

Functional expression of high-affinity receptor for immunoglobulin E on mast cells precedes that of tryptase during differentiation from human bone marrow-derived CD34 progenitors cultured in the presence of stem cell factor and interleukin-6

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Summary

Background CD34⁺ progenitor cells develop into tryptase⁺, CD117⁺ mast cells when cultured in the presence of recombinant human stem cell factor (rhSCF). However, spontaneous IgE receptor (FcεRI) expression during human mast cell development is not well examined.

Objective Here, the expression and function of FcεRI in and on human bone marrow-derived mast cells (HBMMCs) during development were investigated.

Methods and Results At 4 weeks of culture, predominant cells expressed high-affinity IgE receptor α chain (FcεRIα) on the cell surface determined by flow cytometry, but CD117 was less expressed. Immunocytochemistry with antitryptase mAb and anti-FcεRIα mAb revealed intracellular and surface expression of FcεRIα at 2 weeks of culture, but tryptase was less expressed. FcεRIα mRNA transcript preceded that of tryptase mRNA at 2 weeks of culture determined by real-time RT-PCR, and FcεRIα, FcεRIβ, FcεRIγ, and tryptase mRNA increased along with differentiation. FcεRIα cross-link on HBMMC and 4-week-old mast cells/mast cell precursors induced the release of IL-5 and granulocyte macrophage-colony stimulating factor, which was enhanced by rhSCF.

Conclusion These data indicated that HBMMC constitutively and spontaneously expressed functional FcεRI subunits at the early stage of differentiation, probably because of the differences in the ability and functional property of progenitors.

Keywords bone marrow, differentiation, FcεRI, human mast cells

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Introduction

High-affinity receptor for IgE (FcεRI) expressed on mast cells and basophils is a complex of one α chain, one β chain, and two identical disulphide-linked γ chains, and plays pivotal roles in allergic responses. IgE binds to the α subunit of the receptor, and β and γ chains are involved in the signal transduction and bear immunoreceptor tyrosine-based kinase activation motifs in their cytoplasmic tails.

Rodent FcεRI is only expressed on mast cells and basophils [1], whereas expression of human FcεRI extends to monocytes [2], eosinophils (not on cell surface) [3], platelets [4], Langerhans cells [5], polymorphonuclear neutrophils from asthmatic patients [6], and dendritic cells [7]. Transcript for FcεRI subunits and membrane-associated receptors are shown to be apparent by 1 week before granulation in IL-3-

dependent mouse bone marrow cells and human basophils [8]. However, the relationship between IgE receptor expression and mast cell differentiation in humans has not been well elucidated.

Differentiation of human mast cells has well been investigated with progenitors derived from cord blood [9], fetal liver [10], bone marrow [11–13], and peripheral blood [14]. However, high-affinity IgE receptor α chain (FcεRIα) on the cell surface was not detected on human fetal liver-derived mast cells [15], in spite of mRNA for FcεRIα being expressed. FcεRIα was expressed at lower level on mast cells derived from cord blood progenitors [16], and histamine release and increase in intracellular Ca²⁺ were induced by IgE–anti-IgE cross-link [9]. The expression of FcεRIα on cultured mast cells from either cord blood [16] or fetal liver [17] progenitors is transiently up-regulated by IL-4, and is stable in the presence of IL-4 [16]. Also, IgE is shown to enhance FcεRIα expression in both human [18] and mouse model [19]. Human peripheral blood mast cell progenitors express FcεRIα and release histamine through FcεRIα [14], but when FcεRIα is expressed is unknown.

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In the current study, we examined by flow cytometry and immunocytochemistry whether FcεRIα is expressed in and on mast cells/mast cell precursors during differentiation derived from human bone marrow precursors cultured in the presence of recombinant human stem cell factor (rhSCF) + rhIL-6. We also quantified the levels of mRNA for tryptase, FcεRIα, high-affinity IgE receptor β chain (FcεRIβ), and high-affinity IgE receptor γ chain (FcεRIγ) during the culture period. To show that FcεRI is functional, mediator release by cross-link of FcεRIα was investigated.

Materials and methods

Reagents

N,N-dimethylformamide (Aldrich Chemical Company Inc., Milwaukee, WI, USA), peroxidase-conjugated streptavidin (Biosource International, Camarillo, CA, USA), naphthol AS-MX phosphate, L-glutamine, levamisole, collagenase, AEC chromogen kit, and human serum AB (Sigma, St Louis, MO, USA), Ficoll-Paque research grade (Pharmacia Biotech AB, Uppsala, Sweden), Tween-20, Phosphate buffer powder (WAKO Pure Chemical Industries, Ltd, Osaka, Japan), ENVISION polymer, DAB tablets, and casein (DAKO, Glostrup, Denmark) were purchased. Media III, which contains insulin (10 µg/mL), transferrin (10 µg/mL), *N*-2-hydroxyethylpiperazine-*N*-2-ethane-sulphonic acid (4470 µg/mL), NaHCO₃ (2.3 mg/mL), penicillin (100 U/mL), and streptomycin (100 µg/mL), was purchased from Immuno-Biological Laboratories (Fujioka, Japan). rhSCF is a kind gift from AMGEN Inc. (Thousand Oaks, CA, USA). rhIL-6 is from Kirin Brewery Incorp (Takasaki, Japan).

Antibodies

CRA-1, useful for the detection of FcεRIα by either immunocytochemistry or flow cytometric analysis [20, 21], was purchased from Kyokuto Pharmaceutical Indust. Co. Ltd (Ibaraki, Japan). Antitryptase mAb conjugated to alkaline phosphatase (G3/AP) is a kind gift from Prof. Lawrence B. Schwartz in Virginia Commonwealth University (Richmond, VA, USA).

Phycoerythrin (PE)-conjugated mouse anti-human CD117 (YB5.B8), mouse anti-human IL-4 mAb and biotinylated mouse anti-human IL-4 mAb (PharMingen, San Diego, CA, USA), PE-conjugated mouse IgG1, fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins, MOPC 31C (irrelevant mouse IgG1 control), MOPC 141 (irrelevant mouse IgG2b control) (Sigma Chemical Co., St Louis, MO, USA), were purchased. Purified human IgE and goat anti-human IgE polyclonal antibody were purchased from Chemicon (Temecula, CA, USA).

Cell culture

Human bone marrow was collected from patients without any haematological disorders with informed consent and collected in a heparinized tube. The protocol was approved at Institutional Review Board in Gunma University. Bone marrow was incubated with 0.02% collagenase in Dulbecco's

modified Eagle's medium, and then filtered. Cell suspensions were carefully layered above Ficoll-Paque, and centrifuged at 400g for 35 min at room temperature. Cell layer at the interface was transferred into new tubes, and washed three times with phosphate-buffered saline (PBS)/ethylenediamine-tetraacetic acid. CD34⁺ cells were magnetically sorted with CD34 progenitor isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as manufacture protocol described. Sorted cells were washed, resuspended in Media III supplemented with 10% human serum AB, 2 mM L-glutamine and 50 µmol/L 2-mercaptoethanol, and seeded into plastic 48- or 96-well plates (Iwaki Glass, Tokyo, Japan) at a concentration of 5×10^4 cells/mL with a final concentration of 100 ng/mL rhSCF and 50 ng/mL rhIL-6. Cells were cultured in 5% CO₂ at 37°C in a humidified atmosphere. Half of media was exchanged once a week with the same concentration of rhSCF and rhIL-6. A small part of suspended cells were stained by modified toluidine blue stain described by Kimura et al. [22], and cells stained metachromatically were counted by haemocytometer at each time-point.

Flow cytometry

Human bone marrow-derived mast cells (HBMMC)s at 12 weeks of culture or the mixture of mast cells/mast cell precursors at 4 weeks of culture were washed with PBS/1% bovine serum albumin (BSA). Cells were then incubated with either CRA-1 (1 µg/mL) or irrelevant isotype-matched control IgG2b, MOPC141 (1 µg/mL), for 30 min at 4°C. Cells were washed three times with PBS/BSA, and incubated with FITC-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins for 30 min at 4°C. Excess antibody was washed three times with PBS/BSA, and MOPC31C (1 µg/mL) was added to block non-specific binding to rabbit anti-mouse immunoglobulins. After incubation for 15 min at 4°C, cells were incubated with PE-conjugated anti-CD117, or PE-conjugated mouse IgG1 as a control, for 30 min at 4°C without washing out MOPC31C. After washing three times with PBS/BSA, cells were resuspended in sheath fluid, and subjected to flow cytometry. Propidium iodide (PI) was added (at a final concentration of 10 µg/mL) prior to the analysis to exclude dead cells, and fluorescence signal was analysed with EPICS ELITE Flow Cytometer (Coulter, Hialeah, FL, USA). The analysis was performed with at least 5000 cells by WinMDI software (Coulter).

Histological and immunocytochemical stain

Cytospins were prepared after CD34⁺ cells were sorted, or after 2, 4, 8 or 12 weeks of culture, and subjected to either acidic toluidine blue stain or immunocytochemistry. For the single detection of FcεRIα, cytospin preparations were fixed with methanol/0.6% H₂O₂ for 30 min. After washing, they were incubated for 30 min with goat serum (1 : 100 dilution) at room temperature to block non-specific binding, subsequently they were incubated overnight with CRA-1 (1 µg/mL) at 4°C. Excess mAb was washed three times with PBS supplemented with 0.1% Tween 20 (T-PBS), then incubated with ENVISION polymer (peroxidase-labelled, dextran-conjugated anti-mouse immunoglobulins), as described in the manufacture protocol. Positive cells were stained as light

brown with DAB, and nuclei were counterstained with haematoxylin.

For the double immunostain with CRA1 and G3/AP, cytocentrifuge preparations were stained with CRA-1 and developed with ENVISION polymer as described above except for the counterstain of the nuclei. Cytospins were then incubated for 30 min at 4 °C with MOPC31C at a concentration of 1 µg/mL to block non-specific binding to anti-mouse immunoglobulins of ENVISION polymer. G3/AP was then added without washing out MOPC31C, and colour was developed light blue as described previously [23]. After the immunostain, cytospin preparations were analysed by two of the researchers who did not know the procedure. At least 200 cells were analysed at each preparation, and tryptase-expressing cells and FcεRIα-expressing cells were counted.

Real-time quantitative RT-PCR

RNA was isolated from either CD34-sorted cells, or mast cells/mast cell precursors at 2-, 4-, and 12-weeks of culture with RNeasy mini kit according to the manufacturer's instructions (Qiagen Inc., Valencia, CA, USA). Forward and reverse oligonucleotide primers were constructed with Primer Express software (Applied Biosystems, Foster City, CA, USA), and purified by Invitrogen Life Technologies (Tokyo, Japan). The primers were as follows: tryptase 5'-CAGTGGGTGCTGACCGC-3' (nucleotide position 251), and reverse 5'-GCAGTTGCACCTGAGGG-3' (position 323); FcεRIα forward 5'-GCCTTACTGTTCTTCGCTCAG-3' (position 146), and reverse 5'-CCATGGAGGGTTCAAGGAGAC-3' (position 220); FcεRIβ forward 5'-GACCAAGTGCTTTATGGCTTCCT-3' (position 557), and reverse 5'-GTGAGTGACACAGCACTACCAAGTC-3' (position 649); FcεRIγ forward 5'-TGCGAAAGGCAGCTATAACCA (position 174), and reverse 5'-GGTGCTCAGGCCCGTGTA (position 235); glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5'-CTTACCACCATGGAGAAGGC-3', and reverse 5'-GGCATGGACTGTGGTCATGAG-3'. TaqMan probes for use in real-time RT-PCR were also designed with Primer Express software, and prepared by Applied Biosystems. The probe sequences for tryptase, FcεRIα, FcεRIβ, FcεRIγ, and GAPDH were: 5'-CGGACGTCAAGGAT-3' (position 285), 5'-TGGCGTGTAGCAGTCCCTCAGAAACC-3' (position 169), 5'-TTCCACTGAAATTGTAGTGATG-3' (position 581), 5'-CTATGAGAAATCAGATGGTG-3' (position 196), and 5'-CCTGGCCAAGGTCATCCATGACAACCTT-3', respectively. The 5'-reported dye was 6-carboxyfluorescein. The 3'-quencher dye was 6-carboxy-N, N, N', N'-tetramethylrhodamine for FcεRIα and GAPDH TaqMan probes, and non-fluorescent quencher labelled with minor groove binder (Tm enhancer) for the other probes. The real-time RT-PCR method is based on the cleavage of fluorescent dye-labelled probes by the exonuclease activity of Taq-DNA polymerase during PCR and measurement of fluorescent intensity with ABI PRISM® 7700 Sequence Detection System (Applied Biosystems). With this instrumentation, measurements of fluorescence per tube per PCR cycle were made during amplification, and the point at which fluorescence exceeds a predetermined threshold (occurring within the linear region of the amplification curve) determines the cycle number for the sample. Because multiple

measurements are made during each cycle, the analytic software produces fractional cycle numbers for each sample. Using this system, we have determined mRNA levels for tryptase, FcεRIα, FcεRIβ, FcεRIγ, and GAPDH in HBMMC or precursors with the two-step RT-PCR protocol. The final PCR products were run on a 4% agarose gel to confirm that single bands of the correct size (73 bp for tryptase, 75 bp for FcεRIα, 93 bp for FcεRIβ, and 62 bp for FcεRIγ, respectively) for each primer pair were amplified. Each HBMMC or progenitor RNA sample was first reverse transcribed in 96 sample tubes per assay (100 µL per tube) in a reaction buffer containing 1 × TaqMan RT buffer; 5.5 mmol/L magnesium chloride; 500 µmol/L of each of deoxyadenosine triphosphate, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxyuridine triphosphate; 2.5 µM random hexamers; 0.4 U/µL RNase inhibitor, and 1.25 U/µL MultiScribe Reverse Transcriptase. The reverse transcription programme was 25 °C for 10 min (incubation), 48 °C for 30 min (reverse transcription), and 95 °C for 5 min (reverse transcriptase inactivation). PCR was then carried out in duplicate by mixing 25 µL of TaqMan® Universal PCR Master Mix (Applied Biosystems) consisting of 1 × TaqMan Buffer A, 5.5 mM magnesium chloride; 200 µmol/L of each of deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate; 400 µmol/L deoxyuridine triphosphate; 0.01 U/µL AmpErase UNG; and 0.025 U/µL AmpliTaq Gold DNA polymerase; 200 nmol/L of each of forward and reverse primer; 250 nmol/L of each of TaqMan probe (100 nmol/L for GAPDH probe) with 8 µL of template cDNA diluted in RNase-free water in 96 optical tubes. PCR program consisted of 50 °C for 2 min (UNG incubation), and 95 °C for 10 min (AmpliTaq Gold activation), followed by a PCR step (40 cycles each of 15 s of denaturation at 95 °C and 1 min of annealing and extension at 60 °C). RT-PCR products of RNA extracted from IMR90 human fibroblast cell line with GAPDH forward and reverse primer was used for standards of real-time RT-PCR.

Mediator release assay

The release of IL-4, IL-5, and granulocyte macrophage-colony stimulating factor (GM-CSF) from mast cells/mast cell precursors was quantified with ELISA. Briefly, either HBMMC at 12 weeks of culture (more than 95% mast cells) or mast cells/mast cell precursors at 4 weeks of culture (20–32% tryptase-positive cells) were washed three times in serum-free Media III, resuspended in serum-free Media III, and seeded into 96-well culture plates at a concentration of 5×10^5 cells/mL. The cells were sensitized overnight with or without 2 µg/mL of purified human IgE in the presence or absence of 100 ng/mL rhSCF at 37 °C. Cells were transferred into centrifuge tubes, washed with Media III, and then challenged with or without 2 µg/mL goat anti-human IgE in the presence or absence of 100 ng/mL rhSCF for 30 min at 37 °C. After challenge, cells were immediately spun down at 4 °C, and supernatants were collected. IL-4, IL-5, and GM-CSF in supernatant were measured by ELISA using Quantikine immunoassay Kit (R & D Systems Inc., Minneapolis, MN, USA), as manufacturer's protocol described.

For the quantification of histamine, HBMMCs were sensitized with IgE and challenged with anti-IgE as described

above. After the removal of supernatants, cell pellets were resuspended in serum free-Media III, and lysed. Histamine in supernatants and cell pellets were then quantified by radio-immunoassay with Histamine Kit (Immunotech S.A., Cedex, France), as manufacturer's protocol described. Percentage histamine release was calculated as follows:

$$\% \text{ histamine release} = \frac{\text{histamine in supernatants}}{\text{histamine in cell pellets} + \text{supernatants}}$$

Statistics

Some statistical analyses were made with a Student's *t*-test. Results were expressed as means plus or minus standard errors of the means unless otherwise indicated.

Results

Expression of FcεRIα and CD117 on HBMMC at 12 weeks of culture or mast cells/mast cell precursors at 4 weeks of culture in the presence of SCF+IL-6

When CD34⁺ progenitors were cultured for 4 weeks in the presence of rhSCF + rhIL-6, 22 ± 5% of cells showed metachromasia with acidic toluidine blue stain, and were stained immunocytochemically with antitryptase mAb, which was consistent with what was reported previously [24]. Based on the analysis by flow cytometry, 4-week-old cultured cells were divided into two major groups by dotplot analysis with FSC (*forward scatter, cell size*) and SSC (*side scatter, granularity*) as parameters, as shown in Fig. 1a. Cells in gate

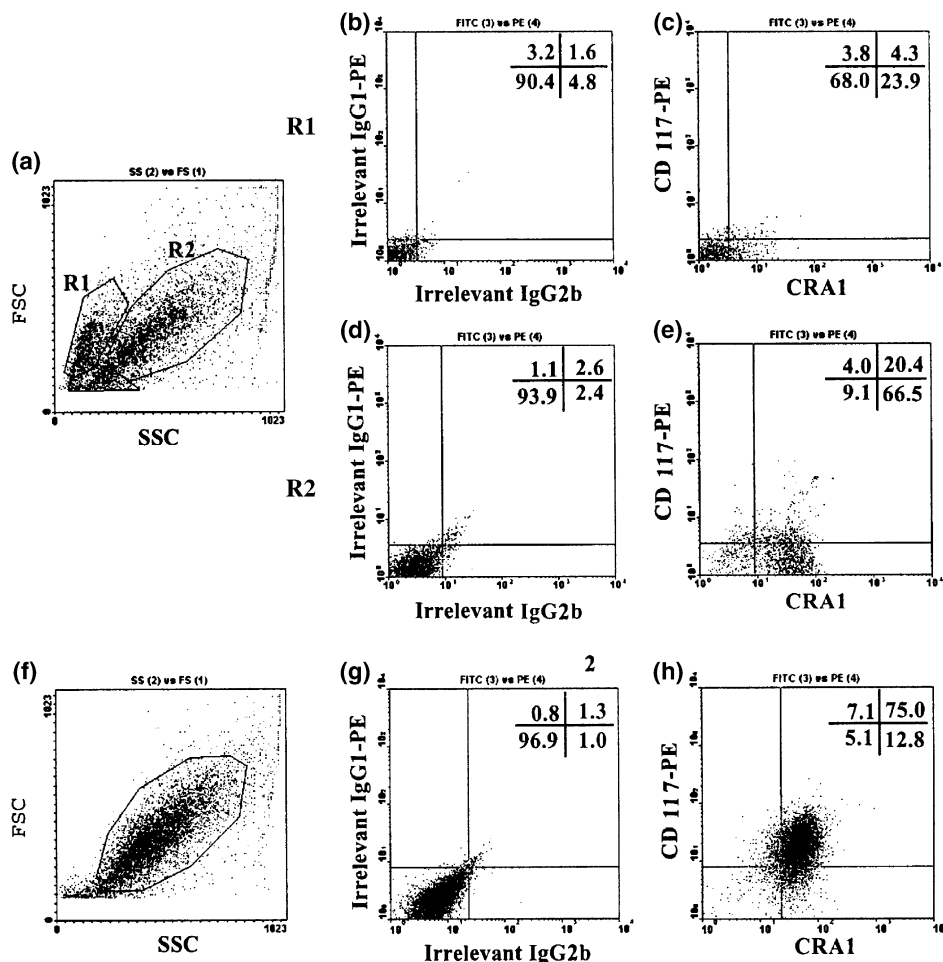


Fig. 1. Surface expression of high-affinity IgE receptor α chain (FcεRIα) and CD117 on human mast cells/mast cell precursors cultured for 4 weeks and human bone marrow-derived mast cell (HBMMC) cultured for 12 weeks in the presence of stem cell factor (SCF) + IL-6. (a) Dotplot analysis of 4-week-old precursors and mast cell/mast cell precursors cultured in the presence of SCF + IL-6 using FSC (*forward scatter, cell size*) and SSC (*side scatter, granularity*) as parameters. Two major groups of cells are gated: R1 as a progenitor group, and R2 as mast cell/ mast cell precursors. (b) Cells in R1 are sequentially labelled with irrelevant IgG2b (isotype-matched control)/fluorescein isothiocyanate (FITC)-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins followed by phycoerythrin (PE)-conjugated irrelevant IgG1 as a negative control, and adequate borderlines for quadrant analysis are established on the dotplot. (c) Cells in R1 are sequentially labelled with CRA1/FITC-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins followed by PE-conjugated anti-CD117 mAb, and the same gate as in (b) is drawn for quadrant analysis in the same scale. (d) Cells in R2 are labelled with negative control antibodies, as shown above, and adequate borderlines for quadrant analysis are established. (e) Cells in R2 are labelled with CRA1/FITC-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins and PE-conjugated anti-CD117, and the same gate as in (d) is drawn in the same scale. Predominant cells in R2 are labelled with anti-FcεRIα mAb. (f) Dotplot analysis of 12-week-old HBMMC cultured in the presence of SCF + IL-6 using FSC and SSC as parameters. (g) 12-week-old HBMMC, double labelled with irrelevant IgG2b/FITC-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins and PE-conjugated irrelevant IgG1 as a negative control, are analysed by dotplot (after the exclusion of propidium iodide (PI)-stained cells), and adequate borderlines are established. (h) 12-week-old HBMMCs are labelled with CRA1/FITC-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins and PE-conjugated anti-CD117, and the same gate as in (g) is drawn in the same scale. Predominant cells express both CD117 and FcεRIα on the cell surface. Data are representative of three independent experiments that provide similar results.

R1 are the progenitor group of cells. Cells in gate R2 show the group of immature mast cells/mast cell precursors. Dotplot analysis in R1 as a control with irrelevant IgG2b/FITC-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins and with PE-conjugated mouse IgG1 was first drawn, and borderlines for negative and positive cells for quadrant analysis were determined as shown in Fig. 1b. As shown in Fig. 1c, the ratio of CD117⁺ and FcεRIα⁺ cells on cell surface in gate R1 were 3.3% and 21.8%, respectively, as compared with the control. On the contrary, as compared with the control dotplot (Fig. 1d), 82.1% of mast cells/mast cell precursors at 4 weeks of culture were labelled with CRA1, as shown in Fig. 1e, whereas only 20.7% of cells were labelled with anti-CD117. The expression of CD117 may be masked probably because excess SCF formed CD117–SCF complex, which was consequently internalized [25]. However, the ratio of CD117⁺ cells was similar to tryptase-positive cells. The levels of IL-4 in culture media was less than 2 pg/mL during the culture, which is the lower limit of chemiluminescent enzyme immunoassay (CLEIA), suggesting that IL-4 was neither present in serum nor produced from the cultured cells including other than mast cells, if any.

At 12 weeks of culture, 98 ± 2% of cells developed into tryptase-positive mast cells, and they were classified into one group by dotplot analysis, as shown in Fig. 1f. Cells labelled either with irrelevant IgG2b/FITC-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins or with PE-conjugated mouse IgG1 as a control were gated, and borderlines on dotplot for quadrant analysis were established, as shown in Fig. 1g. Cells labelled with CRA1/FITC-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins and PE-conjugated YB5.B8 were compared with the control, and predominant cells were shown to develop into CD117- and FcεRIα-expressing cells on their surface when cultured with rhSCF and rhIL-6 for 12 weeks (Fig. 1h).

Intracellular localization of FcεRIα and tryptase determined by double immunocytochemistry with CRA1 and G3/AP

When CD34⁺ cells were cultured in the presence of SCF + IL-6 for 12 weeks, predominant cells were differentiated into cells that showed metachromasia by acidic toluidine blue stain (not shown), and tryptase-positive mast cells by immunocytochemical stain using G3/AP (Fig. 2a).

To show the selective localization of FcεRIα in and on HBMMC, immunocytochemical stain with CRA1 was performed. As shown in Fig. 2b, HBMMC at 12 weeks of culture were selectively stained with CRA1, whereas macrophages, which were developed together in a small part with HBMMC (shown by arrow) were not stained, suggesting that CRA1 specifically labelled FcεRIα in and on HBMMC.

Double immunostain with CRA1 and G3/AP was also performed to see when either FcεRIα or tryptase was expressed. Interestingly, a small number of cells at 2 weeks in the presence of SCF + IL-6 were well stained with CRA1, and few number of cells were stained with G3 antitryptase mAb, as shown in Fig. 2c. Incubation of cytospin preparations at 2 weeks with ENVISION polymer did not show any staining intensity, suggesting that undetectable anti-CD34 mAb used for cell sorting was left, if at all, on the cell surface during 2 weeks of culture. When cells were cultured for 4

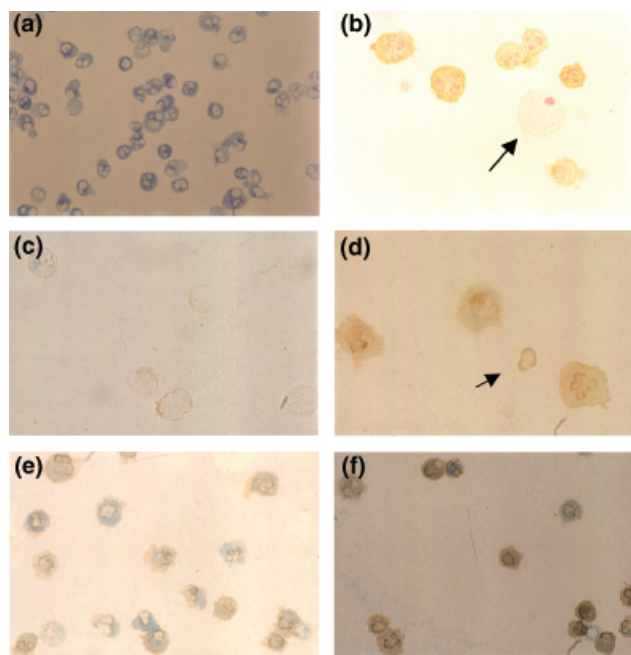


Fig. 2. Immunocytochemical stain of mast cell/mast cell precursors with G3 and/or CRA1. (a) CD34⁺ human bone marrow progenitors were cultured in the presence of recombinant human stem cell factor (rhSCF) + rhIL-6 for 12 weeks, and cytospins were prepared. Immunocytochemical stain with G3/AP is shown (× 200). (b) Immunocytochemical stain of 12-week-old human bone marrow-derived mast cell (HBMMC) with CRA1, and counterstain by haematoxylin (× 400). Note that HBMMCs were selectively stained with CRA1, but that macrophages, which were grown together with HBMMCs (shown by arrow), were not stained with CRA1. Cytospin preparations at 2 weeks (c, × 200), 4 weeks (d, × 400), 8 weeks (e, × 200), and 12 weeks of culture (f, × 200) were double labelled with both CRA1 (detected as brown red) and G3/AP (detected as light blue). Pictures are representative of three to five independent experiments that provide similar results.

weeks, a large number of cells were stained with CRA1, but only 20–30% of cells were stained with G3/AP (Fig. 2d). Staining intensity with CRA1 around the nucleus was especially strong, and some of cells were only stained around the nucleus (shown by arrow). After 8 or 12 weeks of culture in the presence of SCF + IL-6, predominant cells were stained with both CRA1 and G3, as shown in Figs 2e and f, respectively.

Time course of the number of FcεRIα- and tryptase-expressing cells during culture

The ratio of cells which express either FcεRIα and/or tryptase determined by immunocytochemistry during differentiation is shown in Fig. 3. 24 ± 6% of cells were positive for FcεRIα, and 4 ± 3% of cells were positive for tryptase at 2 weeks of culture. The percentage of positive cells increased to 23 ± 7% for tryptase, and 81 ± 10% for FcεRIα at 4 weeks of culture. At 8 and 12 weeks of culture, predominant cells were positive for both FcεRIα and tryptase.

Quantitative analysis of mRNA for tryptase, FcεRIα, FcεRIβ, and FcεRIγ during culture

The ratio of metachromatic cells stained by toluidine blue used in this study were 0% at 0 week (CD34-sorted cells), 0–6% at 2 weeks, 12–35% at 4 weeks, and 98–99% at 12 weeks.

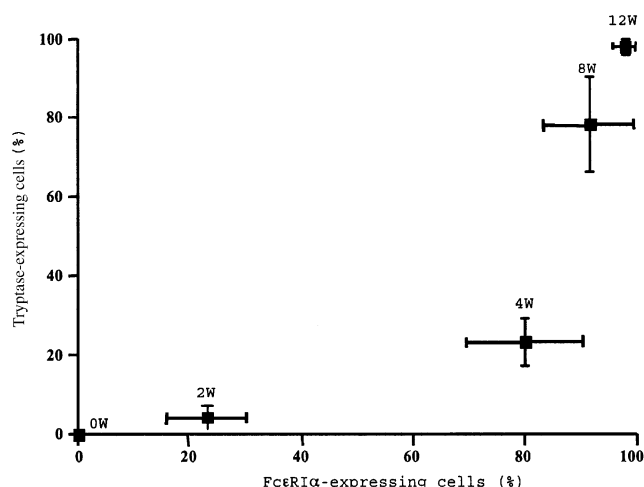


Fig. 3. Time course of the number of high-affinity IgE receptor α chain (Fc ϵ RI α)-expressing and tryptase-expressing cells during differentiation determined by double immunocytochemical stain with CRA1 and G3/AP. Early expression of Fc ϵ RI α prior to granule maturation was observed during bone marrow mast cell differentiation. Note that all of tryptase-positive cells at 2- or 4-week-old mast cells also expressed Fc ϵ RI α , but not all of Fc ϵ RI α ⁺ cells express tryptase, if any. Data are mean $\pm$ SEM in four independent experiments.

As shown in Figs 4a–d, respectively, % mRNA for tryptase, Fc ϵ RI α , Fc ϵ RI β , and Fc ϵ RI γ , divided by GAPDH mRNA (% value of mean mRNA levels as compared with 12-week-old HBMMC is also shown) is 31.5 ± 10.0 (0.4%), 0.8 ± 0.2 (1.7%), 2.3 ± 1.2 (1.2%), and 164.9 ± 112.7 (11.4%) at 0 week, 41.2 ± 30.8 (0.5%), 4.4 ± 2.5 (9.2%), 2.2 ± 1.0 (1.1%), and 68.6 ± 2.8 (4.8%) at 2 weeks, 631.0 ± 313.4 (8.1%), 18.0 ± 4.5 (37.6%), 14.2 ± 6.8 (7.2%), and 249.3 ± 145.0 (17.3%) at 4 weeks, and 7821.8 ± 1333.9 (100%), 47.9 ± 13.8 (100%), 197.3 ± 24.3 (100%), and 1444.1 ± 534.5 (100%) at 12 weeks. The ratio of Fc ϵ RI α mRNA to tryptase mRNA

(Fc ϵ RI α /tryptase) was 0.18 ± 0.06 at 0 week, 2.5 ± 2.3 at 2 weeks, 21.9 ± 18.4 at 4 weeks, and 32.4 ± 5.9 at 12 weeks, suggesting that the transcription of Fc ϵ RI α preceded that of tryptase and that the transcription was not parallel and proportional.

Mediator release

Percentage histamine release from 12-week-old HBMMC after sensitization with IgE and challenge with anti-IgE was increased to $48.2 \pm 5.4\%$ as compared with control ($14.5 \pm 2.5\%$ without IgE and anti-IgE mAb). IL-5 release from HBMMC without IgE cross-link in the presence or absence of rhSCF was below detectable level ($2 \text{ pg}/10^6$ mast cells). Fc ϵ RI α cross-link induced significant IL-5 release ($13.0 \pm 9.6 \text{ pg}/10^6$ mast cells). Although incubation with SCF alone did not show significant IL-5 release from HBMMC, enhanced IL-5 release ($57.4 \pm 18.0 \text{ pg}/10^6$ mast cells) was observed in synergy with Fc ϵ RI α cross-link, as shown in Fig. 5a. Mast cell precursors at 4 weeks of culture also released IL-5 with Fc ϵ RI α cross-link ($4.0 \pm 0.9 \text{ pg}/10^6$ mast cells) and its release was increased synergistically with rhSCF and Fc ϵ RI α cross-link ($14.0 \pm 2.0 \text{ pg}/10^6$ mast cells), suggesting that IgE receptor expressed on mast cell precursor was functional. The mean value of IL-5 release was lower than that from 12-week-old HBMMC, probably because the number of tryptase-positive cells was less and immature, or IgE receptor was not fully functional. GM-CSF was also released from HBMMC by the cross-link of Fc ϵ RI α ($23.0 \pm 3.0 \text{ ng}/10^6$ mast cells), and by incubation with rhSCF ($10.0 \pm 2.0 \text{ ng}/10^6$ mast cells). GM-CSF release by the cross-link of Fc ϵ RI α was enhanced synergistically by incubation with rhSCF ($156.0 \pm 82.4 \text{ ng}/10^6$ mast cells), as shown in Fig. 5b. The value of GM-CSF released from 4-week-old mast cell precursors was $8.0 \pm 2.0 \text{ pg}/10^6$ mast cells (no stimulation),

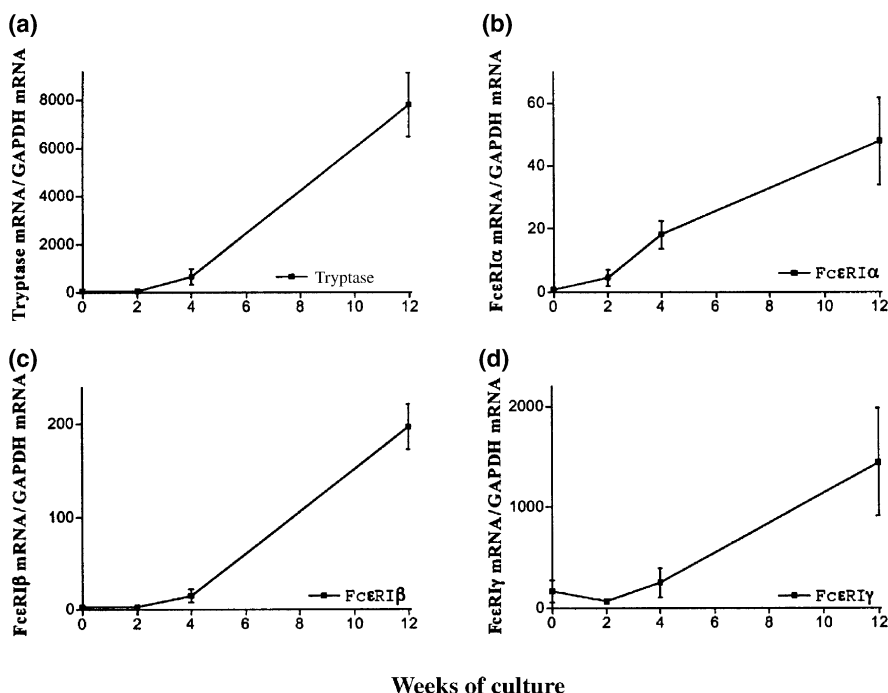


Fig. 4. Quantitative analysis of RNA for tryptase, high-affinity IgE receptor α , β , and γ chains (Fc ϵ RI α , Fc ϵ RI β , and Fc ϵ RI γ) during differentiation. CD34-sorted cells (at 0 week) and cultured bone marrow-derived mast cell/mast cell precursors were harvested at 2, 4, and 12 weeks, and RNA was isolated. Real-time RT-PCR was then performed, and % of expressed mRNA for tryptase, Fc ϵ RI α , Fc ϵ RI β , and Fc ϵ RI γ divided by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA is shown in (a), (b), (c), and (d), respectively. Results are expressed as mean $\pm$ SEM for three independent experiments.

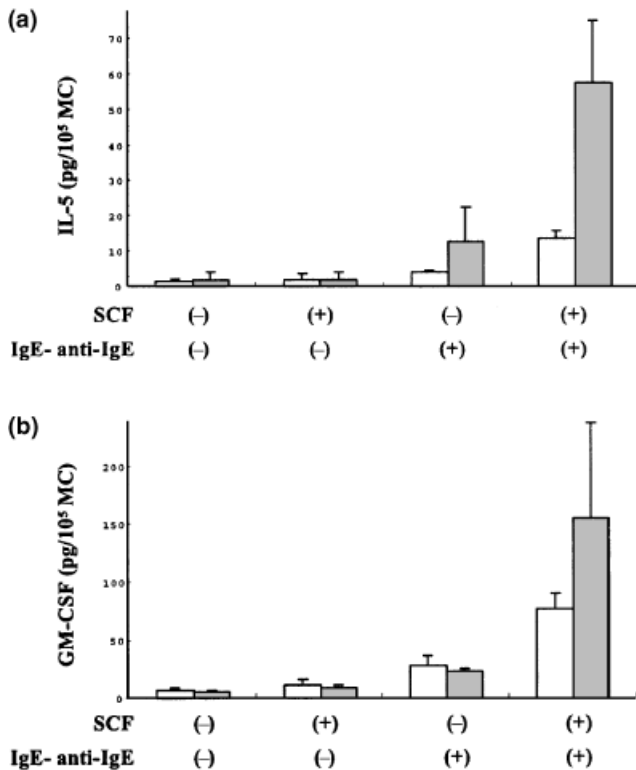


Fig. 5. IL-5 and granulocyte macrophage-colony stimulating factor (GM-CSF) release from either 12-week-old human bone marrow-derived mast cells (HBMMCs) or 4-week-old mast cell precursors. HBMMCs cultured with recombinant human stem cell factor (rhSCF) + rhIL-6 for 12 weeks (shaded area) or 4-week-old mast cells/mast cell precursors (blank area) were sensitized overnight at 37°C with IgE (2 µg/mL) followed by challenge with anti-IgE (2 µg/mL) for 30 min at 37°C in the presence or absence of 100 ng/mL rhSCF. Cells were centrifuged, and then supernatants were collected. IL-5 and GM-CSF in supernatants were quantified by ELISA. Data were mean ± SEM of three independent experiments.

12.0 ± 5.3 pg/10⁶ mast cells (with rhSCF), 29.3 ± 8.3 pg/10⁶ mast cells (FcεRIα cross-link), and 76.7 ± 13.6 pg/10⁶ mast cells (rhSCF and FcεRIα cross-link), respectively. IL-4 was not released by IgE cross-link from both 4- and 12-week-old mast cells/mast cell precursors, and was below detectable level in culture media at each time-point (data not shown).

Discussion

The activation of mast cells via FcεRIα is essential in the immunological responses, such as allergic reactions. However, few studies have been performed for the expression of FcεRIα during human mast cell differentiation, since adequate culture systems for human mast cells derived from progenitors that constitutively express FcεRIα were not easily obtained.

In murine system, transcript for FcεRI subunits and membrane-associated receptors is shown to be apparent by 1 week before granulation in IL-3-dependent bone marrow cells [8]. On the other hand, KIT + Lineage-murine bone marrow progenitors do not express FcεRI until mast cell granule formation when cultured in the presence of SCF + IL-3 [26].

In human culture systems, FcεRIα is weakly expressed, if any, on the surface of fetal liver-derived [27] or cord blood-derived [9] cultured mast cells in themselves. mRNA for FcεRIα was, however, detectable in fetal liver [27], and cord blood mast cells [9].

IgE is shown to enhance FcεRIα expression in both human cord blood mast cells [18] and mouse bone marrow mast cells [19]. FcεRIα on human cord blood mast cells enhanced by either IL-4 or IgE is functional, since IgE cross-link induced the release of histamine, prostaglandin D₂, leukotriene C₄ [18], and cytokines such as IL-5 and GM-CSF [28]. IgE also up-regulates FcεRI expressed on the surface of human basophils by regulating the rate that receptor is lost from the cell surface [29]. The mechanism of this up-regulation is shown to be the result of a combination of three factors: (1) stabilization of the receptor at the cell surface, which prevents receptor internalization and degradation [19]; (2) use of a preformed pool of receptor comprising recycled and recently synthesized receptors [30]; and (3) continued basal level of protein synthesis [1].

We have recently established the mast cell culture system from human bone marrow [24]. When CD34⁺ progenitors from human bone marrow were cultured in the presence of rhSCF + rhIL-6 for 12 weeks, more than 95% of cells became tryptase-positive, CD117⁺ mast cells (HBMMCs). What is characteristic is that HBMMCs are rich in chymase as well as tryptase in the granules, express FcεRIα on the cell surface spontaneously without any activation, even in the absence of IL-4, and that CD13 is reduced during differentiation, suggesting that HBMMC is more mature than any other progenitor-derived human cultured mast cells.

In the current study, we focused on the kinetics of the expression of FcεRIα during differentiation. As shown in Fig. 1h, predominant cells express CD117 and FcεRIα on the cell surface of 12-week culture in the presence of SCF and IL-6. Interestingly, as shown in Fig. 1e, FcεRIα was expressed on HBMMC surface at the early stage of differentiation, in spite of the fact that CD117 was less expressed (gated as R2). FcεRIα expression was less on the cell surface of the progenitor group (gated as R1) (Fig. 1c). The expression of FcεRIα in and on HBMMC detected by immunocytochemical stain, as shown in Figs 2c and d, showed abundant FcεRIα protein around the nucleus, suggesting that the major mechanism of the early expression of FcεRI is not dependent on the protection from degradation, but is due to abundant protein synthesis. The mechanism of how FcεRIα is transferred from the nucleus to the cell surface is not fully understood. Recently, it has been shown that endoplasmic reticulum quality control modulates export from the endoplasmic reticulum of newly synthesized αγ2 and αβγ2 receptors [31]. Investigations to see how intracellular FcεRIα is localized in the cells were performed by flow cytometry with perforated cells incubated with low concentration of saponin. HBMMCs were more selectively labelled with CRA1 with saponin treatment. However, 4-week-old mast cells/mast cell precursor failed to be labelled intracellularly with CRA1 because of cell lysis in the same concentration of saponin (data not shown).

The up-regulation of FcεRI expression is not entirely dependent on ligand mediation, however, it seems likely that the other mechanisms are not common between rodent and

human. In murine system, for instance, IL-4 inhibits FcεRI expression on bone marrow mast cell precursors at a concentration of 2.5 ng/mL. The inhibition was dose dependent, and resulted in decreased sensitivity to IgE-mediated stimulation, and IgE-mediated increase in FcεRI expression. On the other hand, IL-4 is required for the up-regulation of FcεRIα expression derived from either human cord blood [16] or human fetal liver [17] progenitors. IL-4 in culture media was then quantified with ELISA. It was, however, below 2 pg/mL in both raw culture media and discarded media after 1 week of culture, which was not effective for the expression of FcεRIα, suggesting that FcεRIα on HBMMC was expressed independent of either ligand or IL-4.

Real-time RT-PCR revealed early transcript for FcεRIα, and little for tryptase in CD34⁺ cells, as shown in Fig. 4. The data that FcεRIα mRNA is less in CD34-sorted cells is consistent with the observations that FcεRIα is less expressed at the precursor level, as shown in Fig. 1b. Tryptase mRNA at 0 and 2 weeks of culture was negligible, and increased as cells were differentiated into mast cells. Human mast cells are shown to arise from CD34⁺, c-kit⁺, and CD13⁺ progenitor cells derived from bone marrow and peripheral blood [32]. Moreover, mast cells in human bone marrow were shown to be negative for CD34 [33]. Mast cells are defined as tryptase-positive cells [34], but our real-time RT-PCR and immunocytochemistry data revealed that few tryptase-expressing cells are present in CD34-sorted cells, if any.

The transition of the tryptase mRNA/FcεRIα mRNA ratio was shown not to be parallel or proportional, but increased as cells are differentiated, suggesting that the expression of FcεRIα precedes that of tryptase at both protein and mRNA levels. It is reported by Iida et al. that peripheral blood-derived mast cells expressed five times higher levels of mRNA for FcεRIα than cord blood-derived mast cells [35]. The selective down-regulation of FcεRIα mRNA may occur at the precursor level.

FcεRIα on HBMMC and mast cell precursors at 4-week culture was functional since IgE-mediated IL-5 and GM-CSF release, as shown in Fig. 5, was observed. IL-5 and GM-CSF release was enhanced in synergy with rhSCF, although SCF itself did not induce such cytokines release. SCF was not a direct secretagogue for rat bone marrow-derived cultured mast cells, but it significantly enhanced IgE-mediated secretion of the granule constituents rat mast cell protease-II [36]. IL-5 expression in mature human mast cells isolated from intestinal tissue was also enhanced by incubation with SCF [37], which is consistent with our data. SCF may not be only a differentiation factor, but an enhancer for mast cell cytokine release.

It is shown that cultured mouse mast cells lacking FcεRIα are deficient in FcεRIγ mRNA [38]. FcεRIγ functions as an autonomous activation module, whereas FcεRIβ has the functional properties of an amplifier [1]. As shown in Figs 4c and d, HBMMC express mRNA for both FcεRIβ and FcεRIγ as well as FcεRIα from the early stage of differentiation, suggesting that IgE-mediated mediator is released through the full range of the FcεRI subunit.

It is well known that mast cells are derived from haematopoietic progenitors, and recruited into tissues partially via αvβ3 integrin [39], and become mature. A newly developed mAb, designated 2C7, raised against *in vitro*-

derived human mast cells appears to identify mature mast cells and a population of FcεRIα (+), chymase (−), and tryptase (−) cells in a variety of human tissues [40]. It is possible to speculate that immature mast cells localize in tissues, and are detected by 2C7. Our studies clearly show that FcεRI is expressed in and on HBMMC earlier than the maturation of mast cells independent of either ligand or IL-4 *in vitro*, probably because of the different potential functions of adult bone marrow progenitors. Further *in vivo* mechanisms remain to be determined.

In conclusion, HBMMC express functional IgE receptor subunits at the early stage of differentiation, which may be therapeutic targets for allergic disorders and other diseases with which mast cells are associated.

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