

Brief report

Human acute stem cell leukemia with multilineage differentiation potential via cascade activation of growth factor receptors

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The morphologic, immunophenotypic, genotypic, genomic, and functional features of an undifferentiated acute leukemia with stem cell features are reported. At light and electron microscopy, the leukemic population was represented by primitive progenitor cells with no evidence of differentiation. The blasts were CD34⁺, AC133⁺, CD71⁻, HLA-DR⁻, CD38⁻/^{dim+}, CD90⁺, CD117^{dim+}, flt3⁺; did not express B, T, or

myeloid-associated antigens; and showed a germline configuration of the immunoglobulin and T-cell receptor. Genomic profiling documented the expression of early stem cell and myeloid-associated genes. Receptors for early-acting hemopoietic growth factors (HGFs) were detected, while receptors for unilineage HGF were not expressed. Incubation with the flt3 or Kit ligand induced the expression of uni-

lineage HGF receptors, allowing these cells to respond to their respective ligands. Growth without differentiation was sustained only in the presence of early-acting HGF, namely flt3 ligand, while early and unilineage HGF gave rise to all types of hemopoietic colonies. (Blood. 2002;99:4634-4637)

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Introduction

The use of monoclonal antibodies allows one to identify the lineage affiliation and level of differentiation of virtually all acute leukemias (ALs).^{1,2} The possibility that AL may display multilineage differentiation potential has been suggested for t(9;22) and t(4;11) acute lymphoblastic leukemia.³⁻⁷ The high frequency of CD34 positivity⁸⁻¹⁰ points to the close relationship of AL with early hemopoietic progenitor cells. Considerable interest has focused on acute myeloid leukemia with minimal signs of differentiation¹¹⁻¹³; no study, however, has addressed the multilineage potential of human AL or investigated the genomic profiling and true “stem cell” capacity of such early leukemic cells. Here, we report a very undifferentiated stem cell leukemia in which the lineage affiliation could not be defined by conventional characterization.

Study design

Case report

An 82-year-old woman was referred to the Department of Cellular Biotechnologies and Hematology, University of Rome, Italy, in May 1997 because of mild anemia, progressive asthenia, and malleolar edema. The peripheral blood count showed the following: hemoglobin, 80 g/L [8.07 g/dL]; white blood cell count, $55 \times 10^9/L$; polymorphonuclear cells $2.5 \times 10^9/L$; undifferentiated lymphoidlike cells, $52.5 \times 10^9/L$; platelets, $184 \times 10^9/L$. A diagnosis of undifferentiated stem cell leukemia was made. At this stage, the patient gave her informed consent to the storage of viable pathologic cells. In view of the age of the patient, vincristine plus prednisone was administered on an outpatient basis. At 1 month later, a complete recovery of peripheral parameters was achieved, and the patient

was put on maintenance treatment. In January 1999, a recurrence was documented. The patient was restarted on vincristine plus prednisone, but died 15 days later.

Electron microscopy, immunophenotype, and hemopoietic growth factor receptor characterization

Ultrastructural examination and immunophenotyping were performed on peripheral blood mononuclear cells. Thin sections were stained with toluidine blue, and ultrathin sections were stained with uranyl acetate and lead citrate; sections were observed under a Philips CM10 transmission electron microscope (FEI, Acht, The Netherlands). Myeloperoxidase (MPO) activity was investigated as described.¹⁴

Immunophenotypic characterization was conducted by means of reagents against the following: CD34, surface (s)CD13, cytoplasmic (cy)CD13, CD33, CD11a, CD11b, CD11c, CD14, CD15, CD18, MPO, HLA-DR, terminal deoxynucleotidyl transferase (TdT), CD10, sCD22, cyCD22, CD19, CD23, CD10, CD20, CD79a, immunoglobulin-κ (Igκ), Igλ, sCD3, cyCD3, CD2, CD7, CD5, CD31, CD38, CD117, CD41a, CD61, CD62, CD54, CD56, CD90, glycoporphin A, AC133, multidrug resistance (MDR-1) (BD Pharmingen, San Diego, CA). Analyses were performed by flow cytometry with a FACS Scan Flow cytometer (Becton Dickinson, Bedford, MA).

The expression of different hemopoietic growth factor (HGF) receptors was investigated by means of reagents and procedures previously reported.¹⁵

Genotypic characterization and genomic profiling

The configuration of the IgH chain gene region was analyzed by single-step polymerase chain reaction,¹⁶ while the T-cell receptor-γ (TCR-γ) gene was analyzed through the amplification of the TCR-γ locus V-J junction region by heteroduplex analysis.¹⁷

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For genomic profiling, total RNA was extracted by means of TRizol reagent (Gibco BRL, Life Technologies, Grand Island, NY) and further purified with the use of SV Total RNA Isolation System (Promega, Madison, WI). The protocol for sample preparation and microarray processing is available from Affymetrix (www.Affymetrix.com; Santa Clara, CA). Genomic profiling was carried out by means of the Affymetrix U95Av2 gene chip, which contains 12 600 gene sequences. To calculate gene expression levels and for normalization with other acute leukemia samples, the d-chip program was used.¹⁸ Genes whose expression levels were higher than 100 were considered expressed.

Cell-growth, cell-cycle, apoptosis, and clonogenic activity

Recombinant human interleukin-3 (rhIL-3) (2×10^6 U/mg), rh granulocyte-macrophage colony stimulating factor (rhGM-CSF), and rhIL-6 (2×10^8 U/mg) were supplied by Genetics Institute (Cambridge, MA); rh erythropoietin (rhEpo) (1.2×10^5 U/mg) and bovine basic fibroblast growth factor (rhbFGF; 2×10^7 U/mg) by Amgen (Thousand Oaks, CA); rh flt-3 ligand (FL) (1.9×10^6 U/mg) and rh c-kit ligand (KL) (1×10^5 U/mg) by Immunex (Seattle, WA); rhG-CSF (1×10^8 U/mg) and rhM-CSF (6×10^7 U/mg) by R&D Systems (Minneapolis, MN); rh thrombopoietin (rhTpo) was generously provided by Genentech (San Francisco, CA). Iscoves modified Dulbecco medium (IMDM) (GIBCO, Grand Island, NY) was prepared weekly.

Leukemic cells were grown in liquid suspension in IMDM containing 10% fetal calf serum (GIBCO) and various combinations of HGFs. The cell number was evaluated at each day of culture after trypan blue staining.

Cell-cycle analysis was carried out on nuclei stained with propidium iodide, as described.¹⁹

Results and discussion

Morphologic and immunophenotypic characterization

The leukemic population was uniformly composed of highly undifferentiated small- to medium-sized blasts. All cytochemical reactions were negative. Ultrastructural examination showed a large, centrally placed nucleus with prominent nucleoli. The cytoplasm contained small amounts of free ribosomes, a small Golgi apparatus, and endoplasmic reticulum cisternae. No MPO activity was detected.

The immunophenotype showed that (1) leukemic cells were CD34⁺ and did not express MPO; (2) CD34⁺ cells displayed a phenotype similar to that associated with most normal immature hemopoietic progenitors, CD33⁻, HLA-DR⁻, flt3⁺, CD90⁺, c-kit^{dim+}, CD38^{-/dim+}, AC133⁺; and (3) CD34⁺ cells did not display membrane markers of the erythroid, megakaryocytic, monocytic, granulocytic, B and T lineages.²⁰⁻²² TdT was expressed on 27% of cells. MDR-1 was negative (Figure 1A).

The immaturity of the blasts was further supported by the pattern of transcription factor expression: GATA-2⁺, Tal-1⁻, GATA-1⁻.²³

Genotypic characterization and genomic profiling

The IgH and TCR- γ gene regions were germline. To more precisely elucidate the nature of the leukemia, a genomic profiling was carried out. While the conventional lymphoid- and myeloid-related genes were not expressed, a set of early stem cell and myeloid-associated genes, particularly of the mixed lineage leukemia (MLL) family, was found (Table 1). CD34 and flt3 were expressed at high levels.

HGF receptor expression

HGF receptor analysis showed that (1) untreated blasts exhibited a characteristic pattern of HGF receptor expression, in which recep-

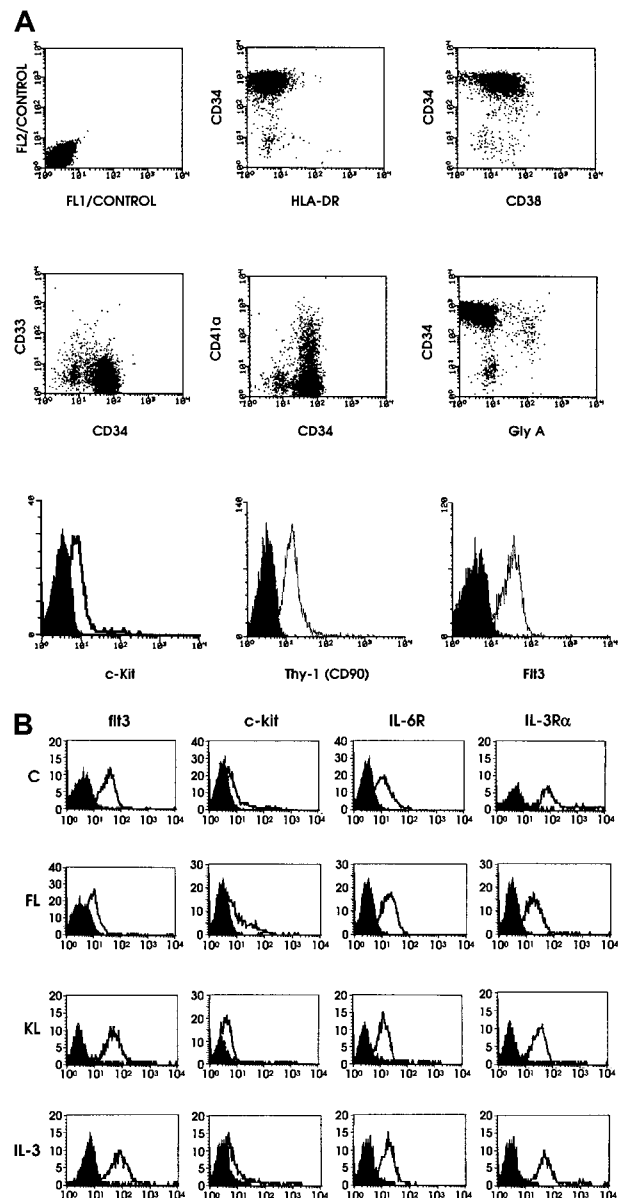


Figure 1. Flow cytometric analysis of leukemic blasts and results of HGF receptor expression. (A) Flow cytometric analysis of leukemic blasts. Flow cytometry of leukemic blasts was performed by using a panel of monoclonal antibodies selected to define the stem cell leukemia phenotype. Representative results are shown. (B) Representative results on HGF receptor (HGFR) expression. Expression of flt3, c-kit, IL-6R, and IL-3R α chain on leukemic blasts grown either in the absence of HGFs (control) or in the presence of FL, KL, or IL-3.

tors for early-acting and multilineage HGF were expressed, while receptors for late-acting HGF were virtually absent; (2) blasts grown for 5 days with FL exhibited the expected down-modulation of flt3; no modifications in c-kit, IL-6R, or IL-3R α ; and a significant rise in the expression of all 4 unilineage HGF receptors (Figure 1B); and (3) incubation for 5 days with KL induced a similar phenomenon, with some quantitative differences, consisting of the induction of higher levels of EpoR and lower levels of G-CSFR and c-fms compared with those elicited by FL (Figure 1B).

In vitro growth factor response

Studies performed with the use of a single HGF showed that only FL, but not other HGFs, had the capacity to sustain the proliferation (Figure 2A) and cell cycling (Figure 2B) of

Table 1. Genomic profile

Gene description	Map	Locus link ID	Level of expression
Human myeloid differentiation	1q21	4 170	839
Human stem cell progenitor homeobox	10q26	27 287	154
Human myeloid elf-1	Xq26	2 000	622
Myeloperoxidase	17q23	4 353	284
Myelodysplasia/myeloid leukemia factor 2	12p13	8 079	317
CD34	1q32	947	860
Human stem cell leukemia exon 6			124
Human AML1b protein	21q22.3	861	481
Fli3	13q12	2 322	1 417
Breakpoint cluster region	22q11.23	613	934
MLL, translocated to 2	4q21	4 299	558
MLL, translocated to 3	9p22	4 300	319
MLL, translocated to 4	6q27	4 301	176
MLL, translocated to 7	Xq13.1	4 303	355
Myeloid cell nuclear differentiation antigen	1q22	4 322	125
MLL, septinlike fusion	17q25	10 801	1 259
Myeloid differentiation primary response gene, 88	3p22	4 615	273
Clathrin-8 assembly lymphoid-myeloid leukemia gene	11q14	8 301	656
SET translocation (myeloid leukemia associated)	9q34	6 418	1 071
Control genes			
AFFX-HUMGAPDH	12p13	2 597	4 511
AFFX-HSACO (β -actin)	7p15-p12	60	3 161

elf-1 indicates E74-like factor 1; AML, acute myeloid leukemia; MLL, mixed lineage leukemia; AFFX, affimetrix; HUMGAPDH, human GAPDH; HSACO, β -actin.

leukemic cells and protect them from apoptosis (data not shown). The combination of different HGFs revealed several peculiar findings: (1) The combination of late-acting HGFs was insufficient to support the growth (Figure 2A-B) and to protect from apoptosis leukemic cells (data not shown). (2) Similarly,

multilineage HGF added alone or in combination with late-acting HGF neither stimulated the proliferation of leukemic cells (Figure 2A-B) nor protected them from apoptosis (data not shown). (3) Different combinations of early-acting HGF stimulated the proliferation of leukemic cells only when FL was present in the HGF cocktail (Figure 2A-B). (4) The combination of all early, multilineage, and late-acting HGFs maximally stimulated leukemic blast growth (Figure 2A-B).

In parallel, we evaluated the capacity of HGFs to induce the differentiation of leukemic blasts. Single HGF did not allow blasts to differentiate: blasts grown for 1, 2, or 3 weeks in the presence of FL maintained a blast morphology (Figure 2C) and did not display lineage-specific membrane antigens. Interestingly, the addition of early-acting HGFs induced, after 3 weeks of culture, a predominant monocytic differentiation, as shown by morphologic (Figure 2C) and immunophenotypic criteria (the cells became CD14⁺). Addition of KL to multilineage plus unilineage HGFs elicited the differentiation of leukemic blasts into the monocytic and erythroid lineages (Figure 2C), while the addition of KL to unilineage HGFs elicited a selective erythroid differentiation (Figure 2C). The addition of FL to multilineage HGFs in combination with unilineage HGFs or to unilineage HGFs alone induced the differentiation of leukemic cells toward all 4 myeloid hemopoietic lineages (Figure 2C).

Taken together, these findings confirm the close stem cell association of this case and further pointed to its likely early myeloid affiliation. It should also be noted that we observed the induction of endothelial cell markers (ie, CD105, CD106, CD62E, and CD62P) after in vitro culture of leukemic cells in the presence of FL and vascular endothelial growth factor.

Further studies on other cases of undifferentiated AL are required to verify whether the atypical pattern of HGF receptor expression and response to HGF and multilineage differentiation potential here reported represents a peculiar property of this leukemia subtype.

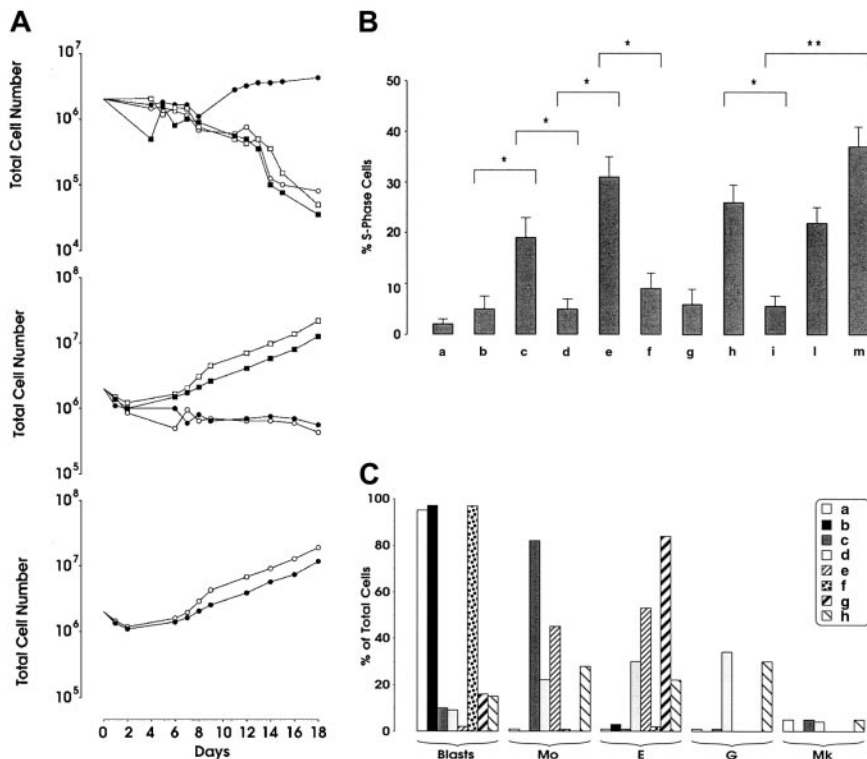


Figure 2. Cell-growth and cell-cycle analysis of leukemic blasts and differentiation of leukemic blasts in liquid suspension culture supplemented with different HGFs.

(A) Cell-growth analysis of leukemic blasts. Leukemic blasts were grown in liquid suspension cultures in the presence of different HGFs. Top panel: (○) control; (●) FL; (□) KL, and (■) IL-3. Middle panel: (○) Epo, Tpo, M-CSF, and G-CSF; (●) IL-3, GM-CSF, Epo, Tpo, M-CSF, and G-CSF; (□) IL-6, bFGF, IL-3, GM-CSF, Epo, Tpo, M-CSF, and G-CSF; (■) KL, IL-6, bFGF, IL-3, GM-CSF, Epo, Tpo, M-CSF, G-CSF. Bottom panel: (○) FL, IL-3, GM-CSF, Epo, Tpo, M-CSF, and G-CSF; (●) KL, IL-3, GM-CSF, Epo, Tpo, M-CSF, and G-CSF. Mean values of 3 independent experiments are shown. (B) Cell-cycle analysis of leukemic blasts. Leukemic blasts were grown for 5 days in liquid suspension in the presence of different HGFs. (a) Control; (b) KL; (c) FL; (d) Epo, Tpo, M-CSF, and G-CSF; (e) FL, Epo, Tpo, M-CSF, and G-CSF; (f) KL, Epo, Tpo, M-CSF, and G-CSF; (g) IL-3, GM-CSF, Epo, Tpo, M-CSF, and G-CSF; (h) FL, KL, IL-6, and bFGF; (i) KL, IL-6, and bFGF; (j) FL, IL-6, bFGF, IL-3, GM-CSF, Epo, Tpo, M-CSF, and G-CSF; and (m) KL, IL-6, bFGF, IL-3, GM-CSF, Epo, Tpo, M-CSF, and G-CSF. Mean + SD of 3 independent experiments is presented. * $P < .05$. ** $P < .01$. (C) Differentiation of leukemic blasts in liquid suspension culture supplemented with different HGFs. Cells were identified on cytopins stained with May-Grünwald Giemsa and were classified as blasts, erythroid (E), monocytic (Mo), granulocytic (G), or megakaryocytic (Mk) cells. (a) Control; (b) KL; (c) FL, KL, IL-6, and bFGF; (d) FL, IL-6, bFGF, IL-3, Epo, Tpo, M-CSF, and G-CSF; (e) KL, IL-6, bFGF, IL-3, Epo, Tpo, M-CSF, and G-CSF; (f) Epo, Tpo, M-CSF, and G-CSF; (g) KL, Epo, Tpo, M-CSF, and G-CSF; and (h) FL, Epo, Tpo, M-CSF, and G-CSF. A representative experiment is presented.

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