Expression and Functional Role of the Proto-oncogene c-kit in Acute Myeloblastic Leukemia Cells

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The c-kit proto-oncogene encodes a receptor tyrosine kinase that is thought to play an important role in hematopoiesis. In a series of human acute myeloblastic leukemia (AML), the expression of the c-kit proto-oncogene and its product was studied by means of Northern blot and immunoblot analyses. The c-kit mRNA was expressed in 20 of 25 cases of AML, and in those cases the product of the c-kit proto-oncogene was detected by immunoblotting with anti-c-kit antibody. The expression of c-kit transcripts and protein was barely detectable in normal bone marrow cells as a control. The expression of c-kit transcript did not correlate with the French-American-British classification nor clinical manifestations. In 6 of 11 cases that expressed c-kit product, AML cells were found to proliferate in response to recombinant human stem cell factor (rhSCF), the ligand for c-kit, and the synergistic stimulation of AML cells was observed by rhSCF and granulocyte-macrophage colony-stimulating factor. Immunoblotting with anti-phosphotyrosine antibody showed that the c-kit receptor protein was detectably phosphorylated in 7 of 12 cases tested before the stimulation with rhSCF, while the rhSCF treatment resulted in an increased tyrosine phosphorylation of c-kit in AML cells. These results indicate that c-kit proto-oncogene is expressed in most cases of AML and is functional in terms of supporting proliferation.

The c-kit proto-oncogene encodes a transmembrane tyrosine kinase receptor that is a member of receptors for platelet-derived growth factor (PDGF) and colony-stimulating factor-1 (CSF-1).2,3 The c-kit proto-oncogene has been reported to be allelic with the murine white locus, that is thought to play an important role in hematopoiesis. In human and murine hematopoietic cell lines, c-kit has been shown to be expressed and secreted by mast cells. Therefore, we examined the expression of c-kit in a series of AML cases by using Northern and immunoblotting techniques. The expression of c-kit transcripts and protein was barely detectable in normal bone marrow cells as a control. The expression of c-kit transcript did not correlate with the French-American-British classification nor clinical manifestations. In 6 of 11 cases that expressed c-kit product, AML cells were found to proliferate in response to recombinant human stem cell factor (rhSCF), the ligand for c-kit, and the synergistic stimulation of AML cells was observed by rhSCF and granulocyte-macrophage colony-stimulating factor. Immunoblotting with anti-phosphotyrosine antibody showed that the c-kit receptor protein was detectably phosphorylated in 7 of 12 cases tested before the stimulation with rhSCF, while the rhSCF treatment resulted in an increased tyrosine phosphorylation of c-kit in AML cells. These results indicate that c-kit proto-oncogene is expressed in most cases of AML and is functional in terms of supporting proliferation.

Materials and Methods

Reagents. rhSCF was a gift from Dr Kristzina M. Zsebo (Amgen Inc, Thousand Oaks, CA). Highly purified rh granulocyte-macrophage CSF (rhGM-CSF) and interleukin-3 (rhIL-3) were gifts from Drs Steve Clark and Gordon Wong (Genetics Institute, Cambridge, MA). Rabbit anti-c-kit serum was kindly provided by Dr Axel Ullrich (Max-Planck Institut für Biochemie, Martinsried, Germany). This antiserum was generated against a synthetic peptide corresponding to C-terminal 16 amino acid residues of c-kit. The anti-phosphotyrosine antibody is a murine monoclonal antibody (MoAb) generated against phosphotyrosine as the immunogen and was generously supplied by Dr Brian Drucker (Dana-Farber Cancer Institute, Boston, MA). This antibody is specific for tyrosine-phosphorylated proteins and does not cross-react with phosphoserine, phosphothreonine, phosphostigmine, or tyrosine sulfate.

Leukemic cells. Blood or marrow samples were obtained from 24 adult patients with AML and one adult patient with biphenotypic leukemia. All samples were obtained after informed consent. The diagnosis of leukemia was established by morphology and cytochemical staining and cases were classified according to French-American-British (FAB) criteria.2,3 Two patients were classified as FAB M1, five as M2, two as M3, five as M4, seven as M5, one as M6, one as M7, and one as biphenotypic. As controls, normal bone marrow cells from volunteers and tonsillar cells were used. Leukemic cells and mononuclear cells from normal bone marrow and tonsils were isolated with Ficoll-Hypaque (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation, and mononuclear...
cells were cryopreserved until use in 10% dimethyl-sulfoxide in the vapor phase of liquid nitrogen. Samples selected for study were shown to contain greater than 90% blasts and to have a viability greater than 90% after thawing.

Cell lines. M07E, a human GM-CSF- and IL-3-dependent cell line, was obtained from Dr Steve Clark (Genetics Institute) and was originally established by Avanzi et al.22 From the peripheral blood of an infant with acute megakaryocytic leukemia. M07E 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. HEL, a human erythroleukemia cell line; THP-1, a human monocyctic leukemia cell line, and KU-612, a human chronic myelogenous leukemia cell line were obtained from the Japanese Cancer Research Resources Bank (Tokyo), and these cell lines were adapted to grow and maintained in RPMI-1640 supplemented with 10% FCS.

Detection of c-kit mRNA by means of Northern blot analysis. Total cellular RNA was isolated with a guanidine isooxyanate method in combination with cesium chloride modification,24 and equal amounts of RNA (15 μg) were size-fractionated by electrophoresis through 1.0% agarose gels containing 2.2 mol/L formaldehyde. Before transfer to nitrocellulose papers, the ethidium-stained gels were visualized under ultraviolet illumination to determine the position of the ribosomal RNA bands, and to verify that equal amounts of RNA had been loaded. c-kit probe was obtained from American Type Culture Collection (ATCC; Bethesda, MD). The probe consisted of the 1.3-kb Ssr1 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 with specific activities of 1 to 5 × 106 cpm/μg in 10% dextran sulfate, 50% formamide, 4X SSC (standard saline citrate), 1X Denhardt’s solution, and 10 μg/mL salmon sperm DNA for 24 hours at 42°C. The filters were washed and autoradiographed at −70°C with two intensifying screens. As controls, normal bone marrow (BM) cells and tonsillar mononuclear cells (MNC) were used. The level of expression of c-kit transcripts in M07E cells was arbitrarily set at 3+; the levels of expression in leukemic cells were then quantified relative to the levels expressed in M07E on a scale of 1+ to 3+, with 3+ representing an equal to a one-fifteenth level of expression in leukemic cells were then quantified relative to the levels expressed in M07E cells was arbitrarily set at 3+; the levels of expression in a leukemic cells were then quantified relative to the levels expressed in M07E cells was arbitrarily set at 3+; the levels of expression in a leukemic cells were then quantified relative to the levels expressed in M07E cells.

Stimulation with rhSCF and cell lysis. The frozen cells were thawed, diluted slowly, and washed with RPMI-1640 medium containing 0.5% bovine serum albumin. In some experiments, the cells were divided into two equal groups, after which one group was exposed to 100 ng/mL of rhSCF and the other to medium at 37°C for 15 minutes. In our previous study on M07E cells,9 the maximum tyrosine phosphorylation of c-kit product was observed 15 minutes after treatment with rhSCF at a concentration of 100 ng/mL. Subsequently, 10% cells were washed with cold phosphate-buffered saline (PBS) and 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 [Nakalai Tesque, Kyoto, Japan]) containing protease and phosphatase inhibitors (1 mmol/L phenyl methylsulfonyl fluoride [PMSF; Sigma, St Louis, MO], 0.15 U/mL aprotinin [Sigma], 10 mmol/L EDTA, 10 μg/mL of 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 centrifugation at 10,000g for 15 minutes at 4°C. The cell lysates were frozen at −80°C until use.

Gene electrophoresis and immunoblotting. Cell lysates (30 μg) were mixed 2:1 with 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 Corp, Tokyo, Japan). After electrophoresis, proteins were electrophoretically transferred from the gel onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA) in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol at 0.4 A 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 rabbit c-kit peptide antisera diluted 1:2,500 in TBST. The blots were washed four times in TBST, incubated for 2 hours with alkaline phosphatase-conjugated antirabbit IgG (Promega Corp, Madison, WI) diluted 1:2,000 in TBST, and washed three times in TBST. Antibody reactions were developed for 10 to 30 minutes in a source containing 100 mmol/L Tris-HCl pH 9.5, 100 mmol/L NaCl, 5 mmol/L MgCl2, 330 μg/mL Nitro blue tetrazolium (Promega Corp, Madison, WI), and 150 μg/mL 5-bromo-4-chloro-3-indolyl phosphate. Enzymatic color development was stopped by rinsing the filters in deionized water. In some experiments, anti-phosphotyrosine MoAb (1.5 μg/mL in TBST) was used as the primary antibody, and alkaline phosphatase-conjugated antiose IgG (Promega) as the secondary antibody. The level of expression of c-kit protein or tyrosine phosphorylation in M07E cells was arbitrarily set at 3+; the levels of expression in a leukemic cells were then quantified relative to the levels expressed in M07E cells.

Immunoprecipitation. The lysates from leukemic cells were preclarified with 2 μL of normal rabbit serum and protein A-Sepharose beads for 2 hours at 4°C. The preclarified lysates were then incubated with 2 μL of rabbit c-kit peptide antisera and protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) to collect the antigen-antibody complexes. The immunoprecipitates were washed three times with lysis buffer containing protease and phosphatase inhibitors as described previously. This was followed by SDS-PAGE and immunoblotting performed with either anti-c-kit or anti-phosphotyrosine antibody.

Cell proliferation assay. To quantitate the proliferation of cells, MTt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma] rapid colorimetric assay was used as previously described with a minor modification.23 Briefly, triplicate aliquots of cells (3.0 × 104 cells suspended in 100 μL of RPMI-1640 medium supplemented with 10% FCS) were cultured in 96-well flat bottom microtiter plates for 72 hours at 37°C in the presence or absence of 100 ng/mL of rhSCF. In some experiments, rhGM-CSF (10 ng/mL) or rhIL-3 (10 ng/mL) was used in combination with rhSCF to determine the synergistic effects of the factor on rhSCF-induced proliferation of AML cells. MTt was added for the final 4 hours of culture (10 μL of 5 mg/mL solution of MTt in PBS). After 72 hours of culture, 100 μL of acid isopropanol (0.04N HCl in isopropanol) was added to all wells, mixed, and the optical density (OD) was measured on a Microelisa plate reader (Corona Electric Co, Ibaragi, Japan) at 550 nm. This assay was found to be more reproducible and reliable than "H-thymidine incorporation or cell enumeration as described previously,26 although equivalent results are obtained with all three assays.

RESULTS

Expression of c-kit transcripts in AML cells. Expression of c-kit gene in AML leukemic cells was examined by means
Table 1. Expression of c-kit mRNA and Protein in AML Cells

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*Types of leukemias were determined by clinical, morphologic, and cytochemical criteria in accordance with the FAB classification.
†Bone marrow (BM) or peripheral blood (PB).
‡Levels of mRNA and protein were quantified relative to the levels expressed in M07E as described in Materials and Methods.
§Overt AML that developed after the lapse of MDS.
∥NT, not tested.

To identify c-kit receptor protein, immunoblots with rabbit anti-c-kit peptide antiserum were performed in 19 cases. In all cases expressing c-kit transcript, the product of c-kit proto-oncogene, which is a 145- to 165-Kd protein, was detected in the lysates of AML cells (Table 1). However, as shown in Fig 2 some variation in signal intensity was seen as described under Northern blots. M07E expressed a higher dose of c-kit product than any of the AML cases. In most cases, the intensity of the c-kit band in the Western blot roughly corresponded with that of the transcript in the Northern blot. In contrast, little or no expression of c-kit protein was detected in the lysates of normal BM cells.

Proliferative response of AML cells to rhSCF. The effects of rhSCF on in vitro proliferation of AML cells was evaluated by MTT assay in 11 cases that express c-kit product (Table 2). Six of the 11 cases showed proliferative response to rhSCF at a concentration of 100 ng/mL. Although proliferative response in most cases was lower...
cells were used. As controls, normal BM cells (BM) and M07E cells were used.

than that of M07E, cells from some cases (case nos. 3 and 24) showed a remarkable proliferative response, as high as that of M07E cells. Preliminary study showed that rhSCF induced a dose-dependent proliferation of AML cells over the range of 0.1 to 100 ng/mL. The dose response of AML cells to rhSCF was comparable with that of M07E cells. Despite c-kit expression, some cases such as case 1 did not proliferate in response to rhSCF. This would be attributable to degradation of c-kit protein in the preparation of leukemic cells, or to structural abnormality of the c-kit product in AML cells.

GM-CSF or IL-3 is known to promote the growth of leukemic clonogenic cells in vitro and is considered to contribute to the unrestricted growth of AML cells in autocrine or paracrine mechanisms. Therefore, we examined the effects of rhGM-CSF or rhIL-3 on rhSCF-induced proliferation of AML cells. In most cases tested, the highest proliferation was observed in combination of rhSCF with rhGM-CSF or rhIL-3 (Table 2).

Tyrosine phosphorylation of c-kit protein in AML cells in response to rhSCF. Activation of transmembrane tyrosine kinase receptors is associated with an increase in tyrosine phosphorylation of the receptor. We examined the phosphorylation state of c-kit product in 12 cases of AML that expressed c-kit product. The product of c-kit was immunoprecipitated from cell lysates, and the immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody or anti-c-kit antibody. As shown in Fig 3, c-kit was clearly immunoprecipitated in these cases. Tyrosine phosphorylation of c-kit was faint in these cases, but detectable in 7 of 12 cases before stimulation with rhSCF (Fig 3). In addition, c-kit was immunoprecipitated from cell lysates obtained before and 15 minutes after rhSCF stimulation; this c-kit was immunoprecipitated with anti-phosphotyrosine antibody. As shown in a representative blot in Fig 4, treatment of AML cells with rhSCF resulted in an increase of tyrosine phosphorylation of c-kit protein in four of five cases tested. In these experiments, the same amounts of c-kit were immunoprecipitated before and after rhSCF stimulation.

**DISCUSSION**

AML is a clonal disorder of myeloid progenitor cells resulting in the progressive accumulation of leukemic cells that fail to differentiate. Several research groups have shown that hematopoietic growth factors are required for in vitro growth of AML cells in most cases. In some cases human, murine, and avian myeloid leukemia cells can become factor-independent through autocrine or paracrine mechanisms involving CSFs. In addition to CSFs, it is suggested that some molecule(s) associated with stromal cells or with the extracellular matrix secreted by them is responsible for the growth of myeloid leukemic cells. The product of c-kit proto-oncogene is implicated as an indispensable molecule for stromal-dependent proliferation of normal hematopoietic cells, such as cells of erythroid and mast cell lineages as well as stem cells. This conclusion is supported by data obtained largely from W/Wv and Sf/Sf mutant mice by using in vivo transplantation and coculture experiments. Only limited information is avail-
able about human cells. We previously showed that a human myeloid leukemia cell line, M07E, was found to express c-kit product and c-kit ligand to induce proliferation of M07E cells in association with c-kit phosphorylation and activation. These results led us to investigate the pathophysiological role of c-kit in human AML.

In the present study, we found expression of c-kit RNA and protein in 80% of AML cases. The amount of transcripts varied widely from case to case, and the amount and expression of c-kit did not appear to correlate with either the FAB classification or the clinical manifestations. Also, there was no significant difference in c-kit expression between primary AML and AML developed from MDS. Recently, faint expression of c-kit transcripts in normal BM cells has been reported, but in this experiment c-kit expression was barely identified in them. This may be due to the low proportion of blast cells in our samples.

In a substantial fraction of AML cases that express c-kit, rhSCF induced proliferation of leukemic cells. Furthermore, the rhSCF-induced proliferation of AML cells was significantly augmented by the addition of rhGM-CSF or rhIL-3. Considering CSF involvement in abnormal growth of AML cells, c-kit ligand may cooperate synergistically with CSFs in inducing leukemic cell growth in vivo.

The treatment of AML cells with rhSCF resulted in an increase in tyrosine phosphorylation of c-kit product. Considering the findings that other tyrosine kinase receptors, such as PDGF or CSF-1 receptors, are minimal or absent in resting cells, and the tyrosine phosphorylation induced by ligand correlates with receptor activation, it is likely that rhSCF induces activation of c-kit product expressed on AML cells. However, in some cases, such as cases 4 and 12, the proliferative response was not correlated with the induction of c-kit tyrosine phosphorylation: rhSCF did not stimulate the proliferation of the cells from those cases, while tyrosine phosphorylation of c-kit protein was induced after the treatment with rhSCF. It is not known yet how the c-kit phosphorylation might function in those cases, but the c-kit product may be functionally active because rhSCF acts synergistically with GM-CSF or IL-3 to stimulate the proliferation of the AML (case 12) cells. It is possible that the response of AML cells to rhSCF may not be proliferative in a part of AML cases. However, the present study does not exclude the possibility that unresponsiveness of cells from some AML cases might be due to a neoplastic change that makes them be refractory to SCF stimulation because of a defect distal to c-kit receptor in the signal transduction pathway.

In addition, although the signal was faint, tyrosine phosphorylation was observed in freshly prepared AML cells even in the absence of rhSCF stimulation. Tyrosine phosphorylation of c-kit observed in unstimulated AML cells may have been induced in vivo by interaction of leukemic cells with the cells that express c-kit ligand or with the secreted form of c-kit ligand, and the interaction may
c-kit EXPRESSION AND FUNCTION IN AML

Contribute in part to the excessive growth of AML cells. It is possible that c-kit in some cases of AML may be constitutively activated in the absence of a ligand, although this has not been examined in the present study. Point mutations in the human CSF-1 receptor result in phosphorylation on tyrosine residues in the absence of a ligand and cause morphologic transformation, anchorage-independent growth, and tumorigenicity in nude mice.\(^{23,40}\) Although leukemic cells from some patients with AML produce CSF-1 and express c-fms,\(^{24}\) CSF-1 is not a very potent growth factor for AML cells. If present, structural abnormalities of c-kit could contribute to excessive proliferation of AML cells.

In summary, the results presented here show that c-kit is expressed in most AML cases and suggest that c-kit product may have some function in promoting the proliferation of myeloid leukemic cells in vivo. Functional characterization and molecular identification of c-kit product in AML cells will not only provide important insights into fundamental mechanisms underlying regulation of normal hematopoiesis, but also provide further understanding of abnormal growth of AML cells.

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Expression and functional role of the proto-oncogene c-kit in acute myeloblastic leukemia cells

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