Expression of the Hematopoietic Growth Factor Receptor FLT3 (STK-1/Flik2) in Human Leukemias

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Normal expression of the hematopoietic growth factor receptor FLT3 (STK-1/Flik2) is limited to CD34+ stem/progenitor cells. We have evaluated the expression of FLT3 by RNase protection assay and Western blotting in 161 primary bone marrow (BM) samples from patients with leukemia. FLT3 RNA was found to be expressed at a higher level than in normal BM controls in 33 of 33 B-lineage acute leukemias, 11 of 12 acute myeloid leukemias (AMLs), and 3 of 11 T-cell acute leukemias (T-ALLs). Expression of FLT3 RNA was also observed in some cases of blast crisis CML. The FLT3 signal resulted from expression on the leukemic blasts, and was not caused by increased FLT3 expression on normal CD34+ stem/progenitor cells in the leukemic samples. To determine if FLT3 protein was also overexpressed, proteins were extracted from leukemic BM samples and screened by Western blotting with anti-FLT3 antisera. FLT3 protein was not detected in normal BM controls, but was found in 14 of 14 B-lineage AMLs, 36 of 41 AMLs, and 1 of 4 T-ALLs. Stimulation of patient samples with FLT3 ligand resulted in autophosphorylation of the FLT3 receptor, suggesting the receptor is functional in these cells. These data show that FLT3 RNA and protein are aberrantly expressed by AML and ALL cells in that CD34 expression and FLT3 expression are no longer synchronous, and suggest the possibility that overexpression of FLT3 could play a role in the survival and/or proliferation of malignant clones in acute myeloid and lymphoid leukemias.

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1089

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tors resulted in the factor-independent growth of these normally IL-3-dependent cell lines. Point mutations at codons 301 and 969 have been found within the gene for c-fms in some cases of myelodysplastic syndrome and AML. However, no functional studies were performed with these mutant receptors. Constitutive activation of c-kit or c-fms, in the absence of their ligand, could contribute to the leukemic phenotype.

Expression of FLT3 has been shown in samples from AML and ALL patients by Northern blot analysis. However, expression of a gene at the message level may not translate into proportionate overexpression of protein prod-

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**Fig 1.**
FLT3 (STK12/Flk2) EXPRESSION IN HUMAN LEUKEMIAS

ucl.30,31 Therefore, we investigated the expression of FLT3 in cases of human acute leukemia by the techniques of RNase protection and Western blotting. We have shown previously that the expression of FLT3 in normal BM is restricted to CD34+ stem/progenitor cells. Although leukemic blasts often comprise the majority of cells in affected marrow, normal cells are also present. Therefore, it was possible that increased FLT3 expression in samples of BM from patients with leukemia could also result from an increased number of FLT3 receptors/normal CD34+ cell or an overall increase in the number of normal CD34+ cells. To conclusively show that FLT3 was expressed by the leukemic blasts, we immunoaffinity-depleted the remaining normal CD34+ cells from samples of leukemic BM obtained from cases where the leukemic blast cells were CD34+, and examined the expression of FLT3 by the leukemic blasts.

MATERIALS AND METHODS

Patient samples. BM samples from normal adults and either adult or pediatric patients with leukemia at diagnosis were collected according to protocols approved by the Joint Committee on Clinical Investigation of the Johns Hopkins Medical Institutions in accordance with the policies of the United States Department of Health and Human Services. Samples were routinely immunophenotyped for cell surface markers.32 Leukemic blast cells were classified as CD34+ if >10% of cells in the sample expressed CD34 by fluorescence-activated cell sorter (FACS) analysis. Marrow mononuclear cells were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) sedimentation. CD34-depleted cell fractions were indirectly purified by two rounds of immunomagnetic separation.33 Briefly, mononuclear cells were incubated with the anti-CD34 antibody HPCA-1 (My10). The cells were incubated with sheep-antimouse IgG1-coated immunomagnetic microspheres (Dynal, Great Neck, NY), and the CD34+ (antibody-bound) cells were removed with a magnet. The remaining cells were classified as CD34−. Fractionated populations were routinely immunophenotyped and analyzed by flow cytometry.

RNA protection and RNase protection analysis. Total RNA from pediatric and adult BM mononuclear cells was extracted using the guanidium thiocyanate method.34 The RNase protection assay was performed as described on approximately 5 μg of RNA.35 The specific FLT3 probe used in the assay was created by cloning a 5′ fragment of FLT3 extending from nucleotide (nt) 70 through 269 into pBluescript II KS− (Stratagene Cloning Systems, La Jolla, CA) downstream of the T3 promoter. Transcription from this promoter produced a 330-nt transcript with 130 nt of vector sequence, followed by 200 nt of antisense FLT3. A β-actin probe was included in the hybridization reactions as an internal control for RNA loading. Protected fragments were resolved by electrophoresis on 6% polyacrylamide sequencing gels. After exposure to film, gels were exposed to a phosphorimaging screen ( Molecular Dynamics, Sunnyvale, CA). Bands were quantified using the Image Quanti program, and normalized relative to the actin signals.

Protein preparation and Western analysis. Proteins were extracted from adult BM mononuclear cells as previously described.30,36 Briefly, samples were solubilized by sonication in alkylating buffer (6 mol/L guanidine HCl, 250 mmol/L Tris-HCl/pH 8.5, and 10 mmol/EDTA, supplemented with 1% vol/vol 2-mercaptoethanol and 1 mmol/L phenylmethylsulfonyl fluoride), alkylated with iodoacetamide, and dialyzed sequentially into 4 mol/L urea and 0.1% weight/vol sodium dodecyl sulfate (SDS). Approximately equal amounts of protein (representing 400,000 cells) in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer were electrophoresed and transferred to nitrocellulose. For ligand stimulation experiments, BM mononuclear cells were incubated for 10 minutes at 37°C with or without 200 ng/mL of human FLT3 ligand (Immunex Corp, Seattle, WA) under serum-free conditions, and proteins were extracted as described above. The unstimulated lanes were overloaded aproximately threefold to show the dramatic differences in phosphorylation levels on ligand stimulation. Western blotting was performed as described37 with the following primary antibodies: IM 140, an affinity purified rabbit polyclonal IgG raised against a peptide from the kinase insert region of FLK23; IM 133, an affinity purified rabbit polyclonal IgG raised against a peptide from the kinase insert region of c-fms38; a rabbit polyclonal IgG raised against the c-terminal domain (amino acids [aa] 958-976) of human c-kit (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), and murine monoclonal anti-phosphotyrosine (clone 4G10; Upstate Biotechnology Inc, Lake Placid, NY). After washing, the filters were incubated with a horse-radish peroxidase-conjugated secondary antibody (Amersham, Arlington Heights, IL) and detected by ECL (Amersham). All Western blots were exposed to film for 1 hour unless noted otherwise in the figure legend. To control for differences in loading, blots were subsequently reacted with a murine monoclonal IgM specific for histone H1 (the kind gift of Dr James A. Sorace, V.A. Hospital, Baltimore, MD). For antibody blocking experiments, the antibody was incubated for 1 hour at room temperature with a fourfold excess of immunizing peptide before use.

RESULTS

FLT3 message is overexpressed in human leukemias. Samples of BM obtained from 66 patients with a diagnosis of either acute or chronic leukemia before treatment were analyzed by RNase protection and representative samples are shown in Fig 1A through D. Protected probe of the expected size, indicating the expression of FLT3 message, was detected in the myeloid leukemia cell line ML-1 (Fig 1A, C and D, lane 7; Fig 1B, lane 6) and in normal BM controls (Fig 1A and D, lane 10; Fig 1B, lane 8; Fig 1C,
The probe was not protected from digestion by no RNA or 20 µg of tRNA (Fig 1A through D, lanes 4 and 5). Overexpression of FLT3 message compared to normal BM was detected in 11 of 12 AML cases (Fig 1A, patient samples in lanes 11 through 22), 33 of 33 B-lineage ALL cases (Fig 1B, patient samples in lanes 9 through 29; others not shown), and 3 of 11 T-ALL cases (Fig 1C, patient samples in lanes 9 through 18; others not shown). There were no immunophenotypic differences between the T-ALL samples that did and those that did not express FLT3. We also screened both chronic and blast crisis phase CML samples for the presence of FLT3 transcripts by RNase protection (Fig 1D). Expression was higher than in normal marrow in one sample of CML in myeloid blast crisis (lane 19). Immunophenotyping analysis showed that 9 of 12 AML samples were CD34+ and expressed FLT3, 2 of 12 samples expressed FLT3 and were CD34+, and the remaining sample was CD34+ and did not express FLT3. Among B-lineage ALL samples in which CD34 expression was known, 13 of 26 samples were CD34+ and expressed FLT3, whereas 13 of 26 samples were CD34+ and expressed FLT3. One of the three T-All samples that expressed FLT3 was CD34+, whereas the other two were CD34+. Of the FLT3 negative samples two out of eight were CD34+. Relative FLT3 RNA levels (determined by phosphorimaging with actin as an internal control for loading differences) ranged to 8.2-fold higher than that of normal marrow for CML samples, 3.4-fold higher for T-ALLs, 22.4-fold higher for AML, and 657.5-fold higher for B-lineage ALLs. On average, B-lineage ALL samples expressed 107.6-fold higher levels of FLT3 than the normal marrow control.

FLT3 is overexpressed by leukemic blasts. We have shown previously that the expression of FLT3 in normal BM is restricted to CD34 stem/progenitor cells. Although leukemic blasts often comprise the majority of cells in affected marrow, normal cells are also present. Therefore, it was possible that FLT3 expression in samples of BM from patients with leukemia could result from an increased number of FLT3 receptors/normal CD34+ cell, or an overall increase in the number of normal CD34+ cells. To conclusively show that FLT3 was expressed on the leukemic blasts, we double-depleted the normal CD34+ cells from samples of BM obtained from cases in which the leukemic blast cells were immunophenotyped as CD34+ and examined the expression of FLT3 by the leukemic blasts. Figure 2 shows samples from three patients in which we were able to obtain adequate amounts of RNA enabling loading of equal quantities of total and CD34 depleted RNA (all patients were B-lineage ALLs with staining positive for CD19, CD10, and HLA-DR and negative for CD4, CD7, and CD34). By FACS analysis, double-depletion with immunomagnetic beads removed greater than 90%, and left undetectable numbers of CD34+ cells remaining in the blast cell fraction. If the FLT3 signal resulted from expression on the remaining normal CD34+ cells, the band intensity should be diminished by greater than 90%. As is evident visually, the expression of FLT3 RNA was detected in the CD34 depleted fraction and the undepleted fraction at approximately equal levels. Quantitation by phosphorimaging analysis after normalization to the internal actin signal confirmed that each of the CD34 depleted samples was within ±15% of the signal from the undepleted total BM sample. Quantitation of samples from five other patients (not shown because of large differences in loading) also confirmed that in no cases did the CD34 depletion result in a significant depletion of FLT3 signal. Thus, the overexpression of FLT3 originates from the leukemic blasts, rather than the CD34+ cells in the samples.

FLT3 protein is overexpressed in AML and ALL cells. Because protein expression does not necessarily correlate with the amount of RNA expressed, we wanted to determine whether FLT3 protein was overexpressed compared to normal BM controls. Proteins were extracted from 95 fresh samples of leukemic BM, representing 75 individual patients. Samples contained on average 69.6% blasts (range: 10% to 100% blasts). Each sample was screened between one and three times on separate blots by Western analysis using anti-FLT3 antibody, and representative blots are shown in Figs 3 and 4. Expression of FLT3 polypeptide, corresponding to a doublet of 130 and 160 kD (double arrows), was observed in ML-I cells (Fig 3A, lane 21, positive control; shorter exposure, additional panel), but not in CEM cells (Fig 3A, lane 23, negative control). NIH 3T3 cells transduced with the full-length FLT3 gene also express products of these sizes. Preincubation of the antibody with a fourfold excess of immunizing peptide inhibited specific binding (data not shown). Blots were routinely probed for histone H1 as a control for loading differences (Fig 3B).

Expression of FLT3 protein was not detectable in normal BM controls (Fig 3A, lanes 19 and 20). FLT3 protein was
Fig 3. Expression of FLT3 protein in human leukemias. (A) Western blotting performed with anti-FLT3 antibody showing 130-kD and 160-kD forms of the receptor (arrows). Lanes 1 through 6, AML; lanes 7 and 8, T-ALL; lanes 9 through 11, B-lineage ALL; lane 12, CML in accelerated phase; lanes 13 and 14, blast crisis CML; lane 15, chronic phase CML; lane 16, no sample; lane 17, AML in clinical remission; lanes 18 and 19, normal BM controls; lane 20, no sample; lane 21, ML-1 cells (positive control); lane 22, no sample; lane 23, CEM cells (negative control). Additional panel: ML-1 cells, lane 21, 5 minutes exposure. (B) To control for differences in loading, the blot in (A) was probed with anti-histone H1 antibody.

FLT3 protein expression by AML blasts was not restricted to any specific French-American-British (FAB) classification (Table 1). FLT3 protein was also expressed in one of four samples of T-ALL (Fig 3A, lanes 7, 8; Fig 4A, lanes 4 through 6, 13; Table 2) and 14 of 14 samples of B-lineage ALL (Fig 3A, lanes 9 through 11; Fig 4A, lanes 1 through 3, 7 through 12, 14 through 16; others not shown; Table 2), including one case of Burkitt’s leukemia (Fig 4A, lane 14; Table 1).

Fig 4. Expression of FLT3 protein in ALL and CLL. (A) Western blotting performed with anti-FLT3 antibody showing 130-kD and 160-kD forms of the receptor (arrows). Lanes 1 through 3, 7 through 12, 15, and 16, B-lineage ALL; lanes 4 through 6, and 13, T-ALL; lane 14, Burkitt’s leukemia; additional panel: lanes 17 through 19, CLL samples. Bracketed samples were obtained from the same patient: lane 2, presentation; lane 3, relapse; lane 4, presentation; lane 5, remission; lane 6, relapse; lane 7, presentation; lane 8, relapse; lane 9, presentation; lane 10, relapse. (B) To control for differences in loading, the blot in (A) was probed with anti-histone H1 antibody.
Expression of FLT3 protein was also analyzed in a series of 13 CML samples (Fig 3A, lanes 12 through 15; others not shown; Table 2). Expression of FLT3 was detected in 1 chronic phase CML sample (data not shown), 1 mixed blast crisis CML sample (Fig 3A, lane 13) and 1 lymphoid blast crisis CML sample (Fig 3A, lane 14). In addition, FLT3 expression was observed in 2 of 3 samples of chronic lymphocytic leukemia (CLL panel, Fig 4A, lanes 9). Expression of FLT3 protein was also analyzed in a series of 13 CML samples (Fig 3A, lanes 12 through 15; others not shown; Table 2). These results show that FLT3 protein is expressed at a higher level than in normal BM cells in 88% of AML cases, 100% of B-lineage ALL cases, and 25% of T-lineage ALLs (Table 2).

A total of 9 samples were screened by both RNase protection and Western blotting. These samples represented 2 patients with AML, 1 patient with Burkitt’s lymphoma, 1 patient with T-ALL, and 5 patients with B-lineage ALL. All 9 patients expressed FLT3 and thus there was 100% concordance between the two assays. In cases in which samples were screened by either RNase protection or Western blotting, but not both (152 samples), the percentages of patients that expressed FLT3 were in agreement between the two assays, except in cases where the number of samples was small (Table 2). We also screened 5 patients by Western blotting at presentation and after relapse (2 AMLs, not shown; 3 B-lineage ALLs, Fig 4A, lanes 2 and 3, 7 and 8, 9 and 10), and 1 patient at presentation, during remission, and after relapse (T-ALL) (Fig 4A, lanes 4 through 6). Expression of FLT3 was detected at both presentation and relapse for 5 of 6 samples. One sample (T-ALL) expressed FLT3 at low but detectable levels at presentation (lane 4), became undetectable during remission (lane 5), and expressed high levels of FLT3 after relapse (lane 6).

The expression pattern of FLT3 differs from that of c-kit and c-fms. To compare the expression pattern of FLT3 to that of c-kit and c-fms, the blot in Fig 3A was probed with antibodies to c-fms and c-kit. The expression pattern of these receptors differed from that of FLT3 (data not shown), consistent with the expression data in the literature for these receptors. Overexpression of c-kit protein compared to normal BM was observed in some samples of AML and CML in blast crisis, but was not detectable in ALL samples. Expression of c-fms was detected in both normal and leukemic BM samples.

FLT3 ligand stimulates the autophosphorylation of FLT3 receptor in primary human leukemia cells. It was still possible that the overexpressed protein shown in these leukemic samples was nonfunctional. The protein could be expressed in a form that does not make it to the cell surface or might not possess the ability to respond to its ligand. Other type III receptors to which FLT3 is most closely related include the c-kit and c-fms receptors. These receptors possess an intrinsic kinase activity that resides in the carboxy terminus of the protein. Their kinase activity is activated on binding of ligand and results in autophosphorylation. Therefore, we decided to test the functionality of the FLT3 receptor expressed by the leukemic blasts by stimulating the cells with FLT3 ligand and then examining the receptor for phosphorylation. Two patient samples of primary B-lineage ALL cells were incubated without, or stimulated with FLT3 ligand under serum-free conditions for 10 minutes at 37°C, and cell lysates were prepared as described. Samples were analyzed.

<table>
<thead>
<tr>
<th>Patient Sample</th>
<th>No. FLT3 Positive By RNase Protection (%)</th>
<th>No. FLT3 Positive By Western Blotting (%)</th>
<th>Total No. Of Patients FLT3 Positive (%)</th>
</tr>
</thead>
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<tr>
<td>AML</td>
<td>11/12 (92)</td>
<td>36/41 (88)</td>
<td>47/53 (89)</td>
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<tr>
<td>B-Lineage ALL</td>
<td>33/33 (100)</td>
<td>13/13 (100)</td>
<td>46/46 (100)</td>
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<td>CML, chronic</td>
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<td>0/2 (0)</td>
<td>0/2 (0)</td>
</tr>
<tr>
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<td>1/1 (100)</td>
<td>2/7 (29)</td>
<td>3/8 (38)</td>
</tr>
<tr>
<td>CML, blast crisis</td>
<td>0 (0)</td>
<td>2/3 (67)</td>
<td>2/3 (67)</td>
</tr>
</tbody>
</table>
by SDS-PAGE followed by Western blotting with anti-FLT3 antibody (Fig 5A), and after stripping the blot, with antiphosphotyrosine antibody (Fig 5B). Stimulation with FLT3 ligand resulted in the appearance of a phosphorylated band at 160 kD corresponding to the glycosylated, cell surface form of FLT3 (Fig 5B, lanes 6 and 8). The unstimulated lanes were loaded with approximately threefold more protein (compare lanes 1 and 3 v 2 and 4), thus making the relative intensity of the phosphorylated bands, which are only visible in the stimulated samples even more impressive. Thus, the overexpressed FLT3 receptor seems to be functional because it is expressed on the cell surface where it is able to bind FLT3 ligand and activate its kinase activity.

DISCUSSION

We have shown that FLT3 RNA is overexpressed compared to normal BM controls in 100% of B-lineage ALLs, 92% of AMLs, and 27% of T-ALLs. We have also shown that the overexpression of FLT3 RNA results in a similar pattern of overexpression of FLT3 protein. Overall, including samples assayed either by RNase protection or Western blotting, FLT3 is expressed at a higher level than normal BM in 100% of B-lineage ALLs, 89% of AMLs, and 27% of T-ALLs. This is in close agreement with a previously published results, which showed that high levels of FLT3 RNA were expressed in 100% of B-lineage ALLs, 92% of AMLs, and a number of T-ALLs.29 We detected overexpression of FLT3 RNA or protein in three cases of blast crisis CML, representing mixed, lymphoid, and myeloid blast crisis. This is also in agreement with the previous study, which showed that FLT3 RNA is expressed in blast crisis CML irrespective of phenotype.29 In contrast to the previous study, we did detect FLT3 expression in one chronic phase CML sample. However, this patient entered blast crisis within several months of sampling. Additionally, FLT3 protein expression was observed in one case of Burkitt’s leukemia and two of three cases of CLL. The expression pattern of FLT3 is distinct from that of c-kit, which is expressed at a high level in AMLs and CMLs in myeloid blast crisis but not in B-lineage or T-cell ALL.12-17 and c-fms, which is expressed in some AMLs and T-ALLs.18-23 The finding of FLT3 expression in what was believed to be a remission sample of an AML patient is intriguing because this expression pattern may be indicative of a marker of relapse. However, this observation must be tested in a prospective fashion with adequate samples to prove its validity.

Expression of FLT3 is normally limited to CD34+ cells. Importantly, we have shown that FLT3 RNA is expressed on leukemic blasts in cases of AML and ALL, irrespective of CD34 expression. Taken together, these observations suggest that overexpression of FLT3 in AMLs and ALLs compared with normal BM is the result of aberrant expression of FLT3 which is no longer synchronous with CD34 expression. Availability of an anti-FLT3 antibody directed at cell-surface epitopes of FLT3 should allow the further characterization of FLT3 expression on leukemic blast cells, and will provide a means of determining FLT3 expression on a per-cell basis compared with normal marrow cells.29 In addition, we have shown that the stimulation of leukemia cells with FLT3 ligand results in autophosphorylation of the FLT3 receptor, suggesting active signal transduction in these cells. Whether coexpression of FLT3 and FLT3 ligand resulting in an autocrine or paracrine loop occurs in the same leukemia cell remains to be investigated. However, considering the wide pattern of expression of FLT3 ligand mRNA, it is possible that cells expressing FLT3 may have access to a readily available ligand. The data suggests that overexpression of FLT3 may be involved in the maintenance/proliferation of malignant clones in cases of acute leukemia.

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REFERENCES

12. Ikeda H, Kanakura Y, Tamaki T, Kurii A, Katayama H,


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CE Carow, M Levenstein, SH Kaufmann, J Chen, S Amin, P Rockwell, L Witte, MJ Borowitz, CI Civein and D Small