A Novel Human Myeloid Leukemia Cell Line, NKM-1, Coexpressing Granulocyte Colony-stimulating Factor Receptors and Macrophage Colony-stimulating Factor Receptors

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ABSTRACT

A novel human myeloid leukemia cell line, NKM-1, was established from a patient with acute myeloid leukemia (FAB classification M2). The cells were positive for myeloperoxidase staining and cluster of differentiation 15 cell surface antigen. Radiolabeled recombinant human granulocyte (G) colony-stimulating factor (CSF) was used, and 60 specific differentiation cell surface antigen. Radiolabeled recombinant human granulocyte-macrophage CSF, or macrophage (M) CSF, NKM-1 cells also expressed M-CSF receptors detected by c-fms mRNA expression. In concordance with the receptor expression, NKM-1 cells proliferated in response to exogenous G-CSF or M-CSF in a dose-dependent manner (0.1-100 ng/ml), while interleukin-3 or granulocyte-macrophage CSF had no effect. Colony-forming capacity of NKM-1 cells in semisolid agar was also enhanced with the addition of 10 ng/ml of G-CSF or M-CSF but decreased at higher concentrations. During CSF stimulation, no remarkable changes were observed morphologically and phenotypically. The stimulatory effect of G-CSF and M-CSF on the cell growth was additive. Neither G-CSF-binding capacity nor c-fms mRNA expression was altered by pretreatment with M-CSF or G-CSF, respectively. This cell line may provide a useful in vitro model for the study of CSF roles in myeloid leukemia cell proliferation.

INTRODUCTION

AML is characterized by excessive proliferation of myeloid precursors and maturation arrest resulting in accumulation of immature hematopoietic cells. In the presence of appropriate growth factors, leukemic cells proliferate in suspension culture or form colonies in semisolid media. Recent studies describing the effects of recombinant human hematopoietic growth factors on AML blasts have revealed that cells from most patients proliferate in response to IL-3 (1-4), GM-CSF (1-9), G-CSF (1-5, 9, 10), or M-CSF (4,11). The ability of a growth factor to exert its biological effect is linked to the expression of receptors specific for that factor on the surface of the cells, although there is no direct correlation between the ability to respond to a CSF and the absolute number of receptors expressed for that factor (12,13). This lack of correlation may be due to the heterogeneity of the leukemic cell population in a patient or to the fact that receptor levels are measured on whole leukemic cell populations, which may not accurately reflect receptor levels for each CSF on relatively rare clonogenic cells. Therefore, it would be advantageous to use a homogeneous cell population when we study how CSFs regulate proliferation of myeloid leukemic cells.

Permanent cell lines of myeloid leukemia cells are considered to be the clonal expansion of hematopoietic stem cells at some stage of maturation. Among established myeloid cell lines, however, proliferative responses to exogenous CSFs were rarely observed. This may be accounted for by the rarity of the expression of CSF receptors on cell lines (13) or by the diverse signal transductions through the binding to its receptors. Here we present a novel human myeloid leukemia cell line, NKM-1, which expresses both G-CSF receptors and M-CSF receptors. The cells proliferate remarkably in response to exogenous G-CSF and M-CSF.

MATERIALS AND METHODS

Case History. The cell line described in this report was derived from a peripheral blood sample of a 33-year-old male with AML. On October 19, 1981, he was admitted to Daido Hospital (Nagoya, Japan) because of gingival bleeding and epigastrical. Physical examination revealed mild splenomegaly and numerous ecchymotic spots. Peripheral blood cell counts at the time of admission were as follows: RBCs, 3.65 × 1012/liter; platelets, 17 × 109/liter; and WBCs, 108 × 109/liter with 74% leukemic blasts. The number of fangic bone marrow nucleated cells was 450 × 109/liter with 88.4% blasts. Both prothrombin time and partial thromboplastin time were markedly prolonged, the plasma fibrinogen level was 95 mg/dl, and the serum fibrinogen degradation products value was 80 µg/ml. AML [FAB classification M2 (14)] with disseminated intravascular coagulation was diagnosed. On the second hospital day, he suddenly went into a coma and computed tomography scanning showed a massive cerebroventricular hemorrhage. He died the same day.

Cell Culture. On October 19, 1981, a heparinized peripheral blood sample was obtained with the patient’s consent and brought to the First Department of Internal Medicine, Nagoya University. The mononuclear cells were separated by Ficoll-Conray gradient centrifugation and plated in Falcon 3013 plastic tissue culture flasks (Falcon, Division of Becton Dickinson, Oxnard, CA) at 105 cells/ml in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 20% FCS (Flow Laboratories, Stanmore, New South Wales, Australia), aqueous penicillin G (100 units/ml), and streptomycin (50 µg/ml) and were incubated at 37°C with a humidified atmosphere of 5% CO2. Cultures were fed once weekly by partial replacement of spent medium with the fresh medium. No conditioned media or feeder cells were used.

Morphological Studies. The cells were stained with MGG, MPO, and dual esterase staining for CAE and NBE according to the standard.

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3 The abbreviations used are: AML, acute myeloid leukemia; IL-3, interleukin-3; CSF, colony-stimulating factor; GM-CSF, granulocyte-macrophage CSF; G-CSF, granulocyte CSF; M-CSF, macrophage CSF; FCS, fetal calf serum; MGG, May-Grünwald-Giemsa; MPO, myeloperoxidase; CAE, naphthol AS-D chloroacetate esterase; NBE, α-naphthyl butyrate esterase; CD, cluster of differentiation; NBT, nitroblue tetrazolium; TPA, 12-O-tetradecanoylphorbol-13-acetate; SB, sodium butyrate; MT, 3,4-(5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Kd, dissociation constant.
procedures. Ultrastructural morphology (conventional thin electron microscopy) and MPO were analyzed as described previously (15).

Cell Surface Markers. Cell surface antigens were determined by an indirect immunofluorescent method using monoclonal antibodies and analyzed by flow cytometry (EPICS PROFILE, Coulter Electronics, Inc., Hialeah, FL) as described previously (15). The following monoclonal antibodies were used for the analysis. CD numbers were used from the designation of the Fourth International Workshop and Conference on Human Leukocyte Differentiation Antigens. 9.6 (CD2), 64.1 (CD3), 10.2 (CD5) and 7.2 (HLA-DR) were provided by Dr. I. A. Hansen (Fred Hutchinson Cancer Research Center, Seattle, WA). IG10 (CD15) was the generous gift of Dr. I. Burnstein, Fred Hutchinson Cancer Research Center. 4A (16) (CD7), NL-1 (17) (CD10), and HPL3 (18) (CD41) were produced locally. B4 (CD19), B1 (CD20), MO1 (CD11b), MY7 (CD13), MY4 (CD14), and MY9 (CD33) were purchased from Coulter Co. Glycophorin A (erythrocytes) (Immunotech, Marseille, France), Leu 11b (CD16) (Becton Dickinson Co.), anti-CD18 (CD18), and anti-low affinity Fcγ receptor (CDw32) (Cosmo Bio Co., Tokyo, Japan) were also used.

Chromosome Analysis. The cultured cells were incubated in the presence of Colcemid (GIBCO Laboratories) at 0.05 µg/ml, for 1 h at 37°C, then treated with 75 mMol/liter KCl hypotonic solution for 30 min at 37°C and fixed by methanol:acetic acid (3:1). Chromosomes were banded by the trypsin-Giemsa methods. Karyotypes were constructed from photographic enlargements according to the Paris Conference classification scheme.

NBT Reduction Test and Phagocytosis. The NBT (Sigma Chemical Co., St. Louis, MO) reduction test was performed according to the method of Park et al. (19). Phagocytosis was tested using immunobeads (Fluoresbrite Plain Microspheres; Polysciences Inc., Warrington, PA) or opsonized zymosan (Sigma Chemical Co.) (15).

Biological Reagents. Recombinant human G-CSF (KRN8601) was obtained at Kirin Brewery Co. (Shibuya-ku, Tokyo, Japan) by a method of Escherichia coli with cloned G-CSF complementatory DNA (20). Recombinant human M-CSF (21) was obtained from Morinaga Milk Industry Co. (Minato-ku, Tokyo, Japan). Recombinant human GM-CSF (BI 71.018) (22) was provided by Hoechst Japan (Minato-ku, Tokyo, Japan) and recombinant human IL-3 (23) was purchased from Genzyme Corp. (Boston, MA). TPA (Paesel GmbH and Co., Frankfurt, West Germany), sodium butyrate (Wako Pure Chemical Co., Tokyo, Japan), dimethyl sulfoxide (Katayama Chemical Co., Tokyo, Japan), and retinoic acid (Sigma Chemical Co.) were used for in vitro stimulation.

Proliferative Response to CSFs. Cells (3 x 10^6) were cultured in flat-bottomed 96-microwell plates (Nunc, Roskilde, Denmark) in serum-free medium (COSMEDIUM-001; Cosmo Bio Co.) which contains 5 µg/ml insulin, 1.5 ng/ml sodium selenite, and 5 µg/ml human transferrin with or without 0.1, 1, 10, and 100 ng/ml CSFs. The viable cells were counted by a trypsin blue exclusion method or quantitated by a colorimetric assay using tetrazolium salt, MTT, as described previously (24). Because the results with the MTT assay were consistently equivalent with the viable cell counts, data in this article represent results using the MTT assay. The plates were read on a scanning multiwell spectrophotometer (SLT 210; SLT-LAB Instruments, Salzburg, Austria) using a test wavelength of 545 nm and a reference wavelength of 650 nm. The results were expressed as absorbance.

Clonogenic Assay. Cells (5 x 10^3) were plated in 96-microwell plates (Nunc) in 100 µl of RPMI 1640 supplemented with 0.3% agar and 10% FCS, with or without various concentrations of G-CSF or M-CSF. After 7 days of incubation at 37°C in a humidified atmosphere of 5% CO2 in air, colonies consisting of ≥20 cells were counted under an inverted microscope. The cultures were dried onto glass slides and then examined microscopically after staining with MGG and MPO. For evaluating clonogenic cell recovery of NKM-1 cells after the exposure to G-CSF or M-CSF in suspension cultures, NKM-1 cells at a concentration of 3 x 10^3/ml were cultivated in COSMEDIUM-001 with or without G-CSF or M-CSF in 3047 multiwell tissue culture plates (Falcon) for the indicated days. After the cells were washed, colony-forming capacity was measured in the absence of CSF as described above. The recovery of clonogenic cells/2 ml in suspension was calculated by multiplying the plating efficiency in agar by the number of cells harvested from the suspension culture (25).

Cell Cycle Analysis. DNA histogram analysis with propidium iodide was performed (26) by staining 1 x 10^6 leukemic blasts/sample with 50 µg/ml propidium iodide (Sigma Chemical Co.). The DNA content of 0.2% Nonidet P-40 (Particulate Data Inc., Elmhurst, IL) and 250 µg/ml RNase (Boehringer-Mannheim, Mannheim, West Germany) for 30 min at 0°C and incubated for another 15 min at 37°C. The nuclei were analyzed by flow cytometry (EPICS PROFILE). The percentage of the cells having S and G2 + M content of DNA was estimated by a program developed by Ortho Diagnostics.

125I-G-CSF-Binding Analysis. The radiolabeling of G-CSF was greatly enhanced by the presence of tyrosine residues at the NH2 terminus and the stability of this activity was not affected by this tyrosination. Cells (5 x 10^6) were incubated with various concentrations of 125I-labeled Tyr-Tyr-recombinant human G-CSF for 3 h at room temperature in RPMI 1640 containing 10% FCS and 20 nm 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma). Cells were spun over a 200-µl FCS cushion in a 400-µl centrifuge tube. The bottom of the tube containing the cell pellet was cut and counted (27). Nonspecific binding was estimated in incubations with a 100-fold excess of unlabeled G-CSF. Effects of other CSFs on G-CSF binding to NKM-1 cells were tested by incubating cells with a fixed concentration of 125I-G-CSF in the presence of unlabeled CSFs. In order to analyze the effect of M-CSF on G-CSF receptor expression, the radioligand assay was performed on NKM-1 cells pretreated with 2 ng/ml M-CSF for 20 h.

Northern Blot Hybridization. Total cellular RNA was extracted by guanidium isocyanate method (28). Ten µg of RNA was electrophoresed in 1% agarose/formaldehyde gel and transferred onto nitrocellulose membranes (Nitroplus 2000; Micron Separation Inc., Westbord, MA). Hybridization was carried out at 42°C using 32P-labeled probes. The membrane was washed 3 times in 2x standard saline citrate and 0.1% sodium dodecyl sulfate for 30 min and in 0.2x standard saline citrate and 0.1% sodium dodecyl sulfate for 30 min at 42°C. The probes used in this study were a 1.4-kilobase human v-fms, pS3M (provided by the Japanese Cancer Research Resources Bank with the developer's consent), and a 1.4-kilobase HLA-B7 probe, pDP001 (a generous gift of Dr. Lloyd J. Old, Memorial Sloan-Kettering Cancer Center, NY).

Statistical Analysis. Data were analyzed by Student's t-test.

RESULTS

Establishment of NKM-1. The cells began to proliferate after 2 weeks of culture and consistently proliferated in single suspension without forming cell clumps or adhesion to the flask. At present, the cells have been in culture in RPMI 1640 medium supplemented with 10% heat-inactivated FCS with a doubling time of 36-48 hours. This cell line was designated NKM-1. Cultures are free from Epstein-Barr virus and Mycoplasma contamination. NKM-1 cells are able to proliferate in serum-free growth media, COSMEDIUM-001, for at least 5 months without changing cell morphology or cell surface phenotypes.

Characterization of Fresh Leukemia Cells and NKM-1 Line Cells. Fresh leukemic cells in the patient's bone marrow ranged from 20-30 µm in diameter and had round or oval nuclei with fine chromatin structure and a few nucleoli. Cytoplasm was basophilic and contained many azurophilic granules and a few vacuoles. No Auer rods were observed. Cytochemical stainings

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showed that almost all blasts were MPO, CAE, and NBE positive. NKM-1 cells were mostly round to ovoid with almost the same modal diameter and had round to ovoid nuclei with fine chromatin and one or more prominent nucleoli. Cytoplasm was slightly basophilic and contained many vacuoles and azurophilic granules (Fig. 1). MPO was strongly positive in almost all the cells. About 20% of the cells were positive for CAE but negative for NBE. Ultrastructural morphological study showed that NKM-1 cells had one round to ovoid nucleus with a smooth to slightly irregular outline, moderately condensed chromatin, and one or more large nucleoli. The cytoplasm contained numerous mitochondria, moderately long segments of rough endoplasmic reticulum, a small Golgi apparatus, and granules (Fig. 2). MPO positivity was noted in granules. Cell surface antigens on the peripheral blood cells of the patient and NKM-1 cells are summarized in Table 1. Both cells were positive for CD15, CD18, and HLA-DR. CD14, CDw32, and CD33 were positive on some of the patient’s cells but weak on NKM-1 cells. T-cell differentiation antigens such as CD2, CD3, CD5, and CD7, B-cell-related antigens such as CD10, CD19, and CD20, antigens for platelets (CD41), and erythrocytes (glycoporphin A) were not demonstrated. Chromosomal analysis of 13 NKM-1 cells showed that the karyotype of the modal number was 47XY,-2,+der(2)t(2;?) (q37;?), -6,+der(6)t(6;?) (p23;?), +8. NBT reduction and phagocytic activities were not detected.

Effects of Chemical Agents. To test whether chemical inducers could initiate differentiation, NKM-1 cells were incubated for 3–7 days with 100 nM TPA, 1 µM retinoic acid, 0.3 mM sodium butyrate, or 1% dimethyl sulfoxide. None of the reagents but TPA had effects of differentiation on NKM-1 cells by morphological and cytochemical studies and cell surface antigen analysis (data not shown). During the 3-day exposure to 100 nM TPA, cells displayed macrophage-like appearance with decreased staining for CAE.

Response to CSFs in Suspension Culture. The capacity of the recombinant human G-CSF, M-CSF, GM-CSF, and IL-3 to stimulate NKM-1 cells was titrated with cells cultured in COS-MEDIUM-001. Viable cells were measured after 1–5 days of cultures using the MTT assay. The cells were in the growth phase at days 1–5. Fig. 3 illustrates growth of CSF-treated cells compared with control cultures after 3 days of cultures. NKM-1 cells showed prominent proliferation in response to G-CSF and M-CSF in a dose-related manner. Neither GM-CSF nor IL-3 affected the cell growth. The cells also proliferated with G-CSF or M-CSF in a dose-dependent manner in RPMI 1640 with 10% FCS, although the proliferation was less apparent; after 3 days of cultures with 100 ng/ml of G-CSF, the cells increased approximately 1.3-fold in absorbance compared with control cultures. The ability of G-CSF and M-CSF to induce cell differentiation was tested. Table 1 presents a phenotypic analysis of NKM-1 cells after stimulation with 10 ng/ml G-CSF for 3 days. Although cells positive for CD18 and HLA-DR diminished in number, few changes were observed in morphological appearance, NBT reduction, or phagocytic capacity. Identical results were obtained using cells stimulated for 7 days.

Effects of CSFs on Colony Formation. Fig. 4 shows the effects of G-CSF and M-CSF on primary colonies of NKM-1 cells in agar. Without addition of CSFs, 31 ± 1 colonies/5 × 10⁵ cells were observed. Morphologically, the colonies were composed of myeloblasts. A greater number of colonies were formed in the presence of G-CSF or M-CSF. The colony sizes also increased with CSFs. The effects of CSFs peaked at 10 ng/ml but

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Fig. 1. Smear of NKM-1 cells stained with MGG. Cytoplasmic granules and vacuoles are demonstrated. Original magnification, × 2000.

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G-CSF-, M-CSF-responsive cell line

Fig. 2. Electron microscopic photograph of NKM-I cells. A round to ovoid nucleus and mitochondria, rough endoplasmic reticulum, and granules are noted. Original magnification, × 3200; bar, 2 μm.

Table 1. Cell surface antigens on NKM-I cells

Cells were stained by an indirect immunofluorescent method and analyzed with a flow cytometer. The negative control showed 5% of positive background. Furthermore, after incubation in COSMEDIUM-001 with or without 10 ng/ml of G-CSF or M-CSF for 3 days, cell surface antigens were examined.

<table>
<thead>
<tr>
<th>CD</th>
<th>PB*</th>
<th>Time 0</th>
<th>No CSF</th>
<th>G-CSF</th>
<th>M-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>14.0</td>
<td>6.2</td>
<td>0.8</td>
<td>0.3</td>
<td>7.3</td>
</tr>
<tr>
<td>CD13</td>
<td>3.3</td>
<td>0.6</td>
<td>1.9</td>
<td>1.9</td>
<td>2.2</td>
</tr>
<tr>
<td>CD14</td>
<td>24.3</td>
<td>4.5</td>
<td>6.6</td>
<td>5.9</td>
<td>3.8</td>
</tr>
<tr>
<td>CD15</td>
<td>88.0</td>
<td>99.2</td>
<td>94.8</td>
<td>92.7</td>
<td>83.0</td>
</tr>
<tr>
<td>CD16</td>
<td>2.4</td>
<td>2.4</td>
<td>2.1</td>
<td>1.1</td>
<td>1.5</td>
</tr>
<tr>
<td>CD18</td>
<td>66.9</td>
<td>58.6</td>
<td>20.4</td>
<td>5.3</td>
<td>3.8</td>
</tr>
<tr>
<td>CDw32</td>
<td>38.6</td>
<td>11.9</td>
<td>8.3</td>
<td>7.5</td>
<td>7.3</td>
</tr>
<tr>
<td>CD33</td>
<td>18.4</td>
<td>6.9</td>
<td>6.1</td>
<td>1.3</td>
<td>7.8</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>59.4</td>
<td>22.9</td>
<td>32.6</td>
<td>7.1</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* PB: Peripheral blood mononuclear cells from the patient at time of diagnosis.

Fig. 3. Effects of CSFs on cell growth of NKM-I. Cells (3 × 10⁴) were cultured in COSMEDIUM-001 with or without CSFs in 96-well microtiter plates. After 3 days of culture, viable cells were measured using the MTT assay. Columns, means of triplicate cultures; bars, ±SD. The absorbance was increased with G-CSF or M-CSF (with 1 ng/ml of G-CSF, P < 0.05; with 10 ng/ml of G-CSF, P < 0.01; with 1, 5, 10, or 20 ng/ml of M-CSF, P < 0.01; compared with the control).

Table 2. Effects of G-CSF and M-CSF on growth of clonogenic cells in suspension

NKM-I cells at a concentration of 3 × 10⁶/ml were cultivated in COSMEDIUM-001 with or without G-CSF or M-CSF. The recovery of clonogenic cells/2 ml in suspension was calculated by multiplying the plating efficiency in agar by the number of cells harvested from the suspension culture.

<table>
<thead>
<tr>
<th>CSF concentration (ng/ml)</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8.9 ± 0.7*</td>
<td>20.8 ± 4.9</td>
</tr>
<tr>
<td>1</td>
<td>10.4 ± 1.5</td>
<td>14.1 ± 0.4*</td>
</tr>
<tr>
<td>10</td>
<td>13.6 ± 2.5*</td>
<td>16.5 ± 1.6*</td>
</tr>
<tr>
<td>100</td>
<td>18.2 ± 3.0*</td>
<td>20.1 ± 2.0*</td>
</tr>
<tr>
<td>M-CSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>11.2 ± 0.7</td>
<td>23.5 ± 5.5</td>
</tr>
<tr>
<td>1</td>
<td>13.6 ± 2.3</td>
<td>19.3 ± 1.9</td>
</tr>
<tr>
<td>10</td>
<td>16.5 ± 1.6*</td>
<td>25.3 ± 5.5</td>
</tr>
<tr>
<td>100</td>
<td>20.1 ± 2.0*</td>
<td>30.6 ± 7.1</td>
</tr>
</tbody>
</table>

* Mean ± SD of three experiments.
* Significantly different from the number without CSFs on the same day (P < 0.01).
* Significantly different from the number without CSFs on the same day (P < 0.05).

Effects of CSFs on cell cycle and [³H]thymidine uptake. In control cultures in COSMEDIUM-001, the percentages of cells entering the S/G₂-M phase of the cell cycle were 24.5/5.5 at time 0 and 25.2/7.7 after 24 h without G-CSF. In contrast, 37.1/7.0% and 37.5/8.6% of cells were in S/G₂-M phase after 24-h stimulation with 1 ng/ml and 10 ng/ml of G-CSF, respectively. This effect of G-CSF was also demonstrated by [³H]thymidine incorporation. The uptake was increased after 24-h exposure to G-CSF, and the increase was detected in cells stimulated with as little as 0.1 ng/ml G-CSF (Fig. 5).

Interaction of G-CSF and M-CSF on growth of NKM-I cells. Because both G-CSF and M-CSF actively influenced cell growth of NKM-1, proliferative potential was measured with the combination of two factors. In a dose range from 0.1–10 ng/ml of M-CSF, the additive increase with G-CSF was evident in suspension cultures (Fig. 6). The additive effects were also observed in colony sizes and numbers and most apparent at 10 ng/ml of G-CSF and M-CSF. The additive effects in suspension cultures were only demonstrated when the two CSFs were added simultaneously. Pretreatment of one of the two CSFs did not decline at higher concentrations. The recovery of clonogenic cells in suspension culture was also measured using cells which had been incubated with G-CSF or M-CSF for 2–4 days (Table 2). The clonogenic cell recovery on day 2 was well correlated with the dose of CSFs in suspension cultures. Conversely, those on days 3 and 4 were decreased at 10 or 100 ng/ml of CSFs (data on day 4 not shown).
G-CSF-, M-CSF- RESPONSIVE CELL LINE

Fig. 5. Dose-response analysis of [3H]thymidine uptake. Cells (6 x 10⁴) were cultured for 20 h in medium containing various doses of G-CSF and pulse labeled with [3H]thymidine for 4 h. Columns, means of triplicate cultures; bars, ±SD. The uptake was increased with G-CSF (with 0.1, 1, or 10 ng/ml of G-CSF compared with G-CSF-untreated cells, P < 0.01).

Fig. 6. Interactions of G-CSF and M-CSF on the proliferation of NKM-1 cells. Cells were plated in microwell plates at 3 x 10⁴/well in COSMEDIUM-001 in the presence of various concentrations of G-CSF (0-100 ng/ml) and 0 (●), 0.1 (□), 1 (●), and 10 (○) ng/ml of M-CSF. The MTT assay was performed after 3 days. Points, means of three experiments; bars, ±SD.

alter the responsiveness of NKM-1 cells to the other CSF (data not shown).

G-CSF Receptors on NKM-1 Cells. The ¹²⁵I-labeled Tyr⁴Tyr-G-CSF was used, and a binding analysis was performed on NKM-1 cells. The specific binding of ¹²⁵I-G-CSF to NKM-1 cells increased depending on the cell number. NKM-1 cells expressed 60 specific binding sites with a calculated Kd of 100 pm (Fig. 7). Scatchard analysis (29) of the data yielded a straight line, indicating a single class of binding sites. No competition was observed with IL-3, GM-CSF, or M-CSF, while unlabeled G-CSF specifically inhibited ¹²⁵I-G-CSF binding. When ¹²⁵I-G-CSF-binding capacity was examined after incubation with M-CSF, the capacity was no different compared with the untreated cells. These results suggest that NKM-1 cells expressed specific receptors for G-CSF which were independent of M-CSF binding and not regulated by M-CSF.

c-fms Expression in NKM-1 Cells. The effects of M-CSF are mediated through binding to surface receptors. The response of NKM-1 cells prompted us to examine c-fms mRNA expression (30). By Northern blot hybridization, a 4.3-kilobase band was detected, indicating that NKM-1 cells expressed M-CSF receptor. The expression was also tested for RNAs from NKM-1 cells which had been incubated with 10 ng/ml G-CSF for 24 h. No differences were observed between G-CSF-treated cells and untreated cells (Fig. 8).

DISCUSSION

We established a myeloid leukemia cell line, NKM-1, from a patient with AML (FAB M2). In addition to the typical myeloid characteristics of positive staining for MPO and CAE with strong CD15 expression, this cell line has unique features: expression of G-CSF receptors and M-CSF receptors and proliferative responses to the two factors. The stimulatory effects were demonstrated by viable cell count, [³H]thymidine uptake, cell cycle analysis, colony forma-
tion, and clonogenic cell recovery. G-CSF and M-CSF had no ability to induce cell differentiation in NKM-1 cells, which was defined by morphological appearance or analysis of surface antigens. This lack of differentiaton might be related to the weak differentiation potential of NKM-1 cells. Colony-forming capacity was stimulated with 1–10 ng/ml of G-CSF or M-CSF but suppressed with higher concentrations (≥20 ng/ml) of CSFs, which was reproducible in all of the experiments performed.

The pattern of responsiveness was also demonstrated in some AML cells (31), which may represent optimal concentrations of CSFs for cell growth. No contaminating inhibitory materials were found in the solutions of colony assay. The clonogenic cell recovery which reflects self-renewal capacity of leukemia stem cells was rapidly increased on day 2 with G-CSF or M-CSF in a dose-dependent manner and decreased on day 3 at higher concentrations of CSFs (10 or 100 ng/ml).

Several myeloid cell lines can be stimulated to differentiate or proliferate in the presence of G-CSF or M-CSF. Purified G-CSF is able to induce murine WEHI-3B D* (20), human HL-60 (32) cells to mature, followed by ultimate suppression of clonogenic cells and terminal differentiation. In general, exogenous CSFs have little effect on long-term proliferation of established cell lines. Some GM-CSF-dependent leukemia cell lines retained their ability to respond to exogenous CSFs (33). Among three GM-CSF-dependent leukemia cell lines, G-CSF can independently support the continuous growth of AML-193 (34) but not of MV4-11 (34) cells or TALL-101 (35) cells. M-CSF also potentiated the proliferation of AML-193 cells only in the presence of suboptimal doses of GM-CSF. Recently, Matsuda et al. (36) reported a murine myeloid leukemia cell line, NFS-60, which is stimulated by murine and human G-CSF to proliferate but not to differentiate. Considering the above findings, one may regard NKM-1 as the first permanent human myeloid leukemia cell line that grows in serum-free medium without adding any CSFs and shows proliferative responses to exogenous G-CSF and M-CSF.

The effects of CSFs are presumably mediated through binding to surface receptors. The 125I-G-CSF used retained full biological activity and receptor-binding capacity. By this labeled G-CSF, normal human neutrophils showed a single receptor type (230 receptors/cell; Kd 30 pm). The number of G-CSF-binding sites/cell was low on NKM-1 cells but the Kd was relatively low compared with that of previously reported myeloid leukemia cells. The prominent proliferative responses of NKM-1 cells may be attributable to abnormalities in G-CSF receptors or postreceptor pathways in NKM-1 cells.

It has been reported that of several human cell lines examined only the Raji cell line expressed c-fms (37, 38). NKM-1 is, to our knowledge, the first human myeloid leukemia cell line with c-fms mRNA expression. Dose-dependent proliferative responses to M-CSF indicate that NKM-1 cells express functional M-CSF receptors, although M-CSF-binding analysis was not performed in the current study. Therefore, the NKM-1 cell line possesses both G-CSF and M-CSF receptors. Only the mouse macrophage cell line J774 (39) was shown to possess both G-CSF and M-CSF receptors (27, 40).

Myeloid leukemia cells are known to express multiple CSF receptors (41), and interaction of CSFs on biological responses has been reported. In NKM-1 cells, M-CSF showed additive effects with G-CSF in potentiating cell growth when two factors were used simultaneously. This could occur in several ways. It may be that NKM-1 cells consist of two populations which express one of the receptors for the two factors or the two receptors appear on a single cell. In either case the cells could be responsive to both CSFs through separate, independent activation pathways or the additive effects might depend on the up-modulation of the growth factor receptor, thus making for more susceptibility to the other growth factor. The latter may not be the case for NKM-1 cells, since the effects were not obvious when G-CSF and M-CSF were added sequentially. Moreover, c-fms mRNA expression was not up-regulated by G-CSF, and pretreatment of M-CSF did not alter 125I-G-CSF-binding capacity. Further studies would be required to elucidate the molecular events underlying cellular responses to CSFs. This cell line may serve as a useful model for these studies.

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A Novel Human Myeloid Leukemia Cell Line, NKM-1, Coexpressing Granulocyte Colony-stimulating Factor Receptors and Macrophage Colony-stimulating Factor Receptors

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