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Elevated platelet activation in patients with chronic urticaria: a comparison between aspirin-intolerant and aspirin-tolerant groups

Sailesh Palikhe, BPharm^{*,†}; Nami Shrestha Palikhe, PhD[†]; Seung-Hyun Kim, PhD[†]; Hye-Soo Yoo, MD^{*}; Yoo Seob Shin, MD, PhD^{*}; and Hae-Sim Park, MD, PhD^{*,†}

^{*} Department of Biomedical Sciences, Graduate School of Ajou University, Suwon, Korea

[†] Department of Allergy and Clinical Immunology, Ajou University, School of Medicine, Suwon, South Korea

[‡] Pulmonary Research Group, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada

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ABSTRACT

Background: Platelets are actively involved in immune inflammatory processes that release inflammatory mediators. Platelet activation has been reported in various inflammatory diseases; however, few studies have described platelet involvement in chronic urticaria (CU).

Objective: To investigate platelet-activation markers, namely P2Y₁₂ receptor and P-selectin expression, and soluble P-selectin level in patients with aspirin-intolerant CU (AICU) and aspirin-tolerant CU (ATCU).

Methods: Forty-eight patients with CU and 25 normal controls were enrolled in this study. Aspirin intolerance in patients with CU was confirmed by an oral provocation test. P2Y₁₂ and P-selectin expressions on platelets were measured using flow cytometry; soluble P-selectin level in plasma was measured by enzyme-linked immunosorbent assay. To study the functional effects of aspirin, platelets were treated with aspirin (2 mmol/L) and the expressions of P2Y₁₂ and P-selectin were compared between the AICU and ATCU groups.

Results: The expression of P2Y₁₂ was significantly higher in patients with CU compared with controls, whereas no significant difference was noted in the expression of P-selectin level. The levels were not significantly different according to urticaria symptom score, symptom control status, and aspirin intolerance. Soluble P-selectin level was significantly higher in the AICU group than in the ATCU group compared with controls. Aspirin did not significantly suppress P2Y₁₂ and P-selectin expressions on platelets in the AICU group, whereas significant suppression was noted in the ATCU group.

Conclusion: These findings suggest that increased platelet activation contributes to skin inflammation in patients with AICU and those with ATCU. The functional difference of platelets in response to aspirin may contribute to persistent skin inflammation in patients with AICU.

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Introduction

Chronic urticaria (CU) is a skin disorder characterized by transient pruritic wheals that recur daily or almost daily for at least 6 weeks.¹ Based on aspirin (acetylsalicylic acid) intolerance, CU is classified as aspirin-intolerant CU (AICU) and aspirin-tolerant CU (ATCU). Aspirin aggravates symptoms in patients with AICU and they present more severe clinical symptoms compared with those with ATCU. Alteration in eicosanoid metabolism after aspirin exposure is a pathogenic mechanism suggested for AICU; however, the differences in pathogenic mechanisms between AICU and ATCU have not been clearly understood.² Mast cells are believed to play a

central role in the progression of CU.³ The activation and release of mediators, especially histamine, can affect cutaneous inflammatory processes by recruiting and activating other inflammatory cells.⁴ Studies have indicated that autoantibodies against α subunits of high-affinity IgE receptors are responsible for the activation and degranulation of mast cells in some subsets of patients with CU; however, the factors that induce mast cells to degranulate are unknown in patients with CU who do not have autoantibodies.⁵ In addition to predominant mast cells, other inflammatory cells may be actively involved in CU pathogenesis, because platelets have been reported to be activated in patients with CU.^{6,7}

Platelets are activated by various physiologic stimuli, including adenosine diphosphate (ADP), and then rapidly release various molecules stored in α or dense granules. The secretory molecules include adhesion proteins, chemokines, cytokines, and coagulation factors that can enhance immune and inflammatory responses.⁸ Platelet activation has been found in various chronic skin inflammatory diseases, including atopic dermatitis and psoriasis.^{8,9} Platelets are essential for

Reprints: Hae-Sim Park, MD, PhD, Department of Allergy and Clinical Immunology, Ajou University, School of Medicine, San 5 Woncheon-dong, Yeongtong-gu, Suwon 443-721, Korea; E-mail: hspark@ajou.ac.kr.

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leukocyte trafficking from blood to tissue in allergic inflammation.¹⁰ This suggests that such platelet activity could influence inflammation responses and that platelets should be considered important effector cells of the skin immune system.

Considering the involvement of platelets in different skin inflammatory diseases, platelets might have a role in CU pathogenesis, particularly in mast cell degranulation. The significance of platelets in CU may be related to proinflammatory effects.¹¹ This study investigated platelet-activation status in patients with CU and compared patients with AICU and those with ATCU by measuring P2Y12 and P-selectin expressions and plasma soluble P-selectin (sPs) level. In addition, the effect of aspirin on platelet activation was compared between the 2 groups.

Methods

Study Subjects

Seventy-three patients were recruited from the Department of Allergy and Clinical Immunology, Ajou University Hospital, Suwon, Korea and classified into 3 groups: patients with AICU ($n = 14$), patients with ATCU ($n = 29$), and normal healthy controls (NCs; $n = 25$). Patients with CU and daily itchy wheals that persisted longer than 6 weeks were classified as having ATCU or AICU based on results from an oral provocation with 500 mg of aspirin, as described previously.¹² A urticaria activity score was applied to evaluate each patient's clinical severity.¹³ Patients with CU who had received antihistamines (H_1) for longer than 2 weeks were classified into controlled ($n = 18$) and uncontrolled ($n = 30$) groups based on disease alleviation. The controlled group consisted of patients with CU whose condition was alleviated after antihistamine therapy, whereas the uncontrolled group consisted of patients with CU whose condition was not alleviated after antihistamine therapy. The NCs had no personal or family history of aspirin or drug hypersensitivity. Written informed consent was obtained from all subjects, and the study protocols were approved by the institutional review board of Ajou University Hospital (AJIRB-GEN-GEN-09-140). Skin prick tests were performed with 55 common aeroallergens (Bencard Co, West Sussex, United Kingdom), and atopy was defined as at least 1 positive reaction to common inhalant allergens. Serum total IgE was measured using the ImmunoCAP system (Thermo Fisher Scientific, Uppsala, Sweden) according to the manufacturer's instructions.

Isolation of Platelet-Rich Plasma

Peripheral blood was drawn from patients and healthy controls into tubes containing an acid-citrate-dextrose solution. To minimize platelet activation, platelet samples were prepared immediately within 2 hours of collection. Whole-blood samples were centrifuged for 20 minutes at 1,500 rpm at 20°C. Platelet-rich plasma (PRP) was aliquoted (200 μ L) and stored at –80°C until the time of analysis, and the remaining PRP was used for platelet isolation. For platelet isolation, PRP was centrifuged at 980g for 15 minutes at 20°C, the supernatant was discarded, and pelleted platelets were harvested. The pellets were stimulated with 2 mmol/L of aspirin or 1 $\times$ phosphate buffered saline (PBS) for 30 minutes at room temperature (RT) in the dark. Subsequently, platelet samples were centrifuged at 980g for 15 minutes at 20°C, and then the supernatant was removed. Then, platelets were fixed with 200 μ L of 4% paraformaldehyde solution and stored at 4°C until analyzed by flow cytometry.

Measurement of P2Y12 and P-Selectin Expression on Platelets

A 100- μ L cell suspension was stained with antihuman P-selectin antibody (CD62P; Santa Cruz Biotechnology, Santa Cruz, California) and anti-P2Y12 antibody (Abcam, Cambridge, Massachusetts) for 30 minutes in the dark at RT. Subsequently, 1 mL of PBS was added,

and the suspensions were centrifuged at 1,500 rpm for 5 minutes at 4°C. The supernatants were removed, and then cells were stained with fluorescein isothiocyanate–conjugated goat antimouse IgG (Santa Cruz Biotechnology) for P-selectin and Alexa Fluor 488–conjugated donkey antirabbit IgG (Invitrogen, Carlsbad, California) for P2Y12 as the secondary antibody and incubated under the same conditions. As before, 1 mL of PBS was added, and the suspensions were centrifuged at 1,500 rpm for 5 minutes at 4°C. The supernatants were removed and the platelets were resuspended with 100 μ L of PBS. The cells were analyzed by flow cytometry and reached a total of 10⁴ cells for each sample.

Measurement of Soluble P-Selectin

To measure the sPs level in PRP, a human sPs-CD62P enzyme-linked immunosorbent assay kit (R&D Systems, Inc, Minneapolis, Minnesota) was used according to the manufacturer's instructions. Briefly, 100 μ L of the standard or sample was added into each well of a 96-well plate. Then, 100 μ L of diluted P-selectin conjugate was added into each well and incubated for 1 hour at RT. The plate was washed 3 times with washing buffer and 100 μ L of substrate solution was added into each well. The plate was incubated for 15 minutes at RT in the dark, and the reaction was stopped by adding 100 μ L of stop solution. The optical density was measured at 450 nm using a microplate reader (Bio-Tek Instruments, Winooski, Vermont).

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 20 for Windows (SPSS, Inc, Chicago, Illinois). Data were expressed as mean $\pm$ SD. Comparison of continuous variables in different subgroups was performed using the Mann-Whitney *U* test. Relations between categorical variables were compared using the χ^2 test. P2Y12 and P-selectin expressions before and after aspirin treatment were evaluated by applying a Wilcoxon signed rank test. Analysis was carried out at a 5% level of significance, and a *P* value less than .05 was considered statistically significant. Post hoc power was calculated using G*Power 3.1 (Heinrich-Heine-Universität, Düsseldorf, Germany).

Results

Clinical Characteristics of Study Subjects

Table 1 presents the clinical characteristics of the study subjects. There was no significant difference in age between the AICU and ATCU groups; however, patients in the AICU and ATCU groups were significantly older than subjects in the NC group ($P = .038$ and $P < .001$, respectively). Duration of disease was significantly longer in the AICU group compared with the ATCU group ($P = .020$). The atopy rate and total IgE serum level were significantly higher in the CU group compared with the NC group. Similarly, the atopy rate and total IgE serum level were significantly higher in the AICU group compared with the ATCU group ($P = .022$ and $P = .049$, respectively).

Expression of P2Y12 and P-Selectin on Platelets

The expression of P2Y12 in patients with CU was significantly higher than in the NC group ($P < .001$). The AICU and ATCU groups showed significantly higher P2Y12 expression compared with the NC group ($P < .001$ and $P < .001$, respectively; Fig 1A). The AICU group tended to show a higher expression, but no significance was noted between the AICU and ATCU groups. For P-selectin expression, no significant differences were noted between the CU and NC groups or between the AICU and ATCU groups (Fig 1B). No significant difference was noted between the controlled and uncontrolled groups for P2Y12 and P-selectin expressions (Fig 2A, B).

Table 1
Comparison of clinical characteristics of study subjects

	AICU (n = 14)	ATCU (n = 29)	NC (n = 25)	P value		
				AICU vs ATCU	AICU vs NC	ATCU vs NC
Age (y), mean $\pm$ SD	34.36 $\pm$ 11.84/14	40.31 $\pm$ 12.27/29	26.12 $\pm$ 2.39/25	.110	.038 ^b	<.001 ^b
Men, n (%)	9/14 (64.29)	9/29 (31.03)	16/25 (64.0)	.038 ^b	.986	.015 ^b
Underlying allergic diseases, n (%) ^a	10/14 (71.43)	24/29 (82.76)	4/19 (21.1)	.392	.004 ^b	<.001 ^b
Duration (mo), mean $\pm$ SD	21.29 $\pm$ 37.82/14	27.62 $\pm$ 37.76/29	NA	.020 ^b	NA	NA
Baseline UAS, mean $\pm$ SD	9.70 $\pm$ 4.06/10	8.87 $\pm$ 5.16/23	NA	.968	NA	NA
Atopy, n (%)	13/14 (92.9)	17/29 (58.62)	9/24 (37.5)	.022 ^b	.001 ^b	.126
Platelet count ($\times 10^9$ /L), mean $\pm$ SD	247.29 $\pm$ 40.68/14	229.52 $\pm$ 51.95/29	NA	.140	NA	NA
Mean platelet volume (fL), mean $\pm$ SD	7.98 $\pm$ 0.67/14	8.11 $\pm$ 0.95/29	NA	.640	NA	NA
C3 (mg/dL), mean $\pm$ SD	112.30 $\pm$ 23.49/10	108.39 $\pm$ 31.63/23	NA	.860	NA	NA
C4 (mg/dL), mean $\pm$ SD	29.50 $\pm$ 7.99/10	26.65 $\pm$ 7.63/23	NA	.529	NA	NA
Total IgE (IU/mL), mean $\pm$ SD	344.36 $\pm$ 227.14/14	222.93 $\pm$ 241.14/29	25.95 $\pm$ 20.53/7	.049 ^b	<.001 ^b	.004 ^b

Abbreviations: AICU, aspirin-intolerant chronic urticaria; ATCU, aspirin-tolerant chronic urticaria; C3, complement 3; C4, complement 4; NA, not assessable; NC, normal control; UAS, urticaria activity score.

^aUnderlying allergic diseases include allergic rhinitis, bronchial asthma, and atopic dermatitis.

^bValues are statistically significant.

Plasma Level of Soluble P-Selectin by Enzyme-Linked Immunosorbent Assay

The sPs level in plasma tended to be higher in patients with CU compared with the NC group ($P = .054$), with no statistical significance; however, the AICU group showed significantly higher sPs levels compared with the NC group ($P = .033$; Fig 3A), whereas there was no significant difference between the ATCU and NC groups and between the AICU and ATCU groups. The controlled group showed significantly higher sPs levels compared with the uncontrolled group ($P = .035$; Fig 3B).

Effect of Aspirin on P2Y12 and P-Selectin Expressions

Aspirin significantly suppressed P2Y12 and P-selectin expressions in the ATCU group ($P = .042$, Fig 4B; $P = .001$, Fig 5B, respectively) when the functional effect of aspirin on platelets was compared between the AICU and ATCU groups; however, no significant suppression was noted in the AICU group.

Discussion

A previous study has shown that platelets are activated in patients with CU, as established by an elevated sPs level and platelet aggregation in the plasma of patients with CU.⁶ In the present study, 2 activation markers on the platelet surface, P2Y12 and

P-selectin, and 1 soluble marker, sPs, were used to assess various aspects of platelet activation in patients with CU. Activated platelets release inflammatory mediators, such as histamine and platelet-activating factor.¹⁴ It has been reported that intradermal injection of platelet-activating factor in healthy volunteers can induce wheal and flare skin reactions independent of mast cell degranulation, indicating that platelet-activating factor can induce urticarial symptoms other than histamine in patients with CU.¹⁵ The factor that induces platelet activation in CU is currently not understood. The P2Y12 receptor plays a central role in platelet activation and its higher expression on platelets increases the inclination toward activation and provides a more likely condition for platelet activation.¹⁶ P-selectin is an adhesive glycoprotein expressed on the platelet surface only after activation; in addition, it is involved in the activation and recruitment of leukocytes to the inflammatory site.⁸ Platelets can migrate extravascularly and accumulate in the inflammatory site in response to various stimuli, such as allergens, and release different inflammatory mediators.¹⁰ In the present study, P2Y12 expression and sPs level were higher in patients with CU, especially in those with AICU, suggesting elevated platelet activity in this disease. There were no significant differences in platelet-activation markers according to urticaria severity or control status. No associations were found between platelet-activation markers and clinical parameters, such as complement level or total IgE level. However, atopy is a predisposing factor for AICU and

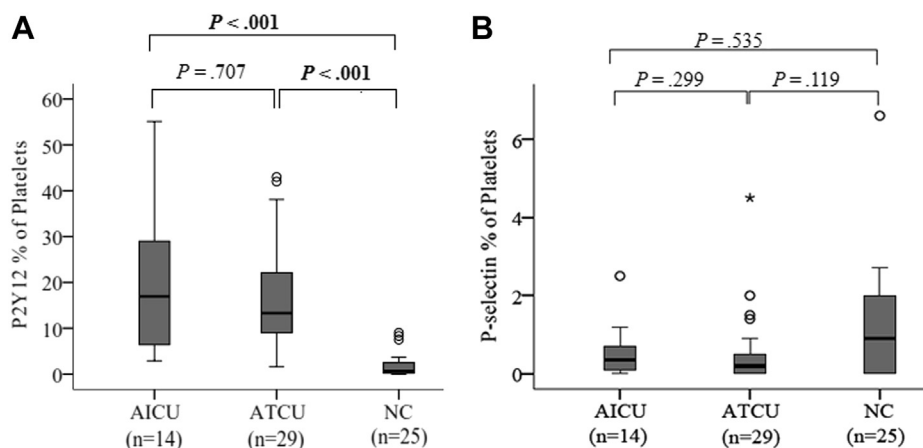


Figure 1. Boxplot analysis of (A) P2Y12 and (B) P-selectin expressions in 3 different study groups. Data are presented as median values (solid lines), interquartile ranges (boxes), and non-outlier ranges (whiskers). Outliers (circles) and extremes (asterisk) are depicted. AICU, aspirin-intolerant chronic urticaria; ATCU, aspirin-tolerant chronic urticaria; NC, normal control.

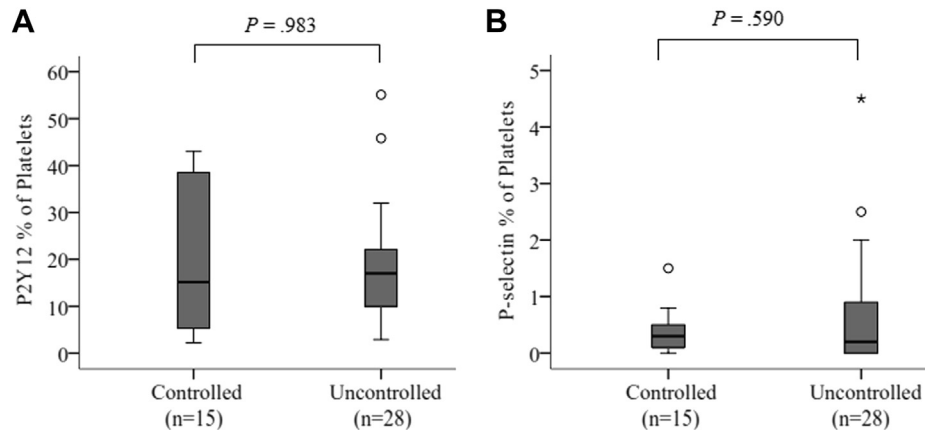


Figure 2. Boxplot analysis of (A) P2Y12 and (B) P-selectin expressions compared between the controlled and uncontrolled groups. Data are presented as median values (solid lines), interquartile ranges (boxes), and non-outlier ranges (whiskers). Outliers (circles) and extremes (asterisk) are depicted.

atopy prevalence was significantly higher in the AICU group than in the ATCU group in the present study, which may increase the platelet-activation level in the AICU group.¹⁷ One potential concern with small samples is that analyses of means can be unreliable, because the means may be disproportionately influenced by outliers. Therefore, the post hoc power calculation between subgroups was performed. More than 80% power was detected in all but 2 subgroup comparisons (66% and 70%). These findings suggest that platelets are activated in patients with CU, particularly in those with AICU, which could promote chronic skin inflammation in CU pathogenic mechanisms.

Platelet activation is comprised of a change in platelet shape, platelet aggregation, and the release of platelet constituents, which can be quantified by measuring the blood levels of relevant platelet metabolic products and the expression of different molecules on the platelet surface.¹⁸ P2Y12 is a purinergic receptor expressed on the surface of platelets.¹⁹ ADP binds with the P2Y12 receptor and amplifies platelet activation initiated by numerous pathways, releasing different molecules stored in α and dense granules that play an important role in inflammation.¹⁶ Platelet-dense granules also contain a high concentration of ADP released at activation, causing further platelet activation by binding to P2Y12.²⁰ In addition, previous studies in patients and mice with dysfunctional P2Y12 have shown that P2Y12 plays a crucial role in platelet storage granule secretion and P-selectin expression.²¹ In the present study, increased

expression of P2Y12 was found in the AICU and ATCU groups compared with the NC group. The detail mechanism of elevated P2Y12 expression in patients with CU is unknown; however, the findings suggest that platelet activation through P2Y12 expression can involve in the pathogenic mechanism of AICU and ATCU.

P-selectin is stored in α granules of platelets and is translocated to the cell surface of activated platelets. It plays an important role in leukocyte recruitment from the circulation to sites of cutaneous inflammation by the formation of platelet-leukocyte aggregates and facilitating leukocyte transmigration.^{8,22} Increase expression of P-selectin on the platelet surface has been reported in various cutaneous inflammatory diseases, such as psoriasis.²³ However, in the present study, P-selectin expression on platelets in the AICU and ATCU groups was not significantly different from that in the NC group. One possible explanation for this finding is that in patients with CU there might be a rapid shedding of P-selectin from the surface of free circulating activated platelets not associated with circulating leukocytes or endothelium. Rapid P-selectin shedding from circulating activated platelets has been described in mice.^{24,25} Soluble P-selectin is the soluble form of P-selectin, which is released in plasma during activation and is thought to be derived predominantly from platelets. Therefore, sPs level is likely to reflect platelet activation.²⁶ In the present study, a higher sPs level was found in patients with CU, notably in the AICU group, reflecting increased platelet activation. This finding is comparable to a

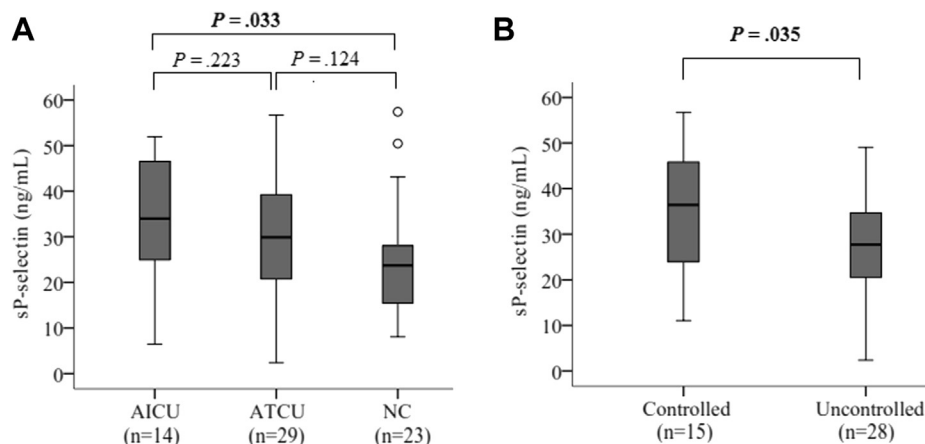


Figure 3. Boxplot analysis of soluble P-selectin (sP-selectin) plasma levels in (A) 3 different study groups and (B) the controlled and uncontrolled groups. Data are presented as median values (solid lines), interquartile ranges (boxes), and non-outlier ranges (whiskers). Outliers (circles) are depicted. AICU, aspirin-intolerant chronic urticaria; ATCU, aspirin-tolerant chronic urticaria; NC, normal control.

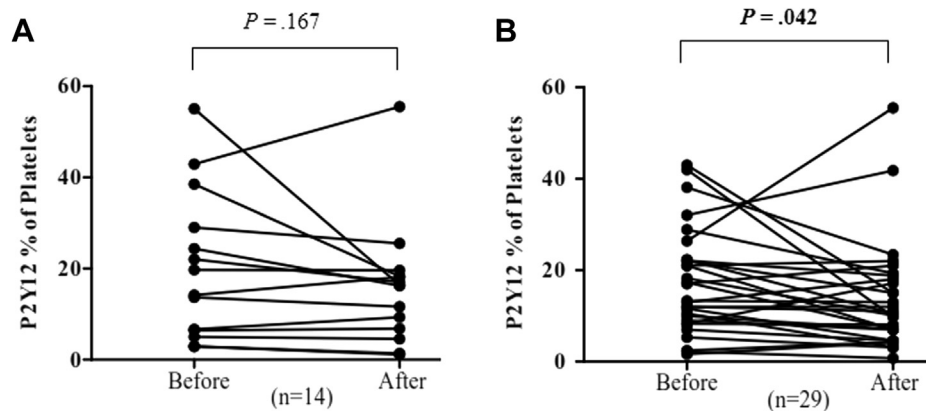


Figure 4. Comparison of P2Y12 expression on platelets before and after aspirin treatment as determined by flow cytometry in patients with (A) aspirin-intolerant chronic urticaria and (B) aspirin-tolerant chronic urticaria. Statistical analysis was performed using the Wilcoxon signed rank test.

previous study that showed that the plasma sPs level in patients with CU was significantly higher compared with that in healthy controls.⁷ Masini et al²⁷ suggested that histamine does not have a direct role in platelet activation. In the present study, higher sPs levels in the controlled group indicated that an alleviation in disease condition was not due to the suppression of platelet activation that can be influenced by antihistamines, which is supported by similar findings showing higher P2Y12 and P-selectin expression in the controlled and uncontrolled groups. Antihistamines may not prevent platelet-mediated mast cell degranulation in CU, but only neutralize the effect of released histamine from mast cells.

Aspirin is an antiplatelet drug widely used in the secondary prevention of coronary heart diseases and stroke. It exerts its effect mainly through irreversible acetylation of the enzyme cyclooxygenase-1 and inhibits the production of prostaglandins (PGs) and thromboxane A₂ from arachidonic acid.²⁸ Functional differences were noted when the effect of aspirin on P2Y12 and P-selectin expression was compared between the AICU and ATCU groups. Li et al²⁸ reported that aspirin did not inhibit agonist-induced platelet activation in vitro, as monitored by platelet P-selectin expression and platelet leukocyte aggregate formation in healthy controls. However, another in vivo study in patients with aspirin-treated cardiovascular disease showed a significant decrease of sPs levels.²⁹ The present study showed that aspirin remarkably suppressed P2Y12 and P-selectin expressions in the ATCU group, but not in the AICU group, which indicates that aspirin might not inhibit cyclooxygenase-1 completely in the platelets of patients with AICU, which results in the synthesis and release of PGE₂. PGE₂ has

been reported to activate mast cells through the activation of prostanoid-3 receptors.^{30,31} These findings suggest that PGE₂ released from platelets of patients with AICU may promote mast cell activation, leading to a more severe clinical course in those with AICU. This is the first study to report different responses of platelets to aspirin in patients with AICU that may be associated with mast cell activation and more severe clinical symptoms.

Previous studies have shown that activated platelets can activate classic and alternative pathways of a complement system. Activation of a complement system is associated with the generation of potent proinflammatory peptides, namely C3a and C5a, that can degranulate mast cells.^{32,33} Therefore, complement activation might be a link between platelet activation and mast cell degranulation in CU. In addition, activated platelet can release ADP and adenosine triphosphate, which can bind to the P₂ purinergic receptor on mast cells, leading to mast cell degranulation.^{34,35} Activated platelets can induce the activation of the coagulation pathway by enhancing the release and reaction of prothrombinase, resulting in thrombin generation.³⁶ Because thrombin can activate protease-activated receptor (PAR) 1 on endothelial cells, generation of thrombin in CU by activated platelets could contribute to increased vascular permeability and amplified inflammatory response in CU. Mast cells express PAR1 and PAR2 receptors.³⁷ Thrombin generated from the coagulation pathway might activate mast cells through PAR1 and coagulation factor Xa might activate these cells through PAR2, amplifying the continuous activation of mast cells in CU.³⁸

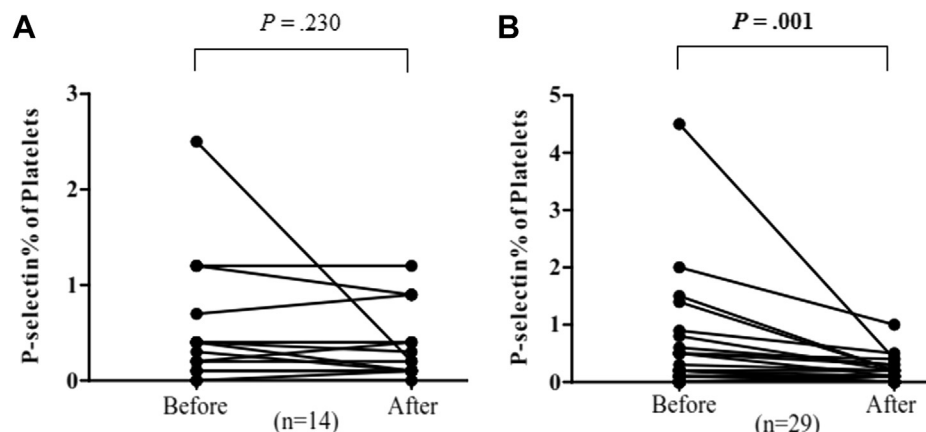


Figure 5. Comparison of P-selectin expression in platelets before and after aspirin treatment as determined by flow cytometry in patients with (A) aspirin-intolerant chronic urticaria and (B) aspirin-tolerant chronic urticaria. Statistical analysis was performed using the Wilcoxon signed rank test.

In conclusion, increased platelet activation in patients with AICU and those with ATCU may contribute to chronic skin inflammation. In addition, the functional difference of platelets in response to aspirin may contribute to more severe and persistent skin inflammation in patients with AICU. However, further studies are needed to clarify the role of the platelet-mast cell interaction in the pathogenesis of AICU and ATCU.

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