STEM CELLS AND DEVELOPMENT
Volume 22, Number 5, 2013
© Mary Ann Liebert, Inc.
DOI: 10.1089/scd.2012.0339

Two-Step Differentiation of Mast Cells from Induced Pluripotent Stem Cells

Tomoko Yamaguchi,[1] Katsuhisa Tashiro,[1] Satoshi Tanaka,[2] Sumie Katayama,[3] Waka Ishida,[4] Ken Fukuda,[4] Atsuki Fukushima,[4] Ryoko Araki,[5] Masumi Abe,[5] Hiroyuki Mizuguchi,[1,6,7] and Kenji Kawabata[1,6]

Mast cells play important roles in the pathogenesis of allergic diseases. They are generally classified into 2 phenotypically distinct populations: connective tissue-type mast cells (CTMCs) and mucosal-type mast cells (MMCs). The number of mast cells that can be obtained from tissues is limited, making it difficult to study the function of mast cells. Here, we report the generation and characterization of CTMC-like mast cells derived from mouse induced pluripotent stem (iPS) cells. iPS cell-derived mast cells (iPSMCs) were generated by the OP9 coculture method or embryoid body formation method. The number of Safranin O-positive cells, expression levels of CD81 protein and histidine decarboxylase mRNA, and protease activities were elevated in the iPSMCs differentiated by both methods as compared with those in bone marrow-derived mast cells (BMMCs). Electron microscopic analysis revealed that iPSMCs contained more granules than BMMCs. Degranulation was induced in iPSMCs after stimulation with cationic secretagogues or vancomycin. In addition, iPSMCs had the ability to respond to stimulation with the IgE/antigen complex in vitro and in vivo. Moreover, when iPSMCs generated on OP9 cells were cocultured with Swiss 3T3 fibroblasts, protease activities as maturation index were more elevated, demonstrating that mature mast cells were differentiated from iPS cells. iPSMCs can be used as an in vitro model of CTMCs to investigate their functions.

Introduction

Mast cells have recently gained attention, because they have been recognized as effector cells not only in allergic disorders, but also in other immune diseases, including autoimmune diseases and chronic inflammatory disorders [1]. Activation of mast cells triggers allergic and inflammatory responses through the release of a wide variety of mediators, such as histamine, arachidonic acid metabolites, and neutral proteases, and regulates immune responses through the production of cytokines and chemokines [2]. Mast cell precursors leave the bone marrow, migrate in the blood, invade tissues, and then proliferate and differentiate into mature cells [3]. Mature rodent mast cells are generally classified into 2 phenotypically distinct populations: connective tissue-type mast cells (CTMCs) and mucosal-type mast cells (MMCs) [3–4]. Each cell type differs with respect to location, staining characteristics, and histamine content. Mouse CTMCs, which are present in the peritoneal cavity and skin, contain heparin and store large amounts of histamine. In contrast, mouse MMCs, which are prominent in the mucosal layer of the gastrointestinal tract, contain chondroitin sulfate E rather than heparin and have relatively low histamine content. Since recent studies have demonstrated that CTMCs are involved in a wide variety of immune responses [5–7], development of an in vitro culture system of CTMCs is needed. Although several mast cell lines and IL-3-dependent bone marrow-derived mast cells (BMMCs) have been used as models to investigate the process of mast cell activation and subsequent production of proinflammatory mediators, these models have limitations in analyzing the functions specific to mature mast cells. Previous studies showed that coculture of BMMCs with Swiss 3T3 fibroblasts in the presence of stem cell factor (SCF) facilitated morphological and functional maturation toward a CTMC-like phenotype [8].

Differentiation of both mouse and human embryonic stem (ES) cells into multiple hematopoietic lineages is now well established as a powerful tool for studying hematopoietic

[1]Laboratory of Stem Cell Regulation, National Institute of Biomedical Innovation, Osaka, Japan.
[2]Department of Immunobiology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan.
[3]Bioresources Research, Laboratory of Common Apparatus, National Institute of Biomedical Innovation, Osaka, Japan.
[4]Department of Ophthalmology and Visual Science, Kochi Medical School, Kochi, Japan.
[5]Transcriptome Research Group, National Institute of Radiological Sciences, Chiba, Japan.
[6]Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan.
[7]The Center for Advanced Medical Engineering and Informatics, Osaka University, Osaka, Japan.

differentiation and lineage restriction, and for generating unlimited numbers of hematopoietic stem and progenitor cell populations for transplantation [9–12]. ES or induced pluripotent stem (iPS) cells into hematopoietic cells have been differentiated by embryoid body (EB) formation or coculture with stromal cells, such as OP9 cells [13–16]. By using these protocols, several groups have previously established methods to generate mast cells from mouse [17–19], cynomolgus monkey [20], and human [21] ES cells. ES cell-derived mast cells could respond to stimulation with antigen and substance P by releasing histamine. However, in most cases, these cells do not develop the large granules and high levels of proteolytic enzymes characteristic of tissue mast cells.

In this study, we generated mast cells from mouse iPS cells (iPSMCs), and characterized them from the point of view of morphology, function, and gene expression. Our results showed that the iPSMCs that were differentiated by coculture with OP9 stromal cells or the EB formation method had characteristics similar to CTMCs. When iPSMCs that were generated on OP9 cells were cocultured with Swiss 3T3 fibroblasts, the iPSMCs exhibited a more functional phenotype.

Materials and Methods

Cell cultures

Two mouse iPS cell clones, 38C2 (a kind gift from Dr. S. Yamanaka, Kyoto University, Kyoto, Japan) [22] and 2A-EGFPTg-4F-01 [23], were used in the present study. These mouse iPS cells were routinely cultured in a leukemia inhibitory factor-containing ES cell medium (Specialty Media) on mytomycin C-treated mouse embryonic fibroblasts (MEFs; Specialty Media), and they were passaged every 2 days using 0.25% trypsin-EDTA (Invitrogen). OP9 stromal cells were cultured in an α-minimum essential medium (α-MEM: Sigma) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen), and 1×nonessential amino acid (NEAA; Invitrogen).

Generation of BMMCs

C57BL/6 mice were purchased from Nippon SLC. Bone marrow cells were prepared from the femurs and tibiae of mice. Cells were cultured in an RPMI 1640 medium containing 10% FBS, 1×NEAA, and 10 ng/mL murine IL-3 (R&D Systems). The culture medium was replaced with a fresh medium every 5 days. After 4 weeks of culture, we confirmed the cellular surface expression of both FcεRI and c-kit (>95% positive).

Differentiation of iPS cells to mast cells

Before coculturing with OP9 cells or EB formation, mouse iPS cells were suspended in an ES cell medium and cultured on a culture dish at 37°C for 30 min to remove MEF layers. In the OP9 cell-mediated differentiation method, iPS cells were transferred onto OP9 cells in 6-well plates at a density of 1×10^4 cells per well. The induced cells were trypsinized on day 7, and 1×10^5 cells were seeded onto fresh OP9 cells with α-MEM supplemented with 20% FBS, 2 mM L-glutamine, 1×NEAA, 30 ng/mL IL-3, and 100 ng/mL SCF (Peprotech). After 7 days, nonadherent cells were reseeded onto fresh OP9 cells. The cells were subcultured every 7 days. We harvested the differentiated cells on day 28 and used them for further analysis.

In the EB-mediated differentiation method, iPS cell-derived EBs were generated by culturing iPS cells on a round-bottom low-cell-binding 96-well plate at 1×10^3 cells per well. iPS cell-derived EBs were collected on day 7, and were transferred to a Petri dish with Differentiation Medium I [Dulbecco's modified Eagle's medium containing 15% FBS, 1×NEAA, 2 mM L-glutamine, 1×nucleosides, 0.1 mM 2-mercaptoethanol, penicillin/streptomycin, 30 ng/mL IL-3, 30 ng/mL IL-6 (Peprotech), and 100 ng/mL SCF]. After 7 days, nonadherent cells were transferred to a culture dish with Differentiation Medium II (Dulbecco's modified Eagle's medium containing 10% FBS, 1×NEAA, 2 mM L-glutamine, penicillin/streptomycin, 30 ng/mL IL-3, and 100 ng/mL SCF). We harvested the nonadherent cells on day 28 and used them for further analysis.

Transmission electron microscopy

BMMCs or iPSMCs were fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4), postfixed with 1% OsO_4, dehydrated by a graded ethanol series, passed through QY-1 (Nisshin EM), and then embedded in Epon-812 (TAAB,). Ultrathin sections (0.06-μm thick) were cut with an ultramicrotome (Leica Microsystems), stained with uranyl acetate–lead citrate, and observed using an electron microscope (H-7650, HITACHI) at 80 kV.

Protease assay

BMMCs or iPSMCs were washed with phosphate-buffered saline (PBS), lysed in PBS containing 2 M NaCl/0.5% Triton X-100, and incubated for 30 min on ice. The lysate was centrifuged at 12,000 rpm for 30 min at 4°C. Activities of granule proteases in the resultant supernatants were measured using their specific chromogenic peptide substrates, such as S-2288 for tryptase (Sekisui medical) and M-2245 for carboxypeptidase A (CPA; Bachem) [24].

β-hexosaminidase release assay

β-hexosaminidase activity was measured as a marker of the granular fraction for evaluation of degranulation. Cells were washed with an HEPES buffer (137 mM NaCl, 20 mM HEPES, 5 mM D-glucose, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 2.4 mM $CaCl_2$, and 0.1% bovine serum albumin) and incubated with the buffer containing compound 48/80 (10 μg/mL; Sigma) or substance P (100 μM; Sigma) for 30 min. In the case of antigen stimulation, mast cells sensitized with 1 μg/mL anti-dinitrophenyl (DNP) IgE (SPE7; Sigma-Aldrich) for 24 h were stimulated with 100 ng/mL DNP-human serum albumin (HSA; Biosearch Technologies) in the presence of lysophosphatidylserine (Lyso-PS; Avanti Polar Lipids).

Coculture of mast cells with Swiss 3T3 fibroblasts

iPSMCs obtained after 28 days of culture with OP9 cells were cocultured with mitomycin C-treated Swiss 3T3 fibroblasts in the presence of 100 ng/mL SCF. BMMCs were

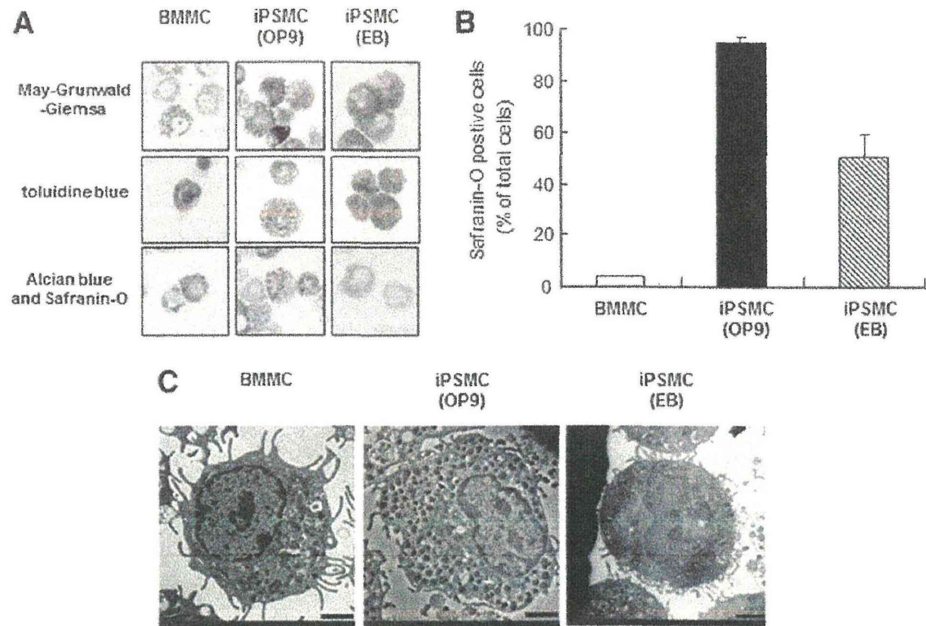

FIG. 1. Morphological characterization of induced pluripotent stem cell-derived mast cells (iPSMCs). **(A)** The iPSMCs, which were differentiated by coculture with OP9 cells or the embryoid body formation method, were harvested on day 28. Cytocentrifuged preparations of bone marrow-derived mast cells (BMMCs) and the iPSMCs were stained with May-Grunwald-Giemsa, toluidine blue, or Alcian blue and Safranin O solutions. **(B)** The ratio of Safranin O-positive cells to total cells was calculated and shown as a percentage. The data represent the means ± S.D. ($n = 4$). **(C)** BMMCs and iPSMCs were visualized by transmission electron microscopy. Scale bar = 2.0 μm.

also cocultured with Swiss 3T3 fibroblasts under the same conditions. The subculture was performed every 4 days. The cells were trypsinized and replated, and nonadherent cells were collected as mast cells and used for further analysis.

Mast cell reconstitution and induction of passive cutaneous anaphylaxis

BMMCs or iPSMCs (5×10^5 cells) were injected subcutaneously into the conjunctivae of mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$

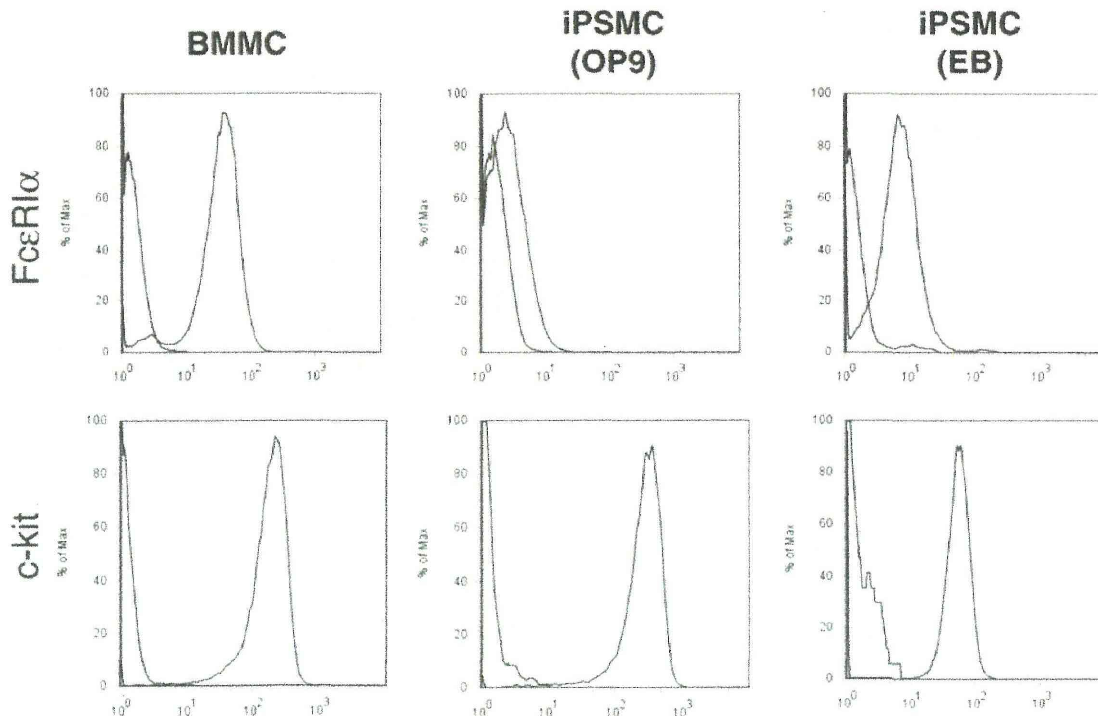

FIG. 2. Flow cytometric analysis of FcεRI and c-kit expression on iPSMCs. BMMCs and iPSMCs were stained with FITC-labeled anti-FcεRI and PE-labeled anti-c-kit antibodies for 30 min on ice. Stained cells were washed, resuspended in 1% fetal bovine serum–phosphate-buffered saline (FBS-PBS), and analyzed by flow cytometry.

mice. To elicit passive cutaneous anaphylaxis reactions, mice were injected subcutaneously into the conjunctiva with 75 ng anti-DNP IgE or saline. Then, 24 h after IgE injection, 100 μg DNP-HSA containing 2% Evan's blue dye was injected intravenously into mice. Thirty minutes later, the mice were killed, and their conjunctivae were excised. Evan's blue dye was extracted from conjunctivae with formamide, and the absorbance was measured at 610 nm.

Results

Generation of mast cells from mouse iPS cells

iPSMCs were generated by the OP9 coculture method or EB formation method as described in Supplementary Fig. S1 (Supplementary Data are available online at www.liebertpub.com/scd). Approximately 6.5×10^6 mast cells could be obtained from 1×10^5 iPS cells by coculturing them with OP9 cells for 4 weeks. In addition, as in the case of BMMCs, iPSMCs can retain their proliferative potential (data not shown).

Next, we performed the staining with May-Grunwald-Giemsa, toluidine blue, Alcian blue, and Safranin O solutions. May-Grunwald-Giemsa staining of the iPSMCs, which were differentiated by coculture with OP9 stromal cells or the EB formation method (Supplementary Fig. S1), revealed that induced mast cells gave rise to a uniform phenotype with rough basophilic granule-containing cells (Fig. 1A, upper). The granules in these cells showed a metachromatic staining pattern when stained with acid toluidine blue (Fig. 1A, middle). We then performed Alcian blue and Safranin O staining, by which mast cells are known to show a specific red color if they are CTMCs and a blue color if they are immature mast cells or MMCs [1]. While BMMCs were Alcian blue positive and Safranin O negative, iPSMCs were positive for both Alcian blue and Safranin O staining (Fig. 1A, [lower], B). Electron microscopic analysis revealed that the iPSMCs differentiated by either method contained more granules than BMMCs (Fig. 1C).

Expression of high-affinity IgE receptor on iPSMCs

Mast cells are known to express c-kit and FcεRI (high-affinity IgE receptor) [1]. We next performed flow cytometric analysis to examine the surface expression of c-kit and FcεRI on iPSMCs. There was no significant difference in c-kit expression levels between iPSMCs and BMMCs (Fig. 2). In contrast, the FcεRIα expression level was significantly lower in the iPSMCs that were generated by coculture with OP9 cells, compared with that in BMMCs. Both c-kit$^+$FcεRI$^+$ and c-kit$^+$FcεRI$^-$ cells showed a granular phenotype by forward and side scatter (data not shown).

FcεRI is a heterotrimer composed of one α-chain and 2 γ-chains or a heterotetramer composed of one β-chain and 2 γ-chains. To evaluate the expression of each FcεRI subunit in iPSMCs, we analyzed mRNA expression levels by reverse transcription and quantitative polymerase chain reaction (RT-PCR). As shown in Supplementary Fig. S2, the expression levels of the mRNAs encoding the FcεRIα, FcεRIβ, and FcεRIγ chains were reduced in the iPSMCs differentiated by either method as compared with the levels in BMMCs.

Phenotypic differences between iPSMCs and BMMCs

To further compare the degree of mast cell differentiation, we measured the tryptase and CPA activities in iPSMCs. The tryptase and CPA activities were elevated in the iPSMCs derived from either method as compared with those in BMMCs (Fig. 3).

Histidine decarboxylase (HDC) is a critical enzyme that is involved in the synthesis of endogenous histamine in mammals [25–26], and is considered to be one of the indices of mast cell maturation [26]. Therefore, quantitative RT-PCR analysis was performed to compare the expression of HDC mRNA levels in iPSMCs and BMMCs (Supplementary Fig. S3). The expression level of HDC mRNA was elevated in the iPSMCs that were differentiated by either method as compared with that in BMMCs.

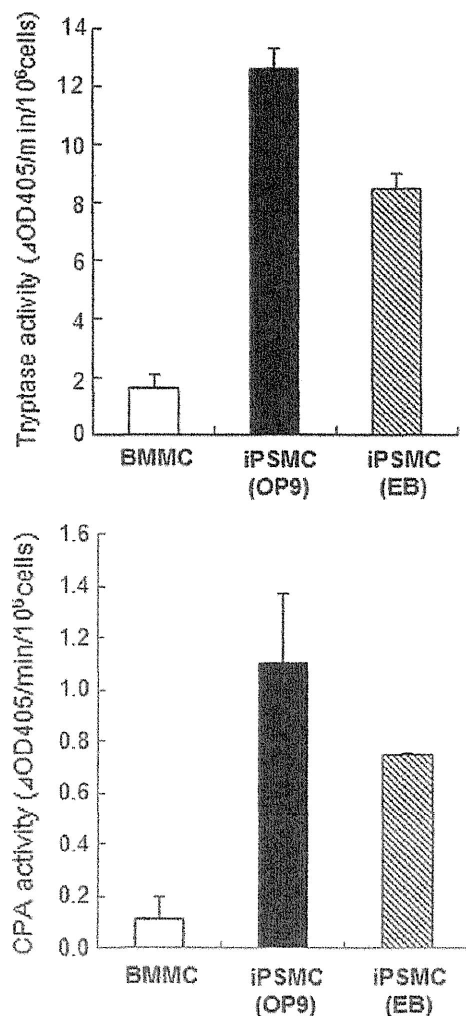

FIG. 3. Tryptase and carboxypeptidase A (CPA) activities in iPSMCs. Cell extracts prepared from BMMCs and iPSMCs were assayed for tryptase and CPA activities as described in the Materials and Methods section. All data represent the means ± S.D. ($n = 4$).

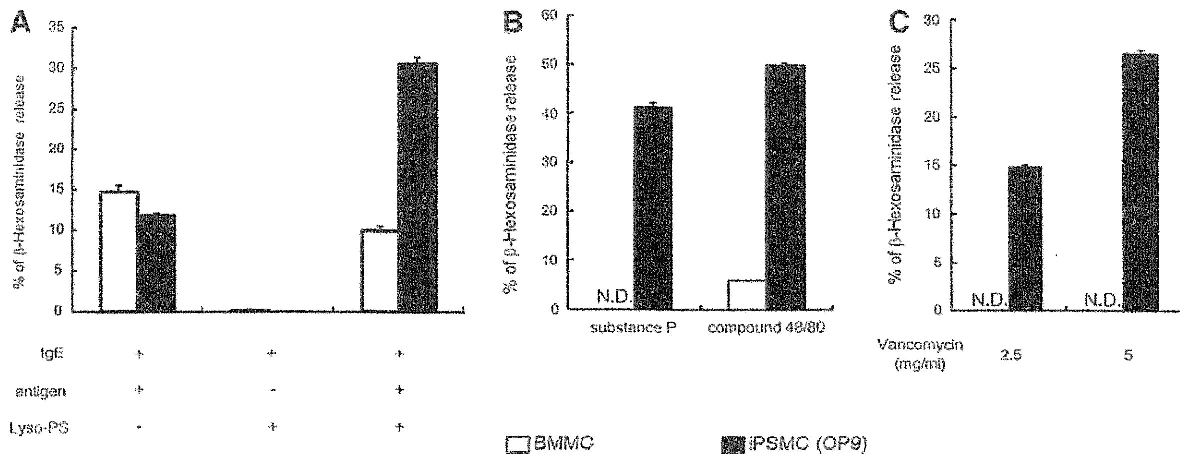

FIG. 4. β-hexosaminidase release from iPSMCs after stimulation with IgE/antigen, compound 48/80, substance P, and vancomycin. **(A)** The exocytotic response was determined by measuring the release of β-hexosaminidase. BMMCs (*open bar*) and the iPSMCs that were cocultured with OP9 cells (*closed bar*) were sensitized with anti-dinitrophenyl (DNP) IgE and stimulated with DNP human serum albumin (HSA) in the presence or absence of Lyso-PS as described in the Materials and Methods section. β-hexosaminidase enzymatic activity was measured in supernatants and cell pellets solubilized with 0.5% Triton X-100 in HEPES buffer. **(B)** BMMCs (*open bar*) and the iPSMCs that were cocultured with OP9 cells (*closed bar*) were stimulated with compound 48/80 or substance P. **(C)** BMMCs (*open bar*) and the iPSMCs that were cocultured with OP9 cells (*closed bar*) were stimulated with vancomycin. All data represent the means ± S.D. ($n = 3$).

Previously, Takano *et al.* demonstrated that *CD81*, a member of the tetraspanin superfamily, is one of the strikingly upregulated genes in BMMCs cocultured with Swiss 3T3 fibroblasts [8]. CD81 is also considered to be a marker of CTMCs. FACS analysis showed that expression of CD81 was elevated in the iPSMCs differentiated by either method as compared with that in BMMCs (Supplementary Fig. S4). In particular, our results revealed that the iPSMCs that were differentiated by coculture with OP9 cells were almost all CD81 positive and showed a homogeneous population.

Degranulation of iPSMCs

We compared FcεRI-mediated degranulation of BMMCs and iPSMCs by measuring the extracellular activity of β-hexosaminidase, a marker enzyme for histamine-containing granules. As shown in Fig. 4A, the iPSMCs that were differentiated by coculture with OP9 cells displayed significantly less release of β-hexosaminidase than the BMMCs in response to IgE-mediated antigen stimulation. Similar results were obtained in iPSMCs that were derived from EB formation methods (Supplementary Fig. S5A). On the other hand, when the iPSMCs that were pretreated with anti-DNP monoclonal IgE were incubated with DNP-HSA in the presence of Lyso-PS, which is known to enhance IgE-mediated degranulation in rat peritoneal mast cells [27], the amount of β-hexosaminidase release was increased.

Responses to cationic secretagogues such as compound 48/80 and substance P are functional characteristics of CTMCs [4]. We next compared the IgE-independent responses between BMMCs and iPSMCs. Stimulation of iPSMCs with compound 48/80 resulted in marked exocytosis of β-hexosaminidase, whereas no or little release of this enzyme was detected from stimulated BMMCs (Fig. 4B and Supplementary Fig. S5B). Similarly, β-hexosaminidase release from iPSMCs was much more markedly elevated by substance P treatment than β-hexosaminidase release from BMMCs (Fig. 4B and Supplementary Fig. S5B). In addition, stimulation of iPSMCs with vancomycin resulted in marked exocytosis of β-hexosaminidase, whereas no or little release of β-hexosaminidase was detected from vancomycin-stimulated BMMCs (Fig. 4C and Supplementary Fig. S5C). These results indicate that the iPSMCs display a CTMC-like phenotype.

Comparison between iPSMCs differentiated by the OP9 coculture and EB formation protocols

We next compared the degree of differentiation between the iPSMCs that were differentiated by the OP9 coculture method and those differentiated by the EB formation method. The expression level of FcεRI was significantly lower in the iPSMCs that were differentiated by coculture with OP9 cells as compared with the iPSMCs that were differentiated by EB formation (Fig. 2). However, the number of Safranin O-positive cells was significantly greater in the iPSMCs that were cocultured with OP9 cells than in the iPSMCs that were derived from the EB formation method (Fig. 1B). In addition, the expression levels of HDC mRNA and CD81 protein were significantly higher in the iPSMCs that were cocultured with OP9 cells than in those that were derived from the EB formation method (Supplementary Figs. S3 and S4). These results showed that the iPSMCs that were cocultured with OP9 cells were more mature than the iPSMCs that were derived from the EB formation method. The iPSMCs that were derived from EB formation were more mature than BMMCs (Figs. 1–3). During the differentiation step, the iPSMCs that were derived from the EB formation method were designated as mast cells I (Fig. 5F). The iPSMCs that were differentiated by coculture with OP9 cells were also designated as mast cells II.

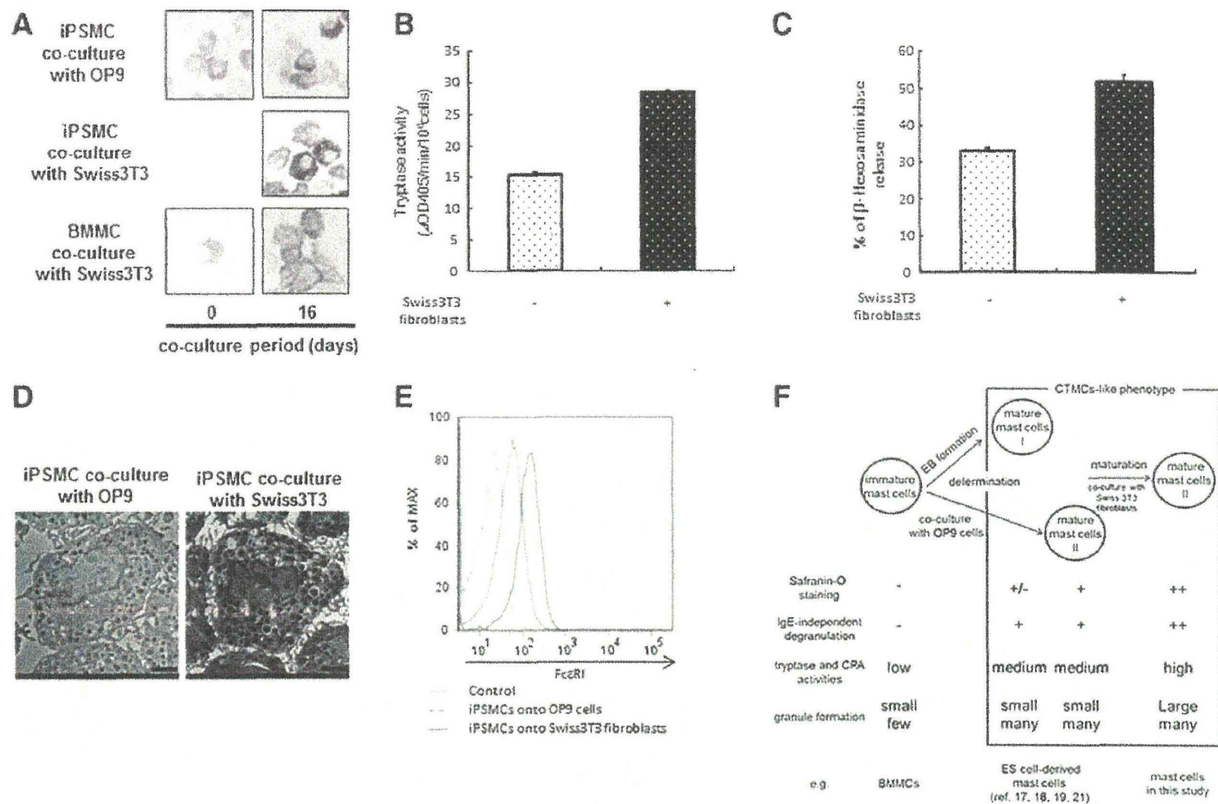

FIG. 5. Maturation of iPSMCs cocultured with Swiss 3T3 fibroblasts in the presence of stem cell factor. **(A)** Cytocentrifuged preparations of the iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts, and the BMMCs that were cocultured with Swiss 3T3 fibroblasts were stained with Alcian blue and Safranin O. **(B)** Granule protease activities of the iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts were measured. **(C)** The exocytotic response was determined by measuring the release of β-hexosaminidase. The iPSMCs that were cocultured with OP9 cells (*open bar*) or Swiss 3T3 fibroblasts (*closed bar*) were stimulated with compound 48/80. **(D)** The iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts were analyzed by transmission electron microscopy. Scale bar=2.0 μm. **(E)** Suspensions of the iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts were stained with FITC-labeled anti-FcεRI antibody for 45 min on ice. The stained cells were washed, resuspended in 1% FBS-PBS, and analyzed by flow cytometry. **(F)** Scheme of 2-step differentiation of mast cells from iPS cells. All data represent the means ± S.D. ($n=3$).

Enhancement of maturation in iPSMCs by Swiss 3T3 fibroblasts

A previous study reported that coculture of BMMCs with Swiss 3T3 fibroblasts in the presence of SCF facilitated morphological and functional maturation toward a CTMC-like phenotype [8]. It is possible that Swiss 3T3 fibroblasts promote the maturation of the iPSMCs (mast cells II) that are generated on OP9 cells. Therefore, we expected to generate more mature iPSMCs by coculturing with Swiss 3T3 fibroblasts. We compared the degree of mast cell maturation of the iPSMCs that were cocultured with OP9 cells or Swiss 3T3 fibroblasts. Although the majority of BMMCs were Alcian blue positive and Safranin O negative, the percentage of Safranin O-positive mast cells was gradually increased up to ~80% on day 16 after coculturing with Swiss 3T3 fibroblasts (Fig. 5A). The staining intensity for Safranin O on the iPSMCs that were cocultured with Swiss 3T3 fibroblasts was stronger than the iPSMCs (mast cells II) (Fig. 5A). Therefore, the iPSMCs that were differentiated by coculture with Swiss 3T3 fibroblasts were found to be more mature than mast cells II and designated as mast cells III (Fig. 5F). We measured the tryptase activity in the iPSMCs (mast cells II) or iPSMCs (mast cells III), and found the elevated tryptase activity in the iPSMCs (mast cells III) relative to the iPSMCs (mast cells II) (Fig. 5B). Similarly, β-hexosaminidase release by compound 48/80 in the iPSMCs (mast cells III) was markedly elevated in comparison with that in the iPSMCs (mast cells II) (Fig. 5C). Electron microscopic analysis of mast cells revealed that the iPSMCs (mast cells III) contained more large granules (Fig. 5D). We performed flow cytometric analysis to examine the surface expression of c-kit and FcεRI on the iPSMCs (mast cells III). After coculturing, iPSMCs (mast cells III) and BMMCs still expressed similar levels of c-kit (data not shown). Remarkably, the expression level of FcεRI was elevated in the iPSMCs (mast cells III) (Fig. 5E). These results showed that there were 2 steps in iPSMC maturation process.

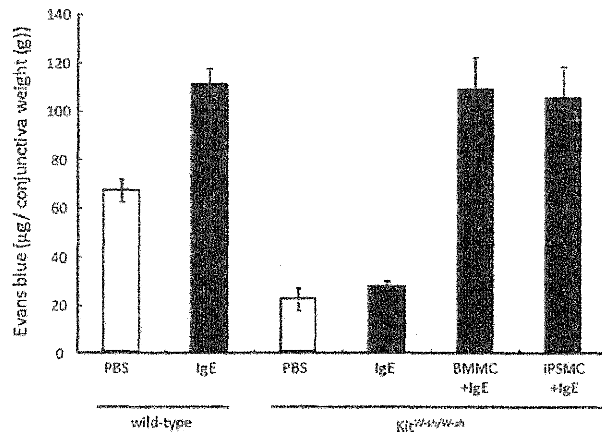

FIG. 6. IgE-dependent passive cutaneous anaphylaxis in mast cell-deficient Kit$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with iPSMCs. BMMCs or iPSMCs were injected subcutaneously into the conjunctivae of mast cell-deficient Kit$^{W\text{-}sh/W\text{-}sh}$ mice. After 6 weeks, mice were subcutaneously sensitized with anti-DNP IgE or saline, followed by induction of passive cutaneous anaphylaxis. After 24 h, mice were intravenously injected with DNP-HSA along with 2% Evan's blue dye. Thirty minutes later, conjunctivae were excised, and Evan's blue dye was extracted. Extravasation of Evan's blue dye was quantified as described in the Materials and Methods section. Results are normalized to average conjunctivae weight and are expressed as mean±S.D. ($n=3$ mice per group).

Mast cell reconstitution and induction of passive cutaneous anaphylaxis

We assessed whether the iPSMCs (mast cells I) with a C57BL/6 background could exhibit passive cutaneous anaphylaxis 6 weeks after injection of iPSMCs or BMMCs into the conjunctiva of mast-cell-deficient (Kit$^{W\text{-}sh/W\text{-}sh}$) mice [28]. Kit$^{W\text{-}sh/W\text{-}sh}$ mice reconstituted with BMMCs or iPSMCs exhibited passive cutaneous anaphylaxis reactions in the conjunctivae as measured by extravasation of Evan's Blue dye (Fig. 6). On the other hand, no passive cutaneous anaphylaxis reactions in the conjunctivae were observed for Kit$^{W\text{-}sh/W\text{-}sh}$ mice that were not reconstituted with mast cells. These results indicate that iPSMCs had the ability to respond to stimulation with IgE/antigen in vivo.

Discussion

In this study, we developed a protocol consisting of mesoderm induction (stage 1), mast cell specification (stage 2), determination of mast cells (stage 3), and maturation of mast cells (stage 4) for mast cell differentiation from iPS cells (Supplementary Figs. S1 and S6). BMMCs have been used extensively as a mast cell model. We compared the degree of maturation in BMMCs and iPSMCs. Expression level of CD81 was higher in the iPSMCs that were differentiated by both methods than in BMMCs (Supplementary Fig. S4). We also showed that IgE-mediated degranulation of iPSMCs was elevated in comparison with that in BMMCs in the presence of Lyso-PS (Fig. 4A and Supplementary Fig. S5A), demonstrating that iPSMCs were more mature than BMMCs. Therefore, both OP9 cells and EB-derived feeder cells might moderately promote the maturation of mast cells. The other possibility is that the maturation of iPSMCs might be accelerated by SCF. SCF is one of the most important cytokines for mast cell maturation [29]. BMMCs are in general generated without SCF. These are reasons why iPSMCs were more mature than BMMCs with respect to their phenotypes and functions.

Cytokines and feeder cells were required to induce the mast cell development from mouse iPS cells. As previously described, IL-3 is known to play an important role in mast cell specification. In contrast, BMMCs are generated without OP9 cells, suggesting that OP9 cells are not necessary for mast cell specification. OP9 cells might promote the maturation of mast cells, possibly by OP9 cell-derived factors, such as IL-4 [30–31], IL-6 [32–34], and nerve growth factor [34]. Therefore, cytokines and OP9 cells are all-essential and have distinct roles in the differentiation of mast cells from iPS cells.

We found a difference in FcεRI expression levels between iPSMCs (mast cell I) and iPSMCs (mast cell II) (Fig. 2). Surface expression level of FcεRI was lower in the iPSMCs (mast cells II), compared with that in the iPSMCs (mast cells I). More recently, Kovarova *et al.* reported that expression of FcεRIα mRNA was lower in human ES cell-derived mast cells that were cocultured with OP9 cells than in human ES cell-derived mast cells that were derived from the EB formation method [21]. These findings were fully consistent with our results. However, our results showed that iPSMCs (mast cells II) were more mature than iPSMCs (mast cells I). These results indicate that the expression levels of FcεRI are not completely correlated with the degree of mast cell differentiation, although the iPSMCs (mast cells III) showed a high level of FcεRI expression (Fig. 5E).

In the present study, we demonstrated that, as in the case of BMMCs, Swiss 3T3 fibroblasts could promote the maturation of iPSMCs (Fig. 5). A recent study has reported that cynomolgus monkey ES cells that are cocultured with the murine aorta-gonad-mesonephros-derived stromal cell line AGM-S1 cells are differentiated into CTMCs [20]. These results suggest that feeder cells, including AGM-S1 and Swiss3T3 fibroblasts, would promote mast cell maturation by similar mechanisms. These feeder cells might contribute to the identification of factors that play a role in mast cell maturation.

While iPSMCs (mast cells II) were almost all Safranin O positive, iPSMCs (mast cells I) included both Safranin O-positive and negative populations, suggesting that immature cells were contained in iPSMCs (mast cells I). Expression levels of CD81 protein and HDC mRNA, and protease activities, were slightly elevated in the iPSMCs (mast cells II) as compared with the iPSMCs (mast cells I). Our results suggest that the iPSMCs (mast cells II) were more mature than iPSMCs (mast cell I). The iPSMCs (mast cells III) exhibited more mature phenotypes, such as large granules and high activity of protease. Taken together, the rank order of maturity in mast cells was the following: iPSMCs (mast cells III) > iPSMCs (mast cells II) > iPSMCs (mast cells I). The precise mechanisms of mast cell maturation process remain to be clarified. Classification of mast cells by using cell surface or internal marker can contribute to clarify the maturation mechanism of mast cells. Further studies are needed to find cell surface or internal marker that can clearly distinguish iPSMCs (mast cells I and II) and iPSMCs (mast cells III).

Our data demonstrate that iPSMCs could functionally respond to IgE stimulation in vivo (Fig. 6). There was no significant difference in Evan's blue extravasation in the Kit$^{W-sh/W-sh}$ mice reconstituted with iPSMCs or BMMCs. Previously, Fukuda *et al.* demonstrated that conjunctiva reconstituted with BMMCs display a CTMC-like phenotype [28]. Therefore, passive cutaneous anaphylaxis reactions were comparable in Kit$^{W-sh/W-sh}$ mice reconstituted with iPSMCs or BMMCs.

Galli's group first reported the identification of mast cell-committed progenitors (MCPs) in adult murine bone marrow [35]. They indicated that MCPs may be directly developed from multipotential progenitors independent of the myeloid pathway. In contrast, Arinobu *et al.* demonstrated that granulocyte/monocyte progenitors gave rise to MCPs [36]. The models of the developmental process in mast cells differed between these 2 reports. To analyze cells at each differentiation step, our differentiation protocol will be useful for clarifying the developmental process of mast cells.

Because of their pluripotency and self-renewal, ES cells and iPS cells are potential cell sources for regenerative medicine and other clinical applications, such as cell therapies, drug screening, toxicology, and investigation of disease mechanisms. Notably, iPS cell-based screening approaches might support the development of personalized medicine and tailor-made treatment plans. Vancomycin, an antibiotic to which methicillin-resistant *Staphylococcus aureus* (MRSA) is sensitive, frequently induces allergic reaction [37]. In this study, the stimulation of the iPSMCs with vancomycin resulted in marked exocytosis of β-hexosaminidase, whereas no or little release of this enzyme was detected from BMMCs (Fig. 4C and Supplementary Fig. S5C). Therefore, iPSMCs would be potential cell sources for drug-allergy-screening system.

We developed a 2-step differentiation protocol of mast cells from iPS cells. In the conventional method, CTMC-like mast cells are produced from bone marrow cells after 45 days of culture. In contrast, the iPSMCs generated on OP9 cells in the present study were produced after 28 days of culture. Thus, homogeneous CTMC-like mast cells can be easily generated from iPS cells by the OP9 coculture method. On the other hand, Swiss 3T3 coculture methods have different advantage from OP9 coculture systems. The iPSMCs that were cocultured with Swiss 3T3 fibroblasts were more mature than the iPSMCs that were generated on OP9 cells. Because each of these methods has its advantages, the protocol should be chosen in accordance with the intended use.

We successfully developed a 2-step differentiation protocol for generating more mature mast cells from mouse iPS cells. The iPSMCs generated in this study exhibit many characteristics distinct from BMMCs. The iPSMCs possessed the characteristics of mature mast cells, including the heparin contents and degranulation, in response to cationic secretagogues and vancomycin. The iPSMCs serve as an excellent model for in vitro studies of CTMCs. Our results could facilitate clarification of the mechanisms that control the development of mast cells.

Acknowledgments

We thank Dr. S. Yamanaka for kindly providing the mouse iPS cell lines 38C2. We would like to thank Misae Nishijima and Mary S. Saldon (National Institute of Biomedical Innovation) for technical assistance. We thank Dr. Keizo Nishikawa (National Institute of Biomedical Innovation) for helpful discussion.

Author Disclosure Statement

The authors have no financial conflict of interest.

References

1. Metcalfe DD, D Baram and YA Mekori. (1997). Mast cells. Physiol Rev 77:1033–1079.
2. Galli SJ, S Nakae and M Tsai. (2005). Mast cells in the development of adaptive immune responses. Nat Immunol 6:135–142.
3. Kitamura Y. (1989). Heterogeneity of mast cells and phenotypic change between subpopulations. Annu Rev Immunol 7:59–76.
4. Stevens RL and KF Austen. (1989). Recent advances in the cellular and molecular biology of mast cells. Immunol Today 10:381–386.
5. Benoist C and D Mathis. (2002). Mast cells in autoimmune disease. Nature 420:875–878.
6. Bryce PJ, ML Miller, I Miyajima, M Tsai, SJ Galli and HC Oettgen. (2004). Immune sensitization in the skin is enhanced by antigen-independent effects of IgE. Immunity 20:381–392.
7. Lu LF, EF Lind, DC Gondek, KA Bennett, MW Gleeson, K Pino-Lagos, ZA Scott, AJ Coyle, JL Reed, et al. (2006). Mast cells are essential intermediaries in regulatory T-cell tolerance. Nature 442:997–1002.
8. Takano H, S Nakazawa, Y Okuno, N Shirata, S Tsuchiya, T Kainoh, S Takamatsu, K Furuta, Y Taketomi, et al. (2008). Establishment of the culture model system that reflects the process of terminal differentiation of connective tissue-type mast cells. FEBS Lett 582:1444–1450.
9. Wang Y, F Yates, O Naveiras, P Ernst and GQ Daley. (2005). Embryonic stem cell-derived hematopoietic stem cells. Proc Natl Acad Sci U S A 102:19081–19086.
10. Karlsson KR, S Cowley, FO Martinez, M Shaw, SL Minger and W James. (2008). Homogeneous monocytes and macrophages from human embryonic stem cells following coculture-free differentiation in M-CSF and IL-3. Exp Hematol 36:1167–1175.
11. Ledran MH, A Krassowska, L Armstrong, I Dimmick, J Renstrom, R Lang, S Yung, M Santibanez-Coref, E Dzierzak, et al. (2008). Efficient hematopoietic differentiation of human embryonic stem cells on stromal cells derived from hematopoietic niches. Cell Stem Cell 3:85–98.
12. Murry CE and G Keller. (2008). Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell 132:661–680.
13. Nakano T, H Kodama and T Honjo. (1994). Generation of lymphohematopoietic cells from embryonic stem cells in culture. Science 265:1098–1101.
14. Chadwick K, L Wang, L Li, P Menendez, B Murdoch, A Rouleau and M Bhatia. (2003). Cytokines and BMP-4 promote hematopoietic differentiation of human embryonic stem cells. Blood 102:906–915.
15. Schmitt TM, RF de Pooter, MA Gronski, SK Cho, PS Ohashi and JC Zuniga-Pflucker. (2004). Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. Nat Immunol 5: 410–417.
16. Vodyanik MA, JA Bork, JA Thomson and II Slukvin. (2005). Human embryonic stem cell-derived CD34+ cells: efficient

production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. Blood 105:617–626.
17. Tsai M, J Wedemeyer, S Ganiatsas, SY Tam, LI Zon and SJ Galli. (2000). In vivo immunological function of mast cells derived from embryonic stem cells: an approach for the rapid analysis of even embryonic lethal mutations in adult mice in vivo. Proc Natl Acad Sci U S A 97:9186–9190.
18. Sugiyama D, M Tanaka, K Kitajima, J Zheng, H Yen, T Murotani, A Yamatodani and T Nakano. (2008). Differential context-dependent effects of friend of GATA-1 (FOG-1) on mast-cell development and differentiation. Blood 111:1924–1932.
19. Wiles MV and G Keller. (1991). Multiple hematopoietic lineages develop from embryonic stem (ES) cells in culture. Development 111:259–267.
20. Ma F, N Kambe, D Wang, G Shinoda, H Fujino, K Umeda, A Fujisawa, L Ma, H Suemori, et al. (2008). Direct development of functionally mature tryptase/chymase double-positive connective tissue-type mast cells from primate embryonic stem cells. Stem Cells 26:706–714.
21. Kovarova M, AM Latour, KD Chason, SL Tilley and BH Koller. (2010). Human embryonic stem cells: a source of mast cells for the study of allergic and inflammatory diseases. Blood 115:3695–3703.
22. Okita K, T Ichisaka and S Yamanaka. (2007). Generation of germline-competent induced pluripotent stem cells. Nature 448:313–317.
23. Jincho Y, R Araki, Y Hoki, C Tamura, M Nakamura, S Ando, Y Kasama and M Abe. (2010). Generation of genome integration-free induced pluripotent stem cells from fibroblasts of C57BL/6 mice without c-Myc transduction. J Biol Chem 285:26384–26389.
24. Tchougounova E, G Pejler and M Abrink. (2003). The chymase, mouse mast cell protease 4, constitutes the major chymotrypsin-like activity in peritoneum and ear tissue. A role for mouse mast cell protease 4 in thrombin regulation and fibronectin turnover. J Exp Med 198:423–431.
25. Ohtsu H, A Kuramasu, S Tanaka, T Terui, N Hirasawa, M Hara, Y Makabe-Kobayashi, N Yamada, K Yanai, et al. (2002). Plasma extravasation induced by dietary supplemented histamine in histamine-free mice. Eur J Immunol 32:1698–1708.
26. Wiener Z, M Andrasfalvy, E Pallinger, P Kovacs, C Szalai, A Erdei, S Toth, A Nagy and A Falus. (2002). Bone marrow-derived mast cell differentiation is strongly reduced in histidine decarboxylase knockout, histamine-free mice. Int Immunol 14:381–387.
27. Hosono H, J Aoki, Y Nagai, K Bandoh, M Ishida, R Taguchi, H Arai and K Inoue. (2001). Phosphatidylserine-specific phospholipase A1 stimulates histamine release from rat peritoneal mast cells through production of 2-acyl-1-lysophosphatidylserine. J Biol Chem 276:29664–29670.
28. Fukuda K, M Ohbayashi, K Morohoshi, L Zhang, FT Liu and SJ Ono. (2009). Critical role of IgE-dependent mast cell activation in a murine model of allergic conjunctivitis. J Allergy Clin Immunol 124:827–833 e2.
29. Zsebo KM, J Wypych, IK McNiece, HS Lu, KA Smith, SB Karkare, RK Sachdev, VN Yuschenkoff, NC Birkett, *et al.* (1990). Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver—conditioned medium. Cell 63:195–201.
30. Yeatman CF, 2nd, SM Jacobs-Helber, P Mirmonsef, SR Gillespie, LA Bouton, HA Collins, ST Sawyer, CP Shelburne and JJ Ryan. (2000). Combined stimulation with the T helper cell type 2 cytokines interleukin (IL)-4 and IL-10 induces mouse mast cell apoptosis. J Exp Med 192:1093–1103.
31. Hamaguchi Y, Y Kanakura, J Fujita, S Takeda, T Nakano, S Tarui, T Honjo and Y Kitamura. (1987). Interleukin 4 as an essential factor for *in vitro* clonal growth of murine connective tissue-type mast cells. J Exp Med 165:268–273.
32. Hu ZQ, K Kobayashi, N Zenda and T Shimamura. (1997). Tumor necrosis factor-alpha- and interleukin-6-triggered mast cell development from mouse spleen cells. Blood 89:526–533.
33. Yuan Q, MF Gurish, DS Friend, KF Austen and JA Boyce. (1998). Generation of a novel stem cell factor-dependent mast cell progenitor. J Immunol 161:5143–5146.
34. Matsuda H, Y Kannan, H Ushio, Y Kiso, T Kanemoto, H Suzuki and Y Kitamura. (1991). Nerve growth factor induces development of connective tissue-type mast cells *in vitro* from murine bone marrow cells. J Exp Med 174:7–14.
35. Chen CC, MA Grimbaldeston, M Tsai, IL Weissman and SJ Galli. (2005). Identification of mast cell progenitors in adult mice. Proc Natl Acad Sci U S A 102:11408–11413.
36. Arinobu Y, H Iwasaki, MF Gurish, S Mizuno, H Shigematsu, H Ozawa, DG Tenen, KF Austen and K Akashi. (2005). Developmental checkpoints of the basophil/mast cell lineages in adult murine hematopoiesis. Proc Natl Acad Sci U S A 102:18105–18110.
37. Horinouchi Y, K Abe, K Kubo and M Oka. (1993). Mechanisms of vancomycin-induced histamine release from rat peritoneal mast cells. Agents Actions 40:28–36.

Address correspondence to:
Dr. Kenji Kawabata
Laboratory of Stem Cell Regulation
National Institute of Biomedical Innovation
Saito-Asagi 7-6-8
Ibaraki, Osaka 567-0085
Japan

E-mail: kawabata@nibio.go.jp

Received for publication June 18, 2012
Accepted after revision October 8, 2012
Prepublished on Liebert Instant Online October 9, 2012

 Biol. Pharm. Bull. 37(1) 81–86 (2014)

Regulation of Histamine Synthesis and Tryptase Expression through Transcription Factors, Growth Factor Independent 1 (Gfi1) and Gfi1b, in Murine Cultured Mast Cells

Azusa Taura,[a] Kazuyuki Furuta,[a] Tomoko Yamaguchi,[b] Kenji Kawabata,[b] and Satoshi Tanaka*,[a]

[a] *Department of Immunobiology, Okayama University Graduated School of Medicine, Dentistry, and Pharmaceutical Sciences; Okayama 700–8530, Japan: and* [b] *Laboratory of Stem Cell Regulation, National Institute of Biomedical Innovation; Osaka 567–0085, Japan.*
Received August 5, 2013; accepted October 11, 2013

Mast cells are involved in various immunological responses, although it remains unknown how their terminal differentiation is regulated. We previously established a culture model that mimics the process of mast cell maturation in the cutaneous tissue and found that growth factor independent 1 (Gfi1) was up-regulated whereas its paralogue Gfi1b down-regulated. Here we investigated the roles of Gfi1 and Gfi1b in the process of mast cell maturation using a murine mast cell line, MC9. Gfi1 and Gfi1b cDNAs were stably expressed in MC9 cells using the recombinant lentivirus. Histamine synthesis was significantly induced by stem cell factor (SCF) alone, whereas tryptase expression was significantly augmented in the presence of both SCF and Swiss 3T3 cells. Since exogenously expressed Gfi1 and Gfi1b might affect their expression levels in MC9 cells, we investigated the relationship between the expression profiles of Gfi1/Gfi1b proteins and maturation indices, such as histamine synthesis and tryptase expression. The comparison suggested that histamine synthesis during the co-culture period was positively regulated by Gfi1b while augmented expression of tryptase was abolished by one-sided expression of Gfi1/Gfi1b. Our findings indicated the involvement of Gfi1 and Gfi1b in the process of murine mast cell maturation.

Key words mast cell; growth factor independent 1; growth factor independent 1b; histidine decarboxylase; tryptase

Mast cells are originated in hematopoietic stem cells in the bone marrow and distributed in nearly all vascularized tissues. Accumulating evidence suggests that mast cells are involved in a wide variety of immunological responses in addition to immediate allergy and protection from parasite infection.[1] When we try to identify the physiological roles of mast cells, it is critical to characterize the nature of local mast cells, because mast cells undergo terminal differentiation in the tissues, in which they are ultimately resident.[2] Interleukin-3 (IL-3)-dependent bone marrow-derived cultured mast cells (BMMCs) are one of the most popular murine culture models, although there are many differences between BMMCs and tissue mature mast cells. Levi-Schaffer *et al.* established a model more similar to mature mast cells distributed in the connective tissues, in which BMMCs were co-cultured with Swiss 3T3 fibroblasts.[3] We modified this method to establish a novel culture model, which shares many characteristics with cutaneous mast cells.[4] In our model, BMMCs were co-cultured with Swiss 3T3 cells in the presence of stem cell factor (SCF) for 16 d. During this process, cultured mast cells acquired many characteristics of mature cutaneous mast cells, such as potentials of degranulation in response to substance P and compound 48/80, and enhanced expression of granule proteases including chymase, tryptase, and carboxypeptidase A. Since these changes observed during the co-culture period reflected at least in part the process of mast cell maturation, we then investigated the gene expression profiles by microarray analysis. We focused on growth factor independent 1 (Gfi1) and its paralogue Gfi1b in this study, because Gfi1 was found in the up-regulated genes in the early phase whereas Gfi1b in the down-regulated genes during the co-culture period, indicating the involvement of these transcription factors in mast cell maturation.

Gfi1 and Gfi1b are transcription repressors, which play important roles in growth and differentiation of various hematopoietic lineages.[5] Gfi1 was found to play critical roles in development and function of hematopoietic stem cells,[6,7] B cells,[8] T cells,[9] neutrophils,[10–12] dendritic cells,[13] and macrophages.[12] On the other hand, Gfi1b was found to be essential for erythrocyte development.[14] However, it remains unknown whether Gfi1 and Gfi1b are involved in regulation of mast cell differentiation and function. In this study we investigate the roles of Gfi1 and Gfi1b using a murine interleukin (IL)-3-dependent mast cell line, MC9, which was co-cultured with Swiss 3T3 in the presence of SCF.

MATERIALS AND METHODS

Antibodies and Reagents The following materials were purchased from the sources indicated; an anti-Gfi1 antibody, anti-Gfi1b antibody and anti-Lyn antibody from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), anti-actin antibody from Millipore (Bedford, MA, U.S.A.), recombinant mouse IL-3 from R&D systems (Minneapolis, MN, U.S.A.), tryptase substrate S-2288 from Chromogenix (Mölndal, Sweden), ViraPower Lentiviral Expression System and blasticidin S from Invitrogen (Carlsbad, CA, U.S.A.), mitomycin C from Sigma (St. Louis, MO, U.S.A.), horseradish peroxidase-conjugated secondary antibodies against rabbit immunoglobulin G (IgG) and mouse IgG from Dako (Carpinteria, CA, U.S.A.). All other chemicals were commercial products of reagent grade. Recombinant mouse SCF was prepared using the baculovirus expression system as described previously.[15]

Preparation of Expression Vectors and Lentiviruses

The authors declare no conflict of interest.

*To whom correspondence should be addressed. e-mail: tanaka@pharm.okayama-u.ac.jp © 2014 The Pharmaceutical Society of Japan