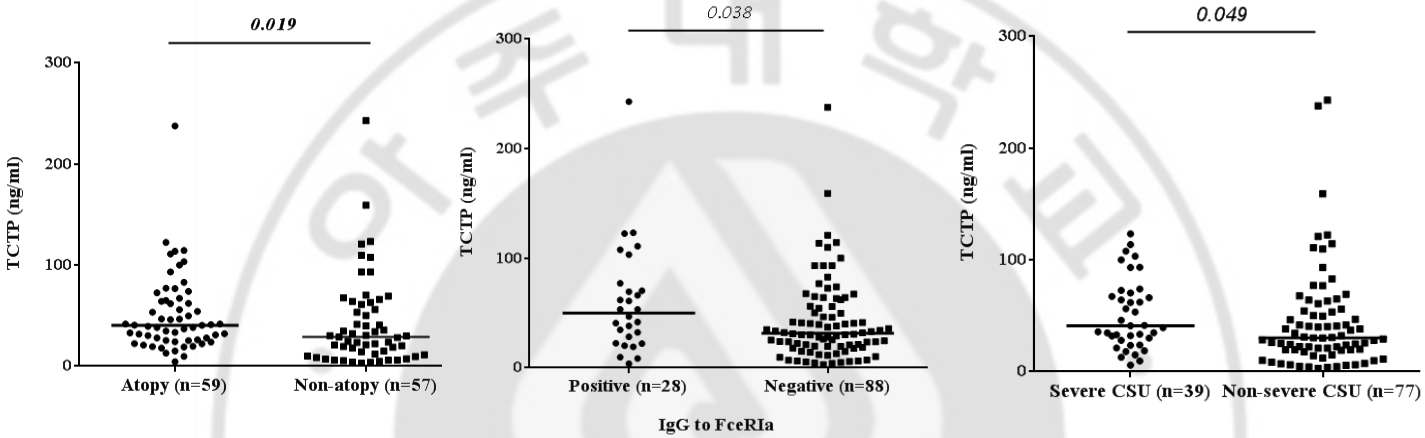

Figure 3. Comparison of serum TCTP levels according to the prevalence of atopy (A), IgG to FcεRIα positivity (B) and urticaria severity (C) in CSU patients.

Table 2. Predictors of log-transformed serum TCTP levels in patients with CSU

Predictors	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Age	-0.009 (-0.016, -0.003)	0.007	-0.004 (-0.011, 0.003)	0.241
Male gender	-0.001 (-0.165, 0.162)	0.987	-0.046 (-0.229, 0.136)	0.619
Atopy	0.192 (0.053, 0.331)	0.007	0.138 (-0.014, 0.291)	0.075
Positivity of IgG to FcεRIα	0.167 (0.005, 0.330)	0.044	0.143 (-0.027, 0.313)	0.098
Severe CSU	0.183 (0.036, 0.330)	0.015	0.175 (0.019, 0.332)	0.028
Log [ECP (μg/L)]	0.373 (0.183, 0.563)	< .001	0.302 (0.117, 0.488)	0.001

Abbreviations: CI, confidence interval; CSU, chronic spontaneous urticaria; ECP, eosinophil cationic protein; FcεRIα, high affinity Fc epsilon RI alpha subunit; IgG, ; NC, healthy control; OR, odds ratio; TCTP, Translationally controlled tumor protein.

D. Basophil activation upon stimulation of monomeric and dimeric TCTPs

To investigate whether monomeric and dimeric TCTPs have the capability to activate basophils, we performed basophil activation tests in a total of 24 patients with CSU, measuring CD203c expression on the surface of basophils, since it had higher sensitivity than CD63 and was a predictor of severe CU in our previous report (Ye et al., 2014). The expression of CD203c on the surface of basophils was significantly increased upon stimulation with dimeric TCTP in a dose-dependent manner (P = 0.01, Figure 4), while monomeric TCTP had no effect on CD203c expression.

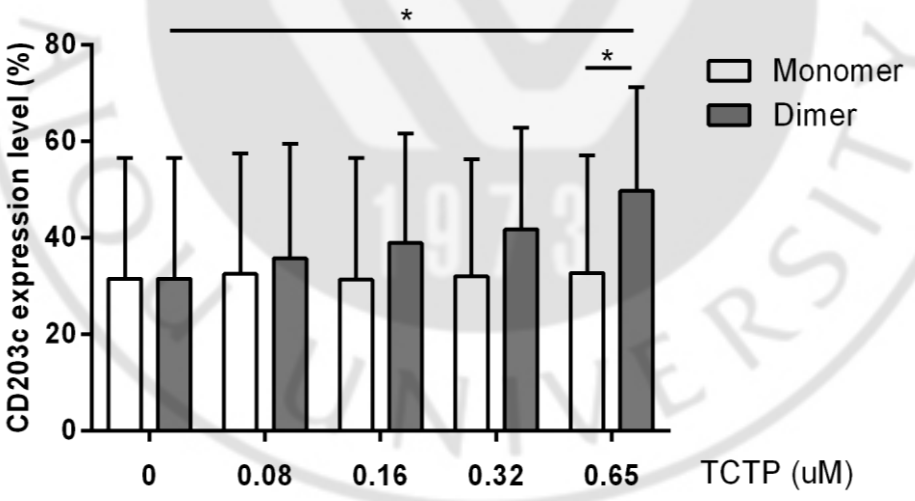

Figure 4. Effects of TCTP on basophil activation. Basophils from 24 CSU

subjects were treated simultaneously with monomeric and dimeric TCTP. Anti-IgE and calcium ionophore Basophil activation was measured by surface CD203c expression using flow cytometry. A statistically significant difference was defined as that with a two-tailed. *P value < .05, **P value < .01, ns, no significant.

E. β-hexosaminidase release from LAD2 cells upon stimulation with TCTP

To confirm the effect of TCTP on human LAD2 cell degranulation, cells were treated with monomeric and dimeric TCTP with or without IgE sensitization. Dimeric TCTP induced a significantly higher level of ß-hexosaminidase release, compared with untreated cells and cells treated with an equal concentration of monomeric TCTP (P < 0.001, Figure 5). With or without IgE sensitization, there was no significant difference in beta-hexosaminidase release from LAD2 cells upon either monomeric or dimeric TCTP stimulation.

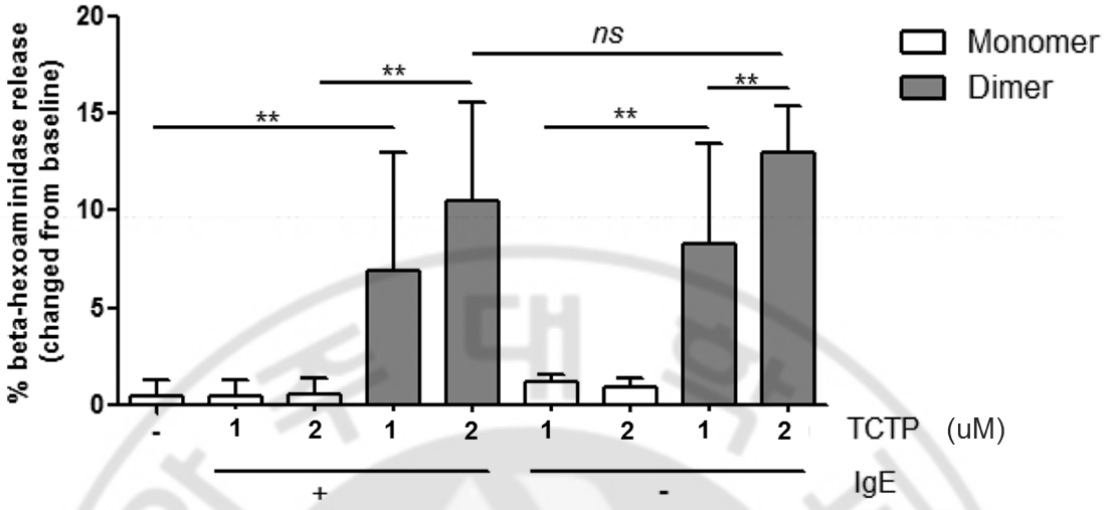

Figure 5. Effects of TCTP on degranulation of LAD-2 cells. Mast cell degranulation was investigated using the β-hexosaminidase release test. The positive control comprised LAD-2 cells sensitized with IgE and stimulated with streptavidin peroxidase (degranulation rate 56.6±4.2%) or only stimulated with calcium ionophore (degranulation rate 65.6±5.4%). A statistically significant difference was defined as that with a two-tailed. **P* value < 0.05, ***P* value < 0.01; ns, no significant.

IV. DISCUSSION

CSU is mast cell driven disease which contributed by several mechanism including infection, coagulation cascade, food intolerance and autoimmunity (Zuberbier et al., 2018). TCTP is expressed in all eukaryotic organisms and tissues that involved in apoptosis, cell cycle/proliferation and cancer (Bommer and Thiele, 2004); however, expression level is various among cell or tissue types. TCTP has been reported to be involved in the pathogenic mechanisms of asthma, immune responses to parasitic infections, atopic dermatitis, and CU (Kashiwakura et al., 2012; Kawakami et al., 2014b; Huang et al., 2017; Jin et al., 2017). TCTP known to form to dimers via disulphide bond revealed to have cytokine-like activity that contains two cysteine residues and Cys172 is suggested to be site of disulphide bond (Kim et al., 2009). While TCTP exist in normal conditions in living body, sturctural modification may occur in an allergic environment rich in oxidants or endogenous and exogenous proteases that able to induce dimerization of TCTP (Kim et al., 2009). Mast cells release proteases, including tryptase and chymase, during degranulation, and studies have shown that serum tryptase levels are increased in CSU and associated with symptom occurrence and increased corticosteroid requirement (Ferrer et al., 2010; Siles et al., 2013). In addition, an imbalance in reactive oxygen species

and antioxidants has been found to worsen allergic inflammation including CSU (Okayama, 2005; Hosoki et al., 2014).

The activation of basophils and mast cells is assumed to be associated with an interaction between TCTP and IgG to FcεRIα (ref). The crystal structure of human TCTP comprises a disulphide-linked dimer that confers an IgE binding site for degranulating mast cells (Doré et al., 2018). In the present study, TCTP were significantly higher in sera of patients positive for IgG autoantibody to FcεRIα than those with negative IgG autoantibody to FcεRIα. Recent study detected higher levels of serum specific IgE antibody against TCTP in CSU patients which was supportive to our study (Huang et al., 2017). TCTP has been found to play a role in immediate hypersensitivity by interacting with the Fab region of immunoglobulin and in mast cell activation in an Fc receptor-dependent manner (Kashiwakura et al., 2012). Moreover, previous studies demonstrated that different signal transduction events are initiated by TCTP on basophils in TCTP responders and non-responders (Vonakis et al., 2003). Therein, TCTP was found to prime basophils through FcεRIα or another adapter molecule that is distinct from IL-3 in TCTP non-responders, whereas TCTP induced secretion directly in TCTP responders (Vonakis et al., 2003). Based on our findings that TCTP could induce degranulation of peripheral basophils from CSU patients, we suspect that the dimerizing process might occur less in TCTP

non-responders than in TCTP responders inducing greater degranulation, despite similar total TCTP levels and Ig-mediated autoimmunity may involve in this view.

Kashiwakura et al. reported that active TCTP interacts with immunoglobilins by its NH_2-terminal 19-residue peptide and H3 region with Fab region and crosslinking IgE to induce degranulation of mast cell via FcεRIα receptor (Kashiwakura et al., 2012). In this study, we demonsrated that dimeric TCTP activates both basophils and mast cells, but nor monomeric one which may exhibiting role of TCTP in development of CSU symptom. However, we found no significant difference in mast cell degranulation upon dimeric TCTP stimulation with or without IgE sensitization and to find mechanism are remaining in further study. To eliminate the controversy regarding whether TCTP dependently or independently activates mast cells via IgE (Wantke et al., 1999), we found dimeric TCTP can activate mast cells independently from IgE, but still exact activating pathway needs to be elucidated. A recent study demonstrated that dimeric TCTP induces IL-8 production in bronchial epithelial cells via the activation of adaptor protein 1 by phosphorylating MAPKs and the NF-kB pathway, which are also involved in mast cell activation, in a LAT (linker of activation of T cell)-dependent manner, thereby generating proinflammatory cytokines(Gilfillan and Tkaczyk, 2006; Lee and Lee, 2018).

Meanwhile, FcεRIα aggregation has been found to lead to LAT phosphorylation through the phosphorylation of Syk, Akt, MEK, and ERK upon exposure to TCTP in TCTP-responders (Vonakis et al., 2003; Gilfillan and Tkaczyk, 2006). Consistent with these results, we speculate that dimeric TCTP may induce basophils/mast cells degranulation via the similar signaling pathway involved.

The release of proteases, leukotrienes, lipid mediators, and histamine from mast cells contributes not only to tissue inflammation but also to recruitment of eosinophils (Shakoory et al., 2004). Mast cells are also sources of IL-5, TNFα, and eotaxin, all of which elicit eosinophil recruitment and stimulation in urticarial lesions (Lampinen et al., 2004; Shakoory et al., 2004). ECP, a typical marker of eosinophil activation, is known to be an indicator of disease severity in CU patients (Lorenzo et al., 1996). Indeed, in the present study, we found that serum ECP levels and urticaria severity were significant predictors of TCTP levels in patients with CSU. ECP has been shown to be concurrently expressed with vascular endothelial growth factor (VEGF) in the lesional skin of CSU patients, and increases in VEGF have been observed in the plasma of CSU patients in association with urticaria severity (Tedeschi et al., 2009). Based on those reports, we hypothesize that there may be a link between TCTP and ECP or basophil/mast cell-induced eosinophil activation leading to more severe urticaria.

V. CONCLUSION

In conclusion, the current study demonstrated that dimerization of TCTP is important for activation of basophils and mast cells in CSU. Atopic status and the presence of IgG autoantibody to FcεRIα were found to be associated with enhanced dimerization of TCTP. In light of these results, we deemed that dimeric TCTP induces basophil activation and mast cell degranulation leading to eosinophil activation and increased urticaria severity in CSU. Accordingly, inhibition of TCTP dimerization may be a potential therapeutic option for CSU patients.

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-국문요약-

만성 자발성 두드러기의 병인기전에서 TCTP의 역할

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배경 및 목적: TCTP는 히스타민 분비인자로도 알려진 물질로서 비만세포의 활성화를 포함한 다양한 기능을 가진 단백질이다. 세포외 TCTP는 비강 및 폐포 세척액, 피부 수포액에서 발견되었고 이는 호염기구로부터 히스타민을 분비하게 함으로써 TCTP가 알레르기 질환의 병인기전에 관여함을 알 수 있었다. TCTP의 이량체화(dimerization)는 사이토카인과 같은 활성을 나타낸다는 보고가 있어, 이량체화된 TCTP가 만성 자발성 두드러기 증상을 일으키는 히스타민과 그 외의 매개물질의 분비를 유도하는데 중요한 역할을 할 것이라 가정하였다.

방법: 116명의 만성 자발성 두드러기 환자 및 70명의 건강한 정상 대조군의 혈청 내 TCTP를 ELISA를 이용하여 측정하여, 만성 자발성 두드러기의 임상지표들과 비교 분석하였다. 만성 자발성 두드러기 환자군의 말초 호염기구에

서 발현되는 CD203c를 측정하여 호염기구 활성화를 평가하였다. 단량체 및 이량체 TCTP를 자극하여 비만 세포주로부터 분비되는 β-hexosaminidase를 측정하였다. Non-reducing Western blot 분석을 통해 만성 자발성 두드러기 환자군 및 정상 대조군의 혈청에서 이량체 TCTP를 측정하였다. 또한, Rapid dot-blot immunoassay 를 이용하여 만성 자발성 두드러기 환자 및 정상 대조군의 혈청에서 고친화성 FcεRIα에 결합된 면역글로불린 G를 측정하였다.

결과: 만성 자발성 두드러기 환자 및 정상 대조군에서 혈청 TCTP 수치의 유의한 차이는 관찰되지 않았다. 그러나, Western blot에서 이량체 TCTP의 강도는 정상 대조군에 비해 만성 자발성 두드러기 환자군에서 더 강하게 나타났다. 혈청 TCTP 수치는 중증 만성 자발성 두드러기 환자 (51.1 ± 31.6 vs 44.6 ± 45.6 ng/ml, *P*=0.049)와 FcεRIα에 대한 면역글로불린 G 양성을 나타내는 환자 (60.4 ± 49.8 vs 42.5 ± 37.6 ng/ml, *P*=0.038)에서 더 높게 측정되었다. TCTP는 혈청 eosinophil cationic protein 와 유의한 양의 상관관계를 보였다 (Spearman's rho 0.341, *P*=0.001). 이량체 TCTP로 자극하였을 때 호염기구의 활성 및 비만

세포주의 탈과립이 모두 증가하였으나, 단량체 TCTP를 자극하였을 때는 이러한 결과가 관찰되지 않았다.

결론: TCTP가 호염기구와 비만세포를 활성화시킬 수 있는 것은 그것의 이량체화 여부에 달려있음을 확인하였다. 따라서, TCTP의 이량체화를 억제하면, 만성 자발성 두드러기의 새로운 치료방법이 될 수 있음을 제시하였다.