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IL-9 Enhances the Growth of Human Mast Cell Progenitors Under Stimulation with Stem Cell Factor[1]

Shigeyuki Matsuzawa,* Kazuo Sakashita,* Tatsuya Kinoshita,* Susumu Ito,‡ Tetsuji Yamashita,§ and Kenichi Koike[2]*†

We examined the effects of IL-9 on human mast cell development from CD34$^+$ cord blood (CB) and peripheral blood cells in serum-deprived cultures. IL-9 apparently enhanced cell production under stimulation with stem cell factor (SCF) from CD34$^+$ CB cells. A great majority of the cultured cells grown with SCF + IL-9 became positive for tryptase at 4 wk. In methylcellulose cultures of CD34$^+$ CB cells, IL-9 increased both the number and size of mast cell colonies grown with SCF. Furthermore, SCF + IL-9 caused an exclusive expansion of mast cell colony-forming cells in a 2-wk liquid culture of CD34$^+$ CB cells, at a level markedly greater than for SCF alone. Clonal cell cultures and RT-PCR analysis showed that the targets of SCF + IL-9 were the CD34$^+$CD38$^+$ CB cells rather than the CD34$^+$CD38$^-$ CB cells. IL-9 neither augmented the SCF-dependent generation of progeny nor supported the survival of 6-wk-cultured mast cells. Moreover, there was no difference in the appearance of tryptase$^+$ cells and histamine content in the cultured cells between SCF and SCF + IL-9. The addition of IL-9 increased numbers of mast cell colonies grown with SCF from CD34$^+$ peripheral blood cells in children with or without asthma. It is of interest that mast cell progenitors of asthmatic patients responded to SCF + IL-9 to a greater extent than those of normal controls. Taken together, IL-9 appears to act as a potent enhancer for the SCF-dependent growth of mast cell progenitors in humans, particularly asthmatic patients. *The Journal of Immunology,* **2003, 170: 3461–3467.**

Mast cells play an important role as primary effector cells in allergic disorders such as asthma, atopic dermatitis, and allergic rhinitis. Ag-specific IgE-mediated degranulation of mast cells leads to the subsequent release of chemical mediators and multiple cytokines. Mast cells originate from pluripotent hemopoietic cells within the bone marrow. Mast cell progenitors depart from the marrow and migrate into the connective or mucous tissues, where they differentiate into the mature form. Stem cell factor (SCF)[3] has been demonstrated to be a major growth and differentiation factor for the human mast cell lineage (1–3). We and others reported that multiple factors may participate in the regulation of the generation and function of human mast cells (4–13).

IL-9 is a cytokine produced by Th2 cells, and acts on various types of cells involved in the allergic immune response. Studies of IL-9-transgenic and -deficient mice clearly showed that IL-9 contributes to airway hyperresponsiveness, mucus hypersecretion, and mast cell infiltration (14–17), suggesting that IL-9 is a candidate gene for asthma. However, it is necessary to evaluate the biological effects of IL-9 on human samples, particularly from patients with allergic disorders, because of functional differences of several cytokines in mast cell development between mice and humans (7, 8, 11, 18–21).

We have recently reported the selective growth of a large number of mast cells from CD34$^+$ human cord blood (CB) under stimulation with SCF in serum-deprived cultures (5). In the present study, we examined the effects of IL-9 on human mast cell development from CD34$^+$ CB or peripheral blood (PB) cells, using this culture system.

Materials and Methods

Cytokines and Abs

Human recombinant IL-9 (~6.5 U/ng) was a gift from Genetics Institutes (Cambridge, MA). Human recombinant SCF, thrombopoietin (TPO), IL-3, GM-CSF, and erythropoietin were generously provided by Kirin Brewery (Takasaki, Japan). Human recombinant IL-4 was purchased from R&D Systems (Minneapolis, MN).

For immunocytochemical staining, purified mAbs for tryptase (MAB1222) and chymase (3D5) were purchased from Chemicon International (Temecula, CA). Purified mAbs for CD41 (SZ.22) and CD2 (T11) were obtained from Immunotech (Westbrook, ME); the mAbs for CD11b (2LPM19c), CD19 (HD37), and glycophorin A (GPA, JC159) were from DAKO (Carpinteria, CA). The control isotype-matched mouse mAbs were also from DAKO.

For flow cytometric analyses and cell sorting, mAbs for CD34 (HPCA-2, FITC) and CD38 (HB7, PE) were purchased from BD Immunocytometry Systems (Mountain View, CA).

For the experiments on the neutralization of IL-9 activity at the receptor level, we used anti-human IL-9R mAb (33423.111; R&D Systems). According to the manufacturer's instruction, the 50% neutralization dose of the mAb was ~2–4 μg/ml, when MO7e cells were stimulated with 2 ng/ml IL-9. Neutralizing anti-human c-*mpl* mAb (AHM21) was a gift from Kirin Brewery (22). One microgram per milliliter of the mAb almost completely inhibited the growth of FDCP-2 cells genetically engineered to constitutively express human c-*mpl* under stimulation with 1 ng/ml TPO.

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[3] Abbreviations used in this paper: SCF, stem cell factor; CB, cord blood; PB, peripheral blood; TPO, thrombopoietin; GPA, glycophorin A; MNC, mononuclear cell; APAAP, alkaline phosphatase-anti-alkaline phosphatase; CFC, colony-forming cell; GF, growth factor; POX, peroxidase.

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Cell preparation

CB samples were aspirated in plastic syringes containing EDTA-2Na from the umbilical vein at normal delivery. Fully informed consent was obtained from the mothers of all neonates before harvesting the specimens.

PB samples (10 ml) were harvested by venous puncture from a total of two males and four females with bronchial asthma aged 10.0 ± 3.2 (5–14) years after obtaining the fully informed consent of each patient and/or the parents. The asthma was defined according to the criteria of the American Thoracic Society. Five age-matched healthy subjects were used as the control group. Based on the guidelines for the diagnosis and management of asthma established by the National Heart, Lung and Blood Institute (23), four cases were classified as mild and the rest as moderate. In moderate cases, oral theophylline with or without a β-adrenergic agonist was given. All medications were withdrawn 12 h before blood sampling. The mean PB eosinophil count was $0.49 \pm 0.05 \times 10^9$/L ($0.42–0.56 \times 10^9$/L), and the total serum IgE concentration was 1041 ± 988 IU/ml (258–2435 IU/ml). All of the patients had positive Radioallergosorbent tests (RAST: grades 3–6) to several Ags including the house dust mite. Levels of specific IgE Abs were estimated by CAP RAST (Pharmacia, Uppsala, Sweden).

CB or PBMCs were separated by density centrifugation over Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), washed twice, and suspended in Ca^{2+}- and Mg^{2+}-free PBS containing 1 mM EDTA-2Na and 2.5% FBS (HyClone Laboratories, Logan, UT). 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 FcR blocking reagent, $0.5–1 \times 10^8$ mononuclear cells (MNCs) were mixed with 100 μl of colloidal superparamagnetic MicroBeads conjugated to a mouse 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, according to the flow cytometric analysis (data not shown).

Cell sorting

CB MNCs (2×10^6) were incubated with both 20 μl of FITC-conjugated anti-CD34 mAb and 20 μl of PE-conjugated anti-CD38 mAb for 30 min at 4°C, as described previously (4). As negative controls, the cells were stained with FITC- and PE-conjugated mouse IgG1 (DAKO). After two washes, $CD34^+CD38^-$ or $CD34^+CD38^+$ cells were individually sorted by a $FACStar^{Plus}$ flow cytometer (BD Biosciences, Mountain View, CA).

For single cell sorting, $CD34^+CD38^+$ or $CD34^+CD38^-$ cells were sorted into individual wells of a 96-well U-bottom tissue culture plate (no. 3077; BD Biosciences) containing 100 μl of serum-deprived culture medium supplemented with 10 ng/ml SCF, 100 U/ml IL-9, and 10 ng/ml TPO, alone or in combination, using the $FACStar^{Plus}$ flow cytometer equipped with an automatic cell deposition unit, as described previously (4, 5). Ninety-nine percent of the wells contained a single cell on the first day of culture.

Suspension cultures

Serum-deprived liquid cultures were conducted in 24-well culture plates (no. 3047; BD Biosciences) using a modification of the technique described previously (4–6, 24). $CD34^+$ cells ($1–2 \times 10^4$/well) were cultured in 2 ml of α-medium supplemented with 1% deionized BSA (Sigma-Aldrich, St. Louis, MO), 300 μg/ml fully iron-saturated human transferrin (~98% pure; Sigma-Aldrich), 16 μg/ml soybean lecithin (Sigma-Aldrich), 9.6 μg/ml cholesterol (Nakalai Tesque, Kyoto, Japan) and SCF (10 or 100 ng/ml), IL-9 (100 U/ml), TPO (10 ng/ml), and IL-4 (20 ng/ml), alone or in combination. The concentrations of TPO and IL-4 used were optimal, as described previously (4, 5). 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(s). 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.

Clonal cell cultures

The mast cell colony assay was conducted in 35-mm Lux suspension culture dishes (no. 171099; Nunc, Naperville, IL) using a modification of the technique described previously (5). The culture consisted of 500 cells/ml, α-medium, 0.9% methylcellulose (Shinetsu Chemical, Tokyo, Japan), 1% BSA, 300 μg/ml fully iron-saturated human transferrin, 16 μg/ml soybean lecithin, 9.6 μg/ml cholesterol, and 100 ng/ml SCF alone or in combination with 100 U/ml IL-9. Dishes were incubated at 37°C in a humidified atmosphere flushed with a mixture of 5% CO_2, 5% O_2, and 90% N_2. After 4 wk, aggregates consisting of 30 or more cells were scored as mast cell colonies. To confirm the in situ identification of mast cells, 30 individual colonies were lifted with a 3-μl Eppendorf micropipette, spread on glass slides using a Cytospin II (Shandon Southern, Sewickly, PA), and stained with the antitryptase mAb or mouse IgG1 using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique. The size of small mast cell colonies (<500 cells) was determined by direct cell counting in situ under an inverted microscope at a magnification of ×150. Colonies consisting of >500 cells were individually lifted with an Eppendorf micropipette and prepared as single cell suspensions. The colony size was estimated by using a counting chamber.

For the assay of GM colony-forming cells (CFCs), erythroid bursts and mixed erythroid CFCs consisting of erythroid cells and cells of lineages other than the erythroid lineage (E/Mix-CFCs), the cells were cultured with a combination of 50 ng/ml SCF, 100 U/ml IL-3, 10 ng/ml GM-CSF, and 2 U/ml erythropoietin (growth factors (GFs)). On day 14, various types of CFCs were scored in situ on an inverted microscope, according to the criteria described previously (25).

Cytochemical and immunologic staining

The cultured cells were spread on glass slides using a Cytospin II, and stained with peroxidase (POX). Reactions with mouse mAbs against tryptase, chymase, CD2, CD11b, CD19, CD41, and GPA were detected using the APAAP method (DAKO APAAP Kit System), as described previously (26).

RT-PCR analysis

RT-PCR was performed according to a modification of the procedure described previously (6). Total RNA was isolated from $CD34^+$ CB cells, $CD34^+CD38^-$ CB cells, $CD34^+CD38^+$ CB cells, 12-wk-cultured mast cells, and MO7e cells, using Isogen (Wako, Osaka, Japan). RT-PCR was performed using an RNA PCR kit (AMV) version 2.1 (Takara Shuzo, Ohtsu, Japan). The primers for amplification were synthesized on the basis of the entire coding region of IL-9Rα (GenBank accession number M84747): 5′-GCAACATCAGTTCTGGCCAC-3′ and 5′-AGAGGGCCT TGTCTCTGGG-3′ for IL-9R; 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 62°C for 2 min, and at 72°C for 1.5 min, with a final 10 min of extension at 72°C in a Gene Amp PCR System 9600 (PerkinElmer/Cetus, Norwalk, CT). PCR products (10 μl) were analyzed on a 2% 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.

Assay of histamine level

Histamine concentrations in cell lysates obtained by the treatment of the cultured mast cells with 0.5 ml of 1% Triton X-100 containing 0.5 M KCl were measured with the Histamine Enzyme Immunoassay kit (Immunotech) as described previously (5, 6). All assays were conducted in triplicate.

Statistical analysis

The results are expressed as means ± SD. To determine the significance of difference between two independent groups, we used the unpaired Student's t test or Mann-Whitney U test when the data were not normally distributed. To compare the size of mast cell colonies, the unpaired Student t test was performed on logarithms of the cell numbers of individual colonies. The paired Student t test was used to assess the significance of difference in the results shown (see Fig. 1*B* and Table II). A level of significance was defined as $p < 0.05$. One-way ANOVA, followed by post hoc contrasts with the Bonferroni limitation, was used for more than three independent groups.

Results

Effects of IL-9 on SCF-dependent mast cell development from $CD34^+$ CB cells

To examine the effects of IL-9 on human mast cell development, 1×10^4 $CD34^+$ CB cells were cultured for 4 wk in wells containing SCF at 10 ng/ml and/or IL-9 at concentrations ranging from 0.1–200 U/ml. The results are shown in Fig. 1*A*. The addition of IL-9 to the culture with SCF gave rise to a dose-dependent increase in the number of progeny, although IL-9 by itself supported no cell growth. The maximal enhancement with IL-9 was obtained at a level of at least 50 U/ml. Thus, IL-9 was used at 100 U/ml in subsequent experiments. The fold-increase in six samples

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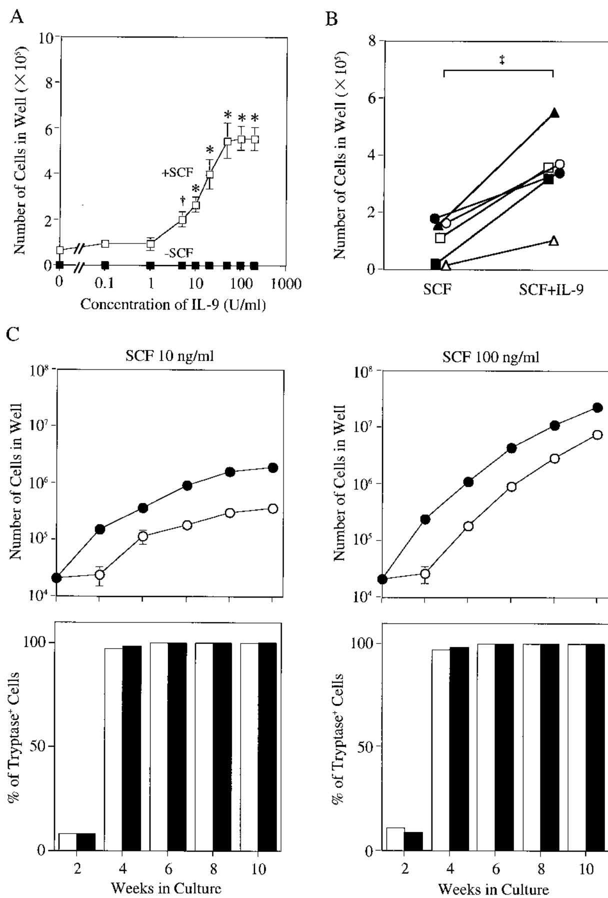

FIGURE 1. Effects of IL-9 on SCF-dependent mast cell development from CD34$^+$ CB cells. *A*, Dose response to IL-9 of total cell growth supported by SCF. CD34$^+$ CB cells (1×10^4) were incubated in wells containing SCF at 10 ng/ml and/or IL-9 at 0.1–200 U/ml. After 4 wk, the viable cells were enumerated. Significantly different from SCF alone (*, $p < 0.0001$; †, $p < 0.001$). *B*, The IL-9-induced magnifications in six CB samples are shown. Total cell numbers generated from 2×10^4 CD34$^+$ CB cells under stimulation with SCF (10 ng/ml) or SCF + IL-9 (100 U/ml) were estimated at 4 wk. Significant difference between the two groups (‡, $p < 0.01$). *C*, The number of viable cells was serially counted every other week. ○, SCF (10 or 100 ng/ml) alone; ●, SCF + IL-9 (100 U/ml). Simultaneously, the relative frequency of tryptase$^+$ cells was examined. □, SCF alone; ■, SCF + IL-9. A representative experiment is shown. Similar data were obtained in two additional experiments.

examined was 8.3 ± 1.1 (2.0–31.4), as presented in Fig. 1*B*. The time-course study showed that the total viable cell numbers in wells containing SCF + IL-9 increased in parallel with the values in wells containing SCF alone after 2 wk of culture (Fig. 1*C*). According to the immunological staining, a great majority of the cultured cells became positive for tryptase at 4 wk in the two groups. In addition, the relative numbers of chymase$^+$ cells in the cultured cells generated by stimulation with SCF (10 ng/ml) + IL-9 increased in parallel with the values in the cells grown with SCF alone: 0.4% at 2 wk, 1.2% at 4 wk, 12.4% at 6 wk, 24.4% at 8 wk, 41.6% at 10 wk in SCF + IL-9 vs 0% at 2 wk, 0.8% at 4 wk, 12.8% at 6 wk, 26.0% at 8 wk, 38.4% at 10 wk in SCF alone. Percentages of cells positive for POX or those of cells reacting with other lineage-specific mAbs (against CD2, CD19, CD11b, CD41, and GPA) were negligible. The histamine concentrations in 1×10^5 6-wk cultured cells were indistinguishable in the presence or absence of IL-9 (79.1 ± 6.3 ng in SCF at 10 ng/ml vs 84.0 ± 18.6 ng in SCF + IL-9). Furthermore, 2-wk exposure to SCF + IL-9 of 30-wk-cultured mast cells grown with 10 ng/ml SCF from CD34$^+$ CB cells did not change the mediator content, as compared with the value obtained by the exposure to SCF alone (data not shown).

From the results shown in Fig. 1*C*, we speculated that the stimulatory effects of IL-9 were restricted to the early stage of mast cell differentiation. To test this premise, we used 6-wk CB-derived mast cells grown with 10 ng/ml SCF as target cells. As presented in Fig. 2, there were no differences in numbers of progeny between SCF and SCF + IL-9. Moreover, IL-9 by itself could not support the survival of 6-wk-cultured mast cells.

To examine the action of IL-9 at the mast cell progenitor level, we performed methylcellulose cell cultures. The results are presented in Fig. 3. IL-9 increased both the number and size of colonies grown by CD34$^+$ CB cells under stimulation with SCF. More than 99% of the constituent cells of 30 pooled colonies were positive for tryptase on stimulation with SCF or SCF + IL-9. We then compared the potential of IL-9 to expand hemopoietic progenitors in the short-term liquid cultures of CD34$^+$ CB cells. After 2 wk, the cultured cells were seeded in dishes containing 100 ng/ml SCF for the assay of mast cell-CFCs, or in dishes containing GFs for the assay of GM-CFCs and E/Mix-CFCs. The results are presented in Fig. 4. SCF increased the number of mast cell-CFCs to 12-fold the input value, while the combination of SCF with IL-9 further amplified the generation of mast cell-CFCs (75-fold the input value). However, SCF + IL-9-mediated increases in GM-CFCs and E/Mix-CFCs were absent, compared with the input values.

Comparison of the ability to generate mast cells from CD34$^+$ CB cells between SCF + IL-9 and SCF + TPO

We previously reported that TPO stimulates the production of human mast cells as well as myeloid cells from CD34$^+$ CB cells in concert with SCF (4). Therefore, the ability of IL-9 to augment the SCF-dependent mast cell generation was compared with that of

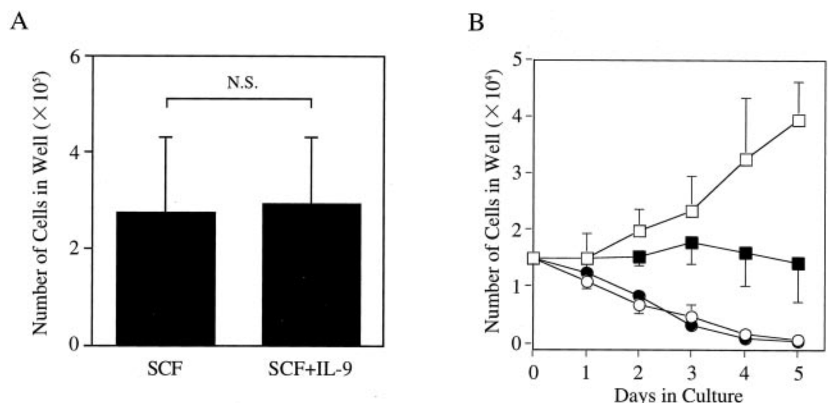

FIGURE 2. Effects of IL-9 on progeny generation and survival of cultured mast cells. *A*, Six-week-cultured mast cells (2×10^4) were incubated in wells containing SCF at 10 ng/ml with or without IL-9 at 100 U/ml. After 2 wk, the viable cells were enumerated. *B*, Six-wk-old cultured mast cells (1.5×10^4 cells) were incubated in wells containing SCF (1or 100 ng/ml) or IL-9 (100 U/ml) for 5 days. The numbers of viable cells were counted every day. No GFs (●), IL-9 (○), SCF at 1 ng/ml (■), SCF at 100 ng/ml (□). Data are the means ± SD of three independent experiments.

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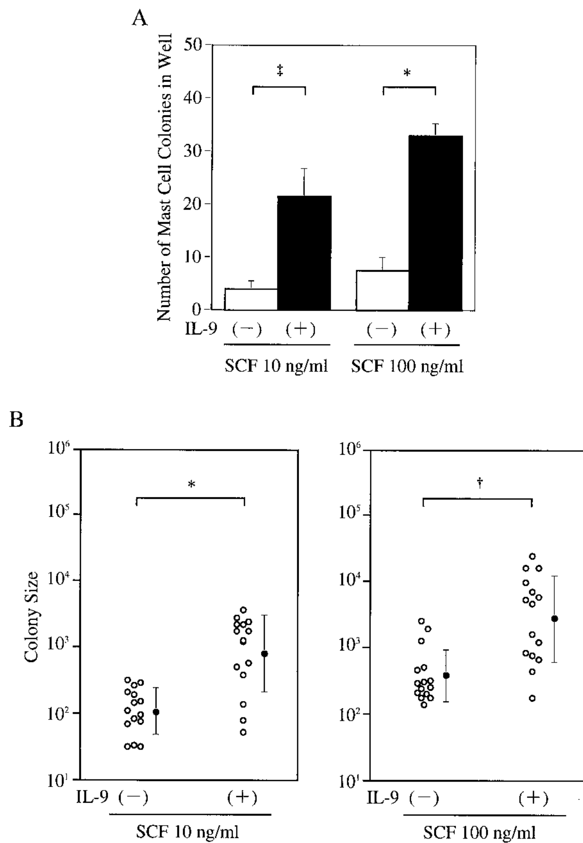

FIGURE 3. Effects of IL-9 on mast cell colony growth by CD34$^+$ CB cells under stimulation with SCF. CD34$^+$ CB cells (500) were cultured in dishes containing SCF (10 or 100 ng/ml) with or without IL-9 (100 U/ml). Numbers of colonies were enumerated at 4 wk. Data are the means ± SD of three independent experiments. *B*, Effects of IL-9 on the size of SCF-dependent mast cell colonies. Mean values and SD for 15 colonies grown with SCF or SCF + IL-9 are indicated. Significantly different from SCF alone (*, $p < 0.0001$; †, $p < 0.0005$; ‡, $p < 0.01$).

TPO. The results are presented in Fig. 5. In 4-wk-cultures of CD34$^+$ CB cells, numbers of tryptase$^+$ cells grown with SCF + IL-9 were comparable to the values obtained by SCF + TPO, while there was a significant difference in numbers of POX$^+$ cells between the two groups. When SCF, IL-9, and TPO were used together, the generation of mast cells was further increased. In contrast with SCF + IL-9, the combination of SCF and TPO increased numbers of GM-CFCs and E/Mix-CFCs as well as mast cell-CFCs in the short-term liquid cultures, compared with the input values (Fig. 4).

Next, we divided CD34$^+$ CB cells into two subsets on the basis of CD38 expression, and compared the ability to form mast cell colonies by various cytokine supplementations between CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ CB cells. As shown in Table I (Clonal cell cultures), in CD34$^+$CD38$^+$ CB cells, the capability of SCF + IL-9 to yield mast cell colonies was equivalent to that of SCF + TPO. When SCF, IL-9, and TPO were added together, the mast cell colony number was significantly more than the two-factor combinations. In contrast, in CD34$^+$CD38$^-$ CB cells, numbers of mast cell colonies grown with SCF + IL-9 were markedly lower than the values obtained with SCF + TPO. The three-factor combination synergistically increased the colony growth. A large proportion of colonies at 4 wk contained tryptase$^+$ cells, irrespective of the type of cytokine supplementation. Similar results were obtained in the single cell culture experiments (Table I, Single cell cultures).

When examined at 4 wk, neutralizing anti-IL-9R Ab at 60 μg/ml reduced the SCF + IL-9 (50 U/ml)-dependent mast cell growth by 2×10^4 CD34$^+$ CB cells to ~55%, with no change in the numbers of mast cells grown with SCF or SCF + TPO: $1.56 \pm 0.42 \times 10^5$ cells in SCF, $1.65 \pm 0.52 \times 10^5$ cells in SCF + anti-IL-9R Ab, $6.28 \pm 0.82 \times 10^5$ cells in SCF + IL-9, $3.54 \pm 0.62 \times 10^5$ cells in SCF + IL-9 + anti-IL-9R Ab, $9.16 \pm 1.56 \times 10^5$ cells in SCF + TPO, and $9.49 \pm 1.97 \times 10^5$ cells in SCF + TPO + anti-IL-9R Ab. Based on the manufacturer's instruction, 60 μg/ml anti-IL-9R Ab was estimated to decrease [^{3}H]thymidine incorporation by MO7e cells in the presence of 50 U/ml IL-9 to ~30%. The discrepancy in the inhibitory effects of anti-IL-9R Ab on the proliferation between MO7e cells and CD34$^+$ CB cells may be due in part to stimulation with IL-9 alone or in combination with SCF. Neutralizing anti-c-*mpl* Ab at 10 μg/ml decreased the progeny generation under stimulation with SCF plus TPO (5 ng/ml) to ~25%, but did not influence the SCF- or SCF + IL-9-induced cell production. Thus, IL-9 activity might be mediated by IL-9R. To further examine the differential action of IL-9 on the two subsets of CD34$^+$ CB cells, we analyzed the IL-9R expression. As presented in Fig. 6, RT-PCR analysis showed that CD34$^+$CD38$^+$ CB cells expressed IL-9R mRNA, whereas the expression of CD34$^+$CD38$^-$ cells was negligible. A weak expression of IL-9R mRNA was noted in 12-wk-cultured mast cells.

IL-4 attenuates the stimulatory effects of IL-9 on SCF-dependent mast cell production from CD34$^+$ CB cells

Because IL-4 has been demonstrated to suppress an early stage of human mast cell growth (7, 8, 11), we examined whether the stimulatory effects of IL-9 on the SCF-dependent mast cell production from CD34$^+$ CB cells was affected by IL-4. The results are presented in Fig. 7. The combination of SCF with IL-4 resulted in an increase in the number of total viable cells at 1 wk as compared with the value obtained with SCF alone, followed by a significant decrease in the progeny number. After 5 wk, total cell numbers rebounded to the input value. The addition of IL-4 to the culture

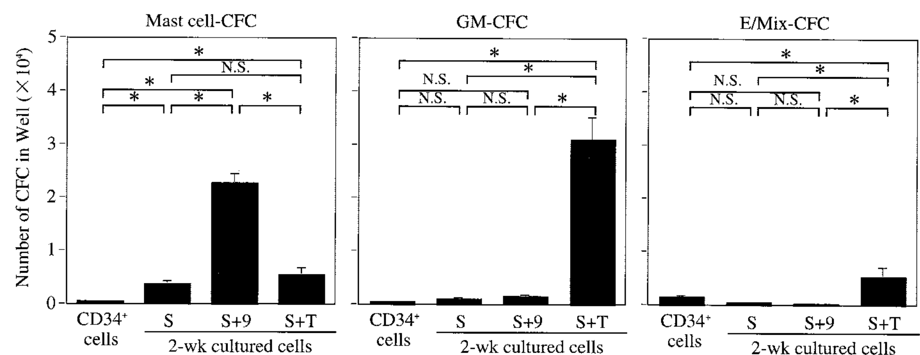

FIGURE 4. A combination of SCF with IL-9 selectively expands mast cell CFCs. CD34$^+$ CB cells (2×10^4) were incubated in wells containing 10 ng/ml SCF, 100 U/ml IL-9, or 10 ng/ml TPO, alone or in combination. After 2 wk, the number of viable cells in each well was counted. Then, 2-wk-cultured cells were seeded in methylcellulose cultures supplemented with SCF or GFs, as described in *Materials and Methods*. Values in a well are expressed as the mean ± SD. *, Significant difference between the groups compared by brackets.

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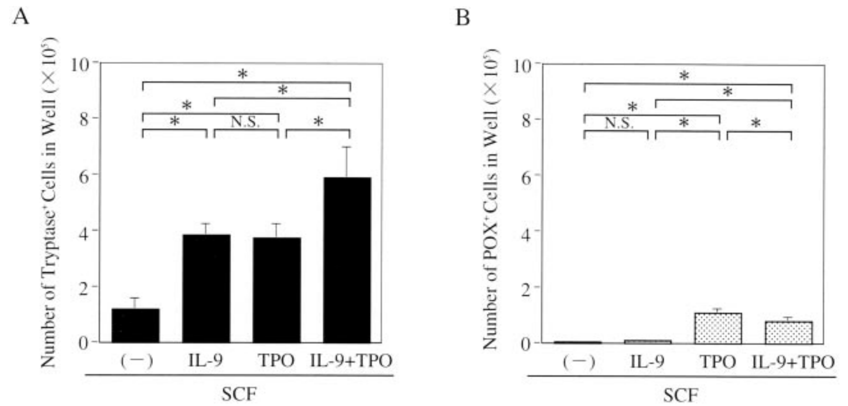

FIGURE 5. Production of mast cells and myeloid cells by CD34$^+$ CB cells under stimulation with SCF, IL-9, or TPO, alone or in combination. CD34$^+$ CB cells (2×10^4) were cultured in wells containing serum-deprived liquid culture medium supplemented with 10 ng/ml SCF, 100 U/ml IL-9, or 10 ng/ml TPO, alone or in combination. Numbers of tryptase$^+$ cells (*A*) and POX$^+$ cells (*B*) were determined at 4 wk. The actual counts of progeny are presented. The results expressed are the means of three independent experiments. *, Significant difference between the groups compared by brackets.

with SCF + IL-9 also caused a three-phase progeny generation. Consequently, the number of 6-wk-cultured mast cells was markedly smaller than the values obtained with SCF + IL-9.

Comparison of the ability to form mast cell colonies by CD34$^+$ PB cells of children with or without asthma

Finally, we examined whether SCF + IL-9 exerted stimulatory action on CD34$^+$ PB cells as well, and whether such proliferative responses were different between asthmatic children and controls, using methylcellulose cultures. The results are presented in Table II. The addition of IL-9 increased numbers of colonies grown with SCF in the two groups (asthmatic groups, $p < 0.05$; control group, $p < 0.01$). A majority of the constituent cells of pooled colonies were positive for tryptase under stimulation with SCF alone or SCF + IL-9 in children with or without asthma. It is of interest that CD34$^+$ PB cells from asthmatic patients generated significantly greater numbers of mast cell colonies in response to SCF + IL-9 than did those of normal controls ($p < 0.01$). A prominent difference was also found in the case of SCF alone ($p < 0.05$).

Discussion

In our serum-deprived liquid cultures, IL-9 apparently enhanced mast cell production under stimulation with SCF from CD34$^+$ CB cells (an ~8-fold increase of the SCF value at 4 wk). In methylcellulose cultures of CD34$^+$ CB cells, IL-9 increased both the number and size of mast cell colonies grown with SCF. Furthermore, SCF + IL-9 caused an expansion of the progenitors that were capable of becoming mast cell-CFCs in the short-term liquid culture of CD34$^+$ CB cells, whose level was markedly greater than the value obtained by SCF alone. However, IL-9 neither augmented the SCF-dependent generation of progeny nor supported the survival of 6-wk-cultured mast cells. Ochi et al. (13) reported that IL-9 augmented the SCF-dependent thymidine incorporation of 4-wk- and 9-wk-cultured mast cells derived from CB MNCs, but the IL-9-mediated amplification was marginal (1.2-fold the SCF response). The frequency of tryptase$^+$ cells and chymase$^+$ cells in the cultured cells grown with SCF + IL-9 increased in parallel with the values in the cells grown with SCF alone. There was no difference in the intracellular histamine level of the cultured mast cells between the two groups. Taken together, IL-9 may potentiate SCF-dependent human mast cell generation at the progenitor level, but does not appear to influence differentiation into the mast cell lineage. We cannot exclude the effects of IL-9 on the function of human mast cells.

Based on our previous report that TPO augments the SCF-dependent proliferation of human mast cell progenitors (4), we elucidated whether the target cells of SCF + IL-9 stimulation were identical to those of SCF + TPO stimulation. In the culture containing CD34$^+$CD38$^+$ cells, the number of mast cell colonies supported by SCF + IL-9 was equivalent to that of mast cell colonies supported by SCF + TPO. When SCF, IL-9, and TPO were added together, the mast cell colony number was significantly larger than with the two-factor combinations. In contrast, the proliferative response of CD34$^+$CD38$^-$ cells to SCF + IL-9 was markedly lower than the value obtained with SCF + TPO. RT-PCR analysis supported the differential action of IL-9 on the two subsets of CD34$^+$

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Table I. *Mast cell colony growth by CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ CB cells under stimulation with SCF, IL-9, or TPO, alone or in combination*

	Clonal Cell Cultures[a]		Single Cell Cultures[b]	
	CD34$^+$CD38$^-$	CD34$^+$CD38$^+$	CD34$^+$CD38$^-$	CD34$^+$CD38$^+$
SCF	0	11.8 ± 4.9	0	6
SCF + IL-9	1.7 ± 1.5	107.5 ± 18.7[c]	2	30
SCF + TPO	16.0 ± 4.0[c]	112.0 ± 13.1[c]	11	35
SCF + IL-9 + TPO	24.2 ± 4.0[c]	145.0 ± 12.8[c]	18	50

Brackets marked [d] in the original: CD34$^+$CD38$^-$ clonal cultures — SCF + IL-9 vs SCF + TPO; SCF + TPO vs SCF + IL-9 + TPO; SCF + IL-9 vs SCF + IL-9 + TPO. CD34$^+$CD38$^+$ clonal cultures — SCF + TPO vs SCF + IL-9 + TPO; SCF + IL-9 vs SCF + IL-9 + TPO.

[a] CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ CB cells sorted by flow cytometry were plated at 150 cells and 750 cells, respectively, in dishes each containing 10 ng/ml SCF, 100 U/ml IL-9, or 10 ng/ml TPO, alone or in combination. The data represent the mean colony number ± SD from quadruplicate dishes.

[b] Single CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ CB cells were sorted into the individual wells of a 96-well culture plate containing various combinations of cytokines. Colonies comprising 30 or more cells were scored at 4 wk. A large portion of colonies contained tryptase$^+$ cells, irrespective of the type of cytokine stimulations. The data represent the number of colonies per 96 cells plated. Two additional experiments gave similar results.

[c] Significantly different from SCF alone.

[d] Significant difference among the two- or three-factor combinations.

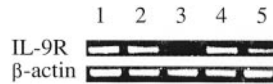

FIGURE 6. Expression of IL-9R mRNA of CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ CB cells. The IL-9R mRNA expression of CD34$^+$CD38$^-$ and CD34$^+$CD38$^+$ CB cells was examined using RT-PCR analysis. The IL-9R cDNA of the cells was amplified by PCR at 35 cycles. *Lane 1*, MO7e cells; *lane 2*, CD34$^+$ CB cells; *lane 3*, CD34$^+$CD38$^-$ CB cells; *lane 4*, CD34$^+$CD38$^+$ CB cells; *lane 5*, 12-wk-cultured mast cells grown with SCF.

cells. These results suggest that SCF + IL-9 stimulates mainly the growth of a mature subset of mast cell progenitors, whereas the target cells of SCF + TPO belong to a primitive subset as well as a mature subset of the progenitors. In addition, mature mast cell progenitors that respond to SCF + IL-9 may not always be identical to those that respond to SCF + TPO. Another difference in the two cytokine stimulations was progeny type: a great majority of cultured cells grown with SCF + IL-9 were positive for tryptase after 4 wk of culture, whereas SCF + TPO yielded significant numbers of myeloid cells as well as mast cells. Thus, IL-9 may act as a potentiator selective for the mast cell lineage in the presence of SCF.

The combination of SCF, IL-9, and TPO caused a synergistic increase in the number of mast cell colonies from CD34$^+$CD38$^-$ CB cells, compared with the values obtained by the two-factor combinations. One possible explanation is the existence of primitive mast cell progenitors requiring the three factors simultaneously at the initiation of the culture for their growth. By the addition of IL-9 on day 2 to the culture containing CD34$^+$CD38$^-$ cells with SCF + TPO, total colony formation was significantly increased. Taking into account the undetectable expression of IL-9R in CD34$^+$CD38$^-$ cells, it is likely that in the presence of SCF, some of the primitive mast cell progenitors are stimulated

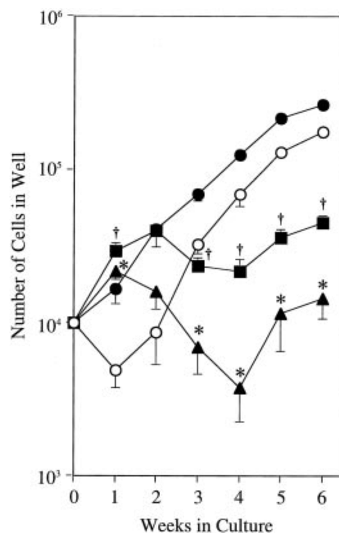

FIGURE 7. Mast cell production by CD34$^+$ CB cells under stimulation with SCF, IL-9, or IL-4, alone or in combination. CD34$^+$ CB cells (1 × 10^4) were cultured in wells containing 2 ml of serum-deprived liquid culture medium supplemented with 10 ng/ml SCF, 100 U/ml IL-9, or 20 ng/ml IL-4, alone or in combination. Numbers of viable cells were serially counted up to 6 wk, and the actual counts of progeny are presented. After 4 wk, >99% of cultured cells reacted with antitryptase mAb in 4 types of cytokine stimulations. Values are expressed as the mean ± SD of three independent experiments. ○, SCF; ●, SCF + IL-9; ▲, SCF + IL-4; ■, SCF + IL-4 + IL-9. *, Significantly different from SCF alone; †, significantly different from SCF + IL-9.

Table II. *Comparison of the ability to form mast cell colonies of CD34$^+$ PB cells from children with or without asthma*[a]

	Number of Mast Cell Colonies	
	SCF	SCF + IL-9
Asthmatic Patients		
1	1.5	5.3
2	3.8	4.8
3	9.0	14.3
4	0.8	3.8
5	7.5	20.5
6	12.8	17.3
Mean ± SD	5.9 ± 4.7[b]	11.0 ± 7.3[b,c]
Normal Controls		
1	1.3	2.0
2	0.8	1.8
3	0.3	0.8
4	0.8	1.5
5	1.3	1.8
Mean ± SD	0.9 ± 0.4	1.6 ± 0.5[c]

[a] CD34$^+$ PB cells from asthmatic patients or normal controls were cultured at 500 cells in dishes each containing 100 ng/ml SCF with or without 100 U/ml IL-9. After 4 wk, aggregates were scored as colonies if the constituent cells numbered 30 or more.
[b] Significantly different from normal controls.
[c] Significantly different from SCF alone.

with TPO in the early stage of the development, and require IL-9 for their subsequent growth.

Our clonal cell culture study showed that CD34$^+$ PB cells of patients with asthma yielded greater numbers of mast cell colonies under stimulation with SCF + IL-9, as compared with the values obtained for normal controls. A significant difference was also found in SCF stimulation. Thus, mast cell progenitors of asthmatic patients may possess superior potential in terms of responsiveness to mast cell growth-promoting cytokines than those of nonallergic subjects. Because numerous cytokines and chemokines are elaborated by resident and inflammatory cells in airways and have many effects on these cells, a variety of autocrine, paracrine, and endocrine mechanisms may operate in asthma (27). In contrast with eosinophil infiltration, there is disagreement concerning the numbers of mast cells in asthmatic mucosa (28, 29). Based on our results that IL-4 and IL-9 exert antagonistic effects on SCF-dependent growth of human mast cell progenitors, the discrepancy may be due in part to a qualitative balance between these Th2 cytokines.

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