Selective Growth of Human Mast Cells Induced by Steel Factor, IL-6, and Prostaglandin E₂ from Cord Blood Mononuclear Cells

Hirohisa Saito,*% Motohiro Ebisawa,* Hiroshi Tachimoto,* Michitaka Shichijo,* Kazumi Fukagawa,* Kenji Matsumoto,* Yoji Iikura,* Takeo Awaji,† Gozo Tsujimoto,† Makoto Yanagida,‡ Hiroya Uzumaki,‡ Gen Takahashi,§ Koichiro Tsuji,§ and Tatsutoshi Nakahata§

To establish the method for generating a large number of mature human mast cells, we cultured cord blood mononuclear cells (CBMC) in several conditions in the presence of Steel factor (SF). Among several cytokines tested, IL-6 enhanced SF-dependent mast cell growth from purified CD34⁺ cells for more than 8 wk in culture. When CBMC were cultured instead of CD34⁺ cells, IL-6 enhanced the mast cell development in the presence but not in the absence of PGE₂. PGE₂ enhanced the SF- and IL-6-dependent development of mast cells from CBMC probably by blocking granulocyte-macrophage CSF (GM-CSF) secretion from accessory cells, because 1) PGE₂ or anti-GM-CSF enhanced the mast cell development induced by SF and IL-6 from CBMC, but not from CD34⁺ cells; 2) GM-CSF inhibited the enhancing effect of IL-6 on the mast cell development from CD34⁺ cells; and 3) PGE₂ inhibited GM-CSF secretion from CBMC. The mast cells cultured in the presence of SF, IL-6, and PGE₂ for >10 wk were 99% pure, and seemed to be functionally mature, because 1) they contained 5.62 µg of histamine and 3.46 µg of trypstat per 10⁶ cells; and 2) when sensitized with human IgE and then challenged with anti-human IgE, the cells released a variety of mediators such as histamine, and an increase in intracellular Ca²⁺ was found in advance of the activation of membrane movement by using a confocal laser-scanning microscope. Electron-microscopic analysis revealed that some of the cultured mast cells are morphologically mature since they filled with scroll granules and contained crystal granules. The Journal of Immunology, 1996, 157: 343–350.

Mast cells play a central role in allergic inflammation by releasing various kinds of cytokines as well as vasoactive mediators (1, 2). Since mast cell heterogeneity has been found in different species and tissues (3, 4), it is expected to use human cells for investigating the role of mast cells in allergic disorders. And, since the number of cells that can be obtained from human tissues is limited, numerous attempts to establish human mast cell culture had been made for a decade after discovery of the method for culturing a large number of mouse mast cells in the presence of mouse IL-3 (5). The development of human mast cells was found in the coculture system of hematopoietic cells; and the c-kit proto-oncogene product (11–13). Stem cell factor, which is also called c-kit ligand or Steel factor (SF), is known to promote colony growth of crude hemopoietic cells (14–16). SF alone, however, induces only minimum proliferation of hemopoietic colonies from purified CD34⁺ cells. In combination with other cytokines, SF strongly stimulates hemopoietic colonies from CD34⁺ cells (17–19). The synergistic effect of SF with other cytokines is hardly detected when crude cell populations are cultured, since accessory cells spontaneously secrete a variety of cytokines (20, 21). Similarly, SF induces development of a substantial number of mast cells from crude hemopoietic cell preparations (8–10). Although human mast cells have been proven to be derived from CD34⁺ cells, SF alone induces only a minimum proliferation of the cells from purified CD34⁺ cells (7). These reports suggest that additional growth factors for mast cell development may be released from accessory cells.

We have recently found in a preliminary experiment (22) that IL-6 enhances the SF-induced development of human mast cells from purified CD34⁺ cells. We have also previously reported that PGE enhances the growth of mast cell colonies and inhibits the growth of granulocyte-macrophage (GM) colonies in the murine system (23). In the present study, therefore, we tried to confirm the effect of IL-6 in several culture conditions, and tested the effect of PGE on the SF- and IL-6-dependent development of human mast cells from crude cord blood mononuclear cells (CBMC) to establish a simple method for generating a large number of human mast cells.

*Division of Allergy and §Department of Pediatric Pharmacology, National Children's Medical Research Center, Tokyo, Japan; †Pharmaceutical Development Laboratory, Kirin Brewery Co., Maebashi, Gunma, Japan; ¶Department of Anatomy, Hironosuke University School of Medicine, Hironosuke, Aomori, Japan; and #Department of Clinical Oncology, The Institute of Medical Science, University of Tokyo, Tokyo, Japan

Received for publication November 15, 1995. Accepted for publication April 22, 1996.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported in part by Pediatric Research Grant 6–04 from the Ministry of Health and Welfare, and by Grant 5114 from the Japan Health Sciences Foundation, 1993.

2 Address correspondence and reprint requests to Dr. Hirohisa Saito, Division of Allergy, National Children's Medical Research Center, 3–35–31, Taishido, Setagaya-ku, Tokyo 154, Japan.

3 Abbreviations used in this paper: SF, Steel factor; CBMC, cord blood mononuclear cells; GM, granulocyte-macrophage.
Materials and Methods

Cell preparation

Heparin-treated umbilical cord blood was obtained under informed consent based on guidance from the hospital, diluted, and layered over lymphocyte separation medium (LSM; Organon Teknika Corp., Durham, NC) at room temperature within 12 h after delivery. Mononuclear cell fractions, which contained lymphocytes (60–80%), monocytes (20–40%), and other cell types (<2%), were obtained after centrifugation. Cellular tryptase was not detected in the CD34+ population from four samples in a preliminary study. The cells were suspended in IBL media I (a modified DMEM component plus 10 μg/ml insulin, 10 μg/ml transferrin, 5 × 10⁻⁵ M-2-μE, 25 mM HEPES, and 2.6 mg/ml Na₂SO₄), which was purchased from IBL Men-eki Seibutsu Kenkyuujo (Gunma, Japan).

Purification of CD34+ cells

Adherent monocytes were removed from CBMC by adhering them to plastic flasks (Falcon 3013; Becton Dickinson, Lincoln Park, NJ) for 1 h at 37°C. The cells were suspended in PBS without Ca²⁺ and Mg²⁺ containing 0.1% human serum albumin at 2 × 10⁷/ml. Dynabeads M-450 CD34 (Nihon Dynal, Tokyo, Japan) were added at 4 × 10⁷/ml to the cell suspension, and they were incubated for 30 min at 4°C. Detachment of Dynabeads M-450 CD34 from the positively selected CD34+ cells was done by incubating the cells with 100 μl of DETACHABEAD CD34 (Nihon Dynal).

Cell culture

The cell preparations were cultured in media I containing 5% FCS (Cansera, Rexdale, Canada), human rSF (Kirin Brewery Co., Mt.ebushi, Japan) at 80 ng/ml, human rIL-6 (Kirin Brewery Co.) at 50 ng/ml, and PGE₂ (GMBH & Co., Frankfurt, Germany) at various concentrations in 75-cm² flasks (Iwaki Glass, Tokyo, Japan) or Falcon’s 24-well flat-bottom plates (Becton Dickinson) at 37°C in 5% CO₂ and 5% O₂ (particle-free). The cells were examined with May-Griinwald and Giemsa staining, or immunocytochemically stained by the tannin-ferrocyanide OsO₄ method (26). The cells were washed and reacting with 0.05% diaminobenzidine-HCl (DAB; Sigma Chemical Co., St. Louis, MO) and 0.03% H₂O₂ in 0.05 M of Tris buffer, pH 7.6, for 10 min at room temperature. The smears were then counterstained with light green solution (Muto Chemical, Tokyo, Japan). Control slides were treated similarly, either with the primary Ab omitted, or in the presence of goat serum instead of the secondary Ab.

Immunophenotypic analyses

Immunoperoxidase tryptase staining was done by using a modification of the method described by Craig et al. (24). Briefly, the smear was fixed with Carnoy’s fixative (60% ethanol, 30% chloroform, and 10% glacial acetic acid) at 4°C. After being kept at room temperature for 2 h, the smears were washed five times with PBS, and incubated with a 1/50 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Organon Teknika Corp.). They were then washed and reacted with 0.05% diaminobenzidine-HCl (DAB; Sigma Chemical Co., St. Louis, MO) and 0.03% H₂O₂ in 0.05 M of Tris buffer, pH 7.6, for 10 min at room temperature. The smears were then counterstained with light green solution (Muto Chemical, Tokyo, Japan). Control slides were treated similarly, either with the primary Ab omitted, or in the presence of goat serum instead of the secondary Ab.

Functional analyses

Cells were sensitized with 1 μg/ml of human myeloma IgE (a generous gift from Dr. Kaminishi Iizhaka, Las Jolla, CA) at 37°C overnight. After washing, the cells were suspended at 1 × 10⁴ total cells/ml in modified Tyrode’s solution (pH 7.4) containing 124 mM NaCl, 4 mM KCl, 0.46 mM Na₂HPO₄, 1 mM CaCl₂, 0.6 mM MgCl₂, 10 mM HEPES, and 0.03% human serum albumin. The cells were prewarmed for 10 min, and challenged with either 1.5 μg/ml of rabbit anti-human IgE (Dako Corp., Glostrup, Denmark) or control Tyrode’s solution at 37°C for 30 min. For TNF-α release, the cells were incubated for 1 to 18 h in the medium used for the mast cell culture. Histamine was measured by an automatic histamine analyzer (Tosoh, Tokyo, Japan) with double column high performance liquid chromatography, as reported (25). Intracellular Ca²⁺ concentrations were determined with an automatic Ca²⁺ analyzer (CAB-100; IDEM, Tokyo, Japan) by staining the cells with 1 μM of fura-2 AM (Microprobe, Funakoshi, Tokyo, Japan). After being washed twice in Tyrode’s solution, cell pellets were resuspended in the solution at 2 × 10⁶/ml. Fluorescence of fura-2 signals between two waves of excitation (340 nm and 380 nm) was measured at 510-nm emission by a Ca²⁺ analyzer (model CAB-100; Japan Spectroscopic Co., Tokyo, Japan) with a temperature-controlled cuvette (37°C) and a magnetically driven stirrer. The mast cells started to move rapidly after anti-IgE challenge. For confocal laser-scanning microscopy, the cultured mast cells were prepared from the albinino 3T3 fibroblast cell line (American Type Culture Collection, Rockville, MD), and they were sensitized with 1 μg/ml of myeloma IgE overnight. After being washed with Tyrode’s solution, the cells were stained with 4 μM of fluo-3 AM (Dojindo, Kumamoto, Japan) for 30 min, washed three times, and examined under an inverted microscope. Olympus GB-200 (Tokyo, Japan). The excitation wavelength was 488 nm, and the emission wavelength was 500 to 530 nm. The levels of TNF-α, IL-5, and GM-CSF were measured with BIOTRAK cytokine ELISA system (Amersham International, Amersham, U.K.). For flow cytometry, the cells were reacted with mAbs recognizing CD2-CD128 (provided from Fifth International Workshop and Conference on Human Leukocyte Differentiation Antigens, 1993, Boston), and were examined with an Epics profile analyzer (Coulter Corp., Hialeah, FL). To measure tryptase levels, the cells were treated with 1 M of NaCl to inhibit the protein nonspecifically sticking to cell membranes or tube walls. After freezing and thawing the cell suspension three times, cellular tryptase levels were determined by using a Pharmacia’s tryptase RIA kit (Uppsala, Sweden).

Electron microscopy

For transmission electron microscopy, cells were prefixed in 2.5% glutaraldehyde in 0.1 M of sodium cacodylate buffer containing 0.1% p-catechil for 1 h at 4°C, and were postfixed in a mixture of 1% OsO₄ and 1.5% K₂[Fe(CN)₆] in the same buffer. The pellets were mordanted by 2% tannic acid 2 h, followed by 1% neutral uranyl acetate solution for 30 min. The epon-embedded sections were examined with an electron microscope (JEM 2000EX, Hitachi, Tokyo, Japan), a Hitachi E-1010 SEM, or a Hitachi S-5200 SEM. For scanning microscopy, cells were prefixed in a mixture of 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M of sodium cacodylate buffer at 4°C, and then conductively stained by the tannin-ferrocyanide OsO₄ method (26). The freeze-dried cells were examined with an ultra-high resolution electron-scanning microscope (JSM-890; JEOL) at 15 or 20 kV.

mRNA isolation and reverse-transcriptase PCR

After challenge with anti-IgE, the cells were being centrifuged for 6 min at 400 × g. After removal of the supernatant, the cell pellet was mixed with RNAzol B (Biotex Laboratories, Houston, TX), which is a modified guanidium-thiocyanate single-step procedure. RNA was extracted from aqueous phase after addition of CHCl₃. The isopropanol-precipitated RNA was washed with 70% ethanol, dried, and resuspended in diethylpyrocarbonate-treated water.

An aliquot of total cellular mRNA was reverse transcribed to cDNA and PCR, expanded by using the first-strand cDNA synthesis kit (Pharmacia Biotech, Tokyo, Japan), according to the manufacturer’s instructions. In this protocol, 8 μl of the RNA extract was denatured at 65°C for 10 min, and mixed with reverse-transcriptase solution containing a reverse transcriptase, dNTP, NotI-d(T)₁₈, and DTT solution. The mixture was incubated at 37°C for 60 min, and inactivated at 65°C for 5 min. The 2-μl solution was mixed with PCR buffer containing 2.5 U of Taq DNA polymerase (Takara Biomedical, Ohtsu, Japan) and each of the following primers: IL-3 (5’ primer, 5’-CCA GGT GGC TTA ACT GCT CTA A; 3’ primer, 5’-GGG TTT TCA GAT GAC ACG TCA G); IL-5 (5’ primer, 5’-AAG TGC ATC ACC TTA CAG G; 3’ primer, 5’-CTC TCT CAT GAT CTT TAG C); IL-5 (5’ primer, 5’-GAC ATT CCC ACA AGT GCA TTA G; 3’ primer, 5’-ATA GGT TTT CTA CTC TCC G); and β-actin (5’ primer, 5’-GGG CTT GGA GCA GAC CAC G; 3’ primer, 5’-ACC ACG TAC TTT CAC GGA G). The PCR reaction was cycled 32 times according to standard protocol: denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min. The cytokine primers were synthesized at the Hitachi Kasei Manufacturing Co. (Ibaragi, Japan) by the courtesy of Dr. M. Mushashi and Dr. T. Akitaya at the Division of Medical Sciences, Hitachi Chemical Research Center (Irvine, CA). The PCR products were analyzed by electrophoresis in 5% agarose gel containing 0.5% ethidium bromide. The fluorescent DNA bands were visualized by UV excitation.

Results

Effect of cytokines

To determine whether other cytokines synergistically act with SF on the development of mast cells, we cultured purified CD34+ cells at 10⁵ to 10⁶/ml for 4 wk. We added IL-3 at 1 ng/ml, GM-CSF at 100 pg/ml, and IL-6 at 50 ng/ml as synergistic factors,
The three separate experiments, although the cell number was not sufficient enough for counting tryptase-positive cells in the cultures. Negative cells were judged to be macrophages by their prominent phagosomes) developed in the presence of SF. IL-6 enhanced the proliferation of the cultures containing SF and IL-6 enhanced the proliferation of tryptase-positive cells in cultures with SF and that in cultures with SF plus cytokines were <0.05 by a double-tailed paired Student's t test. The p value between the number of tryptase-positive cells in cultures with SF and IL-6, and that in cultures with SF, IL-6, and GM-CSF was 0.025.

because they have been reported to enhance the SF-induced early development of hemopoietic cells (17-19, 27). We found that all of the three cytokines enhanced the proliferation of the cells having tryptase-positive granules (after 4 wk in culture, >95% of the tryptase-negative cells were judged to be macrophages by their prominent phagosomes) developed in the presence of SF. IL-6 enhanced the SF-induced development of tryptase-positive mast cells with the highest proportion. Further addition of GM-CSF at 100 pg/ml to the cultures containing SF and IL-6 enhanced the proliferation of tryptase-negative cells, and inhibited the enhancing effect of IL-6 on the SF-induced growth of tryptase-positive mast cells (Fig. 1). In the absence of SF, the growth of tryptase-positive cells was not detected even in the presence of these three cytokines (data not shown). Other than the experiments shown in Figure 1, we have obtained similar results that IL-6, IL-3, and GM-CSF in combination with SF stimulated the growth of tryptase-positive cells in three separate experiments, although the cell number was not sufficient enough for counting tryptase-positive cells in the cultures

**Table 1. Prolonged effect of IL-6, IL-3, and GM-CSF on the SF-dependent development of mast cells**

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Conditions*</th>
<th>4 wk in Culture</th>
<th>8 wk in Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total cells per well</td>
<td>Tryptase-positive cells</td>
<td>Total cells per well</td>
</tr>
<tr>
<td>1&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SF</td>
<td>1.25 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>72.5%</td>
<td>0.35 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF + IL-6</td>
<td>4.55 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>68.0%</td>
<td>3.80 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF + IL-3</td>
<td>13.80 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>12.5%</td>
<td>2.45 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF + GM-CSF</td>
<td>16.00 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>6.5%</td>
<td>1.00 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF</td>
<td>3.50 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>82.5%</td>
<td>6.60 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF + IL-6</td>
<td>5.55 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>75.0%</td>
<td>2.50 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF + IL-3</td>
<td>9.85 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>35.5%</td>
<td>1.20 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>SF + GM-CSF</td>
<td>10.55 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>32.5%</td>
<td>1.25 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> SF, IL-6, IL-3, and GM-CSF were added at 80 ng/ml, 50 ng/ml, 1 ng/ml, and 0.1 ng/ml, respectively.
<sup>b</sup> Non-adherent viable cells were counted under an inverted microscope.
<sup>c</sup> Smears were stained with anti-trypotase and were counter-stained with Light Green solution. The percentages of tryptase-positive cells in total cells were calculated.
<sup>d</sup> CD34<sup>+</sup> cells were seeded at about 10<sup>4</sup> cells per well.

**FIGURE 1.** Effect of cytokines onSF-induced proliferation of tryptase-positive or-negative cells. Purified CD34<sup>+</sup> cells were cultured at 10<sup>6</sup> to 10<sup>7</sup>/ml in the presence of cytokines, as indicated. After 4 wk in culture, the number of cultured cells was counted, the smears were stained for trypotase, and the percentages of cells having tryptase-positive granules (B) or tryptase-negative cells (C) were calculated. Each column and bar represents the mean and SE of four separate experiments, and p values were <0.01 between control and PGE<sub>2</sub> groups.

**FIGURE 2.** Effect of PGE<sub>2</sub> on mast cell development from crude CBMC induced by SF and IL-6. CBMC were cultured at 10<sup>7</sup>/ml in the presence of SF at 80 ng/ml and IL-6 at 50 ng/ml for 4 wk. After 4 wk in culture, the number of cultured cells was counted, the smears were stained for trypotase, and the percentages of tryptase-positive cells were calculated. Each column and bar represents the mean and SE of eight separate experiments, and p values were <0.01 between control and PGE<sub>2</sub> groups.

Stimulated with SF alone (data not shown). The percentages of mast cells remained increasing in the cultures with SF or SF plus IL-6 after 4 wk, whereas it diminished in the cultures with SF plus IL-3 or SF plus GM-CSF after 4 wk (Table 1).

When 10<sup>5</sup> freshly isolated CBMC were cultured in 1 ml of medium I supplemented with 5% FCS, they spontaneously secreted GM-CSF at 31 ± 10 pg/ml in 48 h of incubation. GM-CSF was never detected at day 4 in culture with CD34<sup>+</sup> cells in the presence of SF and IL-6 without PGE<sub>2</sub> (n = 4). PGE<sub>2</sub> was found to inhibit the release of the cytokine as follows: GM-CSF at 12 ± 3 pg/ml, 8 ± 2 pg/ml, and 7 ± 1 pg/ml were respectively released in the presence of PGE<sub>2</sub> at 10<sup>-8</sup> M, 10<sup>-7</sup> M, and 10<sup>-6</sup> M (n = 8; p value between control and PGE<sub>2</sub> groups was p < 0.05). In contrast, PGE<sub>2</sub> enhanced spontaneous secretion of IL-6 from the same mononuclear cells, although the levels of the cytokine were below 1 ng/ml (data not shown).

When crude CBMC were cultured at 10<sup>7</sup>/ml in the presence of SF and IL-6, PGE<sub>2</sub> enhanced the growth of tryptase-positive cells (Fig. 2), and increased the levels of cellular trypotase and histamine in total cultured cells (Table II). PGE<sub>2</sub> also inhibited the growth of tryptase-negative macrophagic cells (data not shown). PGE<sub>2</sub>, however, failed to do so when CD34<sup>+</sup> cells were cultured (Fig. 3).

In the presence of 10<sup>-7</sup> M of PGE<sub>2</sub> and 80 ng/ml of SF, we could obtain 5.05 ± 1.0, 6.1 ± 1.7, 10.4 ± 1.4, and 19.2 ± 2.4 ×
TABLE II. Effect of PGE2 on the increase in cellular histamine and cellular tryptase concentrations in mast cells cultured in the presence of SF and IL-6 for 4 wk

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PGE2 $10^{-6}$ M</th>
<th>PGE2 $10^{-7}$ M</th>
<th>PGE2 $10^{-8}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histamine (ng/well)</td>
<td>51.4 ± 35.2$^a$</td>
<td>75.2 ± 49.9$^c$</td>
<td>119.6 ± 75.2$^c$</td>
<td>110.0 ± 84.8</td>
</tr>
<tr>
<td>Tryptase (ng/well)</td>
<td>81.2 ± 53.2</td>
<td>220.6 ± 189.2</td>
<td>286.4 ± 182.8$^c$</td>
<td>242.2 ± 105.1$^c$</td>
</tr>
</tbody>
</table>

$^a$ Each value indicates the concentration of mediators in the total cultured cells per each well where 1 $\times$ 10$^5$ mononuclear cord blood cells were plated.

$^b$ Each value represents an average ± SEM of five separate experiments.

$^c$ $p < 0.05$ by a double-tailed paired Student's $t$ test.

FIGURE 3. Effect of PGE2 on the SF- and IL-6-induced mast cell development from purified CD34$^+$ cells. Cord blood-derived CD34$^+$ cells were cultured at 10$^3$ to 10$^5$/ml in a well in the presence of SF at 80 ng/ml and IL-6 at 50 ng/ml for 4 wk. After cell count, the smears were stained for tryptase, and then percentages of tryptase-positive cells were calculated. Each column represents the mean of three separate experiments. Tryptase-positive cells were developed at 2.98 ± 2.01 $\times$ 10$^4$ tryptase-positive cells from 10$^5$ mononuclear cord blood cells were plated.

FIGURE 4. Typical mast cells developed in the presence of SF, IL-6, and PGE2 for 12 wk. May-Grunwald Giemsa stain. Note the prominent microvilli-like processes. ×1099.

10$^4$ tryptase-positive cells from 10$^5$ mononuclear cord blood cells after 4 wk in the medium supplemented with IL-6 at 0, 2, 10, and 50 ng/ml, respectively (mean ± SEM; $n = 3$; $p < 0.05$ by Student's paired $t$ test between groups cultured with IL-6 at 10–50 ng/ml and 0 ng/ml). In the absence of PGE2 and the presence of SF, the addition of IL-6 did not affect the number of tryptase-positive cells that arose from 10$^5$ mononuclear cells (3.99 ± 1.88 $\times$ 10$^4$ cells for IL-6 at 50 ng/ml and 3.82 ± 10$^4$ cells for control in three separate experiments).

When 10$^5$ CBMC were cultured for 4 wk in 1 ml of medium provided with SF and IL-6, but not PGE2, the addition of anti-GM-CSF (10 ng/ml) enhanced the growth of tryptase-positive cells (8.03 ± 2.01) $\times$ 10$^4$ for anti-GM-CSF and (15.4 ± 2.01) $\times$ 10$^4$ for control, in four separate experiments. The value represents the mean ± SEM; $p < 0.05$ by paired $t$ test.

Morphologic features

In the presence of SF at 80 ng/ml, IL-6 at 50 ng/ml, and 300 nM of PGE2, the cultured mast cells increased in size as well as in number until day 100 in culture (Fig. 4). Ultrastuctural analysis revealed that most granules of the cultured mast cells were filled with scroll structures, although irregular periodicity was seen in the structures (Fig. 5). We were also able to demonstrate a typical crystal granule in the mast cells (Fig. 6). We used IL-6 at 50 ng/ml, because the cytokine at 100 ng/ml induced atypical characteristics, i.e., the cells had >10 pg of cellular histamine per cell and miltobled nuclei. As has been reported elsewhere (28), the cultured mast cells were stained for tryptase at 98.6 ± 0.4%, and chymase for 18.4 ± 4.4% ($n = 7$).

Functional features

When CBMC were cultured in the presence of SF at 80 ng/ml, IL-6 at 50 ng/ml, and 300 nM of PGE2 for >10 wk, the mast cells contained 5.62 ± 1.88 µg of histamine ($n = 6$) and 3.46 ± 0.89 µg of tryptase ($n = 6$; each value represents mean ± SEM) per 10$^6$ cells. Cells sensitized with 1 µg/ml of human IgE released 52.9% histamine at 30 min, and 1.76 ng of TNF-α per 10$^5$ cells at 6 h after challenge with 1.5 µg/ml of anti-human IgE, whereas the control cells spontaneously released 3.7% histamine and 0.18 ng of TNF-α (Fig. 7). Release of TNF-α reached a plateau at 6 h (data not shown), while the increase in intracellular Ca$^{2+}$ (Fig. 8) and histamine release (data not shown) reached a plateau before 10 min. An increase in intracellular Ca$^{2+}$ could be detected in advance of partial swelling of the plasma membrane by using a confocal laser-scanning microscope (Fig. 9). Some parts of the membrane had ballooned outward and were soon shrinking. The ballooning was repeated from 196 to 476 s after anti-IgE challenge. We could not observe the dynamic movement of the plasma membrane after 8 min. Mast cells also released 3, 85, 98, and 58 pg of IL-5 per 10$^6$ cells at 6 h after challenge with anti-human IgE at 0, 0.15, 1.5, and 15 µg/ml, respectively ($n = 2$). The protein levels of IL-3 and IL-4 were <3 pg/ml at 0, 6, and 18 h when 5 × 10$^5$ mast cells were challenged with anti-human IgE at 1.5 µg/ml. Expression of IL-5 mRNA was observed clearly at 1 h after anti-IgE challenge, while mRNA expression of IL-3 and IL-4 was not detected in samples after challenge (Fig. 10). Before challenge with anti-IgE, neither IL-3, IL-4, nor IL-5 mRNA expression was detected clearly, while β-actin mRNA was demonstrated.

Discussion

SF stimulates proliferation of primitive hemopoietic cells (14–19) and mature mast cells (8–10, 29) through c-kit receptors. However, it is often observed that SF alone exerts a minimum effect on the cell proliferation from purified CD34$^+$ cells (7, 17–19, 27) in the
absence of accessory cells. Several cytokines such as IL-3, IL-6, and GM-CSF have been shown to synergize with SF for proliferation of hemopoietic cells (17–19, 27). Therefore, it was not surprising that these cytokines enhanced the SF-induced proliferation of mast cells probably by stimulating the expansion of primitive hemopoietic cells in the early stage of cell proliferation, as shown in this study. Among these cytokines, only IL-6 stimulated the expansion of hemopoietic cells without inducing marked proliferation of macrophages, which are known to secrete a variety of cytokines such as GM-CSF (20, 21, 30). Subsequent secretion of GM-CSF, which may occur in the presence of exogenous GM-CSF or IL-3, would therefore accelerate the differentiation of primitive hemopoietic cells toward the GM lineage, thereby reducing the differentiation capacity of the cells toward the mast cell lineage in a prolonged period. IL-6 was also effective for proliferation of differentiated mast cells after 4 wk in culture, as well as for the expansion of undifferentiated progenitors. Indeed, we have observed recently that IL-3, IL-4, and IL-5 as well as IL-6 prevent apoptosis of the mast cells cultured for >10 wk (31).

It has been reported by Metcalfe et al. (7, 32) that IL-3 markedly enhanced the SF-dependent mast cell growth from bone marrow-derived CD34+ cells in the early culture period. We had similar
results in the present study, especially in three of the seven experiments showing SF alone failed to induce a substantial amount of mast cells from purified CD34+ cells. In those three experiments, IL-3 seemed to be a powerful synergistic factor for the SF-induced proliferation of mast cells. In one of the seven purified CD34+ samples and in two of ten crude samples with PGE₂ addition, we failed to obtain a substantial number (>10⁶ per sample) of mast cells even in the presence of SF and IL-6, whereas IL-3 alone always induced a substantial amount of basophils (33) from the same cord blood samples (data not shown). It may be related to the number of cytokine receptors present on cord blood-derived CD34+ cells, which may be individually different. In most cases, however, the combination of IL-6 and SF would be the best way for generating highly purified mast cells in a prolonged period.

We have reported previously that PGE₂ enhances the colony formation of mast cells and inhibits GM colony formation in the presence of IL-3-containing medium in murine system (23). In the present study, PGE₂ inhibited proliferation of adherent macrophages by blocking GM-CSF secretion, as has been reported previously by others (29), and it enhanced the development of human mast cells. Since PGE₂ failed to affect mast cell development from purified CD34+ cells, and GM-CSF was never detected until day 4 in culture with CD34+ cells in the presence of SF and IL-6, the enhancing effect of the lipid mediator is probably brought on by modulating cytokine secretion, especially by blocking GM-CSF secretion from accessory cells. Indeed, exogenous addition of anti-GM-CSF Ab enhanced the proliferation of mast cells in the presence of SF and IL-6. IL-6 and granulocyte CSF were released from the 2 × 10⁵ mononuclear cord blood cells at 181 pg and 150 pg in the present study (data not shown in the results) and previous reports (20, 21, 29), and the addition of PGE₂ slightly enhanced the secretion of the cytokines. However, their concentrations were not sufficient for modulating the proliferation of mast cells.

Thus, we established a method for obtaining a large number of human mast cells. We were able to generate >10⁷ mast cells in 2 of 10 samples and 10⁶ to 10⁷ mast cells in 6 of 10 samples with >95% purity when 3 to 8 × 10⁷ crude CBMC were cultured in the presence of SF at 80 ng/ml, IL-6 at 50 ng/ml, and 300 nM of PGE₂ for >10 wk. Similar inconstant results were obtained even by culturing purified CD34+ cells depending on samples. Since the numbers of CD34+ cells, GM colony-forming cells, monocytes, and lymphocytes present in the CBMC were almost constant depending on samples (data not shown), we may have to examine the

**FIGURE 8.** Increase in the intracellular concentrations of Ca²⁺ in the cultured mast cells. The mast cells with 99.5% purity at 12 wk in culture were sensitized with 1 μg of myeloma IgE, and were labeled with 1 μM of fura-2 AM and suspended at 1 × 10⁶ per 1 ml of Tyrode's solution in a cuvette of a Ca²⁺ analyzer, CAF-100. The cells were then challenged with either 1.5 μg (left side) or 15 μg (right side) of anti-human IgE in the analyzer. The two fluorescent intensities were measured at excitation wavelengths of 340 nm and 380 nm, and their ratio was calculated. Similar results were obtained in two other separate experiments.

**FIGURE 9.** Membrane balloonning of the cultured mast cells following an increase in intracellular Ca²⁺. The mast cells at 12 wk in culture were sensitized with 1 μg of myeloma IgE overnight on a fibroblast layer to which the mast cells were fixed, and were labeled with 1 μM of fluo-3, and suspended in a temperature-controlled dish. The cells were then challenged with 15 μg of anti-human IgE. A single cell was scanned every 7 s by using a confocal laser microscope, Olympus GB-200. The mast cell at 0 s (A), 21 s (B), and 399 s (C) after challenge with anti-IgE was respectively shown. Relative Ca²⁺ concentrations were shown in the upper part by colorizing the fluorescent intensities in the following rank order, i.e., red > orange > yellow > green > blue. In the lower part, the same cell was examined by using a phase-contrast technique. Similar results were obtained in three separate experiments, although intracellular Ca²⁺ started to increase at various intervals, from 35 s to >1 min after anti-IgE challenge. The fading of fluo-3 fluorescence was clearly found in the control cells within 5 min.

**FIGURE 10.** IgE-dependent mRNA expression of cytokines. A half-million mast cells at day 150 in culture with >99.9% purity were challenged with 1.5 μg/ml of anti-IgE. After 60 min of incubation at 37°C, the cells were treated with RNAzol B, as shown in Materials and Methods.
expression of c-kit and IL-6R on the CD34+ cells to define differentiation capacity toward the mast cell lineage of the cells. Human mast cells developed in the presence of SF alone often exhibit somewhat immature functional properties (9, 10). The lung mast cells are known to possess 2 to 3.9 μg of histamine and 11 μg of tryptase per 10^6 cells (34, 35). The mast cells cultured in the present method seemed to be functionally mature, because 1) they contained 5.62 μg of histamine and 3.46 μg of tryptase per 10^6 cells; 2) the cultured mast cells had IgE receptors (36), and the cells sensitized with 1 μg/ml of human IgE released 52.9% of the histamine content, 693 ng LTC4, 1.76 ng TNF-α, and 98 pg IL-5 per 10^6 cells when challenged with 1.5 μg/ml anti-human IgE, whereas the control cells spontaneously released 3.7% histamine, 9 ng LTC4, 0.18 ng TNF-α, and 3 pg IL-5; and 3) the reactions were accompanied by an increase in intracellular Ca2+. By using a confocal laser-scanning microscope, some parts of the plasma membrane were found to have ballooned outward following the IgE-dependent increase in intracellular Ca2+. The membrane ballooning was started from 3 min and finished until 8 min after anti-IgE challenge, and its time course was similar with that of histamine release (34), suggesting that the partial swelling of the membrane reflects IgE-dependent degranulation of human mast cells.

The cultured mast cells were positively stained for anti-tryptase and anti-chymase Abs at 99 and 18% (28), respectively. In an electron-microscopic analysis, some of the cells nearly filled with crystalline granules, which are seen frequently in human skin mast cells and in some of the cultured mast cells developed in coculture system with 3T3 fibroblasts (6), were also detectable. These results suggest that the cultured mast cells developed in SF plus IL-6 are morphologically more mature than those developed in SF alone, which are reported to have only incomplete condensation of granule materials (10), and are a mixture of tryptase- and chymase-positive skin-type mast cells and tryptase-positive lung-type mast cells (35).

Since it is hard to obtain 10^7 purified mast cells from human tissues, analyses requiring a large number of pure cells, such as intracellular mechanisms or cytokine production, have not been intensively examined. Contaminated monocytes may produce a variety of cytokines when they adhere to plastic flask (21, 29), and basophils may produce IL-4, as has been reported (38). In the present experiment for cytokine production, cells consisting of >99.9% mast cells and <0.1% fibroblast-like cells were used, and basophils were never detected. In contrast to the report that human lung mast cells produce IL-4 when challenged with anti-IgE (39), we were not able to detect either protein levels or mRNA expression of IL-4. This observation may result from the heterogeneity of human mast cells based on cytokine content (40) or immaturity of the cultured cells. The cultured mast cells, however, produced >1 ng of TNF-α per 10^6 cells, which amount is enough for eosinophil transendothelial migration (41). A substantial amount of IL-5 was also detected. We were able to show that the cultured mast cells expressed IL-5 mRNA after challenge with anti-IgE. In the same samples, however, mRNA expression of IL-3 and IL-4 was not detected. As has been reported elsewhere (28), the cultured mast cells expressed a series of surface CD molecules in keeping with human mast cells in vivo (42, 43), except CD13, CD14, and CD38 present on the cultured cells. Taken together, the present method for generating human mast cells should greatly facilitate investigation of the role of mast cells in human allergic disorders, especially when such studies require large numbers of pure mast cells.

Acknowledgments

We thank Drs. Kimishige Ishizaka and Teruko Ishizaka (La Jolla Institute for Allergy and Immunology, La Jolla, CA) for their critical reviewing of the manuscript. We are grateful to Dr. Shigenobu Shoda (Department of Obstetrics, Gyoda Chuo Hospital, Saitama, Japan) for his continuous support by generously providing the umbilical cord blood. We also thank Dr. Naoya Sakaguchi, Dr. Katsushi Miura, Dr. Akira Akasawa, Mr. Takashi Numazaki, and Mr. Masahiro Kimata (Division of Allergy, National Children’s Medical Research Center, Tokyo, Japan) for their advice on the manuscript and for excellent technical support for the experiments.

References


CULTURED HUMAN MAST CELLS


