



Contents lists available at ScienceDirect

Allergy International

journal homepage: <http://www.elsevier.com/locate/alit>

Original Article

Serum level of hemokinin-1 is significantly lower in patients with chronic spontaneous urticaria than in healthy subjects

Nobuyuki Nishimori^{a, b, e, 1}, Shota Toyoshima^{a, c, e, 1}, Tomomi Sasaki-Sakamoto^{a, d, e}, Koremasa Hayama^{a, b, e}, Tadashi Terui^{b, e}, Yoshimichi Okayama^{a, d, e, *}^a Allergy and Immunology Research Project Team, Nihon University School of Medicine, Tokyo, Japan^b Department of Dermatology, Nihon University School of Medicine, Tokyo, Japan^c Department of Respiratory Medicine, Nihon University School of Medicine, Tokyo, Japan^d Center for Medical Education, Nihon University School of Medicine, Tokyo, Japan^e Center for Allergy, Nihon University Itabashi Hospital, Tokyo, Japan

ARTICLE INFO

Article history:

Received 29 March 2021

Received in revised form
29 March 2021

Accepted 7 April 2021

Available online xxx

Keywords:

Autoantibody

Basophil/mast cell

FcεRIα

Immunoglobulin E

Urticaria

Abbreviations:

AABs, autoantibodies; ASST, autologous serum skin test; CSU, chronic spontaneous urticaria; EPO, eosinophil peroxidase; FcεRIα, FcεRI α-chain; h, human; HBSS, Hank's balanced salt solution; HK-1, hemokinin-1; IMDM, Iscove's modified Dulbecco's medium; LSM, lymphocyte separation medium; MBP, major basic protein; MCs, mast cells; MRGPRX2, Mas-related G protein-coupled receptor X2; NC, non-atopic healthy control; NKA, neurokinin A; NKB, neurokinin B; NK1-R, neurokinin-1 receptor; SCF, stem cell factor; SP, substance P

ABSTRACT

Background: We previously reported upregulation of expression of Mas-related G protein-coupled receptor X2 (MRGPRX2) on mast cells (MCs) in the skin of patients with severe chronic spontaneous urticaria (CSU). Serum levels of substance P (SP) were reportedly significantly elevated, in correlation with the severity of CSU. Hemokinin-1 (HK-1) reportedly induced histamine release from LAD2 cells via MRGPRX2. We aimed to investigate HK-1's role in CSU.

Methods: The concentrations of HK-1 and SP were measured using ELISAs. Skin- and synovium-derived cultured MCs were generated by culturing dispersed skin and synovial cells, respectively, with stem cell factor. MRGPRX2 expression in the MCs was reduced using a lentiviral shRNA silencing technique.

Results: Anti-SP Ab used in the SP ELISA showed 100% cross-reactivity to HK-1, but anti-HK-1 Ab showed 0% cross-reactivity to SP. The serum level of HK-1 was significantly lower in patients with CSU (n = 151) than in non-atopic healthy control (NC) subjects (n = 114). The EC₅₀ of histamine release from MCs induced by HK-1 (5056 nM) was 12-fold higher than by SP (426 nM). Brief pretreatment of MCs with HK-1 at concentrations of 3.0–10 μM significantly reduced histamine release by 0.1 μM SP. However, brief incubation of MCs with HK-1 did not elicit rapid MRGPRX2 internalization.

Conclusions: In NC subjects, high HK-1 concentrations may desensitize MRGPRX2-mediated MC activation, thereby preventing MC degranulation by SP.

Copyright © 2021, Japanese Society of Allergy. Production and hosting by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Chronic spontaneous urticaria (CSU) is defined as the occurrence of daily systemic wheals for at least 6 weeks.¹ Mast cells (MCs) play a key role in the induction of wheal reactions through release of a variety of vasoactive autacoid mediators and functionally diverse proteases, chemokines and cytokines.² We previously reported that expression of Mas-related G protein-coupled receptor X2 (MRGPRX2) — which is a receptor for substance P (SP) — on MCs

* Corresponding author. Allergy and Immunology Research Project Team, Center for Medical Education, Nihon University School of Medicine, Center for Allergy, Nihon University Itabashi Hospital, 30-1 Otaguchi-kamicho, Itabashi-ku, Tokyo 173-8610, Japan.

E-mail address: okayama.yoshimichi@nihon-u.ac.jp (Y. Okayama).

Peer review under responsibility of Japanese Society of Allergy.

¹ These authors contributed equally to this work.

was upregulated in the skin of patients with severe CSU, and that SP activates skin MCs through MRPRGX2, but not via neurokinin-1 receptor (NK1-R).³ The serum level of SP was significantly higher in CSU patients than in healthy subjects^{4–7} and was associated with disease severity.⁴ These results suggest that an SP–MRGPRX2–MC axis plays important roles in the pathogenesis of CSU.

MRGPRX2 encodes for MRGPRX2 (also known as MRGX2), a 37–kDa G protein–coupled receptor (GPCR) that consists of 330 amino acids.⁸ Human MRGPRX2 is expressed in MCs of the connective tissue and small–diameter neurons of the dorsal root ganglion as well as in blood basophils and eosinophils.^{8,9} Human MRGPRX2 is activated by ligands with cationic properties that are endogenous and exogenous compounds with diverse chemical features ranging from peptides and proteins to cysteine proteases and opioids (e.g., antimicrobial host defense peptides, neuropeptides, eosinophil granule proteins, and many US Food and Drug Administration–approved peptidergic drugs).¹⁰

SP, neurokinin A (NKA), neurokinin B (NKB) and hemokinin-1 (HK-1) are members of the tachykinin family.¹¹ TAC1 encodes for SP and NKA, TAC3 encodes for NKB, and TAC4 encodes for HK-1.¹¹ HK-1 has 64% homology to SP and binds to NK1-R and MRGPRX2,¹² but the functions of SP and HK-1 are often different or the opposite.¹¹ HK-1 is expressed in neuronal cells and non-neuronal cells, such as human endothelial cells, inflammatory and immune cells and glial cells, and in the placenta.¹¹ HK-1 is involved in various physiological and pathophysiological functions, including modulation of pain and inflammation, immune regulation, respiratory and endocrine functions, and even tumor genesis.¹¹ Since HK-1 induces scratching, biting and licking in mice,¹³ it might be involved in the itching in CSU.

Commercially available SP EIA kits are known to recognize both SP and HK-1. HK-1 induced histamine release from a human mastocytoma cell line, LAD2, via MRGPRX2.¹² Thus, we hypothesized that HK-1 might play a pivotal role in the pathogenesis of CSU. We compared the serum levels of HK-1 and SP in CSU patients and non-atopic healthy control (NC) subjects. We also looked for associations between the level of HK-1 and various clinical parameters that might point to its involvement in the pathogenesis of CSU. Finally, we compared HK-1 and SP for their ability to induce histamine release from human MCs.

Methods

Ethical considerations

This study was approved by the Ethics Committees of the Nihon University School of Medicine (RK-15908-12 and RK-160112-2). All the subjects provided written informed consent in accordance with the Helsinki Declaration of the World Medical Association.

Patient enrollment

One-hundred-and-fifty-six patients with CSU (113 females and 43 males; ranging in age from 13 to 85 years) were consecutively recruited between May 2011 and December 2019. Five patients were excluded because of treatment with a steroid(s) and/or cyclosporine A within the most recent 3 weeks. Table 1 summarizes the characteristics of the remaining 151 CSU patients (108 females and 43 males; age range: 13–82 years). Most of the patients had been referred from private practice doctors to the outpatient–clinic of Nihon University Hospital. Some patients had already been treated with H1-antihistamines. We also enrolled 114 non-atopic healthy control (NC) subjects (73 females and 41 males; ranging in age from 22 to 64 years) for whom the criterion was no history of urticaria, asthma, allergic disease or autoimmune disease (Table 1).

Table 1

Characteristics of the CSU patients and NC subjects.

	CSU (n = 151)	NC (n = 114)
Demographic details		
Age (year), median (range)	42 (13–82)	31.5 (22–64)
Sex: female (%)	71.5	64
Urticaria		
Duration of illness (month), median (range)	25 (1–517)	N.A.
ASST positive (%)	46.2	0
Severity score, median (range)	28 (0–42)	N.A.
Medication use at time of review (%)		
H1 antihistamine	60 (39.7)	0
H1 antihistamine (double amount)	81 (53.6)	0
H2 antihistamine	62 (41.1)	0
Leukotriene receptor antagonist	75 (49.7)	0
Corticosteroid	0	0
Cyclosporine A	0	0
Trenexame acid	6 (4.0)	0
Omalizumab	2 (1.3)	0
Diaminodiphenyl sulfone	1 (0.7)	0
Suplatast tosilate	2 (1.3)	0
Nil	6 (4.0)	100

ASST, autologous serum skin test; CSU, chronic spontaneous urticaria; N.A., Not applicable; NC, non-atopic healthy control.

Routine blood and urine tests and liver and kidney function were all normal. Pregnant and nursing women were excluded.

Definitions and study protocol

CSU was diagnosed according to the classifications of the European Academy of Allergy and Clinical Immunology (EAACI), the Global Allergy and Asthma European Network (GA²LEN), the European Dermatology Forum (EDF), and the World Allergy Organization (WAO).¹⁴ Subjects were excluded if steroids or immunosuppressive drugs had been used within 3 weeks. The 7–day urticaria activity score (UAS7) is a patient–reported scoring system that captures the severity of pruritus and the number of hives appearing during 1 week.¹⁵ The intensity of pruritus (range: 0 [none] to 3 [severe]) and the number of hives (range: 0 [none] to 3 [>50 hives]) were recorded daily (maximum: 6 points per day). The scores were then summed for 1 week to plot on the UAS7 scale, (0–42). Blood tests, which included the serum IgE level, basophil count, IgG anti-nuclear AAbs, IgG anti-thyroglobulin AAbs and IgG anti-thyroid microsomal AAbs, were performed.

Preparation of serum samples for HK-1 and SP ELISAs

Peripheral blood was drawn from the NC subjects and CSU patients using Aprotinin (1500 units) and EDTA-2Na–containing tubes (NP-EA0305-123D, SRL, Tokyo, Japan). Peptides were extracted from the sera (100 µL) according to the protocol for CEP-column (C-18) (Peninsula Laboratories International, San Carlos, CA, USA). The extracted peptides were freeze–dried in a freeze dryer (FDU-1200, Tokyo Rikakikai, Tokyo, Japan). The freeze–dried peptide specimens were dissolved in 100 µL of PBS and stored at –80 °C until ELISA assays. Thus, the concentrations of HK-1 and SP measured by ELISAs were equivalent to their concentrations in the sera.

Isolation, purification and culture of human skin and synovial MCs

Macroscopically normal human skin and synovial tissue specimens were obtained during surgery. Skin and synovial tissues for the isolation and culture of MCs were obtained from healthy normal donors and patients with osteoarthritis, respectively. Skin

and synovial cells from chopped foreskin and synovial specimens were dispersed by an enzymatic procedure in Iscove's modified Dulbecco's medium (IMDM) containing 1.5 mg/mL collagenase (Sigma–Aldrich, St. Louis, MO, USA), 0.75 mg/mL hyaluronidase (Sigma–Aldrich) and 0.1% BSA, at 37 °C for 1 h.³ The dispersed cells were then centrifuged in lymphocyte separation medium (LSM; MP Biomedicals, Santa Ana, CA, USA). The cells at the LSM interface were collected and washed as described previously.³ The dispersed skin and synovial cells were cultured in serum-free Iscove's methylcellulose medium and IMDM supplemented with 200 ng/mL recombinant human stem cell factor (rhSCF) and 50 ng/mL rhIL-6. On day 42, methylcellulose was dissolved in PBS, and the cells were resuspended and cultured in IMDM containing 0.1% BSA, 100 ng/mL rhSCF and 50 ng/mL rhIL-6 for another 5–6 weeks.

MC activation

MCs were suspended in Hank's balanced salt solution (HBSS) supplemented with 0.1% BSA and 1 mM CaCl₂ for histamine experiments. MCs were activated with HK-1 (Biorbyt, Cambridge-shire, UK) and SP (Sigma–Aldrich), or calcium ionophore A23187 (10^{−6} M, Sigma–Aldrich), at 37 °C for 30 min for measurement of histamine release. For aggregation of FcεRI, MCs were sensitized with 1 µg/mL of human IgE (Calbiochem, San Diego, CA, USA) in IMDM containing 0.1% BSA, 100 ng/mL rhSCF and 50 ng/mL rhIL-6 for 16 h at 37 °C. The cells were washed once and resuspended in HBSS supplemented with 0.1% BSA and 1 mM CaCl₂. The IgE-sensitized MCs were next stimulated with anti-human IgE Ab (DakoCytomation, Glostrup, Denmark) for 30 min for measurement of histamine release, and then cell-free culture supernatants and cell pellets were harvested to measure the histamine levels. In some histamine assay experiments, IgE-sensitized MCs were pretreated with indicated concentrations of SP, HK-1 or compound 48/80 (Sigma–Aldrich) for 15 min in HBSS supplemented with 0.1% BSA and 1 mM CaCl₂, or for 24 h in IMDM containing 0.1% BSA, 100 ng/mL rhSCF and 50 ng/mL rhIL-6 (1st activation). The cell supernatants were removed, and the cells were resuspended in HBSS supplemented with 0.1% BSA and 1 mM CaCl₂. Equivalent numbers of MCs were prepared in wells to measure the total histamine content, and these MCs were incubated with medium alone. Other MCs were activated with indicated concentrations of SP or anti-human IgE for 30 min (2nd activation). The cell supernatants and cell pellets were collected for histamine assay.

Mediator assays

Histamine, SP and HK-1 released from MCs were measured using, respectively, Histamine EIA kit (MBL, Nagoya, Japan), SP ELISA kit (R&D Systems, Minneapolis, MN, USA) and HK-1 ELISA kit (Peninsula Laboratories International).

RNA isolation and RT-PCR

Total RNA was isolated from synovium- and skin-derived cultured MCs, and real-time quantitative RT-PCR was performed as described previously.³ A human gene-specific primer and probe set for MRGPRX2 and GAPDH were designed using the Assay-on-Demand service (ThermoFisher Scientific, Waltham, MA, USA).

To examine TAC1 and TAC4 mRNA expression in the skin, we studied 12 CSU patients (8 females and 4 males; age range: 17–72 years) and 9 nonatopic control (NC) subjects (5 female and 4 male subjects; age range, 60–90 years). The characteristics of the patients with CU and NC subjects are summarized in Table 2. Skin tissue

Table 2

Characteristics of the CSU patients and NC subjects of skin specimens.

	CSU (n = 12)	NC (n = 9)
Demographic details		
Age (year), median (range)	40 (17–72)	86 (60–90)
Sex: female (%)	66.7	55.6
Urticaria		
Duration of illness (month), median (range)	2 (1–240)	N.A.
ASST positive (%)		0
Severity score, median (range)	31.5 (5–42)	N.A.
Medication use at time of review (%)		
H1 antihistamine	4 (33.3)	0
H1 antihistamine (double amount)	5 (41.7)	0
H2 antihistamine	3 (25.0)	0
Leukotriene receptor antagonist	3 (25.0)	0
Corticosteroid	0	0
Cyclosporine A	0	0
Trenexame acid	1 (8.3)	0
Omalizumab	0	0
Diaminodiphenyl sulfone	1 (8.3)	0
Suplataste	0	0
Nil	2 (16.7)	100

ASST, autologous serum skin test; CSU, chronic spontaneous urticaria; N.A., Not applicable; NC, non-atopic healthy control.

sections were obtained by using punch biopsy specimens from wheal regions on the arms of 12 patients with CU. Skin specimens from the arms of NC subjects were obtained from macroscopically normal skin tissue from surplus skin grafts. Total RNA was extracted from skin specimens (10 mg) using a High Pure miRNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). It was then reverse-transcribed to cDNA using 10 mM dNTP mix, oligo-dT primers, Super Script IV and nuclease-free water (Invitrogen), followed by amplification with specific primers (Invitrogen) and Gotaq DNA polymerase purchased from Promega (Madison, WI, USA). The PCR conditions were as follows: initial denaturation for 10 min at 95 °C, 40 cycles of denaturation for 30 s at 95 °C, annealing for 1 min at 63 °C and amplification for 1 min at 72 °C. After 7-min terminal amplification at 72 °C, the PCR products were subjected to 2.0% agarose gel electrophoresis. Semi-quantitative PCR was performed using GeneAtlas (Astec, Fukuoka, Japan). The following three primer sets were used: TAC1 Forward 5'-CGACAGCGACCAGATCAAGGAGG-3', TAC1 Reverse 5'-TGCATTGCACTCTTCAT-3' TAC4 Forward 5'-TCTCTCTCTGTGTCTCTCTCTCT-3', TAC4 Reverse 5'-CATTTATTGAGTGCCTACTGTGTGCT-3'; and GAPDH Forward 5'-TGATGACATCAAGAAGGTGGTGAAG-3' and GAPDH Reverse 5'-TCCTTGGAGGCCATGTGGGCCAT-3'. The relative intensities of TAC4 mRNA bands to GAPDH mRNA bands were determined using a densitometer (FAS-1V, Nippon Genetics, Tokyo, Japan).

Statistical analysis

To identify variables associated with CSU or a specific CSU patient group, we compared continuous variables between the NC subjects and CSU patients or between two different patient groups using the Mann–Whitney *U* test. To compare categorical variables, a 2-sided Fisher exact test was used. More than three groups were compared using two-way ANOVA and Tukey's multiple comparisons test. Spearman rank correlation coefficients were calculated to determine the strength of correlations between continuous variables. *P* values were considered significant at *P* < 0.05. Data analyses were performed using Prism version 7 (GraphPad Software, San Diego, CA). See the [Supplementary Methods](#) for additional information regarding the experimental protocols.

Results

Comparison of HK-1 and SP concentrations between NC subjects and CSU patients

We first checked for cross-reactivity between HK-1 and SP for (1) the anti-HK-1 Ab used in the HK-1 ELISA kit purchased from Laboratories International (Fig. 1a) and (2) the anti-SP Ab used in the SP ELISA kit purchased from R&D Systems (Fig. 1b). The anti-HK-1 Ab used in the HK-1 ELISA kit was specific to HK-1, but the anti-SP Ab used in the SP ELISA kit showed 100% cross-reactivity to HK-1. Using the HK-1 and SP ELISA kits, we compared the concentrations of HK-1 and SP between the 114 NC subjects and 151 CSU patients. The HK-1 concentration was significantly lower

in sera from the CSU patients (median 125.8 pg/mL) than in the NC subjects' sera (median 217.7 pg/mL, Fig. 1c, $P < 0.0001$), but the SP concentration was not (Fig. 1d). The HK-1 concentrations in sera from moderate and severe CSU patients were significantly lower than in the NC subjects (Fig. 1e, $P < 0.01$ and $P < 0.0001$). However, the serum SP level did not differ significantly between the NC and CSU patients, regardless of the latter's disease severity (Fig. 1f).

Next, Spearman rank correlation coefficients were calculated to determine the strength of correlations between the HK-1 and SP concentrations and each of the clinical parameters (Table 3). There were no significant correlations with the urticaria severity score; clinical data, including the serum IgE level, eosinophil count, basophil count or serum levels of IgG AAbs to thyroid peroxidase,

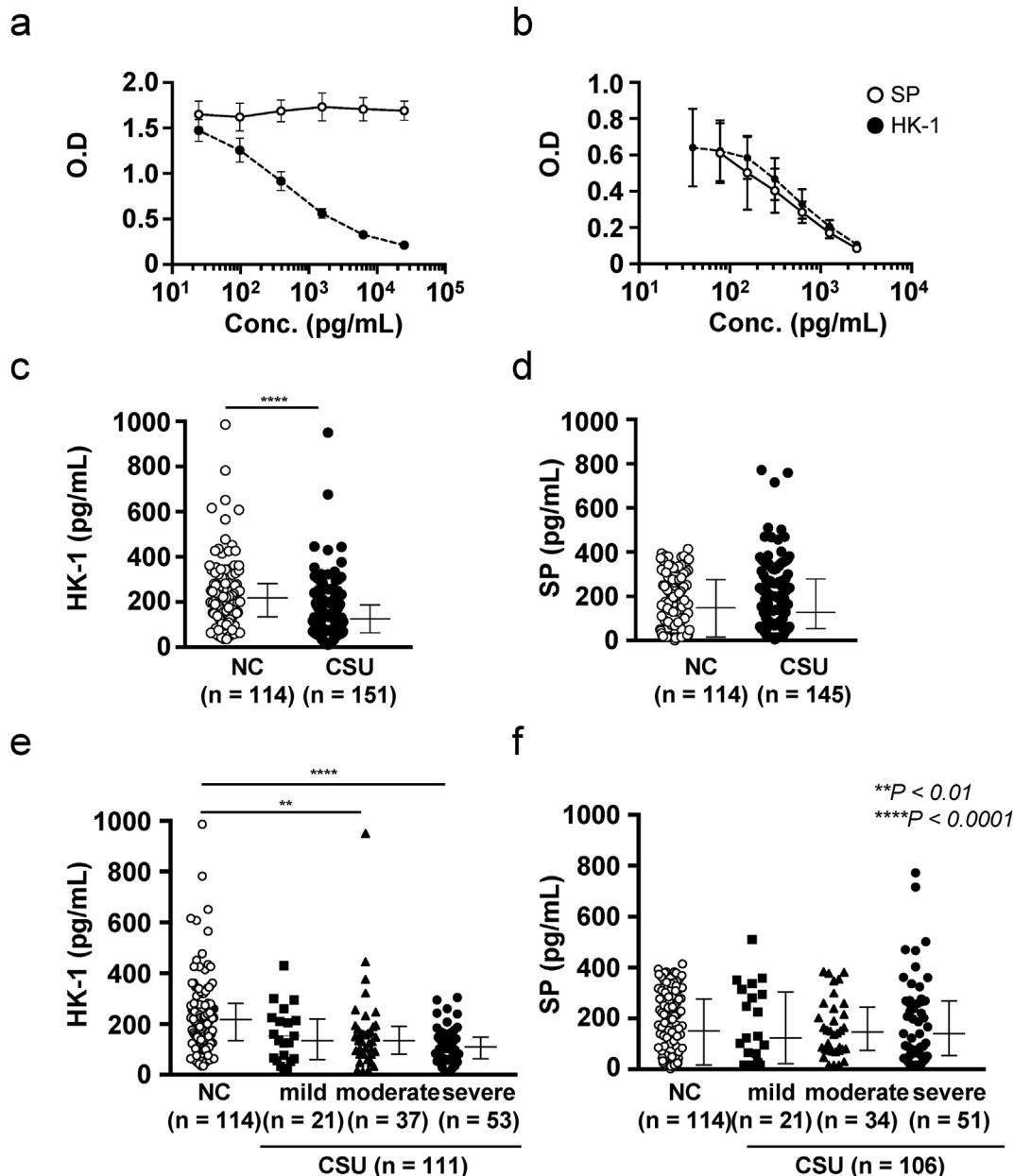


Fig. 1. Comparison of concentrations of measured HK-1 and SP between NC subjects and CSU patients. (a) Standard curve analyses of HK-1 (closed circles) and SP (open circles) using the HK-1 ELISA kit (Peninsula Laboratories International). Data are the means $\pm$ SEM ($n = 3$). (b) Standard curve analyses of HK-1 (closed circles) and SP (open circles) using the SP ELISA kit (R&D Systems). Data are the means $\pm$ SEM ($n = 3$). Comparison of concentrations of (c) HK-1 and (d) SP between NC subjects and CSU patients (scatter plot). The medians and inter-quartile ranges are also shown. Comparison of (e) HK-1 and (f) SP concentrations between NC subjects and CSU patients, categorized by disease severity. ** and **** indicate $P < 0.01$ and $P < 0.0001$, respectively.

Table 3

Correlations between the serum levels of tachykinins and clinical parameters, or the ability of anti-FcεRIα AAbs and anti-IgE AAbs to induce FcεRI crosslinking in CSU patients.

Tachykinin	Clinical parameter	P value	r	n
HK-1	Duration of illness (months)	0.021	0.188	151
	Severity score, UAS7	0.729	−0.031	127
	Serum IgE level (IU/mL)	0.666	0.037	139
	Blood neutrophils (/mm ³)	0.092	0.153	123
	Blood eosinophils (/mm ³)	0.684	−0.037	123
	Blood basophils (/mm ³)	0.161	0.127	123
	Blood monocytes (/mm ³)	0.898	0.012	123
	Blood lymphocytes (/mm ³)	0.789	0.024	123
	Anti-thyroid peroxidase AAbs (IU/mL)	0.161	−0.199	51
	Anti-thyroglobulin AAbs (IU/mL)	0.668	0.169	9
	Anti-FcεRIα AAbs (μg/mL)	0.445	0.082	90
	Ability of anti-FcεRIα AAbs (FI)	0.858	−0.019	89
	Anti-IgE AAbs (μg/mL)	0.995	−0.001	90
	Ability of anti-IgE AAbs (FI)	0.508	0.071	90
SP	Duration of illness (months)	0.139	0.123	145
	Severity score, UAS7	0.043	0.184	122
	Serum IgE level (IU/mL)	0.051	0.169	134
	Blood neutrophils (/mm ³)	0.286	0.099	117
	Blood eosinophils (/mm ³)	0.081	0.162	117
	Blood basophils (/mm ³)	0.378	0.082	117
	Blood monocytes (/mm ³)	0.876	−0.015	117
	Blood lymphocytes (/mm ³)	0.507	0.062	117
	Anti-thyroid peroxidase AAbs (IU/mL)	0.268	−0.160	50
	Anti-thyroglobulin AAbs (IU/mL)	0.903	0.053	9
	Anti-FcεRIα AAbs (μg/mL)	0.001	0.341	86
	Ability of anti-FcεRIα AAbs (FI)	0.498	−0.074	85
	Anti-IgE AAbs (μg/mL)	0.409	−0.090	86
	Ability of anti-IgE AAbs (FI)	0.408	0.090	86

AAbs, autoantibodies; FI, fold increase; HK-1, hemokinin-1; SP, substance P; UAS7, 7-day urticaria activity score.

thyroglobulin, FcεRIα and IgE; or the ability of anti-FcεRIα AAbs and anti-IgE AAbs to induce FcεRI crosslinking (Table 3). The HK-1 concentration correlated significantly with the duration of illness ($P = 0.021$, $r = 0.188$), but the SP concentration did not (Table 3). The SP concentration correlated significantly with the urticaria severity score ($P = 0.043$, $r = 0.184$; Table 3) and the anti-FcεRIα AAbs concentration ($P = 0.001$, $r = 0.341$; Table 3). The HK-1 and SP levels did not differ significantly between ASST-negative patients and ASST-positive patients (Supplementary Fig. 1a, b).

Evaluation of ability of HK-1 and SP to induce histamine release from human MCs

Next, we evaluated the ability of each of HK-1 and SP to induce histamine release from human synovium-derived cultured MCs. MCs were incubated with 0.01, 0.03, 0.1, 0.3, 1, 3 and 10 μM of each of HK-1 and SP, and histamine release was then measured. As can be seen in Figure 2a, b, the EC₅₀ of histamine release from MCs in response to HK-1 (5056 nM) was 12-fold higher than with SP (426 nM). The maximum percent histamine release in response to HK-1 ($51.5 \pm 12.8\%$) was almost half of that with SP ($90.8 \pm 13.5\%$).

Synovium- and skin-derived cultured MCs were activated by HK-1 through MRGPRX2

HK-1 reportedly induced histamine release from LAD2 cells via MRGPRX2.¹² Therefore, to confirm the function of MRGPRX2 in HK-1-induced activation of synovium- and skin-derived cultured MCs, we knocked down MRGPRX2 expression on those cells using a lentiviral shRNA silencing technique. Figure 3a shows that MRGPRX2 expression was significantly reduced on synovium-derived cultured MCs transduced with MRGPRX2 shRNA compared with on non-transduced and non-targeted shRNA-transduced synovium-derived cultured MCs. Cell-surface MRGPRX2 expression on the MRGPRX2 shRNA-transduced MCs was reduced compared with on the non-transduced and non-targeted shRNA-transduced MCs (Fig. 3b). However, FcεRI expression was not affected by the transduction of MRGPRX2 shRNA (Fig. 3b). After stimulation with HK-1 and SP, we analyzed the degranulation of synovium-derived cultured MCs transduced with non-targeted shRNA and MRGPRX2 shRNA or without viral treatment. Degranulation was significantly reduced in the MCs transduced with MRGPRX2 shRNA compared with the non-transduced and non-targeted shRNA-transduced MCs (Fig. 3c). Using the above shRNA technique, we confirmed that HK-1 activated skin-derived cultured MCs via MRGPRX2 (Fig. 3d).

Desensitization of MRGPRX2-mediated MC activation by high concentrations of HK-1

Since HK-1 and SP are ligands for MRGPRX2 on skin-derived cultured MCs, we hypothesized that high concentrations of HK-1 may desensitize MRGPRX2-mediated MC activation, resulting in inhibition of activation of the cells by SP. We first incubated

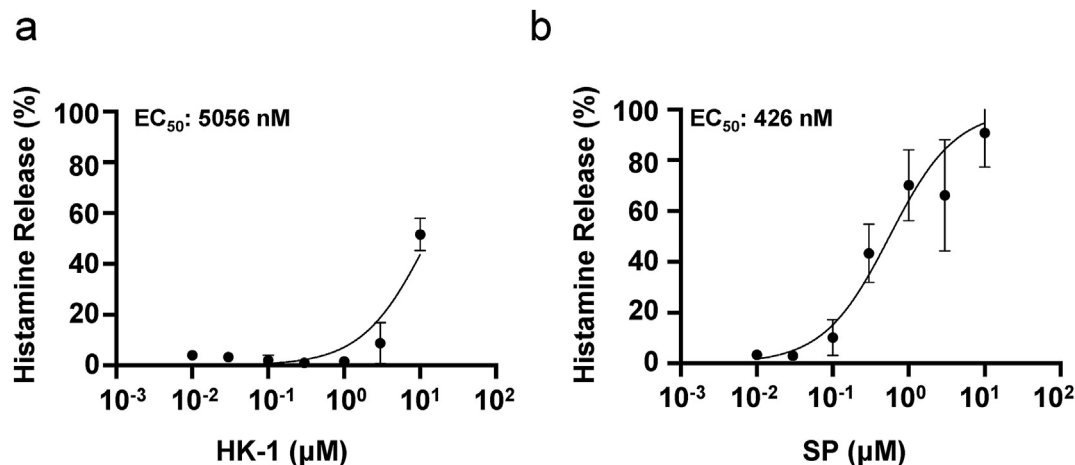


Fig. 2. Evaluation of ability of (a) HK-1 and (b) SP to induce histamine release from human synovium-derived cultured MCs. Data are the means $\pm$ SEM for 5 different donors' MCs.

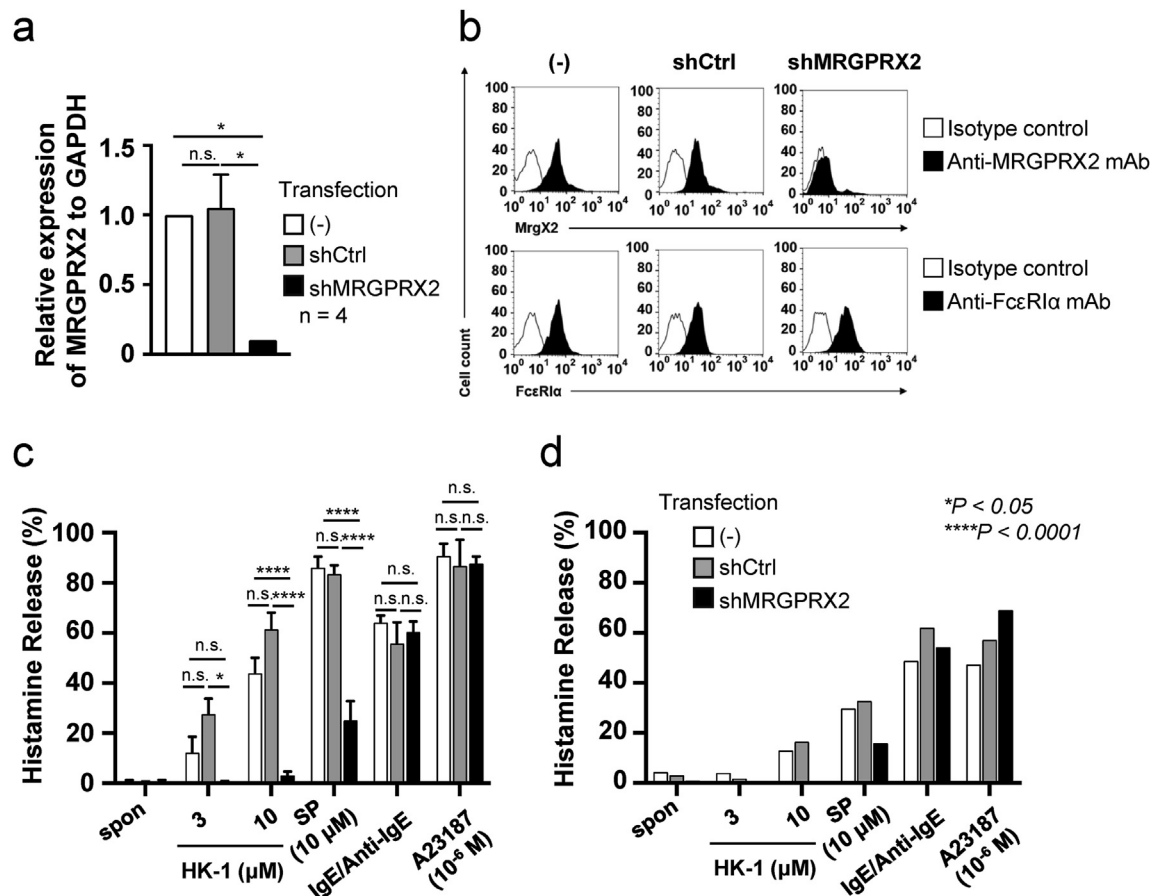


Fig. 3. HK-1-induced histamine release from synovium-derived and skin-derived cultured MCs through MRGPRX2. **(a)** Expression levels of MRGPRX2 mRNA in non-transduced (open column), non-targeted shRNA-transduced (gray column) and MRGPRX2 shRNA-transduced synovium-derived cultured MCs (black column). The expression level of MRGPRX2 mRNA in non-transduced MCs was designated as '1'. The results are the means $\pm$ SEM for 4 different donors. **(b)** Cell surface expression of MRGPRX2 on non-transduced (-), non-targeted shRNA-transduced (shCtrl) and MRGPRX2 shRNA-transduced synovium-derived cultured MCs (shMRGPRX2). The black-filled histograms indicate MCs treated with anti-MRGPRX2 mAb (upper panels) or anti-FcεRI mAb (lower panels). The open histograms indicate isotype Ig-treated MCs. Representative data from 3 experiments using 2 different donors' MCs. **(c)** and **(d)** Effect of diminution of MRGPRX2 on histamine release from MCs in response to HK-1. The white, gray and black columns show, respectively, the histamine release from non-transduced, non-targeted shRNA-transduced and MRGPRX2 shRNA-transduced **(c)** synovium-derived and **(d)** skin-derived cultured MCs. Data are shown as the means $\pm$ SEM ($n = 3-4$ donors, **c**). Representative data from 2 experiments using 2 different donors' MCs (**d**). * and **** indicate $P < 0.05$ and $P < 0.0001$, respectively. A23187, calcium ionophore A23187.

synovium-derived cultured MCs with 1, 3, 5 and 10 μ M HK-1 for 15 min, and then exposed the cells to 0.1 μ M SP for 30 min. The incubation of the MCs with HK-1 induced histamine release from the cells from 0.3 to 40%. HK-1 showed significant inhibition of histamine release induced by 0.1 μ M SP (Fig. 4a). However, pretreatment of MCs with HK-1 or SP did not affect the amount of histamine release induced by anti-IgE Ab (Fig. 4b). Many G-protein-coupled receptors, including MRGPRX2, undergo β -arrestin-initiated internalization upon ligand binding.¹⁶ MRGPRX2 was rapidly diminished in response to 1 μ M of compound 48/80 and 30 μ M of SP on synovium-derived cultured MCs (Fig. 4c, at 30 min of incubation, $P < 0.0001$) and less or no downregulation was found for 3 μ M of SP and 3 and 30 μ M of HK-1 at this time point (Fig. 4c). The downregulation of the curves obtained for compound 48/80, SP and HK-1 stimulation reached a minimum after 24 h (Fig. 4c, at 1 μ M compound 48/80, 3 μ M SP and 30 μ M HK-1, $P < 0.0001$).

Comparison of TAC1 and TAC4 mRNA expression in skin specimens obtained from NC subjects and CSU patients

TAC1 encodes for SP, while NKA and TAC4 encode for HK-1.⁸ We first compared the TAC1 and TAC4 mRNA expression levels in skin specimens obtained from NC subjects and CSU patients by

performing real-time quantitative RT-PCR with primers and probes from the Assay-on-Demand service (Applied Biosystems). However, the CT values for TAC1 and TAC4 mRNA were over 50 (data not shown). The TAC1 mRNA expression levels in skin specimens obtained from NC subjects and CSU patients using RT-PCR were below the detection level (data not shown). Thus, TAC1 mRNA expression in the skin specimens was not detectable by those methods. On the other hand, RT-PCR was able to detect TAC4 mRNA expression in skin specimens from those same sources (Fig. 5a). We analyzed the TAC4 mRNA expression levels semi-quantitatively by measuring the relative intensity of TAC4 mRNA bands to GAPDH bands (Fig. 5b). The TAC4 mRNA expression level did not differ significantly between the skin tissues from NC subjects ($n = 9$) and CSU patients ($n = 12$).

Discussion

This is the first report to clearly demonstrate that (1) the HK-1 serum level was significantly higher in NC subjects than in patients with moderate or severe CSU ($P = 0.007$ and $P < 0.0001$, respectively, Figure 1c), (2) the EC₅₀ of histamine release from MCs by HK-1 (5056 nM, Fig. 2a) was 12-fold higher than that by SP (426 nM, Fig. 2b). Brief incubation of MCs with HK-1 at concentrations of 3.0–10 μ M caused significant inhibition of histamine release in

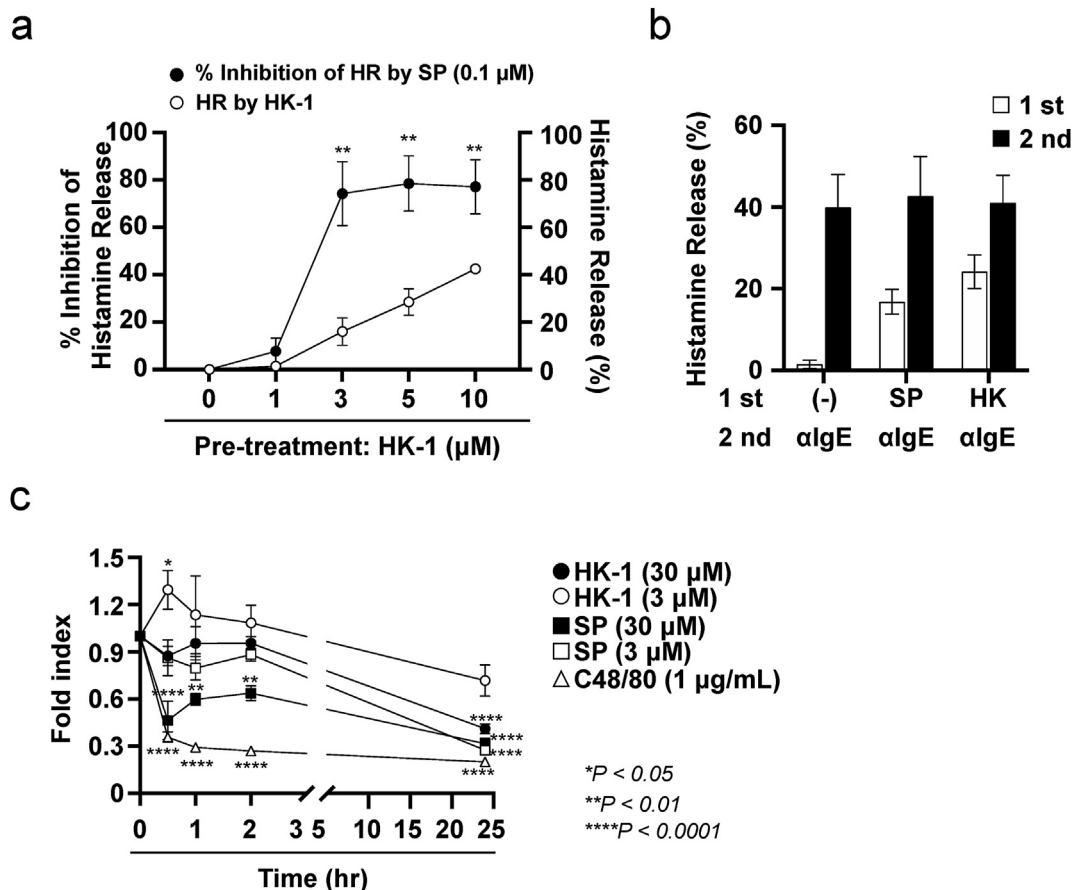


Fig. 4. Desensitization of MRGPRX2-mediated MC activation by HK-1. **(a)** Percentage inhibition of histamine release by 0.1 μM SP after pretreatment of MCs with 1, 3, 5 and 10 μM of HK-1 (closed circles). Histamine release by pretreatment of MCs with 1, 3, 5 and 10 μM of HK-1 is shown (open circles). Results are shown as the means ± SEM of 3 experiments using samples from 3 different donors' MCs. Asterisks indicate a significant difference between histamine release by SP and histamine release by SP after HK-1 pretreatment (** $P < 0.01$). **(b)** Pretreatment with SP or HK has no effect on IgE-dependent histamine release. 1st means the 1st stimulation of human synovium-derived cultured MCs with or without 0.3 μM SP or 3 μM HK-1. 2nd means 2nd stimulation of the MCs with anti-IgE Ab (0.1 μg/mL). Results are shown as the means ± SEM of 5 experiments using 5 different donors' MCs. **(c)** Effects of incubation with SP, HK-1 and compound 48/80 (C48/80) on cell-surface MRGPRX2 expression by synovium-derived, cultured MCs; analyzed by flow cytometry. The MCs were stimulated with 3 and 30 μM of SP, 3 and 30 μM of HK-1 or 1 μg/mL of C48/80 for the indicated times. Fold indexes of MRGPRX2 expression on the MCs are shown (the mean ± SEM for 3 independent donors). MRGPRX2 expression level at 0 h is designated as 1. Asterisks indicate a significant difference between MRGPRX2 expression level at 0 h and MRGPRX2 expression level at each time point (* $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$).

response to 0.1 μM SP (Fig. 4a). However, brief incubation of MCs with HK-1 did not elicit rapid MRGPRX2 internalization. These findings suggest that, in NC subjects, HK-1 may desensitize MRGPRX2-mediated MC activation, thereby preventing MC degranulation by SP.

The results of this study did not confirm those published previously by researchers in Germany, India, China and Iran, who independently reported that the SP serum level was significantly higher in CSU patients than in healthy subjects.^{4–7} The reason for this discrepancy in findings is not clear. The serum levels of SP in Japanese NC subjects and CSU patients were lower than those reported for patients in Germany, China and Iran.^{4,5,7} The detection limit for SP of the ELISA kit we used was around 100 pg/mL (Fig. 1b). The SP levels of both many NC subjects and CSU patients were below 100 pg/mL (Fig. 1d). The lower serum SP levels in Japanese might be one reason why we found no significant difference in that level between NC subjects and CSU patients. The serum SP level correlated significantly with the urticaria severity score ($P = 0.043$, $r = 0.184$; Table 2), suggesting that this might be a new biomarker for the severity of CSU.

The serum HK-1 concentration was significantly lower in the CSU patients than in the NC subjects (Fig. 1c, $P < 0.0001$), and it was also significantly lower in the moderate and severe CSU patients than in

the NC subjects (Fig. 1e). These findings suggest that HK-1 plays a beneficial role in mitigating CSU. Among the clinical parameters examined, only the duration of illness correlated significantly with the HK-1 concentration ($P = 0.021$, $r = 0.188$). The EC₅₀ of histamine release from MCs in response to HK-1 (Fig. 2a) was 12-fold higher than that with SP (Fig. 2b), and the maximum histamine release induced by HK-1 ($51.5 \pm 12.8\%$) was about half of that by SP ($90.8 \pm 13.5\%$). These findings suggest that HK-1 does not exacerbate CSU.

We previously reported that two eosinophil granule proteins, major basic protein (MBP) and eosinophil peroxidase (EPO), inhibit SP-induced skin MC activation, and we suggested that these ligands bind to the same receptor.¹⁷ We then found that MBP and EPO are ligands of MRGPRX2.³ HK-1 is also a ligand of MRGPRX2, which is a receptor for SP. Therefore, we hypothesized that HK-1 might block MRGPRX2-mediated MC activation. Brief incubation (15 min) of MCs with HK-1 at concentrations of 3.0–10 μM caused significant inhibition of histamine release in response to SP (Fig. 4a). However, brief incubation of MCs with HK-1 did not induce rapid MRGPRX2 internalization. Babina *et al.* reported that compound 48/80 potently elicits rapid MRGPRX2 internalization, which is β-arrestin-dependent, whereas SP-triggered internalization is less rapid and complete, indicating less profound β-arrestin coupling.¹⁸ HK-1

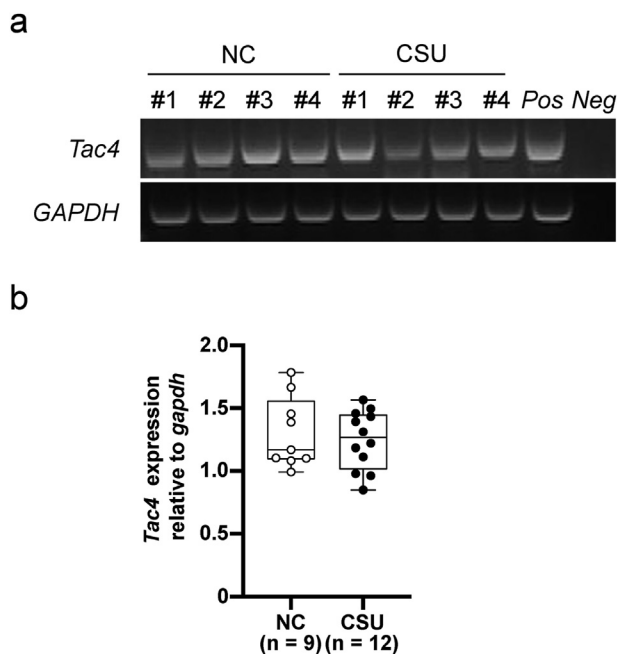


Fig. 5. Comparison of TAC4 mRNA expression in skin specimens obtained from NC subjects and CSU patients. **(a)** Representative data for TAC4 and GAPDH mRNA expression in skin specimens obtained from NC subjects ($n = 4$) and CSU patients ($n = 4$) analyzed by RT–PCR. Pos and Neg indicate positive (Jurkat cells [ATCC TIB-152]) and negative (water only) controls, respectively. **(b)** Comparison of TAC4 mRNA expression in skin specimens obtained from NC subjects ($n = 9$) and CSU patients ($n = 12$). Expression levels are shown as the relative intensity of the TAC4 mRNA band to the GAPDH mRNA band.

caused slower and less complete internalization, suggesting that the mechanism of the internalization might be β -arrestin-independent. Actually, MRGPRX2 can undergo β -arrestin-initiated internalization upon ligand binding, but internalization does not apply to all ligands alike.¹⁶ For example, icatibant, a bradykinin B₂ receptor antagonist, promotes MC degranulation via MRGPRX2, but does not activate β -arrestin.¹⁶ Further studies would be required to clarify the mechanism of inhibition of SP-mediated histamine release by HK-1.

Due to the lack of a specific antibody to SP that does not cross-react with HK-1, SP levels need to be measured by liquid chromatography–tandem mass spectrometry. Another weakness of this study is that we did not determine the cell sources of HK-1 and SP in the NC subjects and CSU patients.

With regard to the cell sources of HK-1 and SP, we previously reported that expression of TAC1 mRNA was below the limit of detection in human synovium-derived cultured MCs before activation, and that the levels of β and γ transcripts of the TAC1 gene were upregulated after Fc ϵ RI aggregation.¹⁹ However, the expression level of either TAC3 or TAC4 mRNA in MCs was below the limit of detection before and after Fc ϵ RI aggregation. Also, 6 h after aggregation of Fc ϵ RI, the amount of SP in the MC pellets was increased.¹⁹ These findings suggest that MCs are a cell source of SP, but not HK-1.

Since the expression levels of TAC4 mRNA in the skin biopsy specimens were quite low, we speculate that to be the reason that we found no significant difference between the NC subjects and CSU patients. For ethical reasons, it was difficult to obtain larger biopsy skin samples (more than 10 mg).

Expression of MRGPRX2 by skin MCs and the levels of MRGPRX2 agonists (e.g., SP, MBP, EPO) are upregulated in the serum and/or skin of CSU patients.^{3,10} For that reason, MRGPRX2 and its agonists have been proposed to be potential biomarkers for progression of

CSU and the response to treatment.¹⁰ In conclusion, SP might play an important role in the pathogenesis of severe CSU, whereas in NC subjects HK-1 may desensitize MRGPRX2-mediated MC activation, thereby preventing MC degranulation by SP.

Acknowledgments

We thank Associate Professor Hideki Fujita and his colleagues in the Department of Dermatology of Nihon University Hospital for providing the human specimens and for their excellent assistance. This work was supported in part by a Grant-in-Aid for Early-Career Scientists (19K17687, awarded to ST) and a Grant-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, Science and Technology (MEXT) of the Japanese Government (20K08811, awarded to YO), a Nihon University Multidisciplinary Research Grant for 2018–2019 (Project No. So18-009, awarded to YO), a Nihon University Research Grant for Social Implementation for 2020 (Project No. Sya20-1201, awarded to Y.O.), and a grant from the MEXT-Supported Program for the Strategic Research Foundation at Private Universities, 2015–2019 (Project No. S1511014, awarded to TT and YO).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2021.05.002>.

Conflict of interest

KH received lecture fee from Mitsubishi Tanabe Pharma. TT received lecture fee from Mitsubishi Tanabe Pharma, Taiho Pharma. The rest of the authors have no conflict of interest.

Authors' contributions

YO designed the study and wrote the manuscript. NN, ST and TSS performed the experiments. NN and ST performed the statistical analyses and prepared the figures and tables. KH and TT organized the collection of clinical data and samples.

References

- Zuberbier T, Aberer W, Asero R, Abdul Latiff AH, Baker D, Ballmer-Weber B, et al. The EAACI/GA(2)LEN/EDF/WAO guideline for the definition, classification, diagnosis and management of urticaria. The 2017 revision and update. *Allergy* 2018;**73**:1393–414.
- Galli SJ, Tsai M. IgE and mast cells in allergic disease. *Nat Med* 2012;**18**:693–704.
- Fujisawa D, Kashiwakura J, Kita H, Kikukawa Y, Fujitani Y, Sasaki-Sakamoto T, et al. Expression of Mas-related gene X2 on mast cells is upregulated in the skin of patients with severe chronic urticaria. *J Allergy Clin Immunol* 2014;**134**:622–33. e9.
- Metz M, Krull C, Hawro T, Saluja R, Groffik A, Stanger C, et al. Substance P is upregulated in the serum of patients with chronic spontaneous urticaria. *J Invest Dermatol* 2014;**134**:2833–6.
- Basak PY, Erturan I, Yuksel O, Kazanoglu OO, Vural H. Evaluation of serum neuropeptide levels in patients with chronic urticaria. *Indian J Dermatol Venereol Leprol* 2014;**80**:483.
- Zheng W, Wang J, Zhu W, Xu C, He S. Upregulated expression of substance P in basophils of the patients with chronic spontaneous urticaria: induction of histamine release and basophil accumulation by substance P. *Cell Biol Toxicol* 2016;**32**:217–28.
- Fadaee J, Khoshkhui M, Emadzadeh M, Hashemy SI, Farid Hosseini R, Jabbari Azad F, et al. Evaluation of serum substance P level in chronic urticaria and correlation with disease severity. *Iran J Allergy Asthma Immunol* 2020;**19**:18–26.
- Zhang L, Taylor N, Xie Y, Ford R, Johnson J, Paulsen JE, et al. Cloning and expression of MRG receptors in macaque, mouse, and human. *Brain Res Mol Brain Res* 2005;**133**:187–97.
- Wedi B, Gehring M, Kapp A. The pseudoallergen receptor MRGPRX2 on peripheral blood basophils and eosinophils: expression and function. *Allergy* 2020;**75**:2229–3342.
- Kuhn H, Kolkhir P, Babina M, Dull M, Frischbutter S, Fok JS, et al. Mas-related G protein-coupled receptor X2 and its activators in dermatologic allergies. *J Allergy Clin Immunol* 2021;**147**:456–69.
- Borbély E, Helyes Z. Role of hemokinin-1 in health and disease. *Neuropeptides* 2017;**64**:9–17.

12. Manorak W, Idahosa C, Gupta K, Roy S, Panettieri Jr R, Ali H. Upregulation of Mas-related G Protein coupled receptor X2 in asthmatic lung mast cells and its activation by the novel neuropeptide hemokinin-1. *Respir Res* 2018;**19**:1.
13. Watanabe C, Mizoguchi H, Bagetta G, Sakurada S. Involvement of spinal glutamate in nociceptive behavior induced by intrathecal administration of hemokinin-1 in mice. *Neurosci Lett* 2016;**617**:236–9.
14. Zuberbier T, Aberer W, Asero R, Bindslev-Jensen C, Brzoza Z, Canonica GW, et al. The EAACI/GA(2) LEN/EDF/WAO Guideline for the definition, classification, diagnosis, and management of urticaria: the 2013 revision and update. *Allergy* 2014;**69**:868–87.
15. Mathias SD, Crosby RD, Rosen KE, Zazzali JL. The minimal important difference for measures of urticaria disease activity: updated findings. *Allergy Asthma Proc* 2015;**36**:394–8.
16. Roy S, Ganguly A, Haque M, Ali H. Angiogenic host defense peptide AG-30/5C and bradykinin B2 receptor antagonist icatibant are G protein biased agonists for MRGPRX2 in mast cells. *J Immunol* 2019;**202**:1229–38.
17. Okayama Y, el-Lati SG, Leiferman KM, Church MK. Eosinophil granule proteins inhibit substance P-induced histamine release from human skin mast cells. *J Allergy Clin Immunol* 1994;**93**:900–9.
18. Babina M, Wang Z, Roy S, Guhl S, Franke K, Artuc M, et al. MRGPRX2 is the codeine receptor of human skin mast cells: desensitization through beta-arrestin and lack of correlation with the FcεRI pathway. *J Invest Dermatol* 2020. <https://doi.org/10.1016/j.jid.2020.09.017>.
19. Okamura Y, Mishima S, Kashiwakura JI, Sasaki-Sakamoto T, Toyoshima S, Kuroda K, et al. The dual regulation of substance P-mediated inflammation via human synovial mast cells in rheumatoid arthritis. *Allergol Int* 2017;**66**:S9–20.