

IL-6 ENHANCES IgE-DEPENDENT HISTAMINE RELEASE FROM HUMAN PERIPHERAL BLOOD-DERIVED CULTURED MAST CELLS

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We examined whether interleukin (IL)-6 exerts the stimulatory effects on the secretion of histamine from human mast cells triggered by crosslinking of the high affinity IgE receptor (FcεRI) with IgE and anti-IgE. As target cells, we used peripheral blood-derived cultured mast cells grown with SCF, because they were superior in FcεRIα expression to cord blood-derived mast cells. Incubation with SCF+IL-6 for 1 week increased the IgE-dependent release as well as intracellular content of histamine in the cultured mast cells, as compared with the values obtained by incubation with SCF alone. The magnitude of these increases was higher than that for priming with SCF+IL-4. A striking difference was also found in the expression of FcεRIα between the two-factor combinations. The addition of IL-6 during FcεRI crosslinking with IgE/anti-IgE in the presence of SCF did not influence histamine secretion. When SCF, IL-6 and IL-4 were used together, a further increase was observed in the anti-IgE-dependent liberation of histamine from the cultured mast cells, compared with the two-factor combinations. These results suggest that IL-6 functions as a secretagogue for the inflammatory mediator of human mast cells in the presence of SCF.

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Human mast cells are distributed throughout the body including the skin, lungs, gut, and nasal mucosa. When antigen-specific IgE molecules bind to mast cells, they confer on these cells the ability to respond to environmental allergens. On exposure to allergen, mast cells are activated through crosslinking of the high affinity IgE receptor (Fcε RI), leading to the subsequent release of chemical mediators and multiple cytokines.

Because only limited numbers of mast cells can be obtained from human tissues, attempts have been made to establish human mast cell cultures using stem

cell factor (SCF), a key factor for the growth and differentiation of mast cells. However, the purity of cultured mast cells grown with SCF alone has ranged from approximately 40% to 85%.¹⁻³ Nakahata *et al.*⁴ reported the growth of large, almost 100%-pure, mast cell populations from human cord blood (CB) cells in a serum-containing liquid culture medium supplemented with SCF plus interleukin (IL)-6. Thereafter, numerous investigators have adopted this culture system to analyze the physiological and pathological characteristics of human mast cells. Affecting how the results are interpreted, however, is the possible role of IL-6.

We have reported a serum-deprived culture method that permits the selective growth of a large number of human mast cells from CD34⁺ CB cells under stimulation with SCF.⁵ The addition of IL-6 to the culture with SCF resulted in a significant decrease in progeny generation, but an increase in cell size, frequency of chymase-positive cells and intracellular histamine content of mast cells. The action of IL-6 appeared to be mediated via the IL-6 receptor/gp130.⁵ In contrast to the relative abundance of information regarding the role of IL-4 in the release of histamine from human mast cells induced by Fcε RI crosslinking, the effects of IL-6 remain unclear. Here we document, we believe for the first time, that IL-6 acts as a potent

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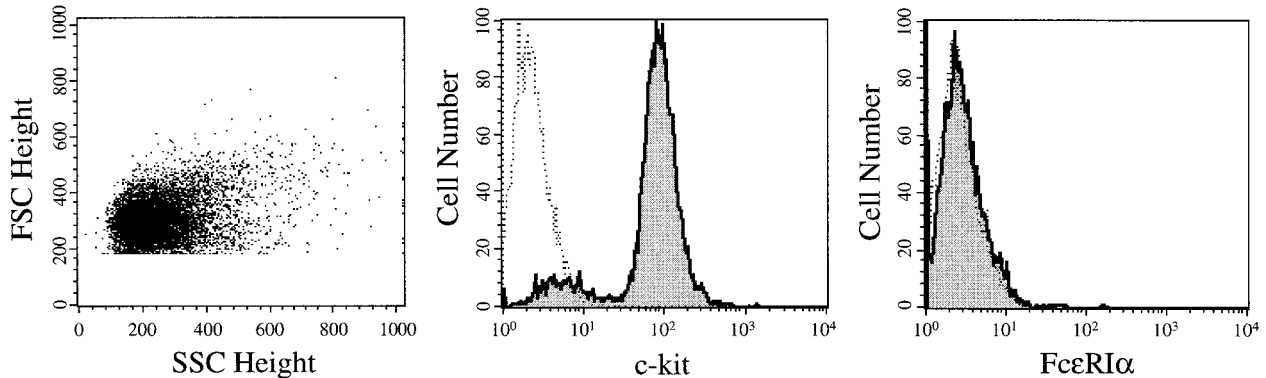
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(A) CB-derived cultured mast cells



(B) PB-derived cultured mast cells

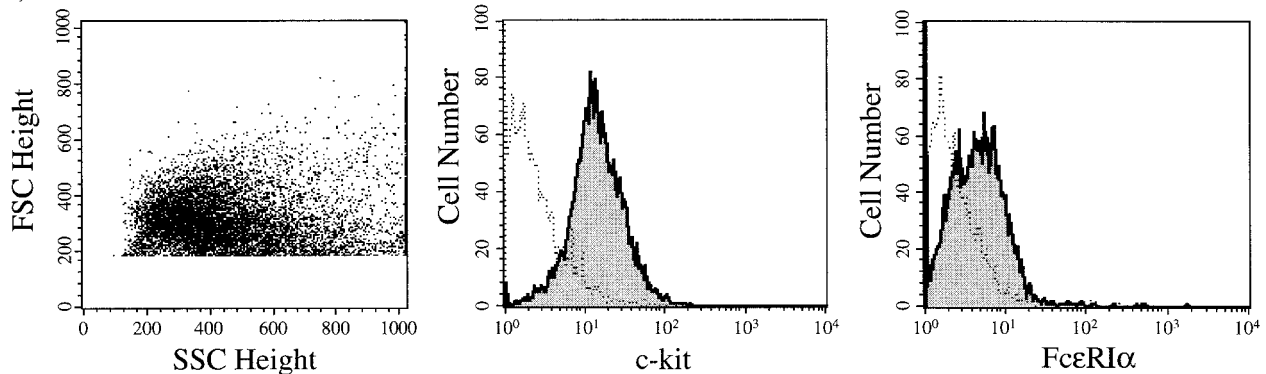


Figure 1. Expression of c-kit and FcεRIα on CB-derived and PB-derived cultured mast cells.

CD34⁺ CB cells and CD34⁺ PB cells were cultured in serum-deprived culture medium supplemented with 10 ng/ml of SCF. After 15 weeks, expression levels of c-kit and FcεRIα in the cells were analyzed by flow cytometry. (—), labeled with PE-conjugated anti-c-kit mAb or anti-FcεRIα mAb followed by FITC-conjugated GAM. (---), labeled with PE-conjugated mouse IgG1 or control mouse IgG2b followed by FITC-conjugated GAM.

stimulator for IgE-dependent histamine release from peripheral blood-derived cultured mast cells.

RESULTS

Different maturation properties between CB-derived and PB-derived cultured mast cells

When CD34⁺ CB cells and CD34⁺ PB cells mobilized with G-CSF were plated at 2×10^4 cells in a well containing serum-deprived culture medium supplemented with 10 ng/ml of SCF, $28.5 \pm 14.8 \times 10^4$ cells ($n=6$) and $5.5 \pm 1.5 \times 10^4$ cells ($n=6$) were present at 6 weeks, respectively. A large proportion (>99%) of the progeny generated by the two sources of CD34⁺ cells after 6 weeks were positive for tryptase. Using 15-week cultured mast cells, we compared maturation properties between the two groups. The results shown in Figure 1 are one representative experiment of three. The expression of c-kit was apparently higher in CB-derived than PB-derived cultured mast cells (the RFI was 30.3 ± 1.2 in CB samples, and 8.0 ± 2.5 in PB

samples, $P<0.001$). In the case of the FcεRIα chain, PB-derived mast cells displayed the antigen at appreciable levels, as compared with the CB-derived mast cells (the RFI was 1.16 ± 0.08 in CB samples, and 2.12 ± 0.13 in PB samples, $P<0.001$). According to the RT-PCR analysis, significant expression of the FcεRIα chain was observed in all four PB samples as well as KU812 cells (data not shown). On the other hand, half of four CB samples expressed FcεRIα chain mRNA at very low levels, and the other half at undetectable levels. PB-derived cultured mast cells (5×10^4) contained larger amounts of histamine than CB-derived mast cells [19.5 ± 10.0 ng in CB samples ($n=4$) vs 69.8 ± 34.9 ng in PB samples ($n=4$), $P<0.05$]. Furthermore, both tryptase and chymase enzymatic activities of 5×10^4 cultured mast cells were superior in PB samples [tryptase, 1827.0 ± 304.4 nmol in CB samples ($n=4$) vs 4264.8 ± 2752.9 nmol in PB samples ($n=4$), $P<0.05$; chymase, 3.4 ± 0.6 nmol in CB samples ($n=4$) vs 30.7 ± 17.3 nmol in PB samples ($n=4$), $P<0.05$].

To examine the ability of 15-week cultured mast cells to release histamine on stimulation with FcεRI

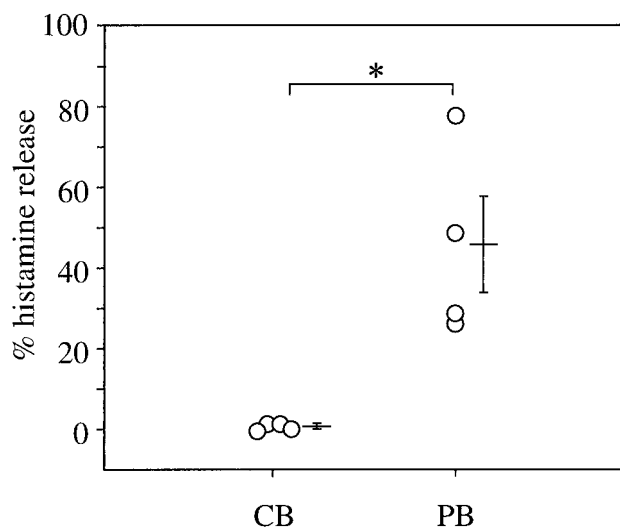


Figure 2. Histamine release induced by FcεRI crosslinking from CB-derived and PB-derived cultured mast cells.

Fifteen-week-old mast cells were replated into serum-containing culture medium supplemented with SCF (10 ng/ml), IL-4 (20 ng/ml), and IL-6 (50 ng/ml). After 1 week, the cells were treated with anti-human IgE after sensitization with IgE in the presence of FBS and the three cytokines. The results are expressed as % histamine release. *, significant difference between the two groups at $P < 0.05$.

crosslinkage, the cells were replated into FBS-containing culture medium supplemented with a combination of SCF (10 ng/ml), IL-4 (20 ng/ml), and IL-6 (50 ng/ml). After 1 week, the cells were treated with anti-human IgE after sensitization with IgE in the presence of FBS and the three cytokines. As presented in Figure 2, % histamine release was significantly higher in PB-derived mast cells than in CB-derived cells. Even in 70-week cultured mast cells derived from CD34⁺ CB cells, both the expression of the FcεRIα chain and % histamine release after the challenge with anti-IgE were still very low (data not shown). We next evaluated whether the existence of FBS influenced the anti-IgE-induced release of histamine from the cultured mast cells. In the two sources of cultured mast cells, the mediator secretion in the serum-containing culture conditions was not different from that in the serum-deprived culture conditions (data not shown). Thus, subsequent experiments of histamine release were performed, using PB-derived cultured mast cells and a serum-deprived culture system.

Effects of proinflammatory cytokines on histamine release induced by FcεRI crosslinking from PB-derived cultured mast cells in the presence of SCF

We first examined whether IL-6 stimulated the release of histamine from PB-derived mast cells. Fifteen-week cultured mast cells were replated into wells containing 10 ng/ml of SCF alone or in combi-

nation with 50 ng/ml of IL-6. After 1 week, the cells were stimulated with IgE and anti-IgE in the presence of the factor(s). As shown in Figure 3, the cultured mast cells preincubated with SCF+IL-6 spontaneously secreted histamine to a greater extent than did those preincubated with SCF alone. Histamine concentrations in the supernatant under stimulation with IgE and anti-IgE were substantially higher in the cells primed with the two-factor combination. The addition of IL-6 to the culture with SCF also increased the intracellular histamine content of the cultured mast cells. Consequently, % histamine release obtained by priming with SCF+IL-6 was similar to the level obtained by priming with SCF. In the cultured mast cells primed with SCF+IL-6, histamine amounts released after FcεRI crosslinking correlated with the mediator content ($\rho = 0.886$, $P = 0.0476$).

Several investigators^{11–16} reported that IL-4 enhances FcεRI-mediated histamine release. Accordingly, we compared the effects of IL-6 on the mediator secretion with those of IL-4. The results are presented in Figure 4. IL-4 upregulated the intracellular histamine content, but not the spontaneous release, in the cells cultured with SCF. Concentrations of histamine in the supernatant after FcεRI stimulation were significantly increased by the addition of IL-4 to the culture with SCF. However, the magnitude of these increases was inferior to that of the SCF+IL-6-primed cultured cells. When % histamine release following immunological stimulation through IgE was calculated, no significant difference was found between SCF and SCF+IL-4. It is of interest that the combination of SCF, IL-4 and IL-6 resulted in a substantial reduction of the spontaneous release of histamine, but an apparent increase in the anti-IgE-dependent release of the mediator, compared with the values obtained with SCF+IL-6. On the other hand, there was no significant difference in the intracellular histamine amount between SCF+IL-6 and SCF+IL-4+IL-6. As a result, the cells preincubated with the three-factor combination exhibited the highest percentage of histamine release after FcεRI crosslinking among those preincubated with four types of cytokine supplementations.

Using the flow cytometric analysis, we analyzed effects of IL-4 and/or IL-6 on the surface expression of FcεRIα of PB-derived mast cells grown with SCF. The results shown in Figure 5 are one representative experiment of four. Incubation with SCF+IL-4 for 1 week caused a slight but significant upregulation of FcεRIα expression, as compared with the level obtained by the incubation with SCF alone (the RFI was 2.10 ± 0.11 in SCF, and 3.87 ± 0.80 in SCF+IL-4, $P < 0.05$). On the other hand, incubation with SCF+IL-6 did not influence FcεRIα expression (the RFI, 1.83 ± 0.24). The cultured mast cells incubated with SCF+IL-4+IL-6

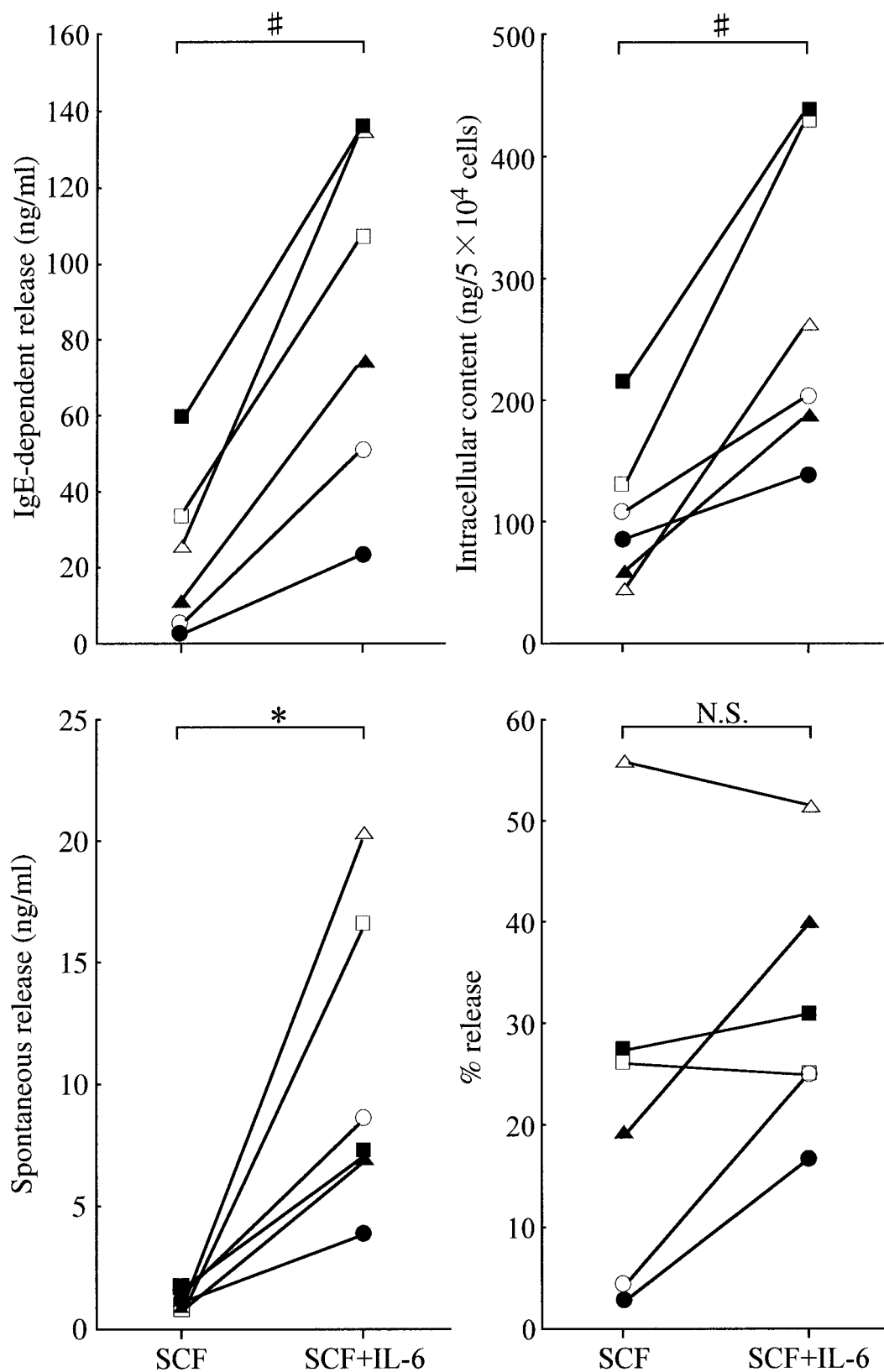


Figure 3. Effects of IL-6 on histamine release induced by Fc ϵ RI crosslinking from PB-derived cultured mast cells.

Fifteen-week PB-derived cultured mast cells were replated into wells containing 10 ng/ml of SCF with or without 50 ng/ml of IL-6. After 1 week, the cells (5×10^4) were stimulated with IgE and anti-IgE in the presence of the factor(s). Data on IgE-dependent and spontaneous histamine release, intracellular histamine content, and % histamine release of six samples are presented individually. * and #, significant difference between the two groups at $P < 0.05$ and $P < 0.01$, respectively. N.S., no significant difference between the two groups.

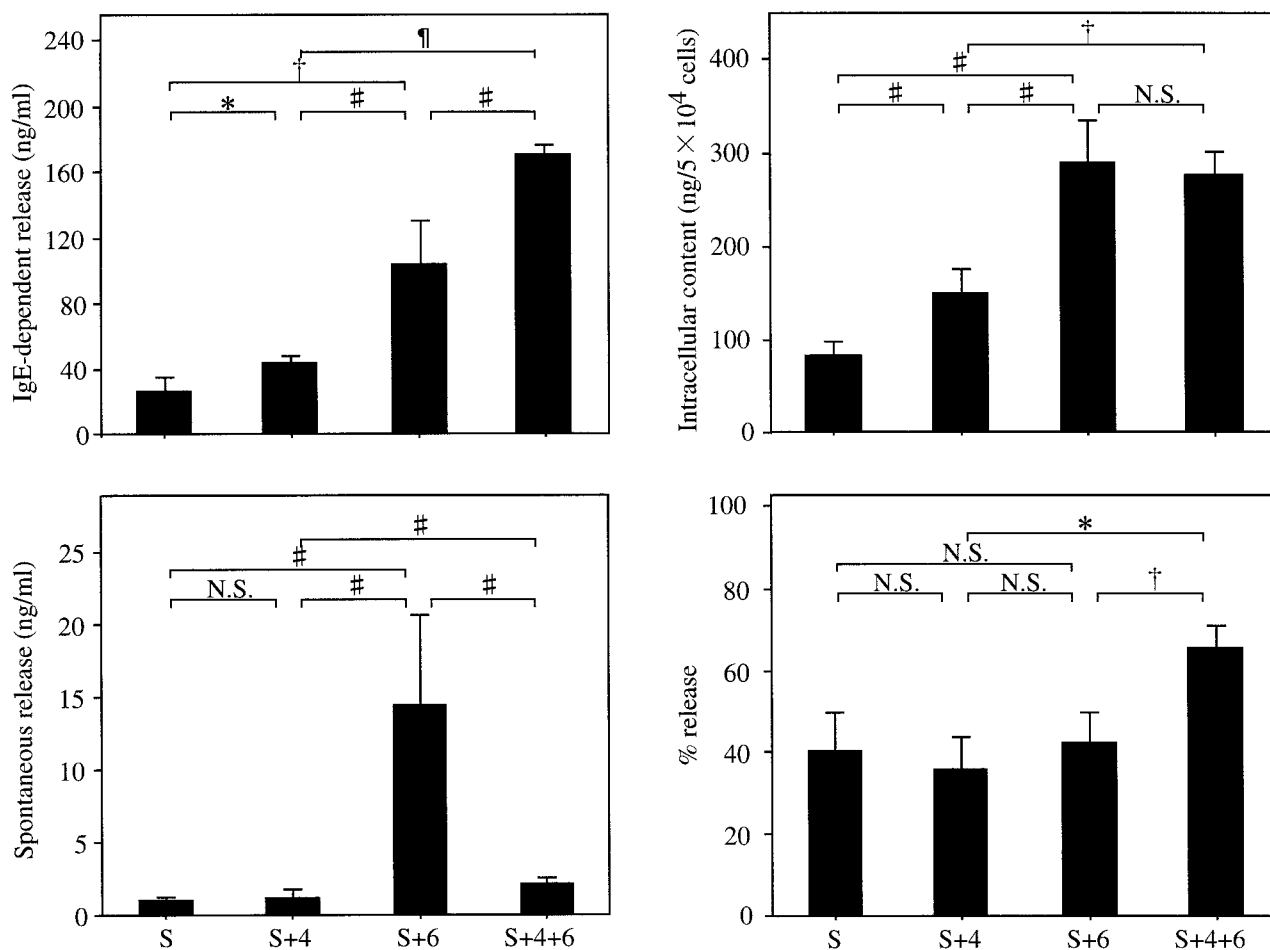


Figure 4. Effects of proinflammatory cytokines on histamine release induced by FcεRI crosslinking from PB-derived cultured mast cells.

PB-derived cultured mast cells were replated into wells containing 10 ng/ml of SCF, 20 ng/ml of IL-4 and 50 ng/ml of IL-6, alone or in combination. After 1 week, the cells (5×10^4) were stimulated with IgE and anti-IgE in the presence of the factor(s). The results shown are the mean $\pm$ SD for six experiments. Significant difference between the two groups (* $P < 0.05$, # $P < 0.01$, † $P < 0.001$, ‡ $P < 0.0001$). NS, no significant difference between the two groups.

expressed FcεRIα at levels (the RFI, 3.47 ± 1.07) similar to the values obtained by the cells incubated with SCF+IL-4.

We finally examined the effects of proinflammatory cytokines on the exocytotic process of histamine release triggered with IgE/anti-IgE. PB-derived cultured mast cells grown with SCF alone were target cells. As presented in Figure 6, the addition of IL-6 or IL-4 during the provocation (IgE for 1 h and anti-IgE for 0.5 h) in the presence of SCF did not influence the histamine release. In this culture period, there was no significant difference in the intracellular mediator content of the cells among three types of cytokine supplementations (data not shown).

DISCUSSION

In the present study, we compared the maturation properties of cultured mast cells grown from CD34⁺

CB cells and CD34⁺ PB cells in serum-deprived culture medium supplemented with SCF alone. Despite an apparent positivity for c-kit and tryptase, there was a significant difference in the tryptase and chymase enzymatic activities of 5×10^4 15-week cultured mast cells between the two groups. Additionally, PB-derived cultured mast cells contained more histamine than CB-derived mast cells. Paralleling the difference in expression of the FcεRIα chain, PB-derived mast cells, but not CB-derived mast cells, substantially released histamine in response to the crosslinkage of FcεRI with IgE and anti-IgE. Part of the data are consistent with the results obtained by Iida et al.¹⁰ using CB-derived and PB-derived cultured mast cells grown with SCF plus IL-6. In our methylcellulose cultures containing 100 ng/ml of SCF, 6-week cultured mast cells derived from CD34⁺ CB cells formed markedly greater numbers of mast cell colonies than did those derived from CD34⁺ PB cells (unpublished data). When the culture of CD34⁺ CB cells with 10 ng/ml of

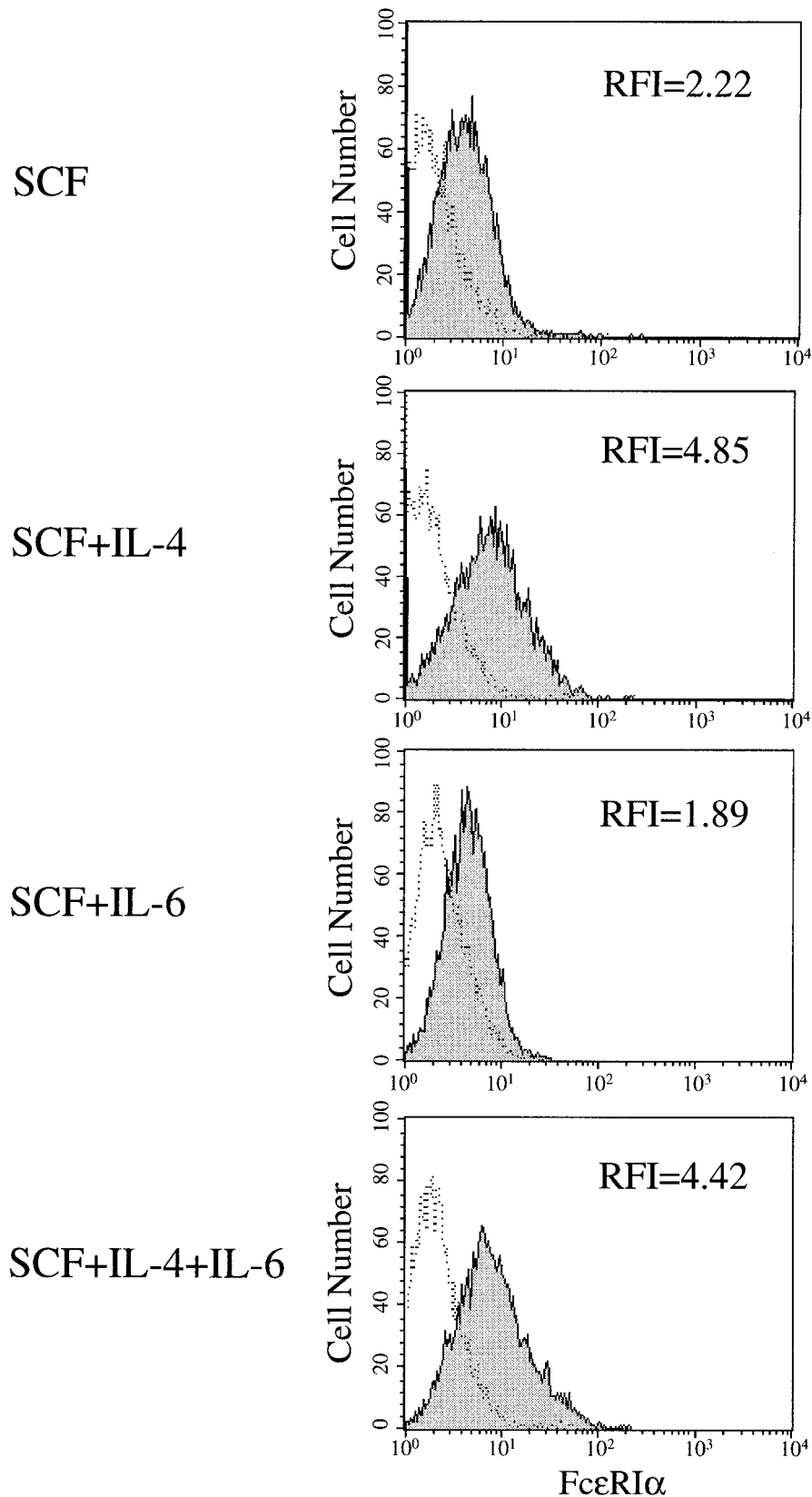


Figure 5. Effects of proinflammatory cytokines on FcεRIα expression in PB-derived cultured mast cells.

PB-derived cultured mast cells were replated into wells containing 10 ng/ml of SCF, 20 ng/ml of IL-4 and 50 ng/ml of IL-6, alone or in combination. After 1 week, FcεRIα expression on the cells was analyzed by flow cytometry. (—), labeled with anti-FcεRIα mAb followed by FITC-conjugated GAM. (---), labeled with control mouse IgG2b followed by FITC-conjugated GAM.

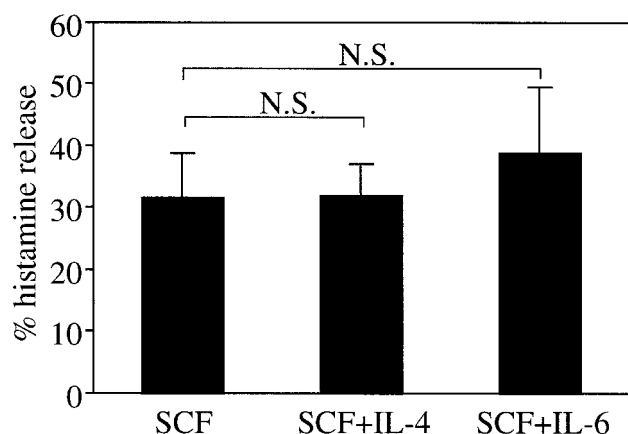


Figure 6. Effects of proinflammatory cytokines on the exocytotic process of histamine release triggered with FcεRIα crosslinking.

PB-derived cultured mast cells (5×10^4) grown with SCF alone were stimulated with IgE and anti-IgE in the presence of 10 ng/ml of SCF, 20 ng/ml of IL-4 and 50 ng/ml of IL-6, alone or in combination. % histamine release was compared. The results shown are the mean $\pm$ SD of three experiments. NS, no significant difference between the two groups.

SCF was continued for up to 50 weeks, the cell production stalled, as described previously.⁵ However, both the expression of the FcεRIα chain and % histamine release after the challenge with anti-IgE were still very low in 70-week cultured mast cells. These results suggest that a decrease in the proliferative potential of mast cells is not always associated with the achievement of histamine releasability in serum-deprived cultures. PB-derived cultured mast cells may provide a more useful tool to clarify the physiological and pathological features of human mast cells than CB-derived cells in this culture condition.

PB-derived mast cells that were incubated with SCF+IL-6 for 1 week secreted a greater level of histamine into the medium in response to IgE crosslinking than those cultured with SCF alone. Priming with SCF+IL-6 also increased the intracellular content of histamine. There was a significant relationship between these two parameters in SCF+IL-6-primed cells. However, the addition of IL-6 during provocation with FcεRI crosslinking did not influence the mediator release, when mast cells cultured with SCF were target cells. Toru *et al.*¹² reported that IL-4 upregulates FcεRI expression in CB-derived cultured mast cells, and possibly leads to an increase in the IgE-binding ability of mast cells. On the other hand, the present study showed that a 1-week culture with SCF+IL-6 did not alter FcεRIα expression. Collectively, IL-6 appears to cooperate with SCF in the IgE-dependent release of histamine from mast cells largely by increasing the synthesis of the mediator.

Consistent with the previous results,^{11–16} histamine concentrations after the challenge with anti-IgE were significantly greater in the supernatants of SCF+IL-4-primed cells, compared to preincubation with SCF alone. Furthermore, preincubation with SCF+IL-4 for 1 week increased the histamine content of the cultured mast cells. Nevertheless, the magnitude of these increases was less than that for priming with SCF+IL-6. Thus, IL-6 may act as a more powerful stimulator for IgE-dependent histamine release from mast cells than IL-4.

When compared with SCF+IL-6, the three-factor combination further increased anti-IgE-dependent liberation of histamine into the medium, but not the mediator content of cultured mast cells. On the other hand, the spontaneous release of histamine was profoundly lower for SCF+IL-4+IL-6 than SCF+IL-6. Consequently, the cultured mast cells preincubated with the three factors exhibited the highest percentage of histamine release in response to FcεRI stimulation among those preincubated with four types of cytokine supplementations. Toru *et al.*¹² and Yamaguchi *et al.*¹⁴ reported that the addition of IL-4 to the culture containing mast cells grown with SCF+IL-6 induced FcεRI expression and elicited greater levels of % histamine release upon crosslinking. Given our data, their findings may be explained by the interactive effects of IL-4 and IL-6 on anti-IgE-induced histamine release from SCF-dependent cultured mast cells. Taken together, IL-6 alone or in combination with IL-4 may contribute to the pathogenesis of allergic disorders as a secretagogue for the inflammatory mediator of human mast cells in the presence of SCF.

In contrast with our results, several investigators^{12,14,16} have reported that CB-derived cultured mast cells express functional IgE receptors in response to IL-4, using a serum-containing culture system supplemented with SCF+IL-6 or with SCF+IL-6+IL-10. Taken together with the results demonstrated by Dahl *et al.*,¹⁷ long-term stimulation with either or both factor(s) present in serum and IL-6 may give rise to the functional maturation of CB-derived mast cells.

Newly synthesized and stored mediators are released from mast cells following the binding of allergens to specific mast cell-associated IgE or non-specific stimulation. The released mediators act on tissue receptors to cause bronchoconstriction, mucosal edema and an immune response. The quantity of these substances secreted from mast cells appears to relate to the initiation or degree of allergic reactions. Therefore, the total amount of histamine as well as % mediator release should be taken into account, when evaluating the potential of mast cells to release the mediator after stimulation with FcεRI crosslinking.

MATERIALS AND METHODS

Cell preparation

Peripheral blood (PB) samples were obtained from normal donors (10.2 ± 6.6 years; range, 4 to 20 years) for allogeneic PB stem cell transplantation. They had no known diseases including allergic disorders, and were not on any medications. All donors and/or their parents provided written informed consent. Donors received G-CSF (Chugai Pharmaceutical Co., Tokyo, Japan) subcutaneously at a dose of 10 $\mu\text{g/kg}$ for 5 consecutive days, and blood sampling was performed on day 5.

CB samples were aspirated in heparinized plastic syringes from the umbilical vein at normal delivery. Fully informed consent was obtained from the mothers of all neonates before harvesting the specimens. Mononuclear cells (MNCs) were separated by density centrifugation over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ, USA), washed twice, and suspended in Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline PBS containing 1 mM EDTA-2Na and 2.5% fetal bovine serum FBS, Hyclone, Logan, UT, USA). CD34-positive cells were enriched using the Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. After treatment with 100 μl of Fc receptor blocking reagent, $0.5\text{--}1 \times 10^8$ MNCs were mixed with 100 μl of colloidal super-paramagnetic MicroBeads conjugated to a mouse monoclonal antibody (mAb) specific for CD34 (QBEND/10), and incubated for 30 min at 4°C. The magnetically labeled cells were separated with a MS^+ / RS^+ column in the magnetic field of a MACS separator (VarioMACS). More than 90% of the isolated cells were CD34-positive, as determined by FACScan flow cytometry (Becton Dickinson).

Cytokines and antibodies

Human recombinant SCF and recombinant IL-6 were generously provided by Kirin Brewery Co. Ltd. (Takasaki, Japan) and Ajinomoto Co. (Kawasaki, Japan), respectively. Human recombinant IL-4 was purchased from R & D Systems (Minneapolis, MN, USA).

For immunocytochemical staining, purified mAb for tryptase (MAB1222) was purchased from Chemicon International (Temecula, CA, USA).

For flow cytometric analyses, mAbs for CD34 (HPCA-2, fluorescein isothiocyanate, FITC) and c-kit (104D2, phycoerythrin, PE) were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, CA, USA), and mAb for human Fc ϵ RI α (CRA-1) from Kyokuto Pharmaceutical Industria (Takahagi, Japan).

Suspension cultures

Serum-deprived liquid cultures were carried out in 24-well culture plates (#3047; Becton Dickinson) using a modification of the technique described previously.^{5,6} Twenty thousand CD34⁺ cells were cultured in each well with 2 ml of α -medium supplemented with 1% bovine serum albumin (BSA), 300 $\mu\text{g/ml}$ of fully iron-saturated human transferrin (approximately 98% pure, Sigma), 16 $\mu\text{g/ml}$ of

soybean lecithin (Sigma), 9.6 $\mu\text{g/ml}$ of cholesterol (Nakalai Chemicals Ltd., Tokyo, Japan) and 10 ng/ml of SCF. The plates were incubated at 37°C in a humidified atmosphere flushed with a mixture of 5% CO_2 , 5% O_2 , and 90% N_2 . Half of the culture medium was replaced weekly with fresh medium containing the factor. The number of viable cells was determined by a trypan-blue exclusion test using a hemocytometer. We presented the actual counts of progeny in the results.

Flow cytometric analysis

For analyzing surface markers on the cultured cells, $1\text{--}2 \times 10^5$ cells were collected in plastic tubes and incubated with appropriately diluted FITC- or PE-conjugated mAb, as described previously.^{5,6} After the cells were washed twice, their surface markers were analyzed with a FACScan flow cytometer using the Lysis 2 software program. Viable cells were gated according to their forward light scatter characteristics and side scatter characteristics. The proportion of positive cells was determined by comparison to cells stained with FITC- or PE-conjugated mouse isotype-matched IgG (Dako). For the analysis of Fc ϵ RI α on the cultured mast cells, 1×10^5 cells were incubated with 20 μl of anti-Fc ϵ RI α mAb for 30 min at 4°C. Mouse isotype mAb was used as a control. The cells were washed three times and stained with FITC-conjugated goat anti-mouse immunoglobulin (GAM, Becton Dickinson) for 15 min. Data were expressed as the relative fluorescence intensity (RFI), as described previously;⁷ $\text{RFI} = \frac{\text{the mean fluorescence intensity of cultured mast cells stained with anti-Fc}\epsilon\text{RI}\alpha \text{ mAb}}{\text{the mean fluorescence intensity of the cells stained with mouse IgG}}$.

Clonal cell cultures

The mast cell colony assay was carried out in 35-mm Lux suspension culture dishes (#171099; Nunc, Naperville, IL, USA), as described previously.^{5,8} After 2 weeks, aggregates consisting of 30 or more cells were scored as mast cell colonies.

Cytochemical and immunologic staining

The cultured cells were spread on glass slides using a Cytospin II. Reactions with mouse mAb against tryptase were detected using the alkaline phosphatase-anti-alkaline phosphatase method (Dako APAAP Kit System, Dako Corp., Carpinteria, CA, USA), as described previously.⁹

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was individually isolated from the cells using Isogen (Wako). RT-PCR was performed using RNA PCR Kit (AMV) Ver.2.1 (Takara Shuzo Co., Ohtsu, Japan). The primers for amplification were designed on the basis of previous reports:¹⁰ 5'-GCCTTACTGTTCTTCGCTCCA-3' and 5'-GTCCAGCTGCCACACTTTGC-3' for Fc ϵ RI α , 5'-CTGGACTTCGAGCAAGAGAT-3' and 5'-TCGTCATACGCCTGCTTGCT-3' for β -actin. The samples were denatured at 95°C for 5 min, then subjected to 35 cycles at 95°C for 1 min, at 54°C for 1 min, and at 72°C for 1 min, with a final 10 min of extension at 72°C in a Gene Amp[™] PCR

System 9600 (Perkin-Elmer Cetus, Norwalk, CT, USA). PCR products (10 µl) were analyzed on a 1.5% agarose gel in TAE buffer (40 mM Tris, 40 mM sodium acetate, 1 mmol/l EDTA, pH 8.4) using a DNA ladder 100-bp marker.

Histamine release assay

Unless otherwise specified, the cultured mast cells grown with 10 ng/ml of SCF were replated into a well containing serum-deprived culture medium supplemented with 10 ng/ml of SCF, 20 ng/ml of IL-4 and 50 ng/ml of IL-6, alone or in combination. Serum-containing culture contained 10% FBS instead of a combination of transferrin, lecithin and cholesterol. After 1 week, a histamine release assay was performed. The cultured mast cells (5×10^4) were sensitized with 1 µg/ml of human myeloma IgE (Chemicon International) at 37°C for 1 h in the presence of the cytokine(s). After two washes with serum-deprived culture medium, the cells were incubated with 10 µg/ml of rabbit anti-human IgE specific for epsilon chains (ICN Biomedicals, Aurora, OH, USA) at 37°C for 30 min in the presence of the cytokine(s). Histamine concentrations in cell lysates obtained by the treatment of the cultured cells with 0.5% Nonidet P-40 and in supernatant were measured with a Histamine RIA Kit (Immunotech, S.A.), as described previously.⁵ All measurements were conducted in triplicate.

The histamine values obtained by FcεRI stimulation were presented as those calculated by subtracting control (spontaneous) release. The percentage of histamine secretion was calculated according to the formula: (the concentration of histamine released from stimulated cells – the concentration of histamine released from unstimulated cells) ÷ total cellular content of histamine.

Tryptase and chymase enzymatic assay

Tryptase and chymase enzymatic activities were measured according to a procedure described previously.¹¹ The cultured cells (5×10^4) grown with SCF were lysed with 0.5 ml of 1% Triton X-100 containing 0.5 M KCl, and sonicated. Aliquots (20 µl) of the samples were incubated with 0.2 mM tosyl-Gly-Pro-Lys-p-nitroanilide (Sigma), 50 mM HEPES (pH 7.6) containing 0.12 M NaCl, 100 µg/ml of a soybean trypsin inhibitor (Sigma), and 1 µg/ml of heparan sulfate proteoglycan (Sigma) in a total volume of 180 µl at 37°C for 1 h. The release of p-nitroaniline was determined spectrophotometrically. As a control, we used p-nitroaniline purchased from Sigma. The addition of phenylmethylsulfonyl fluoride (Nacalai Tesque Inc.) at 1×10^{-3} M reduced levels of tryptase enzymatic activity by 87.3%. For the assay of chymase enzymatic activity, aliquots of the samples (20 µl) were incubated with 0.2 mM succinyl-Ala-Ala-Pro-Phe-MCA (Peptide Institute, Inc., Osaka, Japan), 100 mM Tris (pH 7.8) containing 2 M NaCl and 150 µg/ml of aprotinin (Sigma) in a total volume of 180 µl at 37°C for 1 h. Reactions were stopped by the addition of 200 µl of 10% SDS (Sigma) and 2 ml of 100 mM Tris (pH 9.0). The release of MCA was measured spectrofluorometrically. As a control, we used MCA obtained from Peptide Institute. The addition of chymostatin (Sigma) at 3.3×10^{-8} M to 3.3×10^{-4} M almost completely suppressed the release of MCA.

Statistical analysis

The results are expressed as means ± SD. To determine the significance of difference between two independent groups, we used the unpaired *t*-test or Mann–Whitney U test when the data were not normally distributed. The paired *t*-test was used to assess the significance of difference in the results shown in Figures 3–6. Spearman's rank correlation test was performed to determine the relationship of the two variables. A level of significance was defined as *P* less than 0.05.

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