Differentiation of a Human Eosinophilic Leukemia Cell Line (EoL-1) by a Human T-Cell Leukemia Cell Line (HIL-3)-Derived Factor

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Differentiation of a human eosinophilic leukemia cell line, EoL-1, induced by the culture supernatant of a human ATL cell line, HIL-3 (HIL-3 sup) was compared with differentiation induced by defined cytokines. HIL-3 sup induced EoL-1 cells to express eosinophilic granules and segmented nuclei after 6 to 9 days of incubation. HIL-3 sup also induced the expression of Fcε receptor II (FcεRII/CD23) and an eosinophil differentiation antigen EO-1 mainly on eosinophilic granules (+) cells. Furthermore, HIL-3 sup induced EoL-1 cells to respond to an eosinophil chemotactic factor, platelet activating factor. HIL-3 cells express messenger RNA (mRNA) of interleukin-5 (IL-5), macrophage colony-stimulating factor (M-CSF), and IL-3 but not granulocyte CSF (G-CSF). Granulocyte-macrophage CSF (GM-CSF) and tumor necrosis factor-α (TNF-α) were detected in the HIL-3 sup. Recombinant IL-2 (rIL-2), rIL-3, rIL-4, rIL-5, rM-CSF, and rGM-CSF did not induce eosinophilic granules. rG-CSF induced a few eosinophilic granule (+) cells, and TNF-α, which did not induce eosinophilic granules by itself, enhanced the ability of G-CSF to induce them. However, G-CSF and TNF-α did not induce the expression of FcεRII and EO-1 antigen. Moreover, anti-G-CSF, anti-TNF-α, anti-GM-CSF, anti-IL-3, and anti-IL-5 antibodies did not suppress the effect of HIL-3 sup on the differentiation of EoL-1 cells. All the data suggest that HIL-3 sup contains an unidentified factor that induces differentiation of EoL-1 cells, and that EoL-1 cells and HIL-3 sup provide an important model for the examination of differentiation mechanisms and functions of eosinophils.

T is well known that eosinophils are important effector cells in host defense mechanisms against helminth infections.1,2 Eosinophils in allergy have been considered to play two opposite roles. Histaminease, phospholipase-D2, and arylsulphatase in eosinophils may be scavengers of allergic inflammatory products through inactivation of histamine, slow reactive substance of anaphylaxis, and platelet activating factor (PAF), respectively, whereas major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin, and eosinophil peroxidase may worsen and prolong allergic inflammations by their cytotoxic effects.3,4 Receptors for the Fc portion of IgG, IgA, and IgE have been shown to be involved in the mechanisms of secretion of these substances.5-8 In the proliferation and differentiation of eosinophils, interleukin-1 (IL-1), IL-3, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage CSF (GM-CSF), and IL-5 have been shown to play important roles.9-11 IL-1 and IL-3 are considered to be responsible for eosinophil growth and differentiation at an earlier stage,9 while IL-5 may be responsible for eosinophils at a later stage of differentiation and for prolongation of the life span of mature eosinophils.9,11,12

However, there are still many unanswered questions regarding the proliferation and differentiation of eosinophils and the mechanisms of their effector functions. One of the major problems that makes examinations using eosinophils hard is the difficulty of obtaining a pure eosinophil population. Recently, a human eosinophilic leukemia cell line, EoL-1, has been shown to be a good in vitro model for research on eosinophil differentiation. EoL-1 cells have the cytotoxic features of myeloblasts in normal culture conditions, but they can be induced to differentiate into eosinophilic granule-containing cells but not into other lineage cells under several culture conditions17 and are therefore considered to be committed precursors of eosinophils. This study shows that the culture supernatant of a human adult T-cell leukemia line, HIL-3 causes cytotoxic differentiation of EoL-1 cells. The culture supernatant of HIL-3 cells, which is supposed to contain an undefined eosinophil differentiation factor, also induced the expression of Fcε receptor II (FcεRII/CD23) and an eosinophilic differentiation antigen EO-1 and chemotaxis against PAF.

MATERIALS AND METHODS

Cell lines. A human eosinophilic leukemia cell line EoL-117 and an EBV-transformed human B-cell line RPMI 8866 were maintained in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo) supplemented with 13% fetal calf serum (FCS), 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, referred to as the culture medium in this paper. A human T-cell line HIL-3 was established from peripheral blood mononuclear cells of an adult T-cell leukemia patient with the use of IL-2. HIL-3 cells were maintained in the culture medium supplemented with 5 U/mL of recombinant human IL-2, a gift from Takeda Chemical Industries, Ltd, Osaka.

Reagents. Conditioned medium of HIL-3 cells (HIL-3 sup) was obtained from the supernatant of a 3-day culture of HIL-3 cells at an initial concentration of 1 × 10^6 cells/mL, collected in large amounts, and stored at −40°C until use. Recombinant human IL-3 dissolved in Dulbecco’s phosphate-buffered saline (PBS) with 0.1% FCS at a concentration of 1,000 U/mL and rabbit anti-human IL-3 antibodies (anti-IL-3 ab) were purchased from Genzyme (Boston, MA). Recombinant human GM-CSF dissolved in PBS with 0.1% FCS at a concentration of 100 ng/mL and rabbit anti-human GM-CSF antibodies (anti-GM-CSF ab) were gifts.
from Sumitomo Pharmaceutical, Osaka. Recombinant human granulocyte colony stimulating factor (G-CSF; specific activity was 5 × 10^10 U/mg protein by stem cell assay) dissolved in PBS with 0.1% FCS at a concentration of 1,000 ng/mL and goat anti-human G-CSF antibodies (anti-G-CSF ab) were gifts from Chugai Pharmaceutical, Tokyo. Recombinant human IL-5 dissolved in PBS with 0.1% FCS at a concentration of 100 μg/mL and rabbit anti-human IL-5 antibodies (anti-IL-5 ab) were gifts from Santory, Osaka. Recombinant human tumor necrosis factor-α (TNF-α) and rabbit anti-human TNF-α antibodies (anti-TNF-α ab) were purchased from Genzyme, Boston. Recombinant human macrophage colony stimulating factor (M-CSF) and recombinant human IL-4 were gifts from Ohtsuka Pharmaceutical, Tokyo and DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA, respectively. These factors were all diluted with the culture medium at the time of use. PAF, 1-hexadecy1-2-0-acetylsn-glycero-1-phosphorylcholine, purchased from Bachem Feinchemikalien AG, was dissolved in PBS with 0.1% FCS at a concentration of 10^-4 mol/L and diluted with Hanks balanced salt solution (HBSS; Nissui Pharmaceutical, Tokyo, Japan) at the time of use.

**Analysis of cytokines in HIL-3 sup.** TNF-α contained in HIL-3 sup was determined by a radioimmunoassay with a TNF-α IRMA kit (Medgenix, Belgium) and by a bioassay with L-929 cells.30 GM-CSF in HIL-3 sup was determined by a reverse immunosorbent assay with an Insight GM kit (Medical Resources Limited, Australia), and G-CSF in HIL-3 sup was determined by an enzyme immunoassay.

**Preparation of messenger RNA (mRNA) and Northern blotting.** Crude RNAs were extracted from HIL-3 cells with guanidinium thiocyanate.31 Poly A RNA was purified by oligo(dT) cellulose (Type 5; Collaborative Research, Lexington, MA) column chromatography. Poly A RNA (5 μg) of HIL-3 cells was glyoxalated and electrophoresed in a 1% agarose gel, then transferred to a nitrocellulose filter as described by Macmaster and Carmichael.32 Hybridization was performed as described by Noma et al.

**DNA probes.** The DNA fragments used as probes were: Human IL-3 cDNA, 0.44-kb EcoRI-Xhol fragment of pcD-SRα-IL-330 (provided by Dr K. Arai of DNAX Research Institute of Molecular and Cellular Biology); human IL-5 cDNA, 1 kb BamHI fragment of pH-IL-5-30; human G-CSF, 1.5 kb c ori fragment of pBRG-430 (provided by Dr S. Nagata of Osaka Bioscience Institute); human M-CSF, synthetic 30mer oligonucleotide 5'-TGTTGGTCTGTC-TCTTGGCGGACGAGATGTA-3' corresponding to antisense strand between positions 206 and 235 of the published sequence (provided by Dr K. Motoyoshi of Jichi Medical School). Those DNA fragments, except for human M-CSF synthetic oligonucleotide, were labeled to obtain the specific activity of 500 to 1,200 cpm/pg using random primers31 and α-[32P]dCTP (3,000 Ci/mmol; Amer- sham, Arlington Heights, IL). The oligonucleotide for M-CSF cDNA was labeled by the 5' end labeling method38 using α-[32P]dATP (5,000 Ci/mmol, Amer sham). 

**Cell culture.** EoL-1 cells were suspended in the culture medium at a concentration of 5 × 10^5 cells/mL with or without HIL-3 sup and the several recombinant cytokines. HIL-3 sup was used at a final concentration of 10% and IL-2, IL-4, and M-CSF were used at concentrations of 5 U/mL, 10 U/mL, and 10 ng/mL, respectively. IL-3, IL-5, and GM-CSF were used at concentrations of 10 to 100 U/mL, 10 to 100 ng/mL, and 10 to 100 ng/mL, respectively. G-CSF was used at concentrations of 1, 10, 50, and 100 ng/mL, and TNF-α at 1, 10, and 100 U/mL. One milliliter of the cell suspension was put into a 24-well culture dish (Falcon 3047; Becton Dickinson, Mountain View, CA) and cultured for 9 days at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The culture medium containing HIL-3 sup or the cytokines was renewed, and cell counts were readjusted to a concentration of 5 × 10^5 cells/mL on days 3 and 6. In some experiments, EoL-1 cells were suspended in the serum-free conditioned medium, Cosmedium-001 (Cosmo Bio, Tokyo).

**Morphologic analysis.** Cytospin preparations of the cultured cells were stained with Wright-Giemsa or Luxol-Fast-Blue staining solution. Luxol-Fast-Blue staining was performed with Harris's hematoxylin solution counterstain.39 The percentage of eosinophilic granule-containing cells was determined at a concentration of 20% cells at a magnification of ×400. The cells were also examined by electron microscopy. 

**Assays for Fce receptor II (FceRII/CD23) and eosinophil differentiation antigen (EO-1 antigen) expression.** Expression of FceRII/CD23 and EO-1 antigen, determined by mouse monoclonal antibodies H107 (mouse IgG₁ class,38 a gift from Dr J. Yodoi, Kyoto University) and EO-1 (mouse IgG₂ class),39 respectively, on EoL-1 cells was analyzed by indirect immunofluorescence with fluorescein isothiocyanate (FITC)-conjugated affinity purified goat anti-mouse IgG antibodies (Tago, Burlingame, CA) as the second antibodies, as described elsewhere.37 In each experiment, RPMI 8866 cells were applied in the same manner, as a positive control of FceRII expression. Surface EO-1 antigen was detected by almost the same procedures except that 1,000-times diluted EO-1 antibody-containing ascitic fluid was used, and unrelated mouse monoclonal IgG₁, a gift from Dr S. Nishikawa, Kumamoto University, served as a negative control.

**Effect of anti-TNF-α, anti-G-CSF, anti-IL-3, anti-GM-CSF, and anti-IL-5 antibodies on the induction of eosinophilic granule-containing EoL-1 cells.** The culture medium with additions to a final concentration of 10% HIL-3 sup, 50 ng/mL G-CSF, 10 U/mL TNF-α, or 50 ng/mL G-CSF + 10 U/mL TNF-α was preincubated with sufficient amounts of anti-TNF-α, anti-G-CSF, anti-IL-3, anti-GM-CSF, or anti-IL-5 antibodies for 10 minutes at 37°C with shaking. Then 5 × 10⁶ EoL-1 cells were suspended in 1 mL of these culture medium and cultured for 9 days at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. The culture medium was renewed with the same preincubated ones, and cell counts were adjusted to a concentration of 5 × 10⁶ cells/mL on days 3 and 6. Viable cells were counted in relation to those cultured without antibodies, and the percentage of eosinophilic granule-containing cells was determined.

**Assays for actin polymerization.** Actin polymerization in EoL-1 cells induced by PAF was analyzed by a slight modification of the method of Howard and Meyer.34,35 Briefly, cells were resuspended in HBSS at a concentration of 1 × 10⁶ cells/450 μL and preincubated for 15 minutes at 25°C. The cells were then incubated with or without 10⁻⁶ mol/L PAF at 25°C for 45 seconds or 90 seconds. Cells were fixed with 50 μL of 37% phosphate-buffered formalin, permeabilized, and stained in a single step with HBSS containing 1.65 × 10⁻⁷ mol/L nitrobenzoxadiazol (NBD)-phallacidin (Molecu lar Probes, Junction City, OR) and 100 μg/mL of lysophosphatidylcholine and were analyzed by FACS. In each sample, 10,000 light-scatter-gated cells were analyzed. Fluorescence data were collected with linear amplification, and specific cell immunofluorescence was expressed as channel numbers on a linear scale.

**Fractionation of EoL-1 cells on metrizamide discontinuous gradients.** Ten percent HIL-3 sup-induced EoL-1 cells were fractionated by density gradients by a slight modification of the method reported by Vadas et al.40 Briefly, we made a stock solution of 30% metrizamide (Sigma) in Tyrode's gel (each liter of which contained 1 g of KCl, 8 g of NaCl, 0.05 g of anhydrous Na₂HPO₄, and 0.1% of gelatin) and diluted it with Tyrode's gel to make solutions of 14% (w/v) gelatin. Four solutions were layered on top of 14% metrizamide discontinuous gradients. Each 100 μL of decreasing densities of the metrizamide solutions was placed in a 15 mL centrifuge tube (Falcon 2095; Becton Dickinson). On top of the solution we placed 5 to 10×10⁶ EoL-1
cells in 2 mL of the culture medium. Each tube contained 4 gradient steps (14%, 16%, 18%, and 20%), and the tube was then spun in a centrifuge at 1,200g for 30 minutes at room temperature. Cells were collected carefully at each interface.

Effect of supernatants of EoL-1 cells cultured with or without addition of G-CSF on eosinophilic differentiation of human cord blood mononuclear cells. Mononuclear cells were obtained from heparinized cord blood by the Ficoll-Paque density sedimentation procedure. The cord blood mononuclear cells were washed twice with PBS and suspended in the culture medium at a concentration of 1 × 10⁶ cells/mL. The proportions of contaminating eosinophils and basophils were less than 1%. The mononuclear cells were cultured for 3 weeks at 37°C in a humidified atmosphere of 5% CO₂ and 95% air supplemented with recombinant IL-5 or supernatants of EoL-1 cells cultured for 3 days with or without 50 ng/mL G-CSF. Half the medium was replaced once a week with the same culture medium. Viable cell numbers and their differential counts were documented every week.

Statistical analysis. Student’s t-test was used for statistical analysis.

RESULTS

Effects of HIL-3 sup on morphologic changes of EoL-1 cells. EoL-1 cells have the cytologic characteristics of myeloblasts under the usual culture conditions (Fig 1). Most of them have a round nucleus and no cytoplasmic granules, but less than 3% of unstimulated EoL-1 cells change spontaneously to eosinophilic granule-containing cells. EoL-1 cells, when cultured with HIL-3 sup at a final

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Fig 1. Electron microscopic photographs of EoL-1 cells. Unstimulated EoL-1 cells in normal culture medium (A) ×12,500, (B) ×50,000 amplification. EoL-1 cells cultured with 10% HIL-3 sup for 9 days (C) ×12,500, (D) ×50,000 amplification. Note the segmented nuclei and eosinophilic granules with cores of higher density and matrixes of lower density lacking crystalline cores that are characteristic of mature peripheral blood eosinophils.
concentration of 10%, decreased their cell growth rate, and cell viability, as estimated by trypan blue exclusion, was reduced from day 6 to day 9 (Fig 2), and the proportion of eosinophilic granule-containing cells increased to 24.0% ± 1.5% by day 9 (Fig 3). HIL-3 sup induced eosinophilic granule (+) cell differentiation in a dose-dependent manner; the maximum effect was seen at a final concentration of 10% (data not shown). Higher concentrations of HIL-3 sup decreased cell viability too much and seemed not suitable for examinations (data not shown). Electron microscopic analysis revealed that almost all eosinophilic granules in EoL-1 cells cultured for 9 days with 10% HIL-3 sup were diffuse and membrane-covered. Crystalline cores and matrices, which are characteristics of mature peripheral blood eosinophils, were seldom seen (Fig 1). The features of eosinophilic granules in HIL-3 sup-induced EoL-1 cells were thus similar to those of primary granules in eosinophilic promyelocytes.

Expression of cytokine mRNA and cytokines in HIL-3 cells and HIL-3 sup. Expression of IL-3, IL-5, G-CSF and M-CSF mRNA in HIL-3 cells was analyzed by Northern blot hybridization. As shown in Fig 4, mRNA of IL-5 and M-CSF was expressed in HIL-3 cells. There was little or no expression of IL-3 mRNA, and no expression of G-CSF mRNA. G-CSF was not detectable (less than 60 pg/mL) in HIL-3 sup by an enzyme immunoassay, either. GM-CSF was detected in HIL-3 sup at a concentration of 975.5 pg/mL. TNF-α at concentrations of 1718.2 pg/mL and 32.6

Fig 1. (Cont’d).
Number of viable cells (O, ●) and cell viability (□, ■)

(U/mL) was detected by a radioimmunoassay and by a bioassay, respectively.

Effects of cytokines on morphologic changes of EoL-1 cells. Because HIL-3 sup contained IL-2, TNF-α, and GM-CSF and might contain IL-5 and M-CSF as mentioned above, these defined cytokines as well as IL-3, IL-4, and G-CSF were examined for induction of eosinophilic granules in EoL-1 cells. IL-2, IL-4, and M-CSF did not increase the number of eosinophilic granule-containing cells at concentrations of 5 U/mL, 10 U/mL, and 10 ng/mL, respectively, when used alone or in combination with the other cytokines (data not shown). IL-3, IL-5, and GM-CSF also did not increase the number of eosinophilic granule-containing cells even at full concentrations of 10 to 100 U/mL, 10 to 100 ng/mL, and 10 to 100 ng/mL, respectively, when used alone or in combination with the other cytokines (data not shown). IL-5 (10 ng/mL) did not increase the number of eosinophilic granule-containing cells when used with TNF-α (10 U/mL). G-CSF at a concentration of 50 ng/mL enhanced the proportion of eosinophilic granule-containing cells up to 8.0% ± 1.2% (Table 1). TNF-α did not increase the number of eosinophilic granule-containing cells, but when used in combination with 50 ng/mL of G-CSF, it increased the proportion of eosinophilic granule-containing cells up to 18.0% ± 3.1% (Fig 3). The same experiments were also performed under serum-free conditions. The ability of the cytokines and HIL-3 sup to induce eosinophilic granule-containing cells was also seen in serum-free conditions, although cell growth and proportions of eosinophilic granule (+) cells under serum-free culture conditions were less than those seen under serum-containing culture conditions (Fig 5).

Synergy of G-CSF and TNF-α. As shown in Table 1, G-CSF slightly increased the cell growth of EoL-1 cells in a dose-dependent manner with a maximum of 30 ng/mL. On the other hand, TNF-α suppressed cell growth in a dose-dependent manner, and the number of cells containing vacuoles increased greatly (data not shown). Table 2 shows that the effect of G-CSF + TNF-α on the induction of eosinophilic granule-containing EoL-1 cells was not evident unless they were present simultaneously. The medium in which EoL-1 cells were cultured at an initial concentration of 5 × 10⁶ cells/mL with the addition of G-CSF (50 ng/mL) or G-CSF + TNF-α (50 ng/mL and 10 U/mL, respectively) for 3 days did not show a greater effect on differentiation of EoL-1 cells than did freshly prepared culture medium.
A NOVEL EOSINOPHILIC DIFFERENTIATION FACTOR

Fig 4. Northern blot analysis of cytokine mRNAs in HIL-3 cells. Marker positions of 28 s and 18 s ribosomal RNA on the filters are indicated by lines. cDNA probes used were IL-5 (lane 1), M-CSF (lane 2), IL-3 (lane 3) and G-CSF (lane 4).

containing the same cytokines (data not shown). The culture supernatants of EoL-1 cells did not have any effect on eosinophilic differentiation of cord blood mononuclear cells, either (data not shown).

Expression of FceRII/CD23 and EO-1 antigen on EoL-1 cells. Expression of FceRII/CD23 and an eosinophil differentiation antigen EO-1 on EoL-1 cells cultured with or without 10% HIL-3 sup was examined by flow cytometry. As shown in Fig 5, EoL-1 cells did not express FceRII/CD23 under normal culture conditions. An increase in the population of FceRII/CD23 (+) EoL-1 cells was detectable on day 3 when EoL-1 cells were cultured with 10% HIL-3 sup, and their proportion increased to 20% on day 9. RPMI 8866 cells used for a positive control of FceRII/CD23 expression were always more than 90% positive (data not shown).

Expression of EO-1 antigen, which is expressed on human peripheral blood eosinophils, basophils, and platelets, but not on neutrophils, was also assayed on days 3, 6, and 9. Unstimulated EoL-1 cells did not express this antigen at all. EoL-1 cells cultured with 10% HIL-3 sup began to express this antigen on day 6, and the proportion reached 17% on day 9. Interestingly, the combination of 10 U/mL TNF-α and 50 ng/mL G-CSF did not induce either FceRII/CD23 or EO-1 antigen expression (data not shown), although it induced eosinophilic granule-containing cells, as shown above.

Table 1. Effect of G-CSF and TNF-α on the Induction of Eosinophilic Granule (+) Cells

<table>
<thead>
<tr>
<th>Preincubation Time (d)</th>
<th>Culture Time (d)</th>
<th>Relative Viable Cell Numbers</th>
<th>Eosinophilic Granule (+) Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 G-CSF</td>
<td>6 G-CSF</td>
<td>110 ± 22</td>
<td>7.0 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-α</td>
<td>7.1 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>104 ± 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3.0 ± 1.2</td>
</tr>
<tr>
<td>3 TNF-α</td>
<td>6 G-CSF</td>
<td>102 ± 20</td>
<td>6.5 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TNF-α</td>
<td>58 ± 9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>97 ± 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2.5 ± 1.1</td>
</tr>
<tr>
<td>0 G-CSF</td>
<td>9 Medium</td>
<td>100</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>G-CSF + TNF-α</td>
<td>64 ± 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>17.5 ± 4.2</td>
</tr>
</tbody>
</table>

One milliliter of EoL-1 cells at a concentration of 5 x 10⁶ cells/mL was preincubated for 3 days with a final concentration of 50 ng/mL G-CSF or 10 U/mL TNF-α. The cells were then washed twice with PBS and resuspended at a concentration of 5 x 10⁶ cells/mL in the culture medium containing 50 ng/mL G-CSF or 10 U/mL TNF-α. Cells were cultured again for 6 days with renewal of the culture medium containing the same factors on day 3. Viable cell numbers in relation to those cultured without any additional cytokines for 9 days and percentage of eosinophilic granule-containing cells were determined. Data represent a mean ± 1 SD (n = 3).
was seen at concentrations of to \(10^{-10}\) mol/L PAF, and gradient procedures. 

or not EoL-1 cells show chemotaxis, the actin polymerization in EoL-1 cells cultured with or without 10% HIL-3 sup were stained with either FITC or PE. EoL-1 cells cultured with 10% HIL-3 sup or without HIL-3 sup. The percentages of eosinophilic granule (+) EoL-1 cells cultured with 10% HIL-3 sup are also shown (\(\Delta\)). The data represent a mean ± SD (n = 3).

Assays for actin polymerization. To determine whether or not EoL-1 cells show chemotaxis, the actin polymerization in EoL-1 cells by PAF was examined. As shown in Fig, EoL-1 cells cultured without HIL-3 sup showed a slight actin assembly change when stimulated with PAF. EoL-1 cells cultured with 10% HIL-3 sup for 9 days showed much more actin polymerization. Changes of actin polymerization and depolymerization occurred in a short time; the change returned partially to the unstimulated state after 90 seconds. The change was also temperature-dependent and greatest at 37°C (data not shown). Actin polymerization was seen at concentrations of \(10^{-10}\) to \(10^{-12}\) mol/L PAF, and it was greatest at a concentration of \(10^{-9}\) mol/L PAF (data not shown). The treatment of EoL-1 cells with the combination of TNF-α and G-CSF for 9 days did not induce the enhancement of actin polymerization on EoL-1 cells by PAF (data not shown).

Fractionation of EoL-1 cells by metrizamide discontinuous gradient procedures. EoL-1 cells cultured with 10% HIL-3 sup for 9 days were fractionated by a metrizamide discontinuous gradient. Approximately 30% to 40% of the original cells were recovered in our procedure. The morphology of the cells in the light fraction (top of 14% metrizamide solution) continued proliferation during the culture period, and G-CSF slightly enhanced the proliferation. G-CSF also induced an increase of eosinophilic granule-containing cells induced by G-CSF + TNF-α to the level in the culture with G-CSF alone. On the contrary, anti-TNF-α antibodies did not significantly decrease the effect of HIL-3 sup. The anti-G-CSF antibodies completely suppressed the effect of G-CSF. Anti-IL-3, anti-IL-5, and anti-GM-CSF antibodies did not affect cell growth or the percentage of eosinophilic granule-containing cells after 9 days of culture with 10% HIL-3 sup.

**DISCUSSION**

In this study, we showed that eosinophilic granules were produced in a human eosinophilic leukemia cell line EoL-1 by the culture supernatant of a human adult T-cell leukemia line HIL-3 and by G-CSF + TNF-α. The eosinophilic granules were round, diffuse, and enclosed in a membrane. They seldom showed the crystalline core and matrix configuration characteristic of mature peripheral blood eosinophils. The eosinophilic granules in EoL-1 cells were thus similar to those in eosinophilic promyelocytes. Because eosinophilic granule-containing EoL-1 cells have a seg-
Table 3. Metrizamide Discontinuous Gradient Fractionation of 10% HIL-3-Induced EoL-1 Cells

<table>
<thead>
<tr>
<th>Cell Recovery Rate (%)</th>
<th>Viability (%)</th>
<th>% Eosinophilic Granule (+) Cells</th>
<th>% H107 (+) Cells</th>
<th>% EO-1 Antigen (+) Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top layer of 14% metrizamide</td>
<td>11.0 ± 4.0</td>
<td>88.6 ± 3.2</td>
<td>0.5 ± 0.5</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>14/16% metrizamide interface</td>
<td>16.0 ± 6.2</td>
<td>87.2 ± 1.8</td>
<td>21.5 ± 3.2</td>
<td>6.0 ± 1.4</td>
</tr>
<tr>
<td>16/18% metrizamide interface</td>
<td>5.0 ± 1.5</td>
<td>76.6 ± 4.7</td>
<td>50.0 ± 8.5</td>
<td>5.8 ± 2.2</td>
</tr>
<tr>
<td>18/20% metrizamide interface</td>
<td>1.9 ± 1.6</td>
<td>35.0 ± 10.1</td>
<td>53.4 ± 7.3</td>
<td>14.4 ± 6.3</td>
</tr>
<tr>
<td>Bottom layer of 20% metrizamide</td>
<td>23.8 ± 10.3</td>
<td>1.3 ± 2.0</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

EoL-1 cells were cultured with 10% HIL-3 sup for 9 days and fractionated on metrizamide discontinuous gradients. The cells recovered in each fraction were then analyzed for cell number, viability, eosinophilic granules, and surface expression of FceRII/CD23 and EO-1 antigen. Data represent mean ± 1 SD (n = 3).

Abbreviation: ND, not done.

mented nucleus, they may represent a differentiation stage between eosinophilic promyelocytes and mature segmented

The HIL-3 sup-induced EoL-1 cells expressed FceRII/CD23 and EO-1 antigen on their surfaces, and experiments with metrizamide discontinuous gradient fractionation revealed that FceRII/CD23 and EO-1 antigen were expressed on eosinophilic granule (+) cells but not on eosinophilic granule (-) cells. These results show that these two surface antigens, FceRII/CD23 and EO-1 antigen, are probably differentiation antigens that appear at the stage of eosinophilic promyelocytes or later. On the other hand, the finding that G-CSF + TNF-α did not induce expression of FceRII and EO-1 antigen, although they increased the number of eosinophilic granule-containing cells, suggests the existence of other mechanisms to express FceRII and EO-1. Regarding IgE receptor expression on eosinophils, it has been reported that hypodense, but not normodense, eosinophils express low affinity IgE receptors and a monoclonal anti-FceRII/CD23 antibody H107 may recognize them (Dr M. Capron, personal communication). In our experiments, eosinophils induced by IL-5 in vitro from bone marrow mononuclear cells and cord blood mononuclear cells did not express FceRII/CD23 determined by H107 antibody and BB10 antibody (Akutagawa et al, unpublished data). These results, when combined, suggest that eosinophils induced from precursor cells by IL-5 may need further stimuli to express IgE receptors on their surfaces. The expression of IgE receptors on eosinophils needs further study.

HIL-3 cells were maintained in the presence of recombinant human IL-2, and mRNA of IL-5 and M-CSF was detected in HIL-3 cells. HIL-3 cells also secreted TNF-α and GM-CSF. However, IL-2, IL-4, and M-CSF had no effect on the induction of eosinophilic granules in EoL-1 cells. IL-3, IL-5, and GM-CSF, which have been proved to be involved in the differentiation of eosinophils, had no significant effect on the induction of eosinophilic granule

Fig 8. Effect of G-CSF on cell growth of eosinophilic granule (+) and (-) populations. EoL-1 cells cultured for 9 days with 10% HIL-3 sup were fractionated into a mature, eosinophilic granule (+) cell-containing population (A) and an immature, eosinophilic granule (-) cell population (B) by the metrizamide procedure. The two cell populations were cultured for 5 more days with or without the addition of 50 ng/mL G-CSF. Numbers of total viable cells cultured without G-CSF (O) and with G-CSF (●) and numbers of eosinophilic granule (+) cells cultured without G-CSF (△) and with G-CSF (●) were determined. The data represent a mean ± 1 SD (n = 3). *P < 0.05.
granule-containing cells were determined. Data represent a mean ± 1 SD (n = 3).

**Table 4. Effect of Anti-TNF-α, Anti-G-CSF, Anti-GM-CSF, Anti-IL-3, and Anti-IL-5 Antibodies on the Induction of Eosinophilic Granule (+) EoL-1 Cells**

<table>
<thead>
<tr>
<th>Culture With</th>
<th>Antibodies</th>
<th>Relative Viable Cell Numbers (+) Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% HIL-3 sup</td>
<td>Medium</td>
<td>100</td>
</tr>
<tr>
<td>Anti-TNF-α</td>
<td>102 ± 11</td>
<td>23.5 ± 3.3*</td>
</tr>
<tr>
<td>Anti-GM-CSF</td>
<td>93 ± 3</td>
<td>24.0 ± 1.6*</td>
</tr>
<tr>
<td>Anti-IL-3</td>
<td>95 ± 13</td>
<td>21.0 ± 4.1*</td>
</tr>
<tr>
<td>Anti-IL-5</td>
<td>104 ± 9</td>
<td>24.3 ± 2.8*</td>
</tr>
<tr>
<td>G-CSF</td>
<td>Medium</td>
<td>100</td>
</tr>
<tr>
<td>Anti-G-CSF</td>
<td>90 ± 15</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Medium</td>
<td>100</td>
</tr>
<tr>
<td>Anti-TNF-α</td>
<td>127 ± 20</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>G-CSF + TNF-α</td>
<td>Medium</td>
<td>100</td>
</tr>
<tr>
<td>Anti-TNF-α</td>
<td>114 ± 18</td>
<td>6.5 ± 2.5f</td>
</tr>
<tr>
<td>Anti-G-CSF</td>
<td>87 ± 13</td>
<td>3.0 ± 1.4f</td>
</tr>
</tbody>
</table>

EoL-1 cells were cultured for 9 days with the cytokines and antibodies (Materials and Methods). Viable cell numbers in relation to those cultured without the antibodies and the percentage of eosinophilic granule-containing cells were determined. Data represent a mean ± 1 SD (n = 3).

*P = > .05.

†P = < .01.

(+ ) EoL-1 cells, either. On the contrary, G-CSF and G-CSF + TNF-α increased the number of eosinophilic granule (+ ) EoL-1 cells. Because the effect of HIL-3 sup on the differentiation of EoL-1 cells was not suppressed by the addition of sufficient amounts of antibodies against G-CSF, TNF-α, GM-CSF, IL-3, and IL-5, and because G-CSF was undetectable in HIL-3 sup, all the data suggest that HIL-3 sup contains an undefined factor(s) that induces maturation and/or differentiation of eosinophils. HIL-3 sup also induced preferential growth of eosinophils in cord blood and bone marrow mononuclear cells, and the effect of HIL-3 sup was not decreased by anti-IL-3 and anti-IL-5 antibodies (Akutagawa et al, manuscript in preparation). The HIL-3-derived factor(s) is stable when heated at 65°C for 30 minutes but loses its effect when treated at 100°C for 2 minutes and its molecular weight is greater than 30 Kd (unpublished data). Purification of the factor and detection of the gene that encodes this factor is now in progress.

Regarding the effect of G-CSF and TNF-α, it seemed necessary for these two factors to exist simultaneously for satisfactory induction of eosinophilic granule (+ ) EoL-1 cells. There is a possibility that TNF-α may increase the expression of G-CSF receptors on EoL-1 cells, thus increasing the effect of G-CSF. However, the fact that preincubation of EoL-1 cells with TNF-α for 3 days did not increase G-CSF-induced eosinophil differentiation suggests that this may not be the case. Few eosinophils were seen when cord blood mononuclear cells were cultured with G-CSF, and TNF-α did not have a synergistic effect (Akutagawa, unpublished data).

Whether HIL-3 sup and G-CSF + TNF-α really induce the differentiation of EoL-1 cells or merely prolong the life span of spontaneously differentiated EoL-1 cells is important, but we have no direct evidence to show which is the case. Both G-CSF + TNF-α and HIL-3 sup decreased cell growth and cell viability, suggesting that these factors may not prolong the life span of differentiated cells. Because G-CSF increased slightly the proliferation of immature, eosinophilic granule (- ) EoL-1 cells but had no significant effect on the survival of mature, eosinophilic granule (+ ) EoL-1 cell-containing population, we have no supportive data that show that G-CSF prolongs the survival of spontaneously differentiated EoL-1 cells. The effect of G-CSF on immature EoL-1 cells is presumably transduced via G-CSF receptors. The precise analysis of the G-CSF receptor expression and its regulation on EoL-1 cells during their differentiation processes is the subject of our next experiment.

Regarding the effect of G-CSF on EoL-1 cells, there might be a possibility that G-CSF induces EoL-1 cells to secrete a factor that stimulates the differentiation of EoL-1 cells. However, none of our findings support this possibility.

A comparison of the effects of G-CSF + TNF-α and HIL-3 sup with those of dimethyl sulfoxide (DMSO) and alkaline medium, which were first reported to be effective, on the induction of differentiation of EoL-1 cells was not successful because the latter did not induce any eosinophil differentiation of EoL-1 cells (data not shown). The EoL-1 cells used in our experiments seemed to lose their ability to differentiate when stimulated by DMSO and pH during passage. Although EoL-1 cells are leukemia cells and may not have the characteristics of normal precursors of eosinophils, all data suggest that EoL-1 cells and HIL-3 sup provide a useful model for experiments on the mechanisms of differentiation and the functions of eosinophils.

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A NOVEL EOSINOPHILIC DIFFERENTIATION FACTOR


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