Proliferation of Human Myeloid Leukemia Cell Line Associated With the Tyrosine-Phosphorylation and Activation of the Proto-oncogene c-kit Product

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We investigated the expression, degree of phosphorylation, and activation of the proto-oncogene c-kit product before and after stimulation with the c-kit ligand in a human factor-dependent myeloid leukemia cell line, MO7E. The culture supernatant of the BALB/3T3 fibroblast cell line, which contains the ligand for the murine c-kit product, was found to stimulate proliferation of the MO7E cell line in a dose-dependent manner. The proliferation was significantly inhibited by a tyrosine kinase inhibitor, genistein. An immunoblot technique with a monoclonal antibody specific for phosphotyrosine, showed that there was rapid, dose-dependent tyrosine-phosphorylation of the c-kit product in response to murine c-kit ligand. Furthermore, the murine c-kit ligand increased autokinase activity of the c-kit product in vitro. Similar results were obtained with human stem cell factor (SCF), a recombinant human ligand for the c-kit product. These results suggest that the phosphorylation and activation of the c-kit product are involved in proliferative signals of some human leukemia cells, as well as of normal hematopoietic cells.

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Materials and Methods

Reagents. Recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) was generously donated by Drs Steve Clark and Gordon Wong, Genentech Institute, Cambridge, MA, and recombinant human stem cell factor (rhSCF) by Dr Krisztina M. Zsebo, Amgen, Thousand Oaks, CA. Rabbit c-kit peptide antiserum was kindly provided by Dr Axel Ullrich, Max-Planck-Institut für Biochemie, Martinsried, Germany. This antisera was generated against a synthetic peptide corresponding to c-terminal 16 amino acid residues of c-kit. Antiphosphotyrosine antibody is a murine monoclonal antibody generated against phosphotyramine. This antibody is specific for tyrosine-phosphorylated proteins and does not cross-react with phosphoserine, phosphothreonine, phosphohistidine, or tyrosine sulfate. Chemically defined serum-free medium (ASF-102) was purchased from Ajinomoto (Tokyo, Japan); it contains human transferrin, insulin, and bovine serum albumin. Genistein, a tyrosine kinase inhibitor, was purchased from Extrasynthese (Genay, France).

Cell lines. MO7E, a cell line dependent on human GM-CSF, interleukin-3 (IL-3), or IL-9, was obtained from Dr Steve Clark, Genentech Institute, and was originally established by Dr Luigi Pegoraro and colleagues from the peripheral blood of an infant with acute megakaryocytic leukemia. MO7E cells were cultured in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal calf serum (FCS; Flow Lab, North Ryde, Australia) and 10 ng/mL rhGM-CSF. BALB/3T3, a murine fibroblast cell line, and HEL, a human erythroleukemia cell line obtained from the Japanese Cancer Research Resources Bank, were adapted for growth, and maintained in RPMI 1640 medium supplemented with 10% FCS.

Preparation of fibroblast-conditioned medium. BALB/3T3 fibroblasts were cultured in RPMI 1640 medium supplemented with 10% FCS. After reaching confluence, the medium was aspirated and the adherent fibroblast monolayer washed three times with serum-free RPMI 1640 medium. The culture dishes were filled with fresh ASF-102 medium, and cultured at 37°C in a humidified atmosphere containing 5% CO2 for 10 days. The conditioned supernatant was then harvested, centrifuged at 1,600g for 15 minutes, filtered, and stored at −20°C until use.

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binding sites on the filter were blocked by incubating the membranes in TBS (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl) containing 1% gelatin (Bio-Rad Lab, Richmond, CA) for 1 hour at 4°C. Residual bind sites on the filter were blocked by incubating the membranes in TBS (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl) containing 1% gelatin (Bio-Rad Lab, Richmond, CA) for 1 hour at 25°C. The blots were then washed in TBST (TBS with 0.05% Tween 20) and incubated overnight with antiphosphotyrosine monoclonal antibody (1.5 μg/mL in TBST). After four washings in TBST, the blots were incubated for 2 hours with alkaline phosphatase-conjugated anti-mouse IgG (Promega, Madison, WI) diluted to 1:2,000 in TBST, and washed three times in TBST. Antibody reactions were developed for 10 to 30 minutes in a solution containing 100 mmol/L Tris-HCl, pH 9.5, 100 mmol/L NaCl, 5 mmol/L MgCl₂, 330 μg/mL Nitro blue tetrazolium, and 150 μg/mL 5-bromo-4-chloro-3-indolyl phosphate. Enzymatic color development was stopped by rinsing the filters in deionized water.

<p>| Table 1. Proliferation of M07E Cells Stimulated With GM-CSF or F-CM |
|---------------------------------|------------------|----------------------|------------------------|</p>
<table>
<thead>
<tr>
<th>Factor</th>
<th>MTT Assay (OD at 550 nm)</th>
<th>^3H-Thymidine incorporation (cpm)</th>
<th>Cell No. (x 10^5 cells/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium only</td>
<td>0.081 ± 0.010</td>
<td>2,200 ± 69</td>
<td>8.2 ± 1.3</td>
</tr>
<tr>
<td>GM-CSF (10 ng/mL)</td>
<td>0.253 ± 0.004*</td>
<td>34,000 ± 860*</td>
<td>48.9 ± 4.4*</td>
</tr>
<tr>
<td>F-CM (50% vol/vol)</td>
<td>0.367 ± 0.010*</td>
<td>44,000 ± 910*</td>
<td>64.5 ± 7.2*</td>
</tr>
</tbody>
</table>

Values are mean ± SE of one representative experiment, which consisted of triplicate cultures. At least two separate experiments were performed, and comparable results were obtained.

*P < .01, when compared with control value by t-test.

Described, but with minor modifications. Briefly, triplicate aliquots of M07E cells (1.5 x 10^6 cells suspended in 100 μL of ASF-102 medium containing fibroblast-conditioned medium (F-CM), GM-CSF, or SCF) were cultured in 96-well flat-bottom microtiter plates for 48 hours at 37°C. For the MTT assay, MTT (10 μL of a 5 mg/mL solution of MTT in phosphate-buffered saline (PBS)) was added to all wells, and plates were incubated for 4 hours. Acid isopropanol (100 μL of 0.04N HCl in isopropanol) was added to all wells, and mixed throughly to dissolve the dark blue crystals. The optical density (OD) was then measured on a Microeliza plate reader (Corona Electoric, Ibaragi, Japan) with a test wavelength of 550 nm and a reference wavelength of 630 nm.

For the ^3H-thymidine incorporation assay, each well was pulsed for 4 hours with 1 μCi ^3H-thymidine (specific activity, 2 Ci/mmol; ICN, Costa Mesa, CA). Next, the cells were harvested with an automatic cell harvester (Labo Mash LM101, Labo Science, Tokyo, Japan), and the incorporation was measured with a liquid scintillation counter. For cell enumeration, viable cells were counted either by trypan blue dye exclusion, or under a phase-contrast microscope with a standard hemocytometer. These methods yielded similar results. In some experiments, cell proliferation was assessed in the presence or absence of genistein dissolved in dimethyl sulfoxide (DMSO).

Stimulation with F-CM and cell lysis. Exponentially growing M07E cells were washed three times with RPMI 1640 medium, and incubated in ASF-102 medium for 18 hours at 37°C to eliminate serum and growth factors. The cells (10^6 cells suspended in 1 mL of ASF-102 medium) were then exposed to F-CM, SCF, or GM-CSF at 37°C for various periods (mostly 15 minutes). After stimulation, cells were washed with cold PBS and immediately lysed in 100 μL of lysis buffer (20 mmol/L Tris-HCl, pH 8.0, 137 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40) containing 1 mmol/L phenyl methylsulfonyl fluoride (PMSF Sigma), 0.15 U/mL aprotinin (Sigma), 100 mmol/L EDTA, 10 μg/mL leupeptin (Sigma), 100 mmol/L sodium fluoride, and 2 mmol/L sodium orthovanadate (Sigma) at 4°C for 20 minutes. Insoluble material was removed by 10,000 x g centrifugation at 4°C for 15 minutes. The cell lysates were then frozen at ~80°C until use. In some experiments, cells were treated with genistein for 3 or 15 hours before stimulation with SCF.

Gel electrophoresis and immunoblotting. Cell lysates (10 μL) were mixed with 5 μL of 3x sodium dodecyl sulfate (SDS) sample buffer with 2-mercaptoethanol, heated at 100°C for 5 minutes, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 5% to 20% polyacrylamide gradient gel (Atto, Tokyo, Japan). After electrophoresis, proteins were electrophoretically transferred at 0.4 A from the gel onto a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA) in a buffer containing 25 mmol/L Tris, 192 mmol/L glycin, and 20% methanol. This transfer was conducted for 4 hours at 4°C. Residual binding sites on the filter were blocked by incubating the membrane in TBS (10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl) containing 1% gelatin (Bio-Rad Lab, Richmond, CA) for 1 hour at 25°C. The blots were then washed in TBST (TBS with 0.05% Tween 20) and incubated overnight with antiphosphotyrosine monoclonal antibody (1.5 μg/mL in TBST). After four washings in TBST, the blots were incubated for 2 hours with alkaline phosphatase-conjugated anti-mouse IgG (Promega, Madison, WI) diluted to 1:2,000 in TBST, and washed three times in TBST. Antibody reactions were developed for 10 to 30 minutes in a solution containing 100 mmol/L Tris-HCl, pH 9.5, 100 mmol/L NaCl, 5 mmol/L MgCl₂, 330 μg/mL Nitro blue tetrazolium, and 150 μg/mL 5-bromo-4-chloro-3-indolyl phosphate. Enzymatic color development was stopped by rinsing the filters in deionized water.
Northern blot analysis. Total cellular RNA was isolated with a guanidine isocyanate method combined with cesium chloride modification. Equal amounts of RNA (15 μg) were size-fractionated by electrophoresis through 1% agarose gel containing 2.2 mol/L formaldehyde. Before transfer to nitrocellulose filters, the ethidium bromide-stained gels were visualized under UV illumination to determine the position of the ribosomal RNA band, and to verify that equal amounts of RNA had been loaded. The c-kit probe was obtained from American Type Culture Collection (ATCC, Bethesda, MD). The probe consisted of the 1.3-kb SsfI fragment derived from phckit-171 which encodes part of the extracellular domain of c-kit. The β-actin probe was purchased from Nippon Gene (Toyama, Japan). After prehybridization, the filters were hybridized with random 32P-labeled probes. The filters were then washed and autoradiographed with two intensifying screens. Tonsillar mononuclear cells (MNC) were used as negative, and HEL cells as positive controls.

Immunoprecipitation. The lysates from F-CM–stimulated or unstimulated 10⁷ M07E cells were precleared with 5 μL of normal rabbit serum and protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) for 2 hours at 4°C. The precleared lysates were then incubated with 5 μL of rabbit c-kit peptide antiserum and protein A-Sepharose beads to collect the antigen-antibody complexes. The immunoprecipitates were washed five times with lysis buffer containing protease and phosphatase inhibitors as described above, resuspended in 60 μL of SDS sample buffer, and boiled. Equal volumes of the sample (15 μL per lane) were subjected to SDS-PAGE, and immunoblotting was performed with the antiphosphotyrosine antibody. The same filter was then incubated with a rabbit c-kit peptide antiserum diluted to 1:2,500 in TBST. In this experiment, alkaline phosphatase-conjugated anti-rabbit IgG (Promega) was used as the second antibody.

Immune complex kinase assay. The immunoprecipitates with rabbit c-kit peptide antiserum and protein A-Sepharose beads (from 10⁷ M07E cells stimulated with F-CM for 0, 3, or 15 minutes) were washed once with PBS, twice with 0.5 mol/L LiCl and 50 mmol/L Tris-HCl, pH 7.4, and once with kinase buffer (10 mmol/L MnCl₂, 20 mmol/L Tris-HCl, pH 7.4) at 4°C. Next, the immunoprecipitates were incubated for 10 minutes at 25°C in 40 μL of kinase buffer containing 10 μCi γ-32P-adenosine triphosphate (ATP) (4,500 Ci/mmol, ICN). SDS sample buffer (3×) was added to stop the reaction, followed by boiling at 100°C for 5 minutes. Equal volumes of the sample (15 μL per lane) were subjected to SDS-PAGE, and the gel was stained with Coomassie Blue solution to determine the position of molecular weight markers and to verify that equal amounts of protein had been loaded. The gel was dried with a gel drier, and the radioactive

**Fig 2.** Changes in the protein tyrosine-phosphorylation following F-CM stimulation of M07E cells. M07E cells (10⁷ cells) were stimulated with 50% F-CM for the indicated time, and immediately lysed in 100 μL of lysis buffer. An equal volume of samples was subjected to SDS-PAGE, and phosphotyrosine-containing proteins were identified by immunoblot with antiphosphotyrosine antibody.

**Fig 3.** (A) Proliferation of M07E cells cultured with the indicated concentration of F-CM for 52 hours, and (B) tyrosine-phosphorylation of the 145- to 165-Kd protein stimulated with various concentrations of F-CM for 15 minutes.
ACTIVATION OF c-kit PRODUCT

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Fig 4. Northern blot analysis for c-kit mRNA transcripts. Fifteen micrograms of total cellular RNA was electrophoresed in 1% agarose gel containing 2.2 mol/L formaldehyde. The blots were then hybridized with 32P-labeled cDNA probe for c-kit. Tonsillar mononuclear cells (MNC) were used as negative, and HEL cells as positive controls. The same filter was rehybridized with β-actin probe to verify that an equal amount of RNA was loaded in each lane.

proteins were detected by autoradiography for 2 hours. Densitometric analysis of the autoradiograph was performed with a dual-wavelength flying-spot scanner (CS-9000, Shimadzu, Tokyo, Japan).

RESULTS

Proliferation of M07E cells. Proliferation of M07E cells was examined with the MTT colorimetric method, 3H-thymidine incorporation, and cell enumeration assay. F-CM clearly enhanced the proliferation of M07E cells, as well as of rhGM-CSF (Table 1). Since these three methods yielded comparable results, we assessed cell proliferation in the following experiments mainly with the MTT assay. Next, to investigate whether or not this proliferation was induced by the activation of tyrosine kinase, we examined the effect of a nonspecific tyrosine kinase inhibitor, genistein. A control culture containing DMSO showed no influence on cell proliferation, while genistein significantly inhibited both F-CM- and GM-CSF-induced proliferation of M07E cells in a dose-dependent manner (Fig 1). However, the dose-response experiments with genistein showed the F-CM-induced proliferation of M07E cells to be more susceptible to genistein than the GM-CSF-induced one. It is unlikely that this inhibitory effect resulted from genistein drug toxicity for the following reasons: The viability of M07E cells cultured in ASF-102 medium without growth factors was not affected by the addition of genistein, and more than 80% of the cells remained viable after 15 hours in the presence of genistein (30 μg/mL).

Tyrosine-phosphorylation of a 145- to 165-Kd protein stimulated by F-CM. To determine if F-CM activates tyrosine kinase activity in M07E cells, we investigated the pattern of tyrosine-phosphoproteins by immunoblotting with a monoclonal antibody specific for phosphotyrosine. The onset of F-CM–induced tyrosine-phosphorylation of the proteins, particularly at a molecular weight of 145 to 165 Kd, was observed within 1 minute, and the maximum tyrosine-phosphorylation after 15 minutes (Fig 2). To examine whether the proliferation of M07E cells was related to the tyrosine-phosphorylation of this 145- to 165-Kd protein, these two items were investigated at various concentrations of F-CM. As shown in Fig 3, there was a dose-dependent proliferation of M07E cells in the range from 10% to 50% F-CM. Furthermore, the stimulation of M07E cells with F-CM resulted in a dose-dependent tyrosine-phosphorylation of the 145- to 165-Kd protein with the maximum level at a concentration of 50% F-CM. These results suggest that the tyrosine-phosphorylation of the 145- to 165-Kd protein was involved in the proliferation of M07E cells. BALB-F-CM contains the ligand for c-kit product, and c-kit

Stimulation by F-CM

MW (kD)

200

145

97

+ -

- +

Fig 5. Immunoprecipitates with rabbit c-kit peptide antiserum and protein A-Sepharose beads were prepared from 10⁷ M07E cells, either unstimulated or stimulated with 50% F-CM for 15 minutes. (A) An equal volume of immunoprecipitates was subjected to SDS-PAGE, and immunoblotted with antiphosphotyrosine antibody. (B) The same filter was then reincubated with a rabbit c-kit peptide antiserum.
product is reported to have a molecular weight of 145 Kd; it is therefore probable that this phosphorylation was due primarily to the increased tyrosine-phosphorylation of \( c-kit \) product. To confirm this, we conducted a Northern blot analysis for \( c-kit \) mRNA transcript and an immunoprecipitation study using rabbit \( c-kit \) peptide antiserum for \( c-kit \) product. M07E cells expressed a large amount of \( c-kit \) mRNA (Fig 4), while immunoprecipitated \( c-kit \) protein was found to be tyrosine-phosphorylated by F-CM stimulation at a molecular weight of 145 to 165 Kd (Fig 5). These results clearly demonstrate that F-CM induced tyrosine-phosphorylation of \( c-kit \) product expressed in M07E cells. In addition, we used an in vitro immune complex kinase assay to examine whether F-CM actually changed tyrosine kinase activity of \( c-kit \) product. Densitometric analysis showed that stimulation with F-CM for 3 minutes induced a 1.7-fold, and for 15 minutes a 4.5-fold, increase in autophosphorylation of the \( c-kit \) product (Fig 6). In contrast to F-CM, GM-CSF neither induced tyrosine-phosphorylation of \( c-kit \) product nor activated its autokinase activity (data not shown).

Effect of rhSCF and genistein on M07E cells. To directly demonstrate that the proliferation of M07E cells was mediated through the activation of \( c-kit \) product by \( c-kit \) ligand, we studied the effect of rhSCF on M07E cells. SCF induced a dose-dependent proliferation of M07E cells and a tyrosine-phosphorylation of \( c-kit \) product in the range from 1 to 200 ng/mL (Fig 7; unpublished observation). Further, genistein inhibited both the proliferation of M07E cells and the tyrosine-phosphorylation of \( c-kit \) product induced by SCF (Fig 7). This inhibitory effect on the tyrosine-phosphorylation was dependent on both the concentration of genistein (30 \( \mu \)g/mL > 10 \( \mu \)g/mL) and the length of the treatment with genistein (15 hours > 3 hours) (data not shown). In this experiment, DMSO did not affect either proliferation or tyrosine-phosphorylation, while the inhibitory effect of genistein is unlikely to have resulted from drug toxicity, as explained earlier.
DISCUSSION

In this study, we investigated whether M07E cells, which express early megakaryocytes and erythroid lineage markers, proliferate through the activation of tyrosine kinase, especially of c-kit product.

Stimulation of M07E cells with BALB/3T3 F-CM, which contains SCF, resulted in tyrosine-phosphorylation of the c-kit product, accompanied by an increase in the tyrosine kinase activity. Further, the proliferation of M07E cells roughly paralleled the degree of tyrosine-phosphorylation of c-kit product, while both the proliferation and tyrosine-phosphorylation were inhibited by genistein in a dose-dependent manner. These results indicate that the proliferation of M07E cells was mediated through the activation of c-kit product.

GM-CSF also induced proliferation of M07E cells, and this proliferation was inhibited by genistein. GM-CSF-induced tyrosine kinase activity is important for the factor-dependent proliferation of M07E cells. However, GM-CSF did not induce the tyrosine-phosphorylation of c-kit product. These results demonstrate that GM-CSF did not transactivate c-kit product, and that the GM-CSF-induced proliferative signal was mediated by some tyrosine kinase other than c-kit product.

The physiological role of c-kit product activation is not yet clearly understood, but some information could be obtained from W/W+ mice, which are defective in tyrosine kinase activity of c-kit product. In a murine in vitro culture system, IL-3 in combination with erythropoietin (EPO) was able to stimulate the formation of erythroid bursts from the bone marrow of W/W+ mice, as well as of normal mice. However, such an in vitro effect of IL-3 contradicts its in vivo effect on erythropoiesis. Although W/W+ mice have a normal potential of IL-3 production, W/W+ mice are actually anemic. Furthermore, the anemia of W/W+ mice is not improved by the perfusion of IL-3. These facts suggest that c-kit plays an essential role in constitutive erythroid hematopoiesis in vivo. We have shown that proliferation of M07E cells is stimulated by SCF in vitro. This result suggests that the in vivo proliferation of some leukemia cells is modulated by constitutive hematopoietic mechanisms, such as the interaction of c-kit product and SCF.

SCF alone has only a modest effect on early myeloid and lymphoid cells, but it synergizes with other factors. In particular, SCF stimulates the formation of erythroid bursts from unfracthioned bone marrow cells in the presence of EPO. However, the direct effect of SCF on the formation of erythroid bursts has not been demonstrated, since the involvement of accessory cells or other endogenous growth factors cannot be excluded. In this connection, the results of our study provide evidence that in a human cell population c-kit ligand directly activates the c-kit product in association with proliferation. This also suggests that the response of early erythroid/megakaryocyte progenitors to c-kit ligand might be, at least partially, proliferative.

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