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Dynamics of Plasma and Granule Membrane in Murine Bone Marrow-Derived Mast Cells after Re-stimulation

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Abstract: Mast cells are derived from hematopoietic stem cells and play important roles in allergic responses. Mast cells are long-lived compared with other granular cell types. Since the response of the individual mast cell after FcεRI-induced degranulation is unclear, the aim of this study was to analyze morphological changes in individual mast cells after re-stimulation. To observe plasma and granule membrane dynamics, AcGFP-actb (β-actin) and DsRed-monomer (DRM)-CD63 fusion constructs were introduced into bone marrow-derived mast cells (BMMCs). Furthermore, AcGFP-CD63 and DRM-Cma1 (mMCP-5) were introduced into BMMCs. Re-stimulation resulted in increased β-hexosaminidase release and cytokine mRNA expression similar to those observed during initial stimulation. Moreover, expression of FcεRI on BMMCs 24 h after initial stimulation was similar to that measured before initial stimulation. Changes in morphology of the plasma membrane and colocalization of granules and plasma membrane were observed after initial stimulation. BMMCs returned to normal 120 min after the initial stimulation. These phenomena were also observed in BMMCs after re-stimulation. BMMC chymase content decreased 20 min after stimulation but returned to near normal 24 h after stimulation. These findings suggest that mast cell functions can be maintained and that these cells can be repeatedly degranulated after FcεRI-mediated stimulation.

Keywords: Mast cells, morphology, degranulation, FcεRI, IgE, retrovirus vector.

INTRODUCTION

Mast cells are derived from hematopoietic stem cells; their precursors circulate in the blood and differentiate into mature cells after entering tissues. Mast cells play a pivotal role in IgE-mediated allergic reactions. The high-affinity receptor for IgE (FcεRI) is expressed on mast cells and basophils, and it comprises of an IgE-binding α-subunit, a β-subunit, and a disulphide-linked γ-subunit homodimer [1]. Mast cells respond rapidly to external stimuli. Ca^{2+} flux and tyrosine phosphorylation occur within seconds, the secretion of preformed granules occurs in minutes, and the production of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), occur hours after receptor triggering [2]. FcεRI-induced stimulation of mast cells results in changes in cell-membrane morphology, translocation of granules to the plasma membrane, fusion of granules with the plasma membrane, and degranulation [3-5].

Mast cells release various biologically active compounds that are classified as preformed mediators (histamine, serotonin, proteases, and chondroitin sulfates), newly synthesized mediators (leukotrienes and prostaglandins), and cytokines/chemokines (IL-1, -4, -5, -6, -10, and -13, TNF-α, granulocyte–macrophage colony-stimulating factor (GM-CSF), CC chemokine ligand 2 (CCL2), CCL3, CCL5, and CXC chemokine ligand 2 (CXCL2)) [6-12]. Mast cells in various tissues store different types and amounts of proteases (tryptases and chymases). In humans, mast cells have been categorized into two subtypes based on the presence of tryptase (MC_T cells) or tryptase and chymase (MC_{TC} cells). In mice, mast cells are categorized on the basis of the differences in chymase-like enzymes. The mouse chymase family consists of mouse mast cell protease (mMCP)-1, -2, -4, -5, and -9. Mucosal mast cells preferentially express mMCP-1 and -2, the uterus mast cells express mMCP-9, and the connective tissue mast cells express mMCP-4 and -5. In particular, mMCP-5 (gene name: *Cma1*) is abundantly expressed in mouse bone marrow-derived mast cells (BMMCs) [12-16]. CD63, also known as lysosomal membrane-associated glycoprotein 3 (LAMP3), belongs to the tetraspanin superfamily (TM4SF). CD63 was first described in the granules of resting platelets and on the surface membrane of activated platelets. CD63 is expressed in various cell types (basophils, mast cells, macrophages, and T cells) and is a reliable marker for granule release in basophils and mast cells [17-20].

The fate of mast cells after FcεRI-induced degranulation is unclear, although degranulation is generally viewed as the final stage. However, some reports suggest that mast cells are able to survive and functionally recover after degranulation [21-23]. Mast cells share many features with basophils, including a hematopoietic stem cell origin, expression of FcεRI, and release of histamine; however, mast cells appear to survive longer than basophils do [11]. Mast cells can

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degranulate repeatedly, which is probably facilitated by a prolonged allergic disease. Hence, it is important to investigate mast cells after repeated degranulation. In this study, we examined the morphological changes after antigen-induced stimulation of BMMC. Two plasmids encoding fluorescent fusion proteins, pMXs-AcGFP-actb-IRES-DsRed-monomer (DRM)-CD63 and pMXs-AcGFP-CD63-IRES-DRM-Cma1, were introduced into BMMCs to obtain stably transfected cells. In BMMCs, we observed colocalization of the plasma membrane and granule membrane and translocation of chymase and granule membrane after stimulation. The granules and membranes in BMMCs were visualized using a microscope. Changes in the morphology of the granules and membranes were observed after re-stimulation as well as initial simulation.

MATERIALS AND METHODS

Culture Media

RPMI1640 culture medium supplemented with 10% fetal bovine serum (FBS), 20 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 50 μM 2-mercaptoethanol (2ME) was used for the culture of BMMCs and sensitization of anti-dinitrophenyl (DNP) IgE. Phenol red-free medium (phenol red-free RPMI1640 medium supplemented with 0.5% FBS, 20 mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 50 μM 2ME) was used for the stimulation of DNP-bovine serum albumin (BSA).

Retroviral Transfection and Bone Marrow Cell Culture

To facilitate the generation of fusion proteins, fluorescent agents AcGFP and DRM were used (Clontech, Palo Alto, CA, USA). AcGFP-actb (β-actin) and AcGFP-CD63, in which AcGFP was fused with the N-terminus of actb and CD63, were constructed. DRM-CD63 and DRM-Cma1 (mMCP-5), in which DRM was fused with the N-terminus of CD63 and Cma1, were constructed. AcGFP-actb was inserted upstream of the internal ribosomal entry site (IRES) and DRM-CD63 was replaced by enhanced green fluorescent protein (EGFP) in pMXs-IRES-EGFP retroviral vector (Cell Biolabs, Inc., San Diego, CA, USA) [24]. This construct was termed pMXs-AcGFP-actb-IRES-DRM-CD63. Furthermore, AcGFP-CD63 was inserted upstream of IRES, and DRM-Cma1 was replaced by EGFP. This construct was termed pMXs-AcGFP-CD63-IRES-DRM-Cma1.

Bone marrow cells were collected from the femur of 8−10-week-old C57BL/6 mice (Japan SLC, Inc., Shizuoka, Japan). All the mice were maintained under specific pathogen-free conditions in the animal facility of the Kitasato University School of Science. All the procedures conformed to the guidelines of the Institutional Animal Care and Use Committee of Kitasato University. Bone marrow cells were separated into c-kit-positive lineage-negative (c-kit$^+$ Lin$^-$) cells by using a Moflo XDP cell sorter (Beckman Coulter, Inc., Brea, CA, USA). The sorting efficiency was >98%.

The retroviral packaging cell line Plat-E [25] was transfected with pMXs-AcGFP-actb-IRES-DRM-CD63 or the pMXs-AcGFP-CD63-IRES-DRM-Cma1 vector, and transiently transfected viral supernatants were collected 48 h after transfection. The c-kit$^+$ Lin$^-$ cells were suspended in the virus supernatant containing 100 ng/mL stem cell factor (SCF) and IL-3 (supernatant of the mIL-3-producing cell line CHOmIL-3-3-12-M; provided by T. Sudo, Toray Industry, Inc.) and seeded on plates coated with RetroNectin [26] (Takara Bio Inc., Shiga, Japan). The plates were centrifuged at 1,500 rpm for 1 h at 32°C and incubated at 37°C. To boost transfection efficiency, the medium was replaced on the next day with fresh virus supernatant containing SCF and IL-3. The plates were incubated at 37°C for 3 days. The medium was then replaced with the culture medium containing SCF and IL-3, and the plates were incubated at 37°C for 4−6 weeks. In the cells that were infected with viral vectors expressing the fluorescent fusion proteins AcGFP-actb and DRM-CD63, the plasma membrane and granule membrane appeared green and red, respectively. In the AcGFP-CD63- and DRM-Cma1-infected cells, the granule membrane and chymase appeared green and red, respectively.

Induction of Degranulation

Degranulation of BMMCs was determined by measuring β-hexosaminidase release, as previously described [27], with modifications in the method. In brief, BMMCs were suspended at 1×10^6 cells/mL in the culture medium and sensitized with 1 μg/mL anti-dinitrophenyl (DNP) IgE (clone: 29(4); Yamasa Co., Chiba, Japan) at 37°C for 2 h. The cells were then washed twice, resuspended in the phenol red-free medium, then stimulated with 30 ng/mL DNP-BSA at 37°C for 15 min. The cells were centrifuged at 1,500 rpm for 5 min, and aliquots (50 μL) of the supernatant were transferred into 96-well plates and were allowed to react with 50 μL of 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosamide (β-hexosaminidase substrate) in 0.05 M sodium citrate buffer at 37°C for 1 h. The reaction was terminated by adding 200 μL of 0.05 M sodium carbonate buffer, and the optical density was measured at 405 nm on a microplate reader (Bio Rad Laboratories, Inc., CA, USA). Total β-hexosaminidase activity was determined by lysing the cells with 0.1% Triton X-100. Degranulation is expressed by calculating the β-hexosaminidase release using the following formula:β-hexosaminidase release (%) = (test − negative control)/(positive control − negative control) × 100.

Real-Time RT-PCR

Total RNA was extracted from BMMCs by using the TRIzol reagent (Life Technologies, Grand Island, NY, USA). cDNA was synthesized from 1 μg of total RNA using the Transcriptor First Strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany) and random hexamer primers. Real-time RT-PCR was performed using 1 μL of cDNA in a 20-μL reaction volume containing Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) with IL-6, IL-13, and

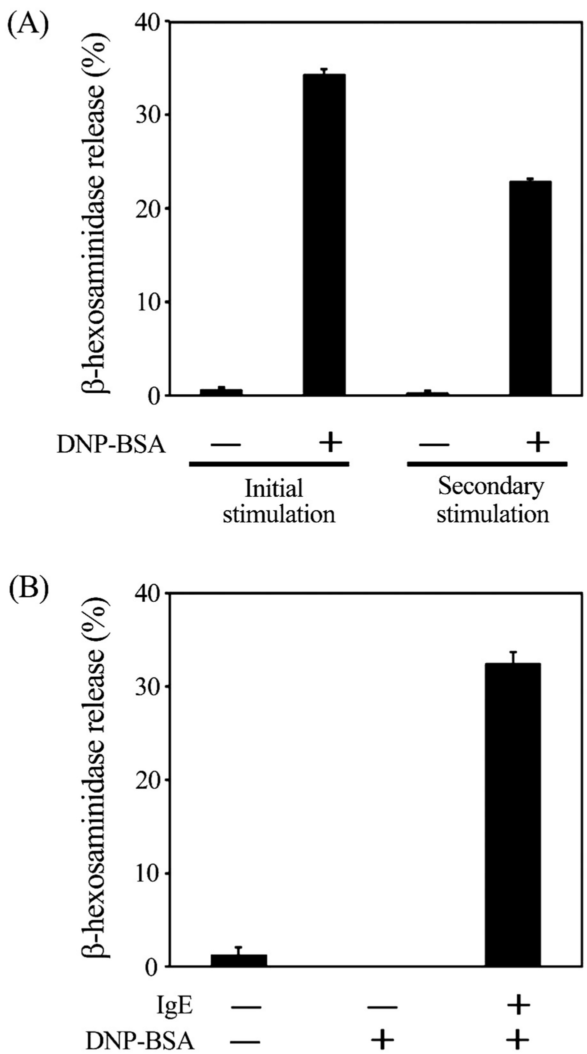

Fig. (1). Degranulation of mast cells by re-stimulation. BMMCs were sensitized by anti-DNP IgE for 2 h and stimulated with DNP-BSA (initial stimulation). (**A**) BMMCs were washed 24 h after initial stimulation and re-sensitized with anti-DNP IgE for 2 h. The cells were washed and re-stimulated with DNP-BSA. (**B**) BMMCs were washed 24 h after initial stimulation and then treated with DNP-BSA with or without sensitization by anti-DNP IgE. β-hexosaminidase release was measured 15 min after stimulation. Values represent mean ± SD (n = 3).

GAPDH RT-PCR primers by using the Mx3000P QPCR System (Agilent Technologies). The GAPDH gene, which was not altered by the experimental conditions, was used as the internal control. Results are reported as relative differences in gene expression compared to the pre-stimulation levels. The specific RT-PCR primers used were as follows: IL-6 (Forward: AGC TGG AGT CAC AGA AGG AGT GGC;Reverse: GGC ATA ACG CAC TAG GTT TGC CGA G), IL-13 (Forward: TTG CAT GGC CTC TGT AAC CGC AAG;Reverse: CCG TGG CGA AAC AGT TGC TTT GTG), and GAPDH (Forward: CAC TCT TCC ACC TTC GAT GC;Reverse: ACC CTG TTG CTG TAG CCG TA).

Flow Cytometry

The cells were resuspended in PBS containing 1% BSA and 0.09% sodium azide and pre-incubated with unconjugated anti-FcγRII/III monoclonal antibody (mAb) (BD Biosciences, San Diego, CA, USA) for 20 min at 4°C to prevent nonspecific binding of other Abs. The cells were then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse FcεRI mAb (FITC-FcεRI) and PECy7-conjugated anti-mouse CD117 (c-kit) mAb (both from Biolegend, San Diego, CA, USA), or with the recommended isotype controls, for 30 min at 4°C. The cells were analyzed on Gallios flow cytometer (Beckman Coulter) by using the Kaluza analysis software (Beckman Coulter).

Morphological Observation

Because BMMCs are non-adherent cells, to immobilize the cells on the floor of the dish, glass-bottomed 35-mm dishes (Matsunami Glass Ind., Ltd., Osaka, Japan) were coated with Cellmatrix (Nitta Gelatin Inc., Osaka, Japan) at room temperature for 1 h. BMMCs were seeded on these dishes in phenol red-free medium, and the dishes were placed on a heated stage and maintained at 37°C in 5% CO_2 atmosphere under a fluorescence microscope (Olympus, Tokyo, Japan). Although the dish was subjected to shaking conditions, the cells that were within the visual field of the microscope were observed as target cells. Images were acquired using a 100× oil immersion objective at 1-min intervals after antigen stimulation and were analyzed using the MetaMorph software (Molecular Devices, Sunnyvale, CA, USA). In other experiments, confocal microscopy was used (LSM510 Meta system; Carl Zeiss, Oberkochen, Germany).

RESULTS

Degranulation and mRNA Expression of Cytokines in BMMCs after Repeated Stimulation

To examine whether mast cells are able to respond to repeated antigen stimulation, β-hexosaminidase release and mRNA expression of cytokines in the BMMCs were examined. BMMCs were sensitized with anti-DNP IgE and stimulated with DNP-BSA (initial stimulation). Twenty-four hours after initial stimulation, the cells were washed and again stimulated with IgE and DNP-BSA (re-stimulation). The release of β-hexosaminidase increased to 34% in response to initial stimulation (Fig. **1A**) and to 24% in response to re-stimulation. After the initial stimulation, BMMCs that were not sensitized with IgE did not show increased β-hexosaminidase release after re-stimulation (Fig. **1B**).

To assess the mRNA expression of cytokines in BMMCs following initial stimulation and re-stimulation, real-time RT-PCR was performed at 1 and 3 h after the stimulation (Fig. **2**). IL-6 and IL-13 mRNA expression levels increased 1 h after the initial stimulation, and this same temporal pattern of increase in the mRNA expression of cytokines was observed after re-stimulation. These results suggest that mast

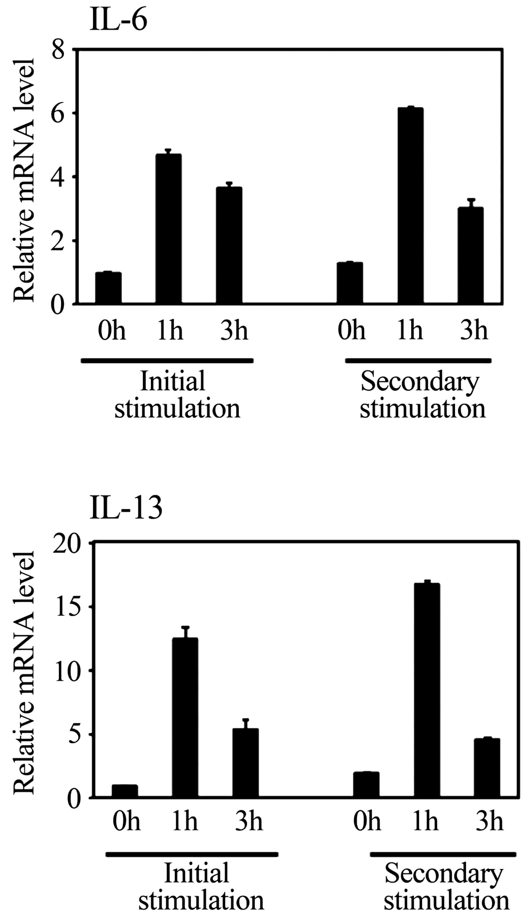

Fig. (2). Expression levels of specific cytokine mRNAs in re-stimulated mast cells. BMMCs were sensitized with anti-DNP IgE for 2 h and stimulated with DNP-BSA (initial stimulation). The BMMCs were washed 24 h after initial stimulation and re-sensitized with anti-DNP IgE for 2 h. The cells were washed and re-stimulated with DNP-BSA. The cells were collected at the specified times. Cytokine mRNA expression levels were determined by real-time RT-PCR (normalized with housekeeping gene GAPDH). Values represent mean ± SD (n = 3).

cells can maintain their function even after antigen-induced degranulation.

Expression of FcεRI in BMMCs

Degranulation was not re-induced in BMMCs by antigen stimulation alone (Fig. **1B**). To examine whether IgE remained bound to FcεRIs on the BMMC cell surface after initial stimulation, surface expression of FcεRI was measured by flow cytometry. BMMCs were sensitized with the specified dose of IgE and were stained with FITC-FcεRI. The fluorescent intensity of FITC-FcεRI was decreased in a dose-dependent manner in BMMCs after IgE sensitization (Fig. **3A**), suggesting that the fluorescent intensity was reduced when IgE was bound to FcεRI in BMMCs. BMMCs were stimulated with IgE and antigen. Twenty-four hours later, these cells were washed and stained with FITC-FcεRI. The fluorescent intensity of FITC-FcεRI was unaltered in the treated BMMCs, 24 h after stimulation as compared to that in the untreated BMMCs (Fig. **3B**), suggesting that FcεRIs were expressed on the cell surface of BMMCs, but IgE did not bind to FcεRI. BMMCs were stimulated with IgE and antigen, and 24 h after stimulation, these cells were washed and sensitized with the specified doses of IgE and stained with FITC-FcεRI. The fluorescent intensity of FITC-FcεRI was dose-dependently reduced in BMMCs after IgE re-sensitization (the binding of FITC-FcεRI to FcεRI was inhibited by IgE) (Fig. **3C**), suggesting that IgE was detached from FcεRI in BMMCs 24 h after the initial stimulation and that IgE could again bind to FcεRI.

Changes in BMMC Morphology

Though BMMCs were repeatedly degranulated by FcεRI-induced stimulation, it was unclear whether BMMCs that had degranulated after the initial stimulation were re-activated after re-stimulation, or whether those that had not degranulated after the initial stimulation responded after re-stimulation. Hence, individual BMMCs were analyzed by time-lapse microscopy after IgE cross-linked activation.

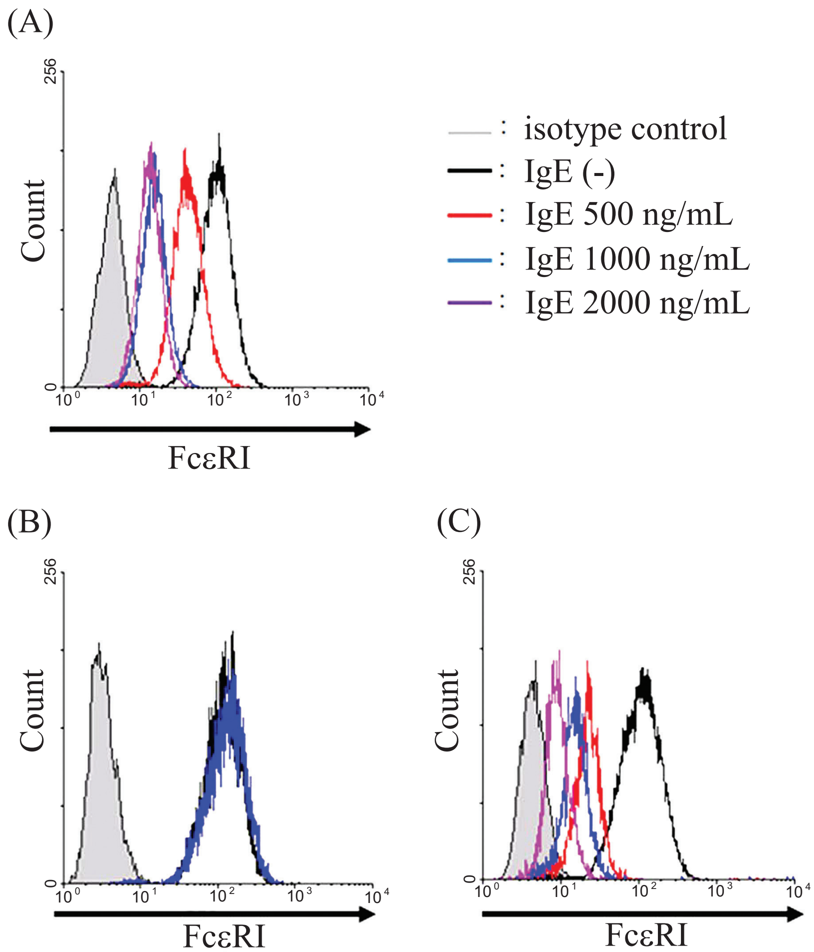

Fig. (3). Expression of FcεRI in BMMCs. (**A**) BMMCs were sensitized with 500, 1,000, or 2,000 ng/mL anti-DNP IgE for 2 h. Then sensitized and non-sensitized BMMCs were stained with FITC-FcεRI. (**B**) BMMCs were sensitized with anti-DNP IgE (1,000 ng/mL) for 2 h. The sensitized and non-sensitized cells were stimulated with DNP-BSA. Twenty-four hours later, the cells were washed and stained with FITC-FcεRI. (**C**) BMMCs were sensitized with anti-DNP IgE for 2 h and then stimulated with DNP-BSA. Twenty-four hours after stimulation, the cells were washed and sensitized with 500, 1,000, or 2,000 ng/mL anti-DNP IgE for 2 h. The sensitized and non-sensitized cells were stained with FITC-FcεRI. The fluorescent intensity was measured by flow cytometry. Data represents results obtained from 3 experiments.

BMMCs were infected with viral vectors expressing the fluorescent fusion proteins AcGFP-actb and DRM-CD63 to examine the location of individual granules in relation to the plasma membrane after antigen-induced stimulation. We continuously monitored individual BMMCs by time-lapse photography. Fig. (**4A** and **B**) indicates that the plasma membrane and CD63-containing granule membrane of the individual BMMCs appeared to be at normal level before stimulation. The CD63-containing granule membranes moved toward the plasma membranes (the plasma membrane and CD63-containing granule membrane were partially colocalized) 5 min after stimulation (Fig. **4D**: arrow), and then returned to normal level 120 min after initial stimulation (Fig. **4F**). Twenty-four hours after the initial stimulation, the monitored BMMCs were washed, resensitized with anti-DNP IgE, and re-stimulated with DNP-BSA. The plasma membrane and CD63 colocalized again, before returning to normal condition after re-stimulation (Fig. **4G−L**).

Next, to visualize chymase, which belongs to the serine protease family, and the CD63-containing granule membrane, pMXs-AcGFP-CD63-IRES-DRM-Cma1 was introduced into the BMMCs. Chymase moved toward the cell surface within few minutes of initial stimulation (Fig. **5A−E**). Chymase levels decreased 20 min after the initial stimulation (Fig. **5F**) and then recovered 24 h after the initial stimulation, although the level was not similar to that observed before the stimulation (Fig. **5G**). Similar pattern of sequential changes were observed after re-stimulation (Fig. **5H−L**). The arrows in Fig. (**5D** and **J**) indicate that chymase had moved toward the cell surface.

DISCUSSION

In this study, pMXs-AcGFP-actb-IRES-DRM-CD63 and pMXs-AcGFP-CD63-IRES-DRM-Cma1 vectors were constructed to observe the intracellular movements of actin,

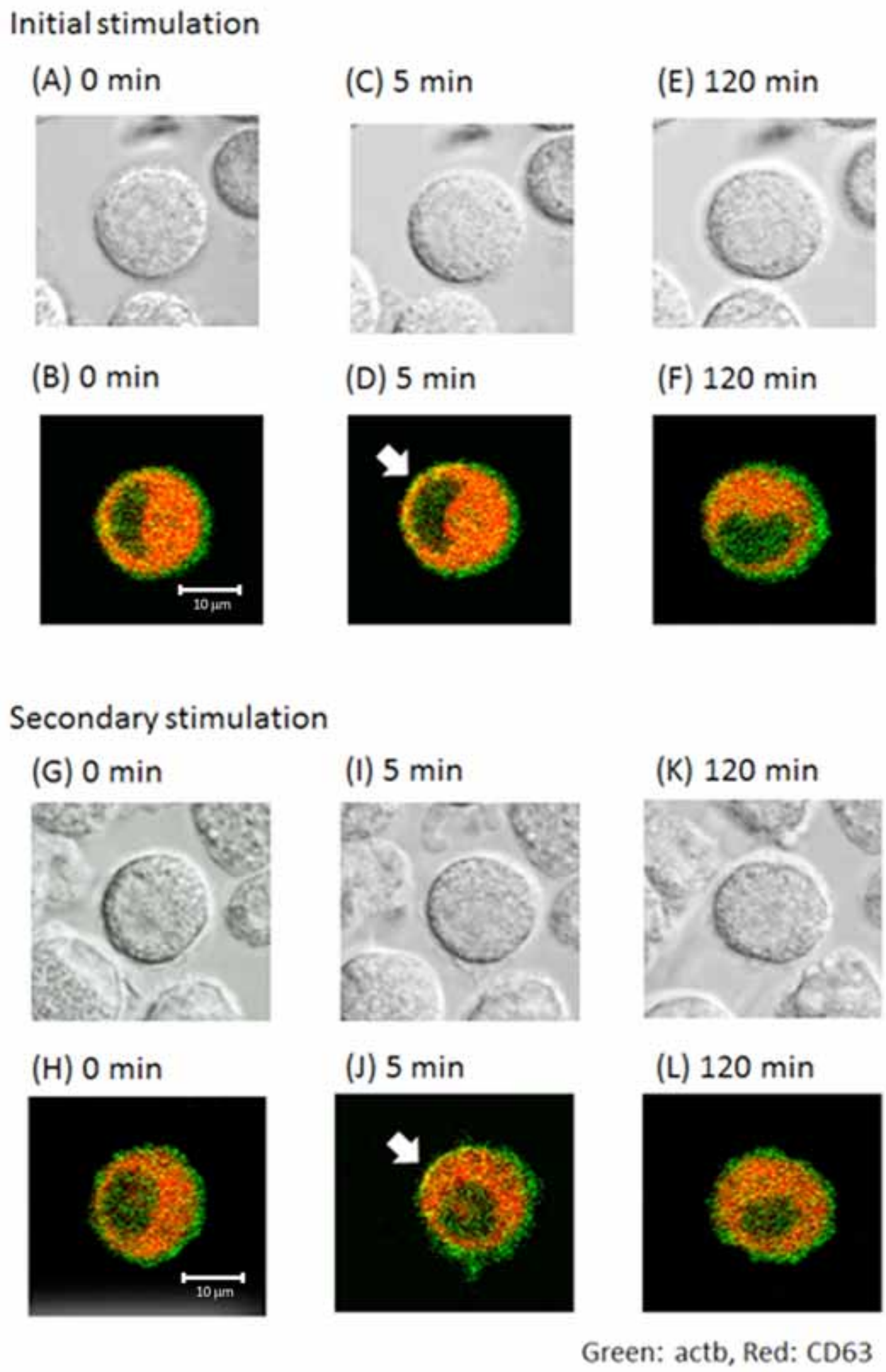

Fig. (4). Observation of BMMCs transfected with pMXs-AcGFP-actb-IRES-DRM-CD63. BMMCs were sensitized with anti-DNP IgE for 2 h and then stimulated with DNP-BSA (**A−F**). At 24 h after initial stimulation, BMMCs were washed and re-sensitized with anti-DNP IgE for 2 h. The cells were washed and re-stimulated with DNP-BSA. (**G−L**). Images were captured before stimulation (**A**, **B**, **G**, **H**) and 5 min (**C**, **D**, **I**, **J**) and 120 min after stimulation (**E**, **F**, **K**, **L**). Arrows indicate the fusion of CD63-containing granules and plasma membrane (**D** and **J**). Data represent results from 3 experiments.

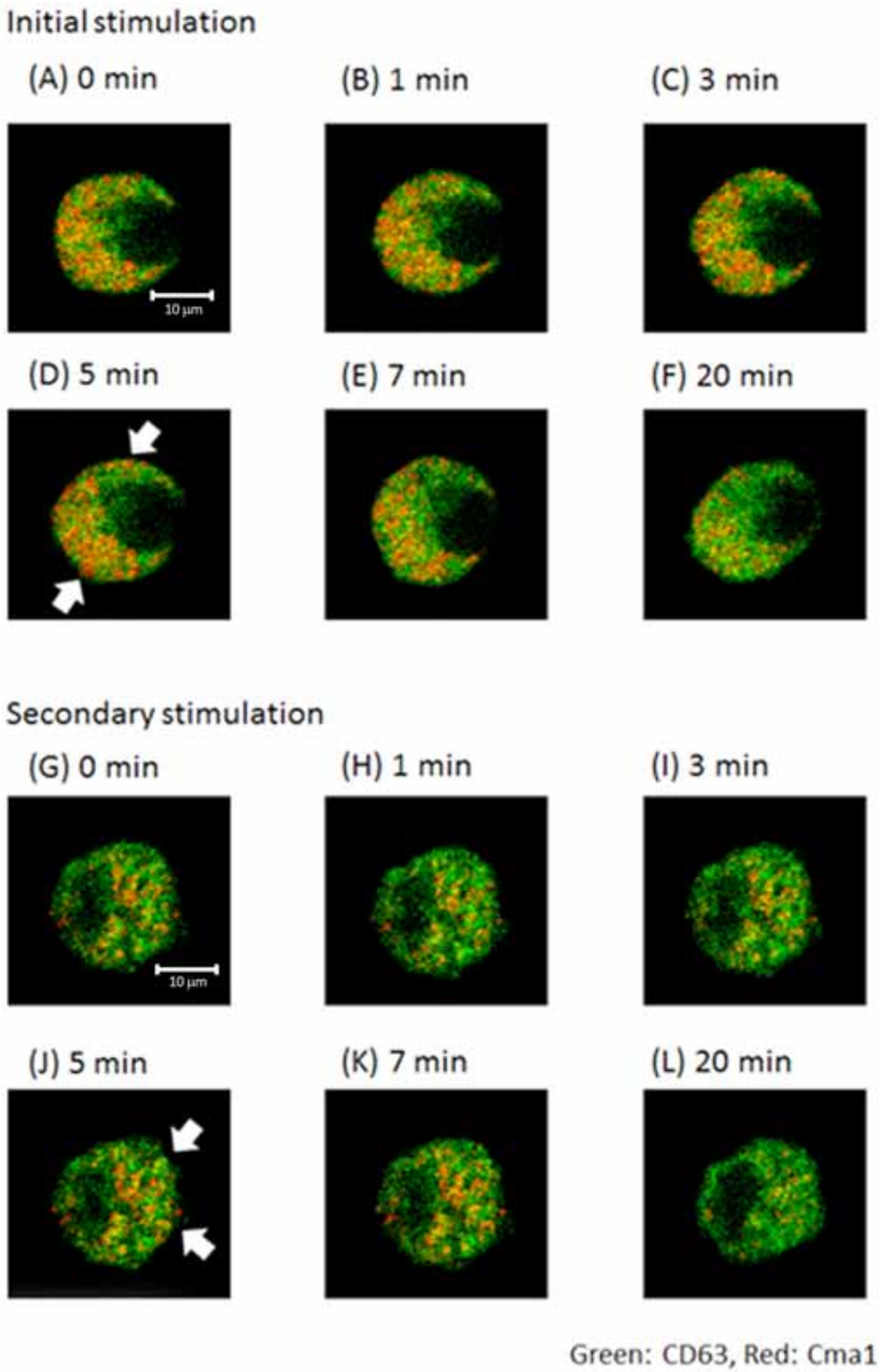

Fig. (5). Observation of BMMCs transfected with pMXs-AcGFP-CD63-IRES-DRM-Cma1. BMMCs were sensitized with anti-DNP IgE for 2 h and then stimulated with DNP-BSA (**A**−**F**). At 24 h after initial stimulation, theindividualBMMCswerewashed and re-sensitized with anti-DNP IgE for 2 h. The cells were washed and re-stimulated with DNP-BSA. (**G**−**L**). Arrows indicate movement of chymase toward the cell surface (**D** and **J**). Data represent results from 3 experiments.

CD63, and chymase after degranulation bythe cross-linking of IgE bound to FcεRI and the antigen. To analyze the dynamics of mast cells after degranulation, these vectors were introduced into BMMCs.

Our results indicate that mast cells can initiate repeated degranulation (Fig. **1** and **2**). To examine whether IgE was bound to FcεRI in BMMCs 24 h after stimulation, BMMCs were stained with FITC-FcεRI. Since the fluorescent intensity of FcεRI was dose-dependently reduced by IgE (Fig. **3A**), the binding of IgE to FcεRI on the cell surface is probably correlated with the fluorescent intensity of FITC-FcεRI. The expression of FcεRI in BMMCs was saturated by1,000 ng/mL of IgE before antigen-stimulation (Fig. **3A**). Although the expression of FcεRI on non-sensitized BMMCs after the antigen-stimulation was unchanged as compared to that before the antigen-stimulation, the IgE binding of FcεRI was not saturated at the same dose (1,000 ng/mL) of IgE 24 h after the antigen-stimulation (Fig. **3C**). Reports by other investigators suggested that IgE induced the upregulation of FcεRI in mouse BMMCs [28, 29]. In this method, BMMCs were sensitized with IgE for 2 h. Therefore, the upregulation of FcεRI by IgE after antigen-stimulation occurs probably earlier than that observed before the antigen-stimulation. However, it is unclear how many IgEs bind to BMMCs after the antigen-stimulation. The expression of FcεRI in BMMCs by FITC-conjugated anti-DNP IgE was not confirmed after IgE binding. These data suggest that BMMCs did not degranulate for the second time in response to the antigen only, because IgE was no longer bound to FcεRI, 24 h after the initial stimulation. It is known that FcεRIs are endocytosed by IgE and antigen stimulation [30, 31]. Therefore, FcεRIs are probably taken up by the cytoplasm after initial stimulation to reappear on the cell surface 24 h later.

It is still unknown whether the response to re-stimulation depends on repeated reactions in the same cell. Therefore, to examine the responses of individual BMMCs to antigen stimulation, BMMCs were transfected with pMXs-AcGFP-actb-IRES-DRM-CD63 or pMXs-AcGFP-CD63-IRES-DRM-Cma1 and observed by time-lapse microscopic imaging to determine the changes in their morphology. We expected that the cell membranes would turn yellow in color after stimulation. FcεRI-induced stimulation of mast cells results in the fusion of granules with the plasma membrane [3]. In this study, although the membrane of BMMCs became partially yellow in color, the whole membrane did not turn yellow. The antigen-induced degranulation of BMMCs may have occurred gradually in this study. In addition, colocalization of granule membrane and plasma membrane was also observed following re-stimulation (Fig. **4J**). Therefore, these results confirmed that the morphological changes in BMMC after initial stimulation were similar to that observed after re-stimulation. Moreover, pMXs-AcGFP-CD63-IRES-DRM-Cma1 was introduced into BMMCs to observe the granule membrane and chymase. The movement of chymase to the periphery of the BMMCs was observed within a few minutes of stimulation. Chymase was reduced 20 min after stimulation (Fig. **5F**) but it recovered after 24 h (Fig. **5G**); this suggested that chymase was released from and reduced by BMMCs by antigen stimulation but recovered to normal levels, 24 h after the stimulation. However, it is unclear what amount of time is required for chymase to recover from the effects of antigen stimulation.

In this study, we observed sequential changes in the morphology of individual BMMCs after repetitive stimulation, demonstrating that mast cells are able to survive and maintain their function after degranulation, and that they are capable of repeated degranulation. In addition, the mast cells returned to the pre-stimulation status 24 h after antigen stimulation. These results also highlight the advantages of using fluorescent fusion proteins to monitor granular release from mast cells.

CONFLICT OF INTEREST

The author confirms that this article content has no conflict of interest.

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Declared none.

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