Increased Level of Basophil CD203c Expression Predicts Severe Chronic Urticaria

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INTRODUCTION

Chronic urticaria (CU) is a common skin disorder characterized by persistent or recurrent itchy wheals that last for at least 6 weeks. The cause of CU is heterogeneous, as many triggering and aggravating factors are involved in its pathogenesis, including physical stimuli, drugs, infections, and autoimmune mechanisms. The clinical phenotypes, severity, and prognosis of CU differ from patient to patient, so that management strategies need to be personalized. Recent guideline suggests a stepwise treatment based on effective symptomatic control with non-sedating antihistamines up to four folds of dosage, which is often efficacious but may not reach well-controlled state in 5% to as many as 50% of CU patients (1, 2). The growing evidence that autoimmunity and altered basophil functions are involved in the pathogenesis of CU has accelerated the introduction of immunomodulating agents for refractory patients (1, 3-5). Interestingly, the need for safe and reliable in vitro diagnostic method to link each mechanism with reasonable therapeutic approaches has increased. Although elevated plasma levels of metalloproteinase (MMP) 9, interleukin (IL) 6 and 18 and D-dimer have been reported as possible biomarkers in association with CU severity, those are not specific for the pathogenesis of CU (6-9). Approximately half of CU patients are characterized by an auto-reactive state with autoantibodies specific for the high-affinity IgE receptor (FceR1α) and IgE itself (10). Although both the sensitivity and specificity of the autologous serum skin test (ASST) are limited, it has been recommended for defining a subgroup with autoreactive CU (10). However, the association between ASST positivity and disease activity is inconsistent.

Basophils are rare granulocytes that resemble tissue mast cells, in that they express FceR1α and release histamine and chemical mediators (11). In active CU patients, basophils infiltrate the urticarial wheals and there is relative basopenia (12-14). While peripheral basophils do not represent cutaneous basophils completely, basophil histamine release (BHR) using healthy donor basophils has been used to evaluate autoreactive CU (15, 16). The basophil activation test (BAT) using flow cytometry has been applied as an in vitro diagnostic method for various allergic diseases (17). CD203c (ectonucleotide pyrophosphatase [E-NPP3]) is a surface marker observed uniquely on basophils and mast cells that is upregulated by anti-IgE antibody and allergens (18). For predicting causative allergens in patients with food, insect sting and drug allergy, the measurement of basophil CD203c expression induced by various kinds of allergens is reported to be useful (17, 19).

To-date, few studies have examined the phenotypes and characteristics of basophils in CU patients (14, 20, 21). Although the expression of the basophil activation markers CD63 and CD69 is increased in CU patients (22), there is to our knowledge no

Increased FceR1α expression with upregulated CD203c expression on peripheral basophils is seen in patients with chronic urticaria (CU). However, there has been no published report on the association between CD203c expression level and clinical disease activity in CU patients. To investigate whether the increase of basophil activation is associated with the disease activity of CU, we measured basophil CD203c expression using a tricolor flow cytometric method in 82 CU patients and 21 normal controls. The relationship between the percentage of CD203c-expressing basophils and clinical parameters was analyzed. The mean basophil CD203c expression was significantly higher in CU patients than in healthy controls (57.5% vs 11.6%, P < 0.001). The basophil CD203c expression in severe CU patients was significantly higher than in non-severe CU (66.5% ± 23.3% vs 54.0% ± 23.3%, P = 0.033). Multiple logistic regression analysis indicated that both ≥ 72% basophil CD203c expression and urticaria activity score (UAS) ≥ 13 were significant predictors of severe CU (P = 0.005 and P = 0.032, respectively). These findings suggest that the quantification of basophil activation with CD203c at baseline may be used as a potential predictor of severe CU requiring another treatment option beyond antihistamines.

Keywords: Chronic Urticaria; Basophil Activation; CD203c Protein, Human
MATERIALS AND METHODS

Subjects
This was a hospital-based, cross-sectional study including 21 healthy controls (NC) and 82 CU patients who had current urticarial symptoms almost daily for at least 6 weeks. CU disease activity was assessed using the urticaria activity score (UAS), which combines pruritus and four characteristics of wheals, including number, distribution range, mean diameter, and duration in total score range, 0-15, with higher scores indicating higher disease activity (23). After 3 months from the enrollment, we reviewed medical records and calculated the medication requirement of each CU patient enrolled. Those patients requiring more than a third level of medication according to the guidelines or systemic steroids to control urticaria were classified as severe CU (10).

Autologous serum skin test and measurements of total IgE and anti-thyroid antibody
Antihistamines and corticosteroids were withdrawn at least 1 week before blood sampling. Intradermal autologous serum skin test (ASST) was performed following the method by EAALEN task force consensus report (10). Serum-induced wheal of diameter greater by at least 1.5 mm than that of a control induced by saline at 30 min was accepted as positive. The levels of total IgE were measured by the ImmunoCAP system (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer’s instructions. Anti-thyroglobulin and thyroid microsomal antibodies were detected by radioimmunoassay (BRAHMS Aktiengesellschaft, Hennigsdorf, Germany).

Sampling and assays
The basophil CD203c expression was measured by flow cytometry. Subjects did not take any medication yet or stopped medication for at least one week before blood sampling. Whole blood was collected in ethylene diamine tetra-acetic acid (EDTA) tubes and red blood cells (RBCs) were lysed with RBC lysis buffer (0.154 M NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.2-7.4). After washing with phosphate-buffered saline (PBS), the resuspended cells were stained with phycoerythrin (PE)-conjugated antihuman CD203c (Beckman Coulter, Marseille, France), fluorescein isothiocyanate (FITC)-conjugated antihuman CD123 (BD PharMingen, San Jose, CA, USA), and allophycocyanin (APC)-conjugated antihuman human leukocyte antigen (HLA)-DR (BD PharMingen), 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). The cells were gated initially based on the dot plot defined by the forward and side scatter, and then a second gate of high FITC-CD123+ cells and low APC-HLA-DR was defined to select the basophil population, analyzing at least 500 basophils. The percent CD203c expression was defined as the percentage of basophils expressing more CD203c than the critical point located at $10^4 < x < 10^6$ in the histogram, which was about 10% of the basophils incubated with buffer only in the normal control. This was determined by defining region M1 on the histogram analysis.

Statistical analysis
The receiver-operating characteristic (ROC) curve was used to determine the optimal cutoff of the percentage of CD203c-expressing basophils used to diagnose CU, and the area under the curve (AUC) with a 95% confidence interval (CI) was computed. Fisher’s test and the Mann-Whitney U-test were used to compare the clinical characteristics of CU, including the positive rate of atopy, autoantibodies, and serum total IgE level. Spearman’s rho was used for the correlation analysis. Logistic regression was used to examine the effect of various factors on the optimal cut-off of basophil CD203c expression and the risk for severe CU. $P$ values $< 0.05$ were considered to indicate statistical significance. All statistical analyses were performed using SPSS for Windows (ver. 12; SPSS, Chicago, IL, USA).

Ethics statement
All of the subjects gave written informed consent at the time of enrollment, and the study was approved by the institutional review board of Ajou University Hospital (AJIRB-GEN-GEN-09-140).

RESULTS

The positive ASST, anti-thyroid autoantibody, and atopy rates were 37.8%, 19.0%, and 39% in the CU patients, respectively (Table 1). The mean white blood cell counts and differential for basophils in peripheral blood were $7,297.5 \pm 2,248.8/\mu L$ and

| Table 1. Clinical characteristics of study subjects |
|-----------------------------------------------|
| Characteristics | CU (n = 82) | NC (n = 21) | $P$ value |
| No. of men (%) | 32 (39.0) | 11 (52.4) | 0.324* |
| Age (yr) | 39.2 ± 13.6 | 29.9 ± 7.0 | 0.003† |
| No. of men (%) | 32 (39.0%) | 4 (19.0%) | 0.124* |
| ASST positivity | 31 (37.8%) | 3 (14.3%) | 0.066* |
| No. of men (%) | 15 (19.0%) | NA | |
| Log[total IgE] | 2.06 ± 0.5 | NA | |
| Baseline expression of CD203c | 57.5 ± 23.9 | 11.6 ± 8.0 | < 0.001 |

*Fisher’s exact test, †Mann-Whitney test. ASST, autologous serum skin test; Log[total IgE], log-transformed serum total IgE levels; CU, chronic urticarial; NC, normal control; Ab, antibody; NA, not assessed.
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0.5% ± 0.2%, respectively. The 23 patients requiring at least third-level treatment according to the guidelines were classified as severe CU. The mean UAS was significantly higher in severe CU (11.5 ± 2.8 vs 9.8 ± 3.2, P = 0.037), while their ASST response, positive anti-thyroid autoantibody and atopy rates did not differ from non-severe CU patients.

In CU patients, the mean percentage of basophil CD203c expression at baseline was 57.5% ± 23.9%, compared with an average activation of 11.6% ± 8.0% of basophils in normal controls (P < 0.001, Fig. 1). There was no significant difference in the baseline basophil CD203c expression according to gender or ASST, antithyroid autoantibodies, or atopy positivity rates among the CU patients. The leukocyte differential of basophils in peripheral blood was significantly correlated with CD203c expression (r = -0.220, P = 0.049) and serum total IgE level (r = -0.237, P = 0.033).

Taking a cutoff of 21.3% CD203c+ basophils as the positivity threshold yielded a sensitivity of 91.4% and a specificity of 90.5% with an AUC value of 0.9663 (95% CI, 0.931-0.995, P < 0.001).

After adjusting for age, gender, and atopy, the logistic regression analysis revealed that the odds ratio for a 21.3% basophil CD203c expression at baseline for detecting CU patients was 166.04 (95% CI, 19.804-1392.071, P < 0.001).

Severe CU patients had significantly higher CD203c expression than the non-severe group (66.5% ± 23.3% vs 54.0% ± 23.3%, P = 0.033). Using the ROC analysis of CD203c expression to determine severe CU, a cutoff of 72.0% CD203c-expressing basophils was chosen as the threshold of severe CU. However, the sensitivity (65.2%) and specificity (71.7%) were not remarkable (95% CI, 0.512-0.782). CD203c-expressing basophils ≥ 72% (P = 0.005) and a UAS ≥ 13 (P = 0.032) were significant, independent predictors of severe CU after adjusting for other factors, including age, gender, atopy, and ASST results (Table 2).

DISCUSSION

This study confirmed previous reports (14, 21) that patients with CU show increased basophil CD203c expression at baseline and suggested that a cutoff basophil CD203c expression of 21.3% be used for determining basophil activation in CU patients, with a very high odds ratio of 166.04. Moreover, this study is the first to suggest that patients with severe CU have higher levels of basophil activation.

We found a significant negative correlation between the number of peripheral basophils and both the percentage of CD203c-expressing basophils and total IgE level in CU patients. Previous investigators suggested that basophils migrate from the circulation into wheals according to the urticarial activity (12). It has

Table 2. Predictors of severe chronic urticaria in the multiple logistic regression analysis

| Variables            | P value | Exp(B)  | 95% CI         |
|----------------------|---------|---------|----------------|
| Age (yr)             | 0.534   | 1.013   | 0.972 - 1.056  |
| Men                  | 0.470   | 1.524   | 0.486 - 4.776  |
| Atopy                | 0.120   | 0.381   | 0.113 - 1.285  |
| ASST                 | 0.489   | 0.650   | 0.192 - 2.199  |
| UAS ≥ 13             | 0.032   | 3.590   | 1.119 - 11.516 |
| BAT-CD203c ≥ 72%     | 0.005   | 5.551   | 1.690 - 18.233 |

95% CI, 95% confidence interval; ASST, autologous serum skin test; UAS, urticaria activity score; BAT-CD203c, percentage of CD203c-expressing basophils.
been demonstrated that serum IgE upregulates the expression of FcεRI on human basophils both in vitro and in vivo (24). In response to cross-linking of IgE and FcεRI, human basophils produce IL-3, which plays a crucial role in their development, survival, and priming for greater expression of activation markers and histamine release in an autocrine manner (14, 25). Various surface markers expressed on human basophils, such as CD63, CD69, and CD203c, have been used to demonstrate the role of basophils in vivo and in vitro (11, 12). Compared with other activation markers, the regulation of CD203c is not limited to the FcεRI-mediated reaction, but is also induced by IL-3. CD203c is thought to be the most useful marker of basophil activation and differentiation (18, 26, 27). The measurement of basophil CD203c expression has been demonstrated to be useful for detecting causative allergenic components in patients with wheat allergy and wheat dependent exercise-induced anaphylaxis, insect sting and amoxicillin allergy (17, 19). If peripheral basophils of CU patients were sensitized by autoantibodies, inflammatory mediators and other extrinsic stimuli during the active urticaria, they would express a high amount of IgE bound to FcεRI on their surface. And then, as like basophils from other allergic diseases are triggered by different stimuliants (19), they upregulate several activation markers including CD203c on their surface. Notably, basophil CD203c expression was significantly higher in severe CU patients whose symptoms were not completely controlled with antihistamines.

There are no convincing predictors of urticarial development after antihistamine treatment or when patients can stop taking immunomodulators. The guidelines emphasize using the UAS in clinical practice to determine disease activity and the response to treatment of CU (10). We also found that a UAS ≥ 13 was a significant determinant of severe CU. However, as one recent study demonstrated (14), the ASST response was not reflected in the basophil CD203c expression or the clinical severity of the CU patients in our series. Flow cytometric BAT for CU patients is usually performed to detect the serologic factors in the patients that trigger histamine release from donor basophils. Recently, several serological markers, such as MMP-9, IL-6, IL-18, C-reactive protein and D-dimer, in association with the clinical severity of CU have been suggested (6-9). Since the coagulation factors, complement, and cytokines associated with chronic low-grade inflammation, in addition to the IgE- and FcεRI-dependent pathways, may activate basophils, a variety of diagnostic tools should be used to further our understanding of CU. This study enrolled 82 CU patients and obtained the optimal cutoff for determining basophil activation in CU patients and identifying severe CU using ROC analysis, although a validation study is needed in a second cohort. Therefore, BAT using CD203c expression can be extended to assess the degree of basophil activation for predicting the clinical severity of and treatment response to CU.

In conclusion, we suggest that the quantification of basophil CD203c expression at baseline is useful for predicting severe CU patients, who may need further treatment beyond antihistamines.

DISCLOSURE

The authors have no conflicts of interest to disclose.

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