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Staphylococcal α -hemolysin does not induce cell damage in murine mast cells but it augments the degranulation induced by Fc ϵ RI cross-linking and ionomycin

Kazuhito Hayashi^a, Saotomo Itoh^{a,*}, Arisa Morikawa^a, Kikuo Onozaki^a, Shinsuke Taki^b, Tsutomu Tsuji^c, Shigeaki Hida^a

^a Department of Molecular and Cellular Health Sciences, Graduate School of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-Dori, Mizuho-ku, Nagoya, 467-8603, Japan

^b Department of Molecular and Cellular Immunology, School of Medicine Shinshu University, 3-1-1 Asahi, Matsumoto, 390-8621, Japan

^c Department of Microbiology, Hoshi University School of Pharmacy and Pharmaceutical Sciences, 2-4-41 Ebara, Shinagawa-ku, Tokyo, 142-8501, Japan

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ABSTRACT

Staphylococcal α -hemolysin (Hla) is a principal small β -barrel pore forming toxin. It targets a variety of mammalian cells including immune cells; however little is known about its effects on mast cells. In this study, we examined whether Hla affects the degranulation of mast cells. Although Hla bound to the surface of bone marrow-derived mast cells (BMMCs) and formed SDS-stable oligomers on the cells, Hla alone induced neither cytotoxicity nor obvious release of a granule enzyme, β -hexosaminidase. However, Hla more than doubled the releases of β -hexosaminidase from BMMCs induced by Fc ϵ RI cross-linking or treatment with ionomycin. The augmentation of the enzyme release by rHla was impaired in the presence of 130 mM of extracellular KCl. The mutants of Hla that lacked pore-formation did not augment the release of the enzyme. These findings demonstrate that Hla is able to enhance the degranulation of mast cells induced by Fc ϵ RI cross-linking and ionomycin, although it alone does not induce the degranulation, and the pore-formation of Hla followed by potassium efflux is involved in the augmentation. These findings propose a previously unrecognized role for Hla in *S. aureus*-associated allergic and inflammatory processes via augmentation of mast cell responses.

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1. Introduction

Staphylococcus aureus (*S. aureus*) is an important pathogen of human; it is a common cause of skin and soft-tissue infections, food-borne diseases, and methicillin-resistant *S. aureus* causes life-threatening infection in hospitalized patients. *S. aureus* is notorious for producing a variety of exotoxins such as superantigens and enterotoxins, hemolysins and leukocidins, that would account for the pathogenicity of the cocci [1]. A pore-forming toxin α -hemolysin (α -toxin, Hla) is a principal arsenal of *S. aureus*. It is expressed in a most of strains and shows toxicity against mammal cells [1]. The secretion of Hla correlates with the morbidity of lung infection,

and vaccination with Hla protects mice from pneumonia [2]. Hla is known as the prototype for small β -barrel pore-forming toxins, that assembles cylindrical heptamers on target cells [3]. Hla induces lysis and death of many types of mammalian cells including erythrocytes, platelets, macrophages, lymphocytes, keratinocytes, and epithelial cells [4]. *S. aureus* frequently colonizes in the skin of atopic dermatitis [5]. Mast cells are thought to be involved in the pathogenesis of atopic dermatitis because of their ability to release a series of inflammatory mediators [6]. Recently it has been reported that δ -toxin and phenol-soluble modulins (PSMs) evoke the activation of mast cells [7,8], suggesting that staphylococcal peptide toxins are presumable determinants for the pathogenesis of atopic dermatitis. However, there are only a few reports about the effects of other staphylococcal exotoxins including Hla on mast cells [8–10].

In this study we examined the interaction of recombinant Hla (rHla) with bone marrow-derived murine mast cells (BMMCs) and the effects of rHla on the viability and degranulation of BMMCs.

Abbreviations: BMMCs, Bone marrow-derived mast cells; DNP, 2,4-dinitrophenol; FLAG, DYKDDDDK; Fc ϵ RI, high-affinity IgE receptor; Hla, α -hemolysin; PSM, Phenol-soluble modulin.

* Corresponding author.

E-mail address: s-itoh@phar.nagoya-cu.ac.jp (S. Itoh).

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2. Materials and methods

2.1. Reagents

All reagents were purchased from Nacalaitesque (Kyoto, Japan), SIGMA (St. Louis, MO), WAKO pure chemicals (Osaka, Japan) unless otherwise specified. Restriction endonucleases and DNA modifying enzymes were from Roche (Basel, Switzerland) and TaKaRa (Osaka, Japan). Oligonucleotides were supplied by Eurofin Genomics (Huntsville, AL) and SIGMA. Antibodies used for flow cytometry were products of Biolegend (San Diego, CA). Staphylococcal δ -toxin (Formyl-MAQDIISTIGDLVKWIIDTVNKFTHK-COOH) was synthesized in the Eurofin Genomics.

2.2. Preparation of bone marrow derived mast cells

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Bone marrow cells recovered from femurs and tibiae, and cultured at 37°C in a humidified atmosphere containing 5% CO₂ in RPMI-1640 medium supplemented with 10% heat-inactivated FBS (Biowest, Nuaille, France), 0.03% sodium glutamate, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-mercaptoethanol, 0.04% NaHCO₃ and IL-3 (5 ng/mL). The media was replaced every 3 days, and after 4 weeks BMMC phenotypes were confirmed by flow cytometry analysis with PE-conjugated anti-FcεRI antibody and APC-conjugated anti-c-Kit antibody.

2.3. Preparation of recombinant Hla and its mutants

The Hla gene was amplified from genomic DNA of *S. aureus* ATCC27733 using PrimeSTAR[®] GxL polymerase (TaKaRa Bio, Osaka, Japan). The amplified fragment was subcloned into pGEM-T Easy (Promega, Madison, WI) and confirmed the sequence using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). The DNA fragment was subcloned into prokaryotic expression plasmid pCold-II (TaKaRa) using BamH I and Hind III sites. The plasmids were transformed into ClearColi[™] that does not express endotoxin (Lucigen, Middletown, WI). The recombinant protein was induced and purified as described previously [11]. Hla(H35L) [12], Hla(PSGS) [13] and C-terminal FLAG-tagged Hla were generated by inverse PCR method as described previously [14]. Primers for the amplification and inverse PCR are listed in Table 1.

2.4. Determination of cytotoxicity induced by rHla

The cytotoxicity of rHla on BMMCs was determined using WST-1 cell counting kit (Dojindo Laboratories, Kumamoto, Japan).

Table 1
Primers used in this study.

Primer name	Sequences
a) Primers for the amplification of Hla	
BamH I-Hla_F	GGA TCC GCA GAT TCT GAT ATT AAT ATT AAA ACC ^a
Hind III-Hla_R	AAG CTT AAT TTG TCA TTT CTT CTT TTT CCC AA
b) primers for inverse PCR	
iHla(H35L)_Frc	CAT GCC ATT TTC TTT ATC ATA AGT G
iHla(H35L)_R	CTC AAA AAA GTA TTT TAT AGT TTT ATC G
iHla(PSGS)_Frc	CAG ACG GTG TAT CAA TCG AAT TTC TTG GAT AG
iHla(PSGS)_R	GTT CTG TTC AAC CTG ATT TCA AAA CAA TTT
iHlaC-term-ns_Frc	ATT TGT CAT TTC TTC TTT TTC CCA A
ipColdIII-ins-GS-FLAG_R	GGG TCC GAC TAC AAG GAT GAC GAT GAC AAG TAG GTA ATC TCT GC TTA AAA GCA CAG

^a Restriction recognition sites are underlined.

BMMCs suspended in RPMI1640 supplemented with 10% FBS and IL-3 (5 ng/mL) were seeded in a 96-well microtiter plate (1×10^5 cells/100 µl/well) in the presence or absence of rHla. After cultivation at 37°C for 24 h, 20 µl of WST-1 solution was added to each well and then incubated for 2 h. Absorbance of each well was measured at 450 nm (reference 750 nm) using iMark Microplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA).

2.5. Flow cytometric analysis

Binding of rHla on BMMCs was determined using a flow cytometer. BMMCs (1×10^7 cells/mL) were incubated with FLAG-tagged Hla (30 µg/mL) in PBS supplemented with 0.5% BSA at 37°C for 30 min, then the cells were stained with FITC-conjugated anti-FcεRI, PE-conjugated anti c-Kit and APC-conjugated anti-FLAG antibody, and analyzed using a flow cytometer (FACS Verse[™], Becton Dickinson).

2.6. Detection of Hla oligomers

BMMCs suspended in modified Tyrode's buffer (2×10^5 cells/100 µl, 10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.1% BSA, pH 7.4) were incubated with 30 µg/mL of the FLAG-tagged rHla at 37°C for 30 min. The cells were recovered by centrifugation and solubilized with 50 µl of laemmli sample buffer without 2-mercaptoethanol. Protein was separated by SDS-PAGE (8%) and transferred to nitrocellulose membrane. The membrane was blocked with BlockAce (Dainippon Pharmaceutical Co. Ltd, Osaka, Japan) for 1 h, sequentially treated with anti-FLAG antibody (Sigma) and HRP-linked anti mouse IgG antibody (Cell Signaling Technology, Beverly, MA), followed by chemiluminescence detection.

2.7. Degranulation assay

BMMCs were pre-cultured in fresh media supplemented with IL-3 (5 ng/mL) in the presence or absence of anti-2,4-dinitrophenol (DNP)-IgE (2 µg/mL, SPE-7, Nippon Biotest Laboratories, Inc., Saitama, Japan) for 16 hr. The cells were washed with modified Tyrode's buffer, and seeded in a 96 well microtiter plate (1×10^5 cells/100 µl/well) in the presence or absence of rHla, ionomycin, δ -toxin and DNP-BSA for 30 min. The microtiter plate was centrifuged at 1400 rpm for 5 min, then supernatant was transferred to a new microtiter plate. The microtiter plate after transferring aliquot of supernatant was used for lactate dehydrogenase (LDH) assay as described below. The activity of β -hexosaminidase in the supernatant was determined using p-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside (WAKO) as substrate. To determine the total amount of β -hexosaminidase activity, cells were lysed with 0.5% Triton X-100. After addition of stop solution (200 mM glycine, pH 10.7), the absorbance of each well was measured at 415 nm (reference 750 nm) using a microplate reader. In some experiments, degranulation assay was performed using the buffer, the concentration of KCl was replaced with NaCl (10 mM HEPES, 5 mM NaCl, 130 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 0.1% BSA, pH 7.4).

2.8. LDH assay

LDH activity released into the buffer was determined using Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories) according to the manufacturer's protocol. Briefly, working solution (50 µL) was added to the well and allowed to react at room temperature for 20 min. The reaction was stopped by adding stop solution, the absorbance of each well was measured at 450 nm (reference

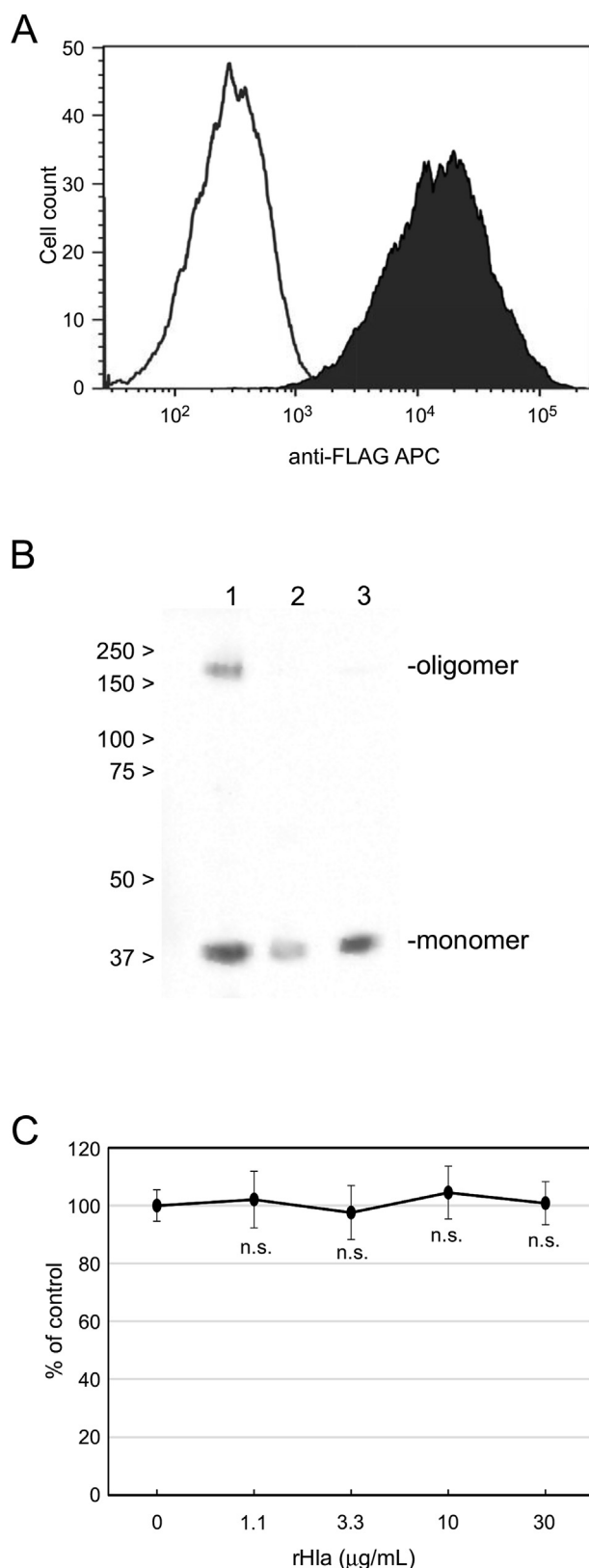


Fig. 1. The binding of rHla to BMMCs and its effect on cell viability.

A. The binding of rHla to BMMCs. BMMCs were treated with FLAG-tagged rHla at 37°C for 30 min, stained with APC-conjugated anti-FLAG antibody and analyzed using a flow cytometer. White filled histogram corresponds to anti-FLAG antibody alone, black filled histogram to the combination treatment of FLAG-tagged rHla and anti-FLAG antibody. **B.** The formation of Hla oligomers. BMMCs were treated with FLAG-tagged Hla (30 $\mu\text{g/mL}$) for 30 min. Cell-bound Hla was recovered and visualized by 8% (w/v) SDS-PAGE

(750 nm) using a microplate reader. To determine the total amount of LDH activity, cells were lysed with 0.5% Triton X-100.

2.9. Statistical analysis

Statistical evaluation was performed using Welch's or Student's t-test (comparisons between two means) and one-way ANOVA (comparisons more than three means).

3. Results

3.1. Recombinant Hla interacts with BMMCs, but does not induce cell death

C-terminal FLAG-tagged rHla bound to BMMCs as determined by flow cytometric analysis (Fig. 1A), and it formed cell-bound SDS-resistant oligomers by incubation with BMMCs (Fig. 1B). The rHla did not induce cytotoxicity in BMMCs at less than 30 $\mu\text{g/mL}$ for 24 h (Fig. 1C), as measured by WST-1 assay.

3.2. Recombinant Hla alone does not induce the degranulation of BMMCs

We examined whether rHla induces the degranulation of BMMCs by measuring the release of a granule enzyme, β -hexosaminidase, from the cells. The release of β -hexosaminidase from untreated BMMCs was $0.43 \pm 0.16\%$ and the enzyme release was increased to $63.0 \pm 2.1\%$ by treatment with 0.25 $\mu\text{g/mL}$ of ionomycin (Fig. 2A). The release of the granule enzyme was not increased by treatment with 1.1–10 $\mu\text{g/mL}$ of rHla, and was only slightly increased to $1.18 \pm 0.19\%$ with 30 $\mu\text{g/mL}$ of rHla (Fig. 2A). The leakage of a cytosolic enzyme, LDH, from BMMCs was not significantly induced by treatment with 0.25 $\mu\text{g/mL}$ of ionomycin and rHla (1.1–30 $\mu\text{g/mL}$, Fig. 2B). Staphylococcal δ -toxin induced the release of β -hexosaminidase from BMMCs as previously reported [7]: The releases of the granule enzyme were 0.48 ± 0.09 , 3.42 ± 0.19 , 5.45 ± 0.19 , 8.88 ± 0.25 and $11.23 \pm 0.09\%$ by treatment with 0, 3.7, 11.1, 33.3 and 100 $\mu\text{g/mL}$ of δ -toxin, respectively (Fig. 2C). The leakage of LDH from BMMCs was also observed by treatment with δ -toxin: The increments of LDH release were 0.55 ± 0.12 , 0.84 ± 0.26 , 1.52 ± 0.07 and $11.0 \pm 0.84\%$ by treatment with 3.7, 11.1, 33.3 and 100 $\mu\text{g/mL}$ of δ -toxin, respectively (Fig. 2D). The release of β -hexosaminidase was not significantly induced by treatment with rHla (30 $\mu\text{g/mL}$) up to 90 min (data not shown).

3.3. Hla augments the degranulation of BMMCs induced by Fc ϵ RI cross-linking and ionophore

We examined the effects of rHla on the degranulation of BMMCs induced by other stimuli. The treatment of anti-DNP-IgE-sensitized BMMCs with DNP-BSA induced the release of β -hexosaminidase: The releases of the enzyme were 1.40 ± 0.24 , 1.11 ± 0.00 , 3.77 ± 0.25 , 6.51 ± 0.21 and 4.99 ± 0.00 by treatment with 0, 0.005, 0.05, 0.5 and 5 $\mu\text{g/mL}$ of DNP-BSA, respectively (Fig. 3A). The releases of the enzyme induced by DNP-BSA from anti-DNP-IgE sensitized cells were significantly increased in the presence of rHla (30 $\mu\text{g/mL}$): The releases of the enzyme induced by the antigen were 2.30, 2.00, 2.00 and 2.04 times by treatment with 0.005, 0.05,

and immunoblotting with anti-FLAG and HRP-conjugated secondary antibody. Lane 1: Cell bound Hla, Lane 2 and 3, FLAG-tagged rHla (3 ng and 10 ng, respectively). **C.** The viability of BMMCs after incubation with indicated concentrations of rHla at 37°C for 24 h. The viability was measured using WST-1 cell counting kit. The data shown were representative of three independent experiments, and the values are expressed as mean $\pm$ SD of triplicate well. n.s. = not significant compared with control.

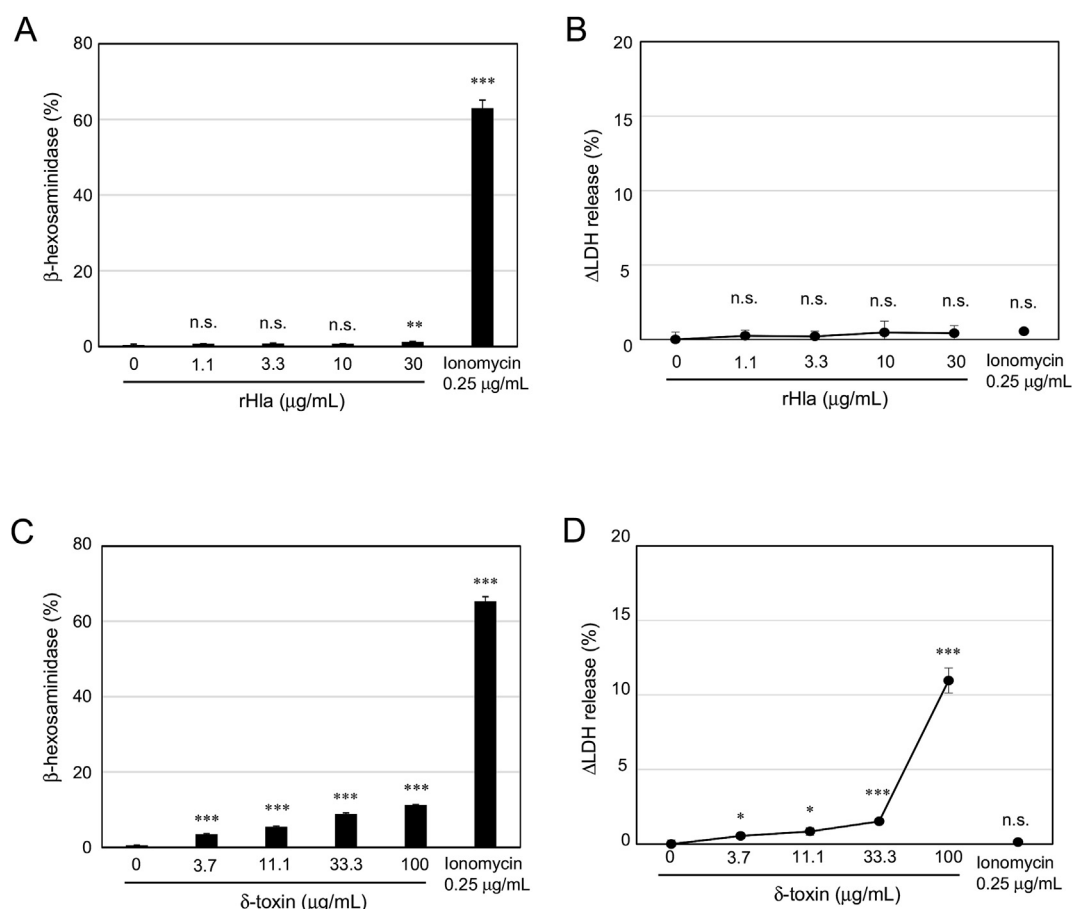


Fig. 2. The releases of β -hexosaminidase and the leakages of LDH from BMMC treated with rHla and δ -toxin.

A, The release of β -hexosaminidase from BMMCs treated with rHla. The cells were treated with indicated concentrations of rHla or ionomycin (0.25 μ g/mL) for 30 min then the β -hexosaminidase in the supernatant was determined. The release of the enzyme was showed % of total β -hexosaminidase content. B, The leakage of LDH from BMMCs treated with rHla. The cells were treated with indicated concentrations of rHla or ionomycin (0.25 μ g/mL) for 30 min then the amount of LDH released into the buffer was determined using Cytotoxicity LDH Assay Kit-WST. The release of the enzyme was showed % of total LDH content, and the data were shown as the difference of LDH release between that from untreated cells and that from rHla treated cells. C, The release of β -hexosaminidase from BMMCs treated with δ -toxin. D, The leakage of LDH from BMMCs treated with δ -toxin. The data shown were representative of three independent experiments, and the values are expressed as mean $\pm$ SD of triplicate well. N.D = not detected. * P < 0.05, ** P < 0.01, *** P < 0.001 and n.s. = not significant compared with control.

0.5 and 5 μ g/mL of DNP-BSA, respectively, in the presence of rHla (Fig. 3A). The releases of β -hexosaminidase induced by ionomycin were also increased in the presence of rHla, and the increments were remarkable at the low concentration of ionomycin: The releases of the enzyme were 2.48, 2.57, 1.77, 1.25 and 1.19 times by treatment with 0.049, 0.074, 0.111, 0.167 and 0.25 μ g/mL of ionomycin, respectively, in the presence of rHla (30 μ g/mL, Fig. 3B). The increments of β -hexosaminidase release were dose dependent of rHla: The releases induced by 0.1 μ g/mL of ionomycin were increased 1.20, 1.16, 1.84 and 2.93 times in the presence of 1.1, 3.3, 10 and 30 μ g/mL of rHla, respectively (Fig. 3C). The increment by 30 μ g/mL rHla was impaired in the buffer containing 130 mM KCl and 5 mM of NaCl: The increments were 1.36, 1.16 and 0.94 times by treatment with 0.049, 0.11 and 0.25 μ g/mL of ionomycin, respectively (Fig. 3D).

3.4. Pore-formation defect mutants of Hla do not augment the degranulation of BMMCs

We prepared two nontoxic mutants of Hla, Hla(H35L) and Hla(PSGS). Hla(H35L) is the mutant of Hla with a H35L substitution [12]. Hla(PSGS) is the mutant of Hla that substituted membrane-spanning stem region to 4 amino acids (PSGS) [13]. C-terminal

FLAG-tagged rHla(H35L) and rHla(PSGS) were able to bind to the surface of BMMCs (Fig. 4A and B, respectively). Hla(H35L) formed SDS-stable oligomers on BMMCs, but Hla(PSGS) failed to form the oligomers (Fig. 4C). The releases of β -hexosaminidase from BMMCs treated with ionomycin (0.0625, 0.125, 0.25 μ g/mL) were not significantly increased in the presence of rHla(H35L) or rHla(PSGS) (Fig. 4D).

4. Discussion

In the present study, rHla was examined for its effects on the activation of BMMCs. FLAG-tagged rHla bound to BMMCs (Fig. 1A) and formed the SDS-resistant oligomers by incubation with BMMCs (Fig. 1B). Recombinant Hla alone did not induce cell death (Fig. 1C), nor induce the leakage of a cytosolic enzyme, LDH (Fig. 2B). These findings indicate that although rHla is able to bind to BMMCs and assemble oligomers on the cells, BMMCs are resistance to Hla-induced cell damage. Recombinant Hla alone did not induce the release of β -hexosaminidase at below 10 μ g/mL, the release of the enzyme was only slightly increased in the presence of 30 μ g/mL of rHla (Fig. 2A). In contrast, *Staphylococcus aureus* δ -toxin induced the release of β -hexosaminidase and leakage of LDH from BMMCs (Fig. 2C and D, respectively). These results were consistent with the

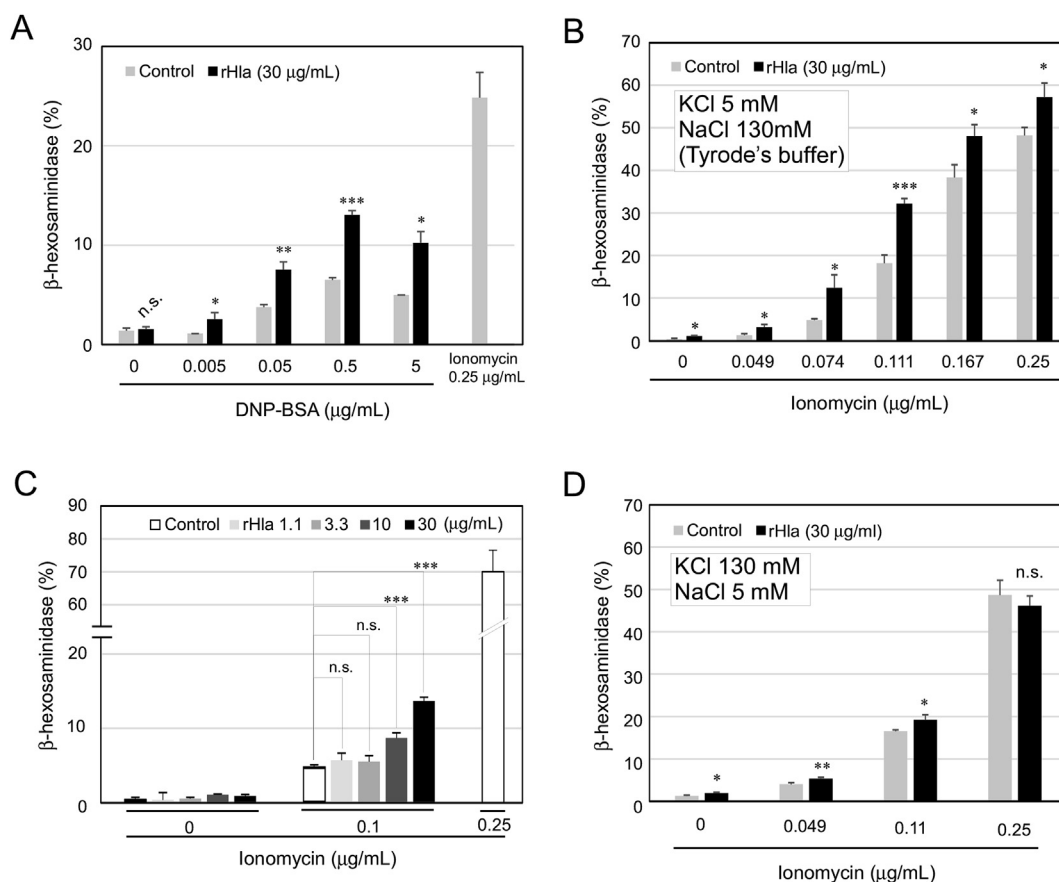


Fig. 3. The effects of rHla on the release of β -hexosaminidase induced by Fc ϵ RI cross-linking and ionophore.

A and B, The effects of rHla on the release of β -hexosaminidase induced by Fc ϵ RI cross-linking and ionophore. IgE-sensitized BMMCs (A) and BMMCs (B) were treated with indicated concentrations of DNP-BSA (A) and ionomycin (B) in the presence or absence of rHla (30 μ g/mL). C, Dose dependency of the augmentation effect of rHla on the β -hexosaminidase release. BMMCs were treated with combinations of indicated concentrations of rHla and ionomycin. D, The effect of rHla on the degranulation of BMMC induced by ionomycin in the buffer containing 130 mM KCl and 5 mM NaCl. After incubation at 37°C for 30 min, the β -hexosaminidase in the supernatant was determined. The data shown were representative of three independent experiments, and the values are expressed as mean $\pm$ SD of triplicate well. *P < 0.05, **P < 0.01, ***P < 0.001 and n.s. = not significant compared with the absence of rHla.

preceding report that δ -toxin and PSM induce the degranulation and leakage of LDH from human mast cell line, on the other hand Hla, γ -hemolysin and PV-leukocidin do not induce the degranulation nor the leakage of LDH from the cells [8].

Interestingly, the release of β -hexosaminidase from BMMCs induced by Fc ϵ RI cross-linking and ionomycin more than doubled in the presence of rHla (Fig. 3A and B, respectively). Recombinant Hla augmented the release in a dose dependent manner (Fig. 3C). As the treatment with rHla did not induce the leakage of LDH in BMMCs, the increased release of β -hexosaminidase does not result from the leakage of cytosolic component due to cell damage. As the release of β -hexosaminidase from rHla alone-treated BMMCs was less than 1.18%, the augmentation of the granule enzyme release by rHla is not a mere additive effect. These results suggest that Hla is able to augment the degranulation of BMMCs in response to Fc ϵ RI cross-linking and ionomycin, although it alone is ineffective to induce the degranulation.

Hla(H35L) and Hla(PSGS) are the mutants that lack pore-forming and hemolytic ability (Refs. 12 and 13). Both mutants were able to bind to BMMCs (Fig. 4A and B) and Hla(H35L) but not Hla(PSGS) formed SDS-resistant oligomers on BMMCs (Fig. 4C). These findings are supported by the previous report that Hla(H35A) forms SDS-stable oligomers [15], and the feature that Hla(PSGS) lacks its stem region that is essential for forming robust β -cylindrical structure of the heptameric pore [13]. Hla(H35L) and

Hla(PSGS) failed to augment the release of β -hexosaminidase induced by ionomycin (Fig. 4D). These findings indicate that pore-formation of Hla, but not binding to the cells nor the formation of oligomers on the cells, is essential for the potentiation of degranulation. The pore formed by Hla is 1.4 nm in diameter and allows the pass of low molecular weight molecules such as Ca^{2+} , K^{+} and ATP [4]. The augmentation of β -hexosaminidase release by rHla was impaired in the presence of 130 mM extracellular KCl (Fig. 3D), the equal concentration of intracellular potassium cation, suggesting that potassium efflux due to pore formation is involved in the augmentation. The pore formation of Hla is insufficient for the induction of degranulation as the treatment with rHla alone did not induce the release of β -hexosaminidase from BMMCs (Fig. 2A). It is likely that the pore formed by Hla does not act just like as ionomycin (Ca^{2+} ionophore) or calcium influx alone is not sufficient for the degranulation of BMMCs.

Little is known about exogenous molecule such a bacterial product that enhances the degranulation of mast cells. It has been reported that cultivation of RBL-2H3 mast cell line with LPS up regulates the expression of calcium release-activated calcium channel protein 1 (Orai1) and stromal interaction molecule 1 (STIM1), and enhances the degranulation of mast cells induced by Fc ϵ RI cross-linking [16]. LPS requires at least 3 h of cultivation to up regulate the channel molecules [16], and LPS shows no effect on the degranulation of mast cells in short term treatment [17]. The

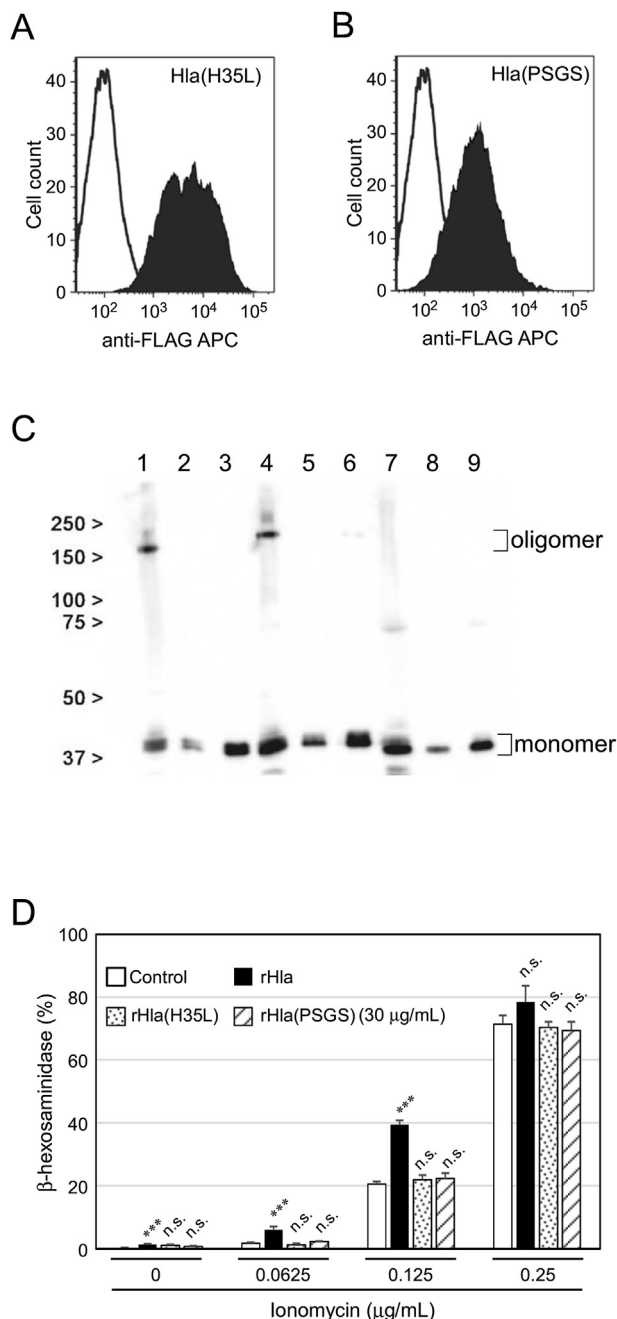


Fig. 4. The effect of pore-formation defect mutants of Hla on the release of β -hexosaminidase in response to ionophore.

A and B, The binding of Hla mutants on BMMCs. BMMCs was treated with FLAG-tagged rHla(H35L) (A) and rHla(PSGS) (B) at 37°C for 30 min, stained with APC-conjugated anti-FLAG antibody and analyzed using a flow cytometer. White filled histogram correspond to anti-FLAG antibody alone, black filled histogram to the combination treatment of FLAG-tagged rHla and anti-FLAG antibody. C, The formation of Hla mutants oligomers. Lane 1–3, rHla; lane 4–6, rHla(H35L); lane 7–9, rHla(PSGS). Lane 1, 4 and 7, Cell bound FLAG-tagged protein; lane 2, 5 and 8; FLAG-tagged protein (3 ng/lane), lane 3, 6 and 8, FLAG-tagged protein (10 ng/lane). D, The effects of Hla mutants on the release of β -hexosaminidase from BMMCs. BMMCs were treated with indicated concentrations ionomycin in the presence or absence of rHla and its mutants for 30 min, then the β -hexosaminidase activity in the supernatant was determined. The data shown were representative of three independent experiments, and the values are expressed as mean $\pm$ SD of triplicate well. ***P < 0.001 and n.s. = not significant, compared with the absence of rHla and its mutants.

effects of Hla on mast cells are different from that of LPS; rHla was able to enhance the degranulation of BMMCs by stimuli in 30 min in this study. The contamination of endotoxin in prokaryotically

prepared recombinant protein often causes artificial results [18]. Recombinant Hla used in this study was prepared using endotoxin-free *E. coli* strain ClearColi™ as host [19], and the augmentation of degranulation was also observed in TLR4-deficient BMMCs (data not shown). Therefore, we exclude the possibility that endotoxin derived from host *E. coli* activates BMMCs.

Mast cells are assumed to play a critical role in the pathogenesis of type I hypersensitivity, and the colonization of *S. aureus* has been associated with atopic dermatitis [5]. There are a few reports indicating that staphylococcal exotoxins directly activate or enhance the function of mast cells. Staphylococcal peptide toxins δ -toxin and PSMs have been reported to activate mast cells individually [7,8], and the degranulation of mast cells evoked by IgE-antigen complex is enhanced by δ -toxin [7]. In this study, rHla alone did not induce cell death, leakage of LDH and degranulation of mast cells (Figs. 1D, 2B and A, respectively), but augments the degranulation induced by other stimuli (Fig. 3A and B). The report that the release of granule enzyme by PSMs and δ -toxin are accompanied with cytolysis [8], is consistent with our study that the leakage of LDH was accompanied with δ -toxin-induced degranulation (Fig. 2C and D). The activation of mast cells by δ -toxin is mediated by formyl peptide receptors [7], on the other hand the augmentation of degranulation by Hla requires the pore forming ability of Hla (Fig. 4D). Therefore, mast cell activation induced by Hla seems to be different from that by δ -toxin and PSMs although they disturb membrane integrity.

Present findings indicate a new role of Hla that augments the degranulation induced by other stimuli. It is possible that the augmentation contributes to the exacerbation of allergic reactions such as atopic dermatitis by the colonization of *S. aureus*. The development of vaccines or neutralizers such as nucleotide aptamer against Hla may be useful for the prevention and treatment of disease that affected by colonization of *S. aureus*.

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Transparency document

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Conflicts of interest

The authors have no conflicts of interest to disclose.

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