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TITLE:

An Ex vivo Mast Cell Degranulation Assay using Crude Peritoneal Exudate Cells and Natural Antigen Stimulation

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SUMMARY:

We have established an ex vivo mast cell degranulation assay carried out by incubating crude peritoneal exudate cells isolated from the mice, treated with a pharmacological agent of interest and administered anti-dinitrophenol (DNP) IgE beforehand, with DNP on a carrier protein.

ABSTRACT:

Mast cell stabilizers are an essential part of allergy medication. Passive systemic anaphylaxis (PSA) is an animal assay widely used for investigating the effect of a pharmacological agent of interest on mast cells in vivo. As the anaphylactic symptoms are primarily attributed to exocytosis of the granules from mast cells, it is conceived that the agent to cause amelioration of the symptoms has a mast cell stabilizing activity. Despite the fact, it is prudent to confirm the activity by directly demonstrating the decline in the functional activity of mast cells following its treatment. In vitro degranulation assays using an immortalized mast cell line or cultured primary mast cells are routinely employed to that end. The results from the in vitro and in vivo assays may not always be akin to each other; however, as treatment conditions (e.g., treatment dose, time, surrounding environments) for the in vitro assays are often distinct from those for the in vivo assay such as PSA. In pursuit of an in vitro (or ex vivo) assay to reflect more closely the effect of a pharmacological agent on mast cells in vivo, we devised the ex vivo mast cell degranulation assay in which crude peritoneal exudate cells (PECs) isolated from the mice, treated with the agent and administered anti-dinitrophenol (DNP) IgE, were incubated directly with DNP on a carrier protein. It turned out that the assay was not only useful in validating the mast cell stabilizing activity of a pharmacological agent indicated by the in vivo assay but also practical and

highly reproducible.

INTRODUCTION:

Mast cells play a central role in allergy[1,2]. When IgE located on the surface of mast cells via interaction with the high-affinity receptor for IgE (FcεRI) encounters a cognate allergen, a signaling cascade is elicited to prompt the release of the granules. As a result, a variety of allergy effector molecules, including monoamines (e.g., histamine, serotonin), cytokines (e.g., TNF-α), and proteolytic enzymes (e.g., tryptase, chymase), are released to cause a series of immunological, neurological and vasomuscular reactions[3,4].

A class of pharmaceuticals is called mast cell stabilizer that alleviates the allergy symptoms by attenuating the mast cell function[5]. Passive systemic anaphylaxis (PSA) is an animal model often used for probing a mast cell stabilizing activity of pharmacological agents. As the anaphylactic symptoms result primarily from the activation of mast cells following interaction of passively transferred hapten-specific IgE with the hapten on a carrier protein injected into the animal later, it is well received that a pharmacological agent of interest bears a mast cell stabilizing activity when its treatment results in amelioration of the symptoms[6]. Still, it is often imperative to directly demonstrate impairment of the mast cell function by the agent in a separate experiment to rule out the possibility that improvement of the symptoms is derived from a mechanism other than suppression of mast cell function.

Mast cell degranulation assay, which is carried out by stimulating mast cells with a chemical reagent or a specific antigen of IgE forming a complex with FcεRI on the surface of mast cells to induce exocytosis of secretory granules (i.e., degranulation), is generally used for determining a mast cell stabilizing activity of a pharmacological reagent in vitro[7]. Several types of cells are used in that assay, including the rat basophilic leukemia (RBL) cell line[8], bone marrow-derived mast cells (BMMC)[9], and peritoneal cell-derived mast cells (PCMC)[10]. While useful as a large number of cells can be easily obtained, RBL is an immortalized cancer cell line whose cellular properties are no longer akin to those of mast cells in the body. Acquiring a sufficient number of BMMC or PCMC, even though their cellular properties may more closely resemble those of mast cells in the body, is often costly and time-consuming.

A degranulation assay using purified primary mast cells is a desirable alternative[11]. Nonetheless, the use of such an assay is not widespread as a facile method for purifying mast cells from animal tissue, particularly from mouse tissue, with a high yield, and purity is not yet available. Moreover, since the concentration and duration of treatment with a pharmacological agent to inhibit the mast cell function in vitro may not always coincide with those in vivo, results obtained with an in vitro degranulation assay may misrepresent those from an in vivo assay such as PSA, and vice versa. Hence, a novel degranulation assay, not only closely mimicking the way of mast cell activation transpiring in vivo but also accurately reflecting effects of a pharmacological reagent exerted on mast cells in vivo, is in high demand. In order to meet those needs, we devised an ex vivo mast cell degranulation assay where mast cells in peritoneal exudate cells (PECs) isolated from the mice, treated with a pharmacological agent of interest and administered IgE specific for dinitrophenol (DNP) beforehand, are stimulated with DNP-conjugated bovine serum albumin

(BSA).

PROTOCOL:

All animal experiments were performed in accordance with the guideline provided by the IACUC (Institutional Animal Care and Use Committee) of Chungnam National University (Animal Protocol Number: CNU-00996).

1. Quantifying mast cell-specific molecules in the lysate of crude PECs

1.1. Isolate the cells from the mouse peritoneal cavity[12].

1.1.1. Anesthetize a mouse (8 weeks old, male, BALB/C) with isoflurane. Euthanize via cervical dislocation.

1.1.2. Place the mouse on a foam block. Wipe the abdomen with 70% ethanol.

1.1.3. Cut the ventral skin longitudinally with blunt edge scissors. Peel off the skin of the mouse using forceps and scissors.

1.1.4. Inject 6 mL of ice-cold Tyrode's B buffer[13] into the peritoneal cavity using a 10 mL syringe with a 26 G needle. Insert the needle gently to avoid pricking any organs.

1.1.5. Massage the abdomen of the mouse for 60-90 s to collect peritoneal cells into Tyrode's B buffer. Do it gently not to damage the blood vessels.

1.1.6. Insert the needle (20 G) attached to a 10 mL syringe bevel up. Aspirate the fluid slowly from the peritoneal cavity (typically 5-6 mL).

1.1.7. Remove the needle from the syringe. Dispense the peritoneal fluid into a 50 mL conical tube. Keep the tube on ice.

1.1.8. Repeat steps 1.1.4 - 1.1.7.

1.1.9. Centrifuge the tube at 300 x *g* for 5 min at 10 °C. Resuspend the cells in 1 mL of 1x red blood cell (RBC) lysis buffer. Keep the cells on ice for 3 min.

1.1.10. Dilute the cell suspension with 2 mL of Tyrode's B buffer. Centrifuge the tube at 300 x *g* for 5 min at 10 °C.

1.1.11. Remove the supernatant. Resuspend the cells in 0.5 mL of Tyrode's A buffer[13].

1.1.12. Count the cells with a hemocytometer. Adjust the cell number to 5 x 10^6/mL with Tyrode's A buffer.

1.2. Prepare mast cell-depleted PECs using a magnetic cell purification system[14].

1.2.1. Centrifuge 5 x 10^5 crude PECs at 300 x *g* for 5 min at 10 °C. Resuspend the pellet in 200 µL of PBSBE cell purification buffer (0.5% BSA, 2 mM EDTA, 1 x PBS, pH 7.4).

1.2.2. Add 1 µL of Fc blocker (0.5 mg/mL) to the cells to prevent non-specific binding of anti-c-kit monoclonal antibody (mAb) to be added next. Keep the cells on ice for 5 min.

1.2.3. Add 1 µL of biotinylated anti-mouse c-kit mAb[15] (0.5 mg/mL). Keep the cells on ice for 10 min.

1.2.4. Add 2 mL of PBSBE buffer. Centrifuge the cells at 300 x *g* for 10 min at 10 °C.

1.2.5. Remove the supernatant. Wash the cells again with 2 mL of PBSBE buffer.

1.2.6. Resuspend the cells in 90 µL of PBSBE buffer. Add 10 µL of streptavidin-conjugated microbeads. Keep the cells on ice for 15 min.

1.2.7. Add 2 mL of PBSBE buffer. Centrifuge the cells at 300 x *g* for 10 min at 10 °C.

1.2.8. Load 500 µL of PBSBE buffer on a medium magnetic column during the spin. Allow the buffer to flow through the column.

1.2.9. Remove the supernatant after centrifugation. Resuspend the cells in 500 µL of PBSBE buffer.

1.2.10. Load the cells on the column. Collect the cells passing freely through the column.

1.2.11. Wash the column with 500 µL of PBSBE buffer. Collect the cells passing through the column again.

1.2.12. Repeat step 1.2.11.

1.2.13. Combine the cells from steps 1.2.9-1.2.11 in one tube. Centrifuge the cells at 300 x *g* for 10 min at 10 °C.

1.2.14. Resuspend the cells in Tyrode's A buffer. Count the cells. Adjust the cell number to 5 x 10^6 cells/mL.

1.3. Prepare the lysate of PECs.

1.3.1. Plate PECs (e.g., 5 x 10^5) in a round-bottom 96 well plate, respectively. Centrifuge the plate at 300 x *g* for 2 min at 10 °C to collect the cells. Remove the supernatant carefully with

pipette.

1.3.2. Add 100 µL of cell lysis buffer to the cell pellet. Resuspend the cells by pipetting up and down several times gently. Keep the plate on ice for 60 min.

1.3.3. Centrifuge the plate at 300 x *g* for 5 min at 10 °C to remove cell debris. Transfer the supernatant (the cell lysate) to a new 96 well plate.

1.4. Measure the enzymatic activity of β-hexosaminidase[16].

1.4.1. Add the cell lysate (50 µL) to prewarmed β-hexosaminidase substrate solution (50 µL) in a 96 well microplate. Mix them gently with a pipette.

1.4.2. Incubate the plate in a 37 °C incubator. Add 50 µL of stop solution (100 mM glycine, pH 10.7) after 30 min to terminate the enzyme reaction.

1.4.3. Read O.D. of the reaction mixtures with a UV-visible absorbance microplate reader in dual wavelength setting; 405 nm for determining the level of the enzyme reaction and 620 nm for automatic background subtraction, respectively.

1.5. Measure the concentration of histamine[17].

1.5.1. Transfer 100 µL of the cleared cell lysates to the anti-histamine mAb-coated plate supplied with the histamine ELISA kit. Perform competitive histamine ELISA assay following the manufacturer's manual.

1.5.2. Read O.D. of the samples with a UV-visible absorbance microplate reader at 450 nm wavelength.

1.6. Determine the ratio of mast cells in PECs with flow cytometry[18].

1.6.1. Transfer 1 x 10^5 crude or mast cell-depleted PECs in a round bottom 96 well plate. Centrifuge the plate at 300 x *g* for 2 min at 10 °C.

1.6.2. Resuspend the cells in 50 µL of FACS buffer. Add 1 µL of anti-mouse c-kit (0.5 mg/mL) and anti-mouse IgE mAbs (0.5 mg/mL).

1.6.3. Vortex the cells briefly. Keep the cells on ice for 20 min in the dark.

1.6.4. Fill the wells with 150 µL of FACS buffer. Centrifuge the plate at 300 x *g* for 2 min at 10 °C. Discard the buffer by quickly flipping the plate.

1.6.5. Resuspend the cells with 150 µL of FACS buffer containing propidium iodide (1 µg/mL). Analyze the cells with a flow cytometer.

2. Mast cell degranulation assay using crude PECs

2.1. Determine the extent of mast cell degranulation (% degranulation).

2.1.1. Inject 3 BALB/C mice (8 weeks old, male) intravenously (i.v.) with 3 μg of anti-DNP mAb (mouse IgE). Isolate PECs one day after Ab injection (refer to step 1.1).

2.1.2. Plate 90 μL of crude PECs (5.5 x 10^6/mL) in a flat-bottom 96 well plate (total 4 wells). Incubate the plate in a 37 °C humidified CO_2 incubator for 30 minutes.

2.1.3. Add 10 μL of DNP-BSA (5 ng/mL in 1x PBS) to the wells containing PECs. Incubate the plate for 10 min in a 37 °C CO_2 incubator.

2.1.4. Centrifuge the plate at 300 x *g* for 5 min at 10 °C immediately after incubation. Take the supernatants (100 μL) carefully with pipette. Save them in a new round-bottom 96 well plate on ice.

2.1.5. Add 100 μL of cell lysis buffer (0.1% Triton X-100 in 1 x PBS, pH 7.4) to the microplate wells containing PECs. Keep the plate on ice for 60 min.

2.1.6. Carry out the β-hexosaminidase assay.

2.1.6.1. Take two sets of the supernatants and the corresponding cell lysates, respectively, out of four that have been stored in ice after the degranulation assay (refer to steps 2.1.4 and 2.1.5). Split the supernatant and the cell lysate into two separate wells (50 μL each) for duplication of the assay.

2.1.6.2. Add 50 μL of β-hexosaminidase substrate solution to each well. Incubate the plate at 37 °C for 30 min. Add 50 μL of stop solution (100 mM glycine, pH 10.7) to the reaction mixture.

2.1.6.3. Read O.D. of the reaction mixtures (refer to step 1.4.3). Calculate the extent of mast cell degranulation as follows.

$$\% \text{ Degranulation} = OD_{supernatant} / (OD_{supernatant} + OD_{lysate}) \times 100\%$$

2.1.7. Carry out the histamine assay.

2.1.7.1. Add 100 μL of 1x PBS to another two wells of the supernatants and the corresponding cell lysates saved after the degranulation to bring the total volume of the samples to 200 μL. Divide each sample into two separate wells in a histamine ELISA plate provided with the histamine ELISA kit.

2.1.7.2. Perform the ELISA assay following the manufacturer's manual. Read O.D. of the samples with a UV-visible absorbance microplate reader at 450 nm wavelength. Calculate the extent of degranulation as in 2.1.6.3.

% Degranulation = $[\text{histamine}]_{sup}$ /($[\text{histamine}]_{sup}$ + $[\text{histamine}]_{lysate}$) X 100%

2.2. Evaluate the effects of antiallergy medications on mast cells exerted in vivo with the ex vivo mast cell degranulation assay.

2.2.1. Administer orally (p.o.) 200 µL of dexamethasone[19] (DEX) and ketotifen[20] (KET), respectively, to mice (6 weeks old, male) once a day for 3 days (6 mice/group).

2.2.2. Inject the mice intravenously (i.v.) with 3 µg of anti-DNP IgE after the 3rd treatment. Divide each group of mice into two separate cages (3 mice/cage); one for PSA assay and the other for ex vivo mast cell degranulation assay.

2.2.3. Carry out PSA assay

2.2.3.1. Inject the mice with DNP-BSA (80 µg) one day after injection of anti-DNP IgE. Measure the body temperature with a rectal thermometer every 15 min for 1 h starting immediately after injection of DNP-BSA.

2.2.3.2. Take the blood one day after injection of DNP-BSA. Measure the levels of MCPT-1 in the serum with ELISA.

2.2.4. Carry out ex vivo mast cell degranulation assay.

2.2.4.1. Isolate PECs from the mice one day after injection of anti-DNP IgE. Count the numbers of isolated PECs.

2.2.4.2. Plate 90 µL of crude PECs (5.5 x 10^6/mL) in a 96 well plate (4 wells per mouse). Incubate the plates in a 37 °C humidified CO_2 incubator for 30 min.

2.2.4.3. Add 10 µL of DNP-BSA (5 ng/mL). Incubate the plate for 10 min at 37 °C humidified CO_2 incubator.

2.2.4.4. Centrifuge the plate at 300 x *g* for 5 min at 10 °C. Take the supernatants (100 µL) carefully, leaving the cells behind in the wells.

2.2.4.5. Add 100 µL of cell lysis buffer to the wells. Incubate the plate on ice for 60 min.

2.2.4.6. Carry out β-hexosaminidase assay and histamine ELISA assay as described in 2.1.6 and 2.1.7. Calculate% degranulation as in step 2.1.6.3.

REPRESENTATIVE RESULTS:

Determining the optimal number of PECs for ex vivo mast cell degranulation assay

Mast cells (c-kit$^+$·IgE$^+$ double positive cells)[15] represent only about 2% of PECs (**Figure 1A**). Estimating the maximum levels of mast cell-specific molecules to be detected in the culture supernatants on the assumption that 100% of the granules were released by mast cells in PECs, we measured the amounts of β-hexosaminidase[16] and histamine[17] in the total cell lysates prepared with different numbers of PECs: 2.5 x 10^5, 5 x 10^5 and 1 x 10^6 PECs, respectively. As shown in **Figure 1B**, significant levels of both β-hexosaminidase and histamine were detected even when the lysate was prepared with 2.5 x 10^5 PECs, and their levels increased proportionally as the number of PECs used for the preparation of the cell lysate increased.

Bearing those results in mind, we decided to use 5 x 10^5 PECs in the ex vivo mast cell degranulation assay for the following reasons. Considering that only a little over 3 x 10^6 PECs were isolated from one mouse (8 weeks old) and that they were to be plated evenly in 4 separate wells in a 96-well microplate (2 wells for β-hexosaminidase and another two wells for histamine assays, respectively), plating 1 x 10^6 PECs per well would likely cause a shortage of the cells. In addition, the results shown above indicated that a sufficient number of mast cells was in 5 x 10^5 crude PECs for carrying out the degranulation assay. That is, according to those results, it was expected that even in the case that only about 30 to 40% of the granules was released by mast cells, the levels of β-hexosaminidase and histamine detected in the culture supernatants after the degranulation assay would be high enough for being accurately quantified.

Next, we tried to confirm that β-hexosaminidase and histamine detected in the lysates of PECs were derived exclusively from mast cells in PECs. To do that, we depleted mast cells from PECs using a magnetic cell purification column (refer to step 1.2). Expectedly, unlabeled PECs that flew through the column were completely devoid of mast cells (**Figure 1C**). Also, expectedly, neither β-hexosaminidase nor histamine were detected in the cell lysate prepared with mast cell-depleted PECs (**Figure 1D**).

Ex vivo mast cell degranulation assay using crude PECs

We next examined the actual levels of β-hexosaminidase and histamine released by mast cells in PECs during culture with a specific antigen of IgE on their surface. To do that, we isolated PECs from the mice injected with anti-DNP-IgE and culture them (5 x 10^5) with DNP-BSA (0.5 ng/mL). As shown in **Figure 2A**, a significant level of β-hexosaminidase was begun to be detected in the culture supernatant within 5 min of culture and increased continually as the culture period was prolonged. Nevertheless, the rate of increase was diminished quickly after 10 min of culture, and the extent of degranulation gradually reached the plateau at around 50% after 30 to 40 min. Similar results were also obtained when the levels of histamine released by mast cells during the culture were examined (**Figure 2B**). Based on those results, we decided to culture PECs with DNP-BSA for 10 min in the following assays.

Verifying the in vivo effects of KET and DEX on mast cell with the ex vivo degranulation assay.

Ketotifen (KET) is an allergy medication with antihistaminergic activity. Different from other

conventional antihistamines, however, it is known as a dual-acting antihistamine that also has a mast cell stabilizing activity in addition to the antihistaminergic activity. The mast cell stabilizing activity of KET has been explored[21,22]; however, mostly in in vitro studies and the studies to show how it has effect on mast cell in vivo are scarce. Dexamethasone (DEX) is another type of allergy medication[23]. DEX is known to impair functional activities of various types of immune cells to suppress a broad spectrum of immune responses[24]. To verify the effect of KET and DEX on the activity of mast cells in vivo, we were to carry out the ex vivo mast cell degranulation assay with PECs isolated from the mice treated with either of them.

Prior to the ex vivo mast cell degranulation assay, we first examined the effects of DEX and KET on anaphylactic reactions elicited by passively transferred DNP-specific IgE and DNP-BSA in PSA model. Expectedly, treatment with either KET or DEX resulted in improvement in anaphylactic symptoms in a dose-dependent manner (**Supplemental Figure 1**), indicating that the functional activity of mast cells was compromised by their treatments.

We also examined the numbers of PECs isolated from the mice treated with either compounds and the ratio of mast cells in PECs (**Figure 3A**). Treatments with KET, regardless of the doses used in the treatments, resulted in no noticeable change in the numbers of PECS isolated from the mice. In contrast, the numbers of PECs isolated from the DEX-treated mice decreased significantly when treated at 4.5 mg/kg dose, indicating that DEX treatment had effect on the viability of peritoneal cells. Still, it must be noted that the ratio of mast cells remained constant at around 2% regardless of the drugs and doses used for the treatment (**Figure 3A**). We also examined the effects of DEX and KET on the levels of β-hexosaminidase and histamine expressed by mast cells in the peritoneal cavity by measuring their amounts in the total cell lysates prepared with the same numbers of crude PECs (**Figure 3B**). Of note, the levels of both β-hexosaminidase and histamine were found to be augmented after treatment with DEX at the dose of 4.5 mg/kg. KET treatments resulted in little change in those levels.

Next, we performed the ex vivo mast cell degranulation assay (**Figure 4**). First, we adjusted the cell density of PECs evenly to 5.5 x 10^6 cells/mL to ensure that the same number of mast cells were used in the assay. PECs (90 μL) were then plated in a 96-well microplate and incubated with DNP-BSA for 10 min. When PECs from the mice treated with the high dose of either DEX or KET were incubated with DNP-BSA, the levels of β-hexosaminidase and histamine detected in the culture supernatants were found to be lowered significantly compared to those detected after incubation of PECs from sham-treated mice with DNP-BSA (**Figure 4**). An inverse correlation was also apparent between the levels of those molecules detected in the culture supernatants and the doses of DEX and KET used for treatments of the mice. Thus, the higher the dose used for the treatment was, the lower the levels of β-hexosaminidase and histamine detected in the culture supernatants after the incubation were.

FIGURE AND TABLE LEGENDS:

Figure 1: Quantifying the levels of mast cell-specific molecules contained by the lysates prepared with different numbers of PECs. (**A**) PECs were stained with FITC-labeled anti-mouse

c-kit plus PE-labeled anti-mouse IgE mAbs and analyzed with flow cytometry. Mast cells (i.e., c-kit$^+$·IgE$^+$ double positive cells) are shown in the upper-right quadrant. (**B**) Cell lysates were prepared with 1 x 10^6 (circle), 5 x 10^5 (square), and 2.5 x 10^5 (diamond) PECs, respectively, and one half of the total lysates was incubated with the substrate of β-hexosaminidase for a period of time as indicated. The extent of color changes by the enzyme reaction was measured with a 96 well microplate reader at 405 nm wavelength. Assays were conducted in duplicate. (**C**) Cell lysates were prepared as in (**B**) and the histamine concentrations in the lysates were measured with ELISA. (**D**) Mast cell-depleted PECs were stained with the mAbs as in (A). (**E**) Cell lysates were prepared with 5 x 10^5 crude (filled bar) and mast cell-depleted (open bar) PECs, respectively, and incubated with the β-hexosaminidase substrate for 30 min before termination of the reactions. (**F**) The concentrations of histamine in the cell lysates prepared as in (**E**) were measured.

Figure 2: Measuring the amounts of mast cell-specific molecules released by mast cells during culture of PECs with DNP-BSA. PECs (5 x 10^5) isolated from the mice injected with anti-DNP IgE (circle) or with just 1 x PBS (triangle) were cultured with DNP-BSA for a period of time as indicated. The levels of β-hexosaminidase (**A, left**) and histamine (**B, left**) detected in the supernatants at each time point were plotted. Extents of degranulation were calculated with the amounts of β-hexosaminidase (**A, right**) and histamine (**B, right**) released to the supernatants and remaining inside the cells (cell lysate), respectively. The following equation was used for calculating the extent of degranulation (%). Degranulation (%) = $[X]_{sup}/([X]_{sup} + [X]_{lysate})$ X 100

Figure 3: Effects of DEX and KET on the viability of mast cells in the peritoneal cavity and the levels of b-hexosaminidase and histamine expressions. (**A**) PECs were isolated from the mice treated p.o. for 3 days with vehicle alone (sham) or with indicated doses of DEX or KET. Total cell numbers were counted with a hemocytometer and the average numbers were calculated and plotted along with standard deviations (**left**). PECs were stained with FITC-labeled anti-c-kit plus PE-labeled anti-IgE mAbs and analyzed with flow cytometry. The ratios of the double positive cells (i.e., mast cells) to total PECs were plotted (**right**). (**B**) PECs were isolated as in (A), and cell lysates were prepared with the same number (5 x 10^5) of PECs. The amounts of β-hexosaminidase (**left**) and histamine (**right**) in those lysates were measured and plotted. All experiments were performed with 3 mice per group. Statistical significances were calculated using one-way anova; $* p < 0.05$.

Figure 4: Ex vivo mast cell degranulation assay using crude PECs isolated from the mice treated with DEX or KET. PECs were isolated from the mice treated p.o. for 3 days with vehicle alone (naïve and sham) or with indicated doses of DEX or KET and then injected i.v. with PBS (naïve) or anti-DNP-IgE. They were then incubated with DNP-BSA for 10 min, and the extents of degranulation were calculated based on the levels of β-hexosaminidase (**A**) and histamine (**B**), respectively, released to the culture supernatants. All experiments were performed with 3 mice per group. Statistical significances were calculated using one-way anova; $* p < 0.05$.

Table 1: Buffer compositions

Supplemental Figure 1: Effects of DEX and KET on the anaphylactic reactions caused by passively transferred anti-DNP-IgE and DNP-BSA. Mice, treated (p.o.) daily for 3 days with the vehicle alone (naïve, sham) or with indicated doses of DEX or KET, were administered (i.v.) PBS (naive) or anti-DNP-IgE mAb. One day after the antibody injection, the mice were injected (i.v.) with PBS (naive) or with DNP-BSA. The body temperatures of the mice measured 30 min after DNP-BSA injection were plotted (**A**). One day after injection of DNP-BSA, the blood was drawn from the mice and the levels of MCPT-1 were measured with ELISA (**B**). The experiment was performed with 3 mice per group. Statistical significances were calculated using one-way ANOVA; * $p < 0.05$.

DISCUSSION:

The finding that mast cell degranulation assay can be carried out with a relatively small number of crude mouse PECs is significant. Even though PECs must be an excellent source of primary mouse mast cells, it is demanding to purify mast cells in PECs. Although a density gradient media such as Percoll[25] has been successfully used for purification of mast cells from rat PECs, its use for purification of mouse peritoneal mast cells has been limited presumably for the difference in the densities of rat and mouse mast cells. Another gradient medium such as Histodenz[26] has been used for purification of mouse peritoneal mast cells with limited success; the outcome of the purification appeared dependent on the conditions of mice (e.g., age). Even a new cell isolation system like magnetic cell purification system led to only a partial purification of mouse mast cells (authors' unpublished data). We show here that purification of peritoneal mast cells is unnecessary for mast cell degranulation assay.

The result that mast cells in crude PECs could be triggered by a cognate antigen of IgE is encouraging in pursuit of an ex vivo assay to faithfully mimic the degranulation event happening in vivo. In addition to mast cells, however, basophils also express FcεRI[27]. Thus, one caveat here is that a small number of basophils in PECs might also contribute to the increase in the concentration of histamine in the culture supernatant during the assay. It seems unlikely, however, as no measurable level of histamine was detected in the culture supernatant when PECs depleted of c-kit-expressing cells (i.e., mast cells) were used in the assay. Those results assured that mast cells were entirely responsible for the release of histamine detected in the culture supernatant after the culture of crude PECs with DNP-BSA.

An intriguing issue in interpreting the result of an in vitro mast cell degranulation assay for probing the inhibition of mast cell function by a pharmacological agent is how to relate such an in vitro result (e.g., IC_{50} of a test compound) to the in vivo result (e.g., ED_{50}) derived from an in vivo animal study such as PSA. Even in the case that primary mast cells are employed, considering that the surrounding environmental conditions for them to encounter during the in vitro and in vivo assays are profoundly different from each other, the results obtained from those studies may not always be akin to each other. Thus, it takes caution to extrapolate the results from the in vitro assay to the in vivo experiment and vice versa. As mast cells used in the ex vivo degranulation assay described in this study are treated with a test compound at the environments same as those for the in vivo assay and incubated with a natural ligand immediately after isolation from the animal, it is thought that the results from the ex vivo assay accurately

reflect how mast cells in the body are affected by the compound during the in vivo assay.

While the main focus of this study was how to use the ex vivo assay to confirm the actual mast cell stabilizing activity of a potential anti-allergy medication in vivo, it must be noted that this assay can also be used in the purpose of examining the effects of specific gene knock-outs on mast cell function. For example, this assay will be useful in examining the effects of the deletion of specific genes critically involved in the differentiation of specific subsets of T cells on mast cell function. In addition, even though we used only passive systemic anaphylaxis as a model system in this manuscript, we presume that the same assay can be carried out with PECs obtained after active immunization of mice.

In summary, this assay has several unique features as follows. First, the assay is expeditious and facile as crude PECs are used. Second, as mast cells are treated with a test compound in the environments where a relevant in vivo animal study is carried out, results from the ex vivo degranulation assay faithfully reflect the effects of the compound exerted on mast cells during the animal study. Third, as the assay is accompanied by flow analysis for the ratio and the number of mast cells in PECs, effects of the test compound on the viability of mast cells in vivo can also be addressed.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Amin, K. The role of mast cells in allergic inflammation. *Respiratory Medicine.* **106** (1), 9-14 (2012).

2 Holgate, S. et al. The anti-inflammatory effects of omalizumab confirm the central role of IgE in allergic inflammation. *Journal of Allergy and Clinical Immunology.* **115** (3), 459-465 (2005).

3 Galli, S. J., Tsai, M. IgE and mast cells in allergic disease. *Nature Medicine.* **18** (5), 693 (2012).

4 Roth, K., Chen, W.-M., Lin, T.-J. Positive and negative regulatory mechanisms in high-affinity IgE receptor-mediated mast cell activation. *Archivum Immunologiae et Therapiae Experimentalis.* **56** (6), 385-399 (2008).

5 Finn, D., Walsh, J. Twenty‐first century mast cell stabilizers. *British Journal of Pharmacology.* **170** (1), 23-37 (2013).

6 Lu, Y. et al. Emodin, a naturally occurring anthraquinone derivative, suppresses IgE-mediated anaphylactic reaction and mast cell activation. *Biochemical Pharmacology.* **82** (11), 1700-1708 (2011).

7 Demo, S. et al. Quantitative measurement of mast cell degranulation using a novel flow cytometric annexin‐V binding assay. *Cytometry: The Journal of the International Society for Analytical Cytology.* **36** (4), 340-348 (1999).
8 Passante, E., Ehrhardt, C., Sheridan, H., Frankish, N. RBL-2H3 cells are an imprecise model for mast cell mediator release. *Inflammation Research.* **58** (9), 611-618 (2009).
9 Fukuishi, N. et al. Does β-hexosaminidase function only as a degranulation indicator in mast cells? The primary role of β-hexosaminidase in mast cell granules. *The Journal of Immunology.* **193** (4), 1886-1894 (2014).
10 Malbec, O. et al. Peritoneal cell-derived mast cells: an in vitro model of mature serosal-type mouse mast cells. *The Journal of Immunology.* **178** (10), 6465-6475 (2007).
11 Arock, M., Le Nours, A., Malbec, O., Daëron, M. in *Innate Immunity* 241-254 (Springer, 2008).
12 Befus, A., Pearce, F., Gauldie, J. Horsewood, P., Bienenstock. J. Mucosal mast cells. I. Isolation and functional characteristics of rat intestinal mast cells. *The Journal of Immunology* **128** (6), 2475-2480 (1982).
13 Yoon, S. C. et al. Anti-allergic and anti-inflammatory effects of aqueous extract of Pogostemon cablin. *International Journal of Molecular Medicien* **37** (1), 217-224 (2016).
14 Mierke, C. T. et al. Human endothelial cells regulate survival and proliferation of human mast cells. *Journal of Experimental Medicien* **192** (6), 801-812 (2000).
15 Hermes, B. et al. Altered expression of mast cell chymase and tryptase and of c-Kit in human cutaneous scar tissue. *Journal of Investigative Dermatology* **114** (1), 51-55 (2000).
16 Wendeler, M., Sandhoff, K. J. Hexosaminidase assays. *Glycoconjugate Journal* **26** (8), 945-952 (2009).
17 Russell, M. et al. Learned histamine release. *Science* **225** (4663), 733-734 (1984).
18 Darzynkiewicz, Z. et al. Features of apoptotic cells measured by flow cytometry. *Cytometry* **13** (8), 795-808 (1992).
19 Kitamura, Y. et al. Dexamethasone suppresses histamine synthesis by repressing both transcription and activity of HDC in allergic rats. *Allergology International*. **55** (3), 279-286 (2006).
20 Grant, S. M., Goa, K. L., Fitton, A., Sorkin, E. M. Ketotifen. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in asthma and allergic disorder. *Drugs.* **40** (3), 412-448 (1990).
21 Cook, E., Stahl, J., Barney, N., Graziano, F. Mechanisms of antihistamines and mast cell stabilizers in ocular allergic inflammation. *Current Drug Targets-Inflammation & Allergy.* **1** (2), 167-180 (2002).
22 Schoch, C. In vitro inhibition of human conjunctival mast-cell degranulation by ketotifen. *Journal of Ocular Pharmacology and Therapeutics.* **19** (1), 75-81 (2003).
23 Franchimont, D. et al. Inhibition of Th1 immune response by glucocorticoids: dexamethasone selectively inhibits IL-12-induced Stat4 phosphorylation in T lymphocytes. *The Journal of Immunology.* **164** (4), 1768-1774 (2000).
24 Simmons, C. P. et al. The clinical benefit of adjunctive dexamethasone in tuberculous meningitis is not associated with measurable attenuation of peripheral or local immune responses. *The Journal of Immunology.* **175** (1), 579-590 (2005).
25 Cavalher-Machado, S. C. et al. The anti-allergic activity of the acetate fraction of Schinus terebinthifolius leaves in IgE induced mice paw edema and pleurisy. *International*

Immunopharmacology **8** (11), 1552-1560 (2008).
26 Dhakal, H. et al. Gomisin M2 inhibits mast cell-mediated allergic inflammation via attenuation of FcεRI-mediated Lyn and Fyn activation and intracellular calcium levels. *Frontiers in Pharmacology.* **10** 869 (2019).
27 Galli, S. J., Wedemeyer, J., Tsai, M. Analyzing the roles of mast cells and basophils in host defense and other biological responses. *International Journal of Hematology.* **75** (4), 363-369 (2002).

Figure

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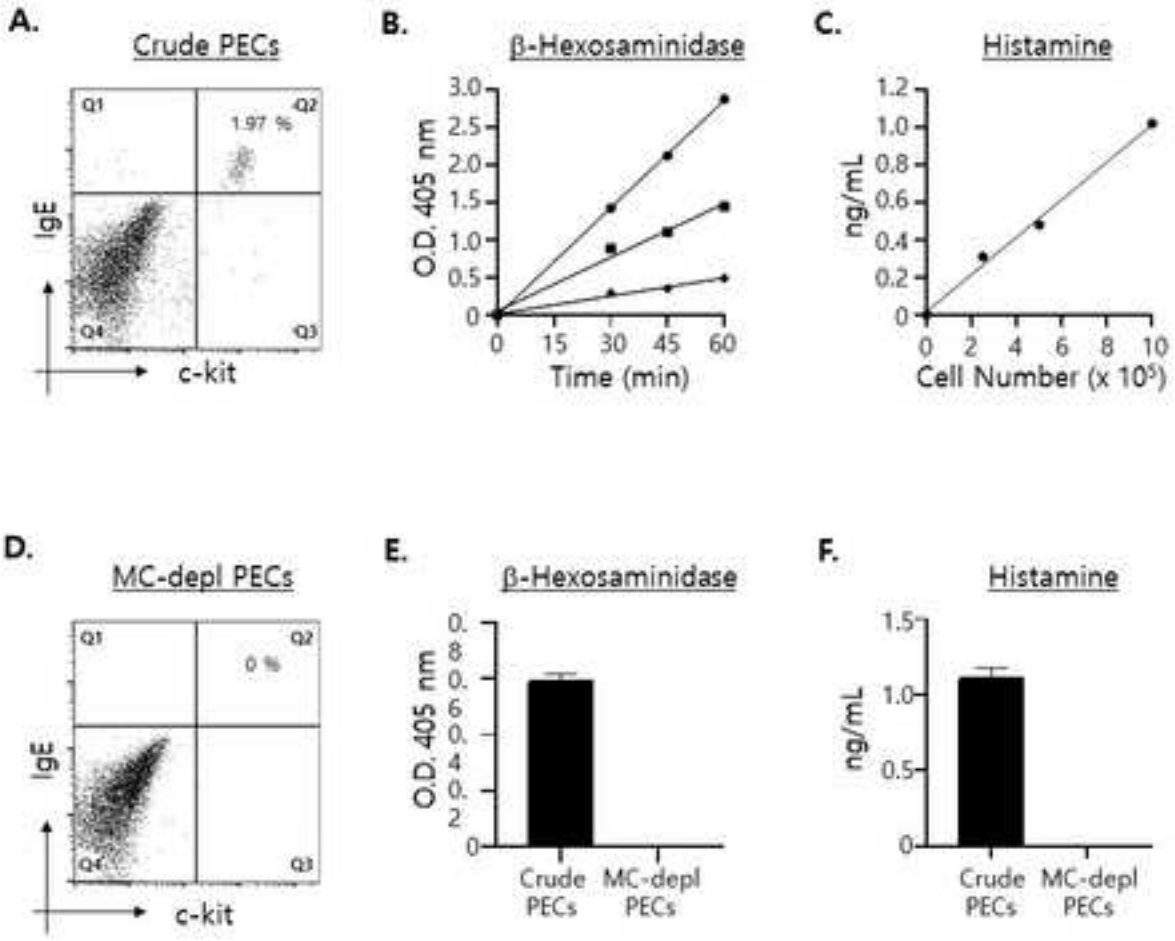

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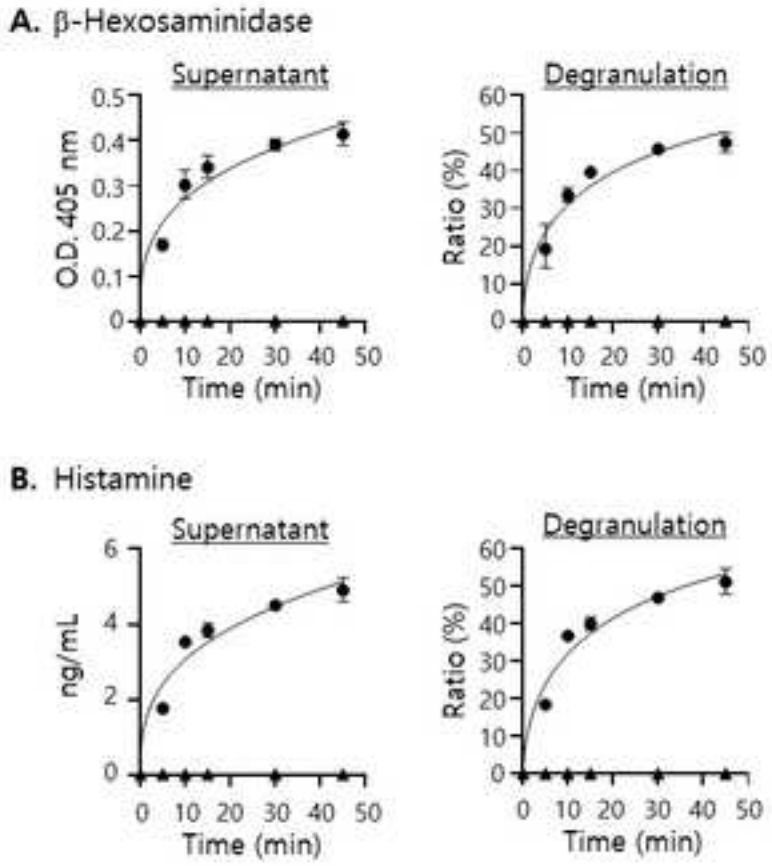

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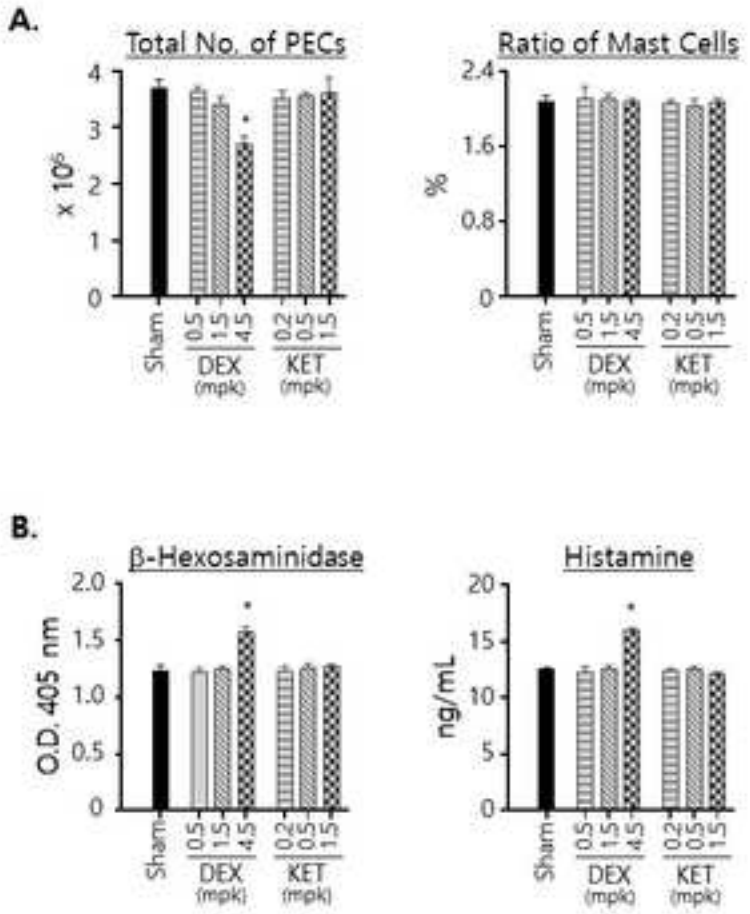

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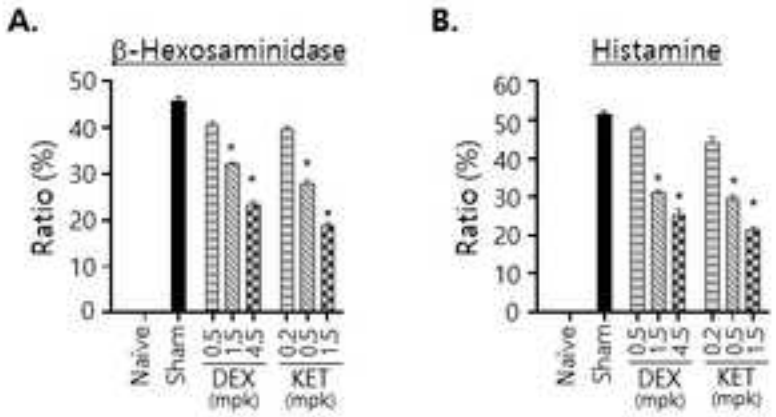

Table

Buffer	Recipe	Comments/Description
0.1 M Na-citrate buffer	45.6 mM Sodium Citrate dihydrate, 54.4 mM Citric Acid, pH 4.5	Store at 2-8℃
10x RBC lysis buffer	80 mg/mL NH_4Cl, 8.4 mg/mL $NaHCO_3$, 3.7 mg/mL EDTA (disodium)	Store at 2-8℃, do not exceed six months
Cell lysis buffer	0.1 % Triton X-100, 1 x phosphate-buffered saline (PBS) pH 7.4	Store at 2-8℃
ELISA stop solution	100 mM glycine, pH 10.7	Normal temperature storage
FACS buffer	1 x PBS, 5 % horse serum, 1 % BSA, 10 mM HEPES, 2 mM EDTA, pH 7.2	Store at 2-8℃, do not exceed two months
PBSBE buffer (*MACS* cell purification buffer)	1x PBS, 0.5 % BSA, 2 mM EDTA, pH 7.2	Store at 2-8℃, do not exceed two months
Tyrode's A buffer	10 mM HEPES, 130 mM NaCl, 5.6 mM glucose, 5 mM KCl, 1 mM $MgCl_2$, 1.4 mM $CaCl_2$, 1 % bovine serum albumin (BSA) pH 7.4	Store at 2-8℃, do not exceed two months
Tyrode's B buffer	137 mM NaCl, 5.6 mM glucose,12 mM $NaHCO_3$, mM KCl, 0.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 0.1 % gelatin,pH 7.4	Store at 2-8℃, do not exceed two months

Table

Click here to access/download;Table of Materials;JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 mL syringe		1757589701	
1.5 mL micro tube	Hisol	MT-15003	
10 mL syringe		1757593161	
15 mL conical tube	Thermo Fisher scientific	14-959-53A	
20xPBS	Tech＆Innovation	BPB-9121-500mL	
4-nitrophenyl-N-acetyl-β-D-glucosaminide	SIGMA	N9376	
5 mL polystyrene round-bottom tube	Life sciences	352003	
50 mL conical tube	Thermo Fisher scientific	14-959-49A	
Aluminium Fiol	BioFact	TS1-3330	
Anti-mouse CD117(c-kit)	Biolegend	135129	keep at 2-8℃
Anti-mouse IgE mAbs	Thermo Fisher scientific	11-5992-81	keep at 2-8℃
Antiti-DNP-IgE	SIGMA	D8406-.2MG	keep at -20℃
Centrifuge	HANIL	396150	
D-(+)-gluouse	SIGMA	G8270	
Dexamethasone	SIGMA	D2915-100MG	
DNP-BSA	Invitrogen	2079360	keep at -20℃
EDTA	Biofact	PB131-500	
Fetal Bovine serum	Thermo Fisher scientific	11455035	
Gelatin	SIGMA	G1890	
Glycine	JUNSEI	27185-0350	
hemocytometer	ZEISS	176045	
HEPES	Thermo Fisher scientific	15630130	
Histamine ELISA kit	Abcam	GK3275957-4	keep at 2-8℃
Hotplate stirrer	Lab teach	zso-9001	
Isoflurance	Troikaa	I29159	

ketotifen fumarate salt	SIGMA	K2628	
MCPT-1 ELISA kit	Thermo Fisher scientific	88-7503-22	keep at 2-8℃
Mouse Fc block	BD Biosciences	553141	keep at 2-8℃
Propidium iodiole	SIGMA	81845	keep at 2-8℃
RBC lysis buffer	Biolegend	420301	
Round-bottom 96 well	SPL-life sciences	30096	
Single use syringe filter	Startoriusag	16555	
Streptavidin microbeads	MilteryiBiotec	130-048-101	keep at 2-8℃
Triton X-100	JUNSEIchemical	49415-1601	
TWEEN **20**	SIGMA	9005-64-5	
Water bath	CHANGSHINSCIENCE	190107	

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Uploaded is the manuscript entitled "*Ex vivo* mast cell degranulation assay featuring the use of crude peritoneal exudate cells and natural antigen stimulation." authored by Hong Son Le, Liu Ye, and Inkyu Hwang.

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