Growth and Differentiation of a Human Megakaryoblastic Cell Line, CMK

By Norio Komatsu, Toshio Suda, Masaaki Moroi, Naomi Tokuyama, Yoichi Sakata, Masayuki Okada, Tsutomu Nishida, Yoshikatsu Hirai, Takeyuki Sato, Akira Fuse, and Yasusada Miura

Recently, a human megakaryoblastic cell line, CMK, was established from the peripheral blood of a megakaryoblastic leukemia patient with Down syndrome. Using this cell line, we studied the proliferation and differentiation of megakaryocytic cells in the presence of highly purified human hematopoietic factors and phorbol 12-myristate-13-acetate (PMA). In a methylcellulose culture system, interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) facilitated colony formation by CMK cells in a dose-dependent manner. The maximum stimulating doses of these factors were 10 and 200 U/mL, respectively. These concentrations were comparable to those that stimulate activity in normal hematopoietic cells. In contrast, granulocyte-colony stimulating factor (G-CSF), macrophage-colony stimulating factor (M-CSF), and erythropoietin (EPO) had no effects on the colony formation of CMK cells. In a liquid culture system, 20% of the CMK cells expressed glycoprotein IIb/IIIa (GPIIb/IIIa) antigen without hematopoietic factors, whereas 40% of the cells expressed GPIIb/IIIa with the addition of IL-3 and GM-CSF. EPO also slightly enhanced expression of GPIIb/IIIa. On the other hand, PMA inhibited growth of CMK cells and induced most of them to express the GPIIb/IIIa antigen. Furthermore, PMA induced CMK cells to produce growth activity toward new inocula of CMK cells. This growth factor (GF) contained colony-stimulating activity (CSA) in normal bone marrow (BM) cells. The activity was believed to be attributable mainly to GM-CSF, since 64% of this activity was neutralized by anti-GM-CSF antibodies and a transcript of GM-CSF was detected in mRNA from PMA-treated CMK cells by Northern blot analysis. These observations suggest that GM-CSF, as well as IL-3, should play an important role in megakaryocytopoiesis.

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Materials and Methods

Preparation of a Human Megakaryoblastic Cell Line. A human megakaryoblastic cell line, CMK, was established from the PB of a patient with acute megakaryoblastic leukemia.7 CMK cells were cultured in a liquid culture system with Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS). One-half of the medium was replaced every three or four days.

Hematopoietic Factors. Recombinant human interleukin-1α (IL-1α) and interleukin-1β (IL-1β) with specific activities of 1.5 x 10^5 and 4.4 x 10^5 U/mg, respectively, were provided by Otsuka Pharmaceutical (Tokushima, Japan). Supernatants of COS cells transfected with cDNA of IL-3 were used as human IL-3. EPO was provided by Sankyo Pharmaceutical Co., Ltd. (Tokyo, Japan). Recombinant human GM-CSF and erythropoietin (EPO) with specific activities of 5.0 x 10^5 and 1.7 x 10^6 U/mg, respectively, were provided by Chugai Pharmaceutical (Tokyo, Japan). Native human M-CSF with a specific activity of 1.4 x 10^6 U/mg was provided by Dr. K. Motoyoshi of Jichi Medical School.

For the anti-GM-CSF antibody, a monoclonal antibody (MoAb) IgG, anti-GM-CSF was purchased from Genzyme (Boston, MA). The half-maximal neutralizing activity for 200 U GM-CSF is achieved with a dilution of 1:62,500. Complete neutralization can be obtained with a dilution of 1:250.

Analysis of Surface Markers by Immunofluorescence. Cell surface antigens were detected by immunofluorescence assay with the following MoAbs: megakaryocyte-directed GPIIb/IIIa (Immunotech, Marseille, France); Plt-1 (Coulter Immunology, Hialeah, FL); myeloid cell- and monocyte-directed MY9 (Coulter) and OKM5 (Ortho Diagnostic Systems, Raritan, NJ); and erythroid cell-directed, anticytochrome-A (GlyA) antibody was donated by Dr. S. Ikemoto of Jichi Medical School. Surface marker studies were performed as follows: CMK cells were incubated for 30 minutes at 4°C with the appropriately diluted MoAbs. After washing procedures, the cells were reincubated with fluorescent goat anti-mouse IgG.
(FITC-GAM) (Tago, Burlingame, CA) for 30 minutes at 4 °C. After a second washing, fluorescence analysis was performed with an Ortho Spectrum III (Ortho Diagnostic Systems).

Colony assay. The colony assay was performed according to a modification of the method of Fauser and Messner. The CMK or human BM cells were suspended in medium containing 0.9% methylcellulose (Dow Chemical, Midland, MI), 30% AB blood-type platelet-poor plasma (PPP), 5 x 10^{-3} mol/L 2-mercaptoethanol (2-ME, Eastman Organic Chemical, Rochester, NY), 1% bovine serum albumin (BSA, Sigma Chemical, St Louis). Purified growth factors (GFs) were added to the culture dishes at various concentrations. One-milliliter aliquots of culture medium containing 250 CMK cells or 2.5 x 10^6 BM cells were cultured in 35-mm non-tissue culture dishes (Falcon, Oxnard, CA) at 37 °C in a 5% CO2 humidified atmosphere. Incubated plates were cultured for nine or 14 days and were counted with an inverted microscope. A colony formed by CMK was defined as a cell cluster containing >100 cells.

Defined reagents. PMA (Sigma Chemical) was stored at -80 °C, in solution, in dimethylsulfoxide (DMSO). Just before use, PMA was diluted in the IMDM culture medium and added to the liquid culture systems.

Measurements of amounts of GPIIb/IIIa. CMK cells were activated with 10 ng/mL PMA and incubated for one to five days as described above. Cells in a flask (4 x 10^6) were harvested every other day, washed twice with Tris buffer saline (TBS), and dissolved in 1% Triton X-100/5 mmol/L EDTA/10 mmol/L N-ethylmaleimide. The supernatants were separated by centrifugation (15,000 g, five minutes). The amounts of protein in the supernatants were measured by Lowry's method with BSA as a standard. Five milligrams of each sample was applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to nitrocellulose paper. The blotted paper was reacted with an antibody which was made against platelet membranes and showed strong reactivity against GPIIb and GPIIIa. The bands were visualized by incubation with 0.02% 3,3'-diaminobenzidine and 0.005% H2O2 in TBS. The stained bands were quantitated by densitometry using a Shimadzu dual-wavelength TLC scanner CS-930 (Shimadzu Seisakusho, Kyoto, Japan), and the total area was calculated by using the total protein amount of the fraction. The relative amounts of GPIIb and GPIIIa were expressed as percentages of the calculated values of nonstimulated cells.

Preparation of PMA-treated CMK-conditioned medium (PMA-CMK-CM). CMK cells in a density of 4 x 10^4/mL were cultured for two days in 25-cm² flasks (Falcon) with 10% FCS and 10 ng/mL PMA at 37 °C in a 5% CO2 humidified atmosphere. The supernatant was harvested and stored at 4 °C until used.

RNA preparation and Northern blot analysis. CMK cells at 1 x 10^6 cells/mL (10 mL/Falcon 3003 dish) in IMDM supplemented with 10% FCS with or without 10 ng/mL PMA were incubated at 37 °C for the indicated period. Poly(A)^+ RNA was prepared by conventional methods, denatured with glyoxal-DMSO, electrophoresed on a 1.2% agarose gel, and blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, FRG). The membranes were prehybridized and hybridized at 42 °C for 18 hours with a nick-translated probe in a solution containing 50% formamide, 5 x SSC (1 x SSC: 0.15 mol/L sodium chloride, 0.015 mol/L sodium citrate), 50 mmol/L sodium phosphate (pH 6.5), 4 x Denhardt's (1 x Denhardt's: 0.2 g/L BSA, 0.2 g/L sodium chloride, 0.2 g/L sodium citrate), 50 mmol/L sodium phosphate (pH 6.5), 4 x Denhardt's (1 x Denhardt's: 0.2 g/L BSA, 0.2 g/L sodium chloride, 0.2 g/L sodium citrate).
RESULTS

Effect of purified hematopoietic factors (IL-3, GM-CSF, G-CSF, M-CSF, EPO, IL-1α and β) on colony formation by CMK cells. We initially investigated the effect of purified recombinant hematopoietic factors on colony formation by CMK cells with the methylcellulose culture method. Small clusters containing <50 were formed when hematopoietic factors were not added (Fig 1 A). Addition of IL-3 or GM-CSF stimulated formation of colonies containing >100 cells in a dose-dependent manner (Figs 1B and 2). The maximum number of colonies formed by IL-3 or GM-CSF were almost equal. M-CSF, G-CSF, EPO, IL-1-α, which is identical to hematopoietin-12 or IL-1-β did not stimulate colony formation.

Effect of hematopoietic factors on expression of surface markers of CMK cells. The number of CMK cells expressing GPIIb/IIIa, PII-1, MY9, OKM5, and GlyA antigens was counted using immunofluorescence assay. As shown in Table 1. GPIIb/IIIa, which is a typical indicator of mature megakaryocytes, was expressed in 20% of the CMK cells without stimuli. Approximately 40% of the CMK cells expressed GPIIb/IIIa with addition of IL-3 and GM-CSF. EPO slightly amplified expression of GPIIb/IIIa. MY9 and OKM5, specific markers of myeloid and monocytic lineages, respectively, were expressed in most unstimulated CMK cells, and the expression did not change with addition of IL-3, GM-CSF, or EPO. GlyA, a specific marker of erythroid lineage, was expressed in few cells and was not enhanced by addition of EPO. Apparent morphologic changes were not observed after treatments with various kinds of CSFs.

Effect of PMA on growth and differentiation of CMK cells. As shown in Fig 1, colony formation was not observed in the presence of PMA. While nontreated CMK cells proliferated logarithmically and reached a plateau at the fifth day of liquid culture (Fig 3), PMA-treated CMK cells lost their proliferative capacity. These PMA-treated CMK cells showed morphologic characteristics of mature megakaryocytes with multilobular nuclei (Fig 1C and D). In accordance with this finding, expression of GPIIb/IIIa was

Table 1. Effect of PMA and Hematopoietic Factors on Expression of CMK Cell Surface Markers

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>GPIIb/IIIa (%)</th>
<th>PII-1 (%)</th>
<th>MY9 (%)</th>
<th>OKM5 (%)</th>
<th>GlyA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>22.6</td>
<td>3.8</td>
<td>99.1</td>
<td>92.7</td>
<td>1.7</td>
</tr>
<tr>
<td>IL-3 (10 U/mL)</td>
<td>43.2</td>
<td>6.5</td>
<td>94.3</td>
<td>89.7</td>
<td>2.6</td>
</tr>
<tr>
<td>GM-CSF (200 U/mL)</td>
<td>40.0</td>
<td>7.3</td>
<td>96.8</td>
<td>86.5</td>
<td>1.5</td>
</tr>
<tr>
<td>EPO (1 U/mL)</td>
<td>33.0</td>
<td>3.5</td>
<td>98.4</td>
<td>91.8</td>
<td>0.2</td>
</tr>
<tr>
<td>PMA (10 ng/mL)</td>
<td>99.5</td>
<td>71.0</td>
<td>99.0</td>
<td>99.5</td>
<td>14.4</td>
</tr>
</tbody>
</table>

CMK cells 5 x 10⁶/mL were incubated for five days with PMA and GFs as shown and prepared for analysis of surface markers; 1 x 10⁶ cells were examined by immunofluorescence in each sample.
increased, i.e., cells positive for GPIIb/IIIa antigen increased from 23% to >99% after addition of PMA (Table 1). The amounts of GPIIb and GPIIIa in the total Triton-soluble protein of CMK cells began to increase on the second or third day after treatment of PMA and were highest on the fourth or fifth day (Fig 4A). The maximum amount of GPIIb and GPIIIa reached 12 and ten times those of nontreated CMK cells, respectively. A precursor form of GPIIb was detected in the Triton-insoluble fraction but not in the Triton-soluble fraction as reported previously. Most of the mature form of GPIIb and all of the GPIIIa were in the Triton-soluble fraction.

Effect of media conditioned by CMK cells on colony formation by CMK cells and normal BM cells. Since two CSFs (IL-3 and GM-CSF) and PMA stimulated expression of GPIIb/IIIa by CMK cells, we tested the possibility of whether PMA-treated CMK cells produce some humoral factors, such as IL-3 or GM-CSF. Initially, we studied the effect of PMA-treated CMK cell supernatant on new inocula of CMK cells by colony assay. As shown in Fig 4B, growth activity of the supernatant was detected after six hours of culturing and reached a plateau at 12 or 24 hours. Even if conditioned medium (CM) of one-day culture was replaced by fresh medium with or without PMA, we could barely detect the growth activity in refreshed CM (data not shown). These results suggest that CMK cells lose their capacity to produce the GF(s) within one day after treatment with PMA. However, the amount of GPIIb/IIIa did not increase during that time (Fig 4A).

When the CM of PMA-treated CMK cells (PMA-CMK-CM) was added to normal BM cells, it stimulated colony formation derived from erythroid (BFU-E), CFU-GM, CFU-Meg, and mixed (CFU-Mix) colony-forming cells. The conditioned medium of untreated CMK cells (CMK-CM) and medium containing PMA (PMA-CM) did not stimulate any hematopoietic progenitor growth (Table 2). These findings indicate that PMA-CMK-CM contains CSF(s) with burst-promoting activity, GM-CSF, and Meg-CSF.

Detection of mRNA of GM-CSF and IL-3 from PMA-treated and nontreated CMK cells with Northern blot hybridization. Figure 5 shows the results of Northern blot analysis of GM-CSF mRNA. After exposure to PMA for six hours, a GM-CSF signal was detected in CMK cells, but was undetectable in original CMK cells. The size of the 1.0-kb GM-CSF transcript corresponded to the expected genomic RNA, suggesting that the GM-CSF signal from PMA-
treated CMK cells is normally transcribed to its product. On the other hand, the IL-3 mRNA was not detectable in original cells or in PMA-treated CMK cells at six or 16 hours (data not shown).

Neutralization of growth activity in PMA-CMK-CM with anti-GM-CSF antibody. The GF(s) produced from PMA-treated CMK cells was believed to be mainly GM-CSF since (a) the factor is a type of pluripoietin, (b) CMK cells respond to GM-CSF, and (c) mRNA of GM-CSF was detected by Northern blot analysis. Table 3 shows the effect of anti-GM-CSF antibody on colony formation by new incula of CMK cells with addition of PMA-CMK-CM, GM-CSF, or IL-3 to the culture. With addition of anti-GM-CSF antibody (1:250 dilution) to the culture, colony formation was obviously reduced in the presence of PMA-CMK-CM (1%) or GM-CSF (200 U/mL), whereas colony formation was not affected in cultures containing IL-3 (10 U/mL). The number of small clusters formed in the absence of stimuli did not decrease with addition of anti-GM-CSF antibody. Moreover, in liquid culture, the growth of CMK cells was not suppressed by anti-GM-CSF antibody (data not shown). These findings suggest that GM-CSF, which is produced from CMK cells after treatment with PMA, is not essential for autonomous growth of CMK cells.

Relationship between expression of GPIIb and its precursor and production of GF(s) from PMA-treated CMK cells. As shown above, PMA could induce CMK cells not only to differentiate into the cells expressing some megakaryocytic properties, such as GPIIb/IIIa antigen, but also to produce GF(s) including GM-CSF. Growth activity released from PMA-treated CMK cells reached a plateau on day 1 of culture, while expression of GPIIb/IIIa was not induced (Fig 4). Therefore, we tested whether GF(s) produced by PMA-treated CMK cells facilitated expression of GPIIb by PMA-treated CMK cells.

After incubation with 10 ng/mL PMA in a 25-cm² flask for one day, the cells were washed three times with PBS and subsequently incubated for four days under various conditions as shown in Table 4. Since the growth activity in PMA-CMK-CM was estimated to be ~10,000 U/mL GM-CSF from the result of colony assay by using CMK cells, we added 10,000 U/mL GM-CSF to the culture instead of PMA-CMK-CM. After we harvested the cultured cells, we performed quantitative analysis of GPIIb and its precursor. As shown in Table 4, there were no differences in the amounts of GPIIb and its precursor form among the various incubation conditions of PMA-treated CMK cells. This result showed that (a) after exposing PMA to CMK cells for

Table 2. Effect of PMA-CMK-CM on Colony Formation by Normal BM Cells

<table>
<thead>
<tr>
<th>Stimulus*</th>
<th>No. of Colonies/2.5 x 10⁶ BM Cells</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>EPO (1 U/mL)</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>EPO + CMK-CM†</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>EPO + PMA-CM</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>EPO + PMA-CMK-CM</td>
<td>99 ± 15</td>
</tr>
</tbody>
</table>

CM was prepared after 48-hour culture. Normal human nonphagocytic BM cells were incubated with CMK-CM, PMA-CM, or PMA-CMK-CM with EPO added. On day 14 of methylcellulose culture, each colony was observed under an inverted microscope. Data are mean ± SD of triplicate cultures.

*CMK-CM, supernatant of untreated CMK cells; PMA-CM, medium containing 10 ng/mL PMA; PMA-CMK-CM, supernatant of 10 ng/mL PMA-treated CMK cells.
†CM 10%.

Table 3. Neutralization of Growth Activity in PMA-CMK-CM With Anti-GM-CSF Antibody

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Anti-GM-CSF Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−</td>
</tr>
<tr>
<td>None*</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>PMA-CMK-CM (1%)</td>
<td>84 ± 11†</td>
</tr>
<tr>
<td>GM-CSF (200 U/mL)</td>
<td>72 ± 1</td>
</tr>
<tr>
<td>IL-3 (10 U/mL)</td>
<td>89 ± 8</td>
</tr>
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</table>

Two hundred fifty untreated CMK cells were plated and cultured for nine days with stimulus and with or without anti-GM-CSF antibody (1:250). Data are mean ± SD of triplicate cultures.

*Clusters (24 ± 1) (a cluster was defined as a cell aggregation consisting of <100 cells) were formed without addition of anti-GM-CSF antibody, whereas clusters (27 ± 1) were formed with addition of anti-GM-CSF antibody.
†Number of colonies formed by CMK cells.
A human megakaryoblastic cell line, CMK, was established from an acute megakaryoblastic leukemia patient with Down syndrome. The original leukemia blasts and CMK cells, although they also have specific markers for myeloid, monocytic, and erythroid lineages, were identified as megakaryoblasts for the following reasons: (a) the platelet peroxidase reaction; (b) the expression of GPIIb/IIIa; and (c) the detection of the mRNA of c-sis gene, the product of which is identical to the B chain of platelet-derived GF.\(^5\)

In this study, we also showed that (a) the cells were promoted to proliferate and express GPIIb/IIIa by addition of hematopoietic factors; (b) almost all the cells expressed the GPIIb/IIIa antigen with the addition of PMA; and (c) after PMA treatment, the cells secreted hematopoietic factors.

By using the colony assay, we showed that IL-3 and GM-CSF enhanced proliferation of CMK cells. This finding is consistent with previous reports that both IL-3 and GM-CSF haveMeg-CSAs.\(^6,7\) Moreover, immunofluorescence analysis of the surface markers of CMK cells with a MoAb against GPIIb/IIIa antigen revealed that they differentiated into the cells having properties of mature megakaryocytes. These findings are compatible with previous reports that IL-3 promotes maturation of a single megakaryocyte and that highly purified small megakaryocytes respond to GM-CSF with significant increase in the thymidine or amino-acid incorporation rates.\(^8\) Whether EPO has an effect on megakaryocytic colony formation is unclear.\(^9,10\) Although EPO did not stimulate colony formation by CMK cells, EPO slightly enhanced the expression of GP-11b/IIIa. This finding supports the observation that megakaryocytes have EPO receptors and show maturation when EPO is added.\(^11\)

PMA, a potent tumor promoter, is mitogenic in human lymphocytes. In certain leukemia cells, such as the HL-60 promyelocytic leukemia cell line, PMA induces terminal differentiation and consequently causes the cells to lose their growth potential.\(^12,13\) Moreover, PMA functions as a source of megakaryocyte potentiator activity in mice. Long, et al reported that PMA not only can stimulate murine megakaryocyte colony formation, but also can induce immature megakaryocytes into large single megakaryocytes.\(^14,15\) Therefore, we examined the effect of PMA on growth and differentiation of CMK cells. After addition of PMA to the liquid culture system, CMK cells lost their proliferation potential and expressed some characteristics of mature megakaryocytes.

We showed that PMA induces CMK cells to produce growth activity toward new inocula of CMK cells. Therefore, we suggest that this is a positive feedback regulation that differentiated CMK cells produce to stimulate growth of their premature ancestors. The conditioned medium of PMA-treated CMK cells could support the formation of BFU-E, GM, Meg, and mixed colonies by normal BM cells, which indicated that the CM contained a few types of CSFs or a pluripoietin, such as IL-3 or GM-CSF.\(^16,17\) Because more than 60% of the activity toward new inocula of CMK cells was neutralized by anti-GM-CSF antibody, these activities were believed to be attributable mainly to GM-CSF. Northern blot analysis revealed that PMA-stimulated CMK cells expressed GM-CSF mRNA, but not that of IL-3. Thus, GM-CSF may play an important role in growth and differentiation of megakaryocytic cells. Similar PMA action has been reported in the case of the human leukemic myeloid cell line HL-60.\(^18\) In CMK cells, the two phenomena occurred sequentially with differentiation; i.e., production of CSF(s) was observed within one day of culture and expression of GPIIb/IIIa was induced more than two days after addition of PMA. However, the existence of CSF(s) produced by CMK cells or GM-CSF is not likely to be essential to subsequent expression of GPIIb/IIIa.

Although CMK is an immortalized cell line, our observation that CMK cells can respond to IL-3 and GM-CSF will be useful in studying IL-3 and GM-CSF receptors and the mechanisms of signal transduction of these factors. Moreover, since PMA can induce CMK cells to express some characteristics of mature megakaryocytes, these cells will also be a useful model in elucidating the biosynthetic process of megakaryocyte platelet-specific proteins.

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