Fms-like Tyrosine Kinase 3 Ligand Stimulation Induces
MLL-Rearranged Leukemia Cells into Quiescence
Resistant to Antileukemic Agents

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Abstract

Fms-like tyrosine kinase 3 (FLT3) is highly expressed in acute lymphoblastic leukemia with the mixed-lineage leukemia (MLL) gene rearrangement refractory to chemotherapy. We examined the biological effect of FLT3-ligand (FL) on 18 B-precursor leukemia cell lines with variable karyotypic abnormalities, and found that nine of nine MLL-rearranged cell lines with wild-type FLT3, in contrast to other leukemia cell lines, are significantly inhibited in their proliferation in a dose-dependent manner by FL. This inhibition was due to induction of the G0-G1 arrest. A marked up-regulation of p27 dose-dependently by FL. This inhibition was due to suppression of its protein degradation and an abrogation of constitutive signal transducers and activators of transcription 5 phosphorylation were revealed in arrested leukemia cells after FL stimulation. Importantly, FL treatment rendered not only cell lines but also primary leukemia cells with MLL rearrangement resistant to chemotherapeutic agents. MLL-rearranged leukemia cells adhering to the bone marrow stromal cell line, which expresses FL as the membrane-bound form, were induced to quiescent state resistant to chemotherapeutic agents. MLL-rearranged leukemia might be involved in maintenance of constitutive signal transducers and activators of transcription 5 phosphorylation were revealed in arrested leukemia cells after FL stimulation. Importantly, FL treatment rendered not only cell lines but also primary leukemia cells with MLL rearrangement resistant to chemotherapeutic agents. MLL-rearranged leukemia cells adhering to the bone marrow stromal cell line, which expresses FL as the membrane-bound form, were induced to quiescent state resistant to chemotherapeutic agents, but their chemosensitivity was significantly restored in the presence of neutralizing anti-FL antibody. The FL/FLT3 interaction between leukemia cells and bone marrow stromal cells expressing FL at high levels should contribute, at least in part, to persistent minimal-residual disease of MLL-rearranged leukemia in bone marrow. [Cancer Res 2007;67(20):9852–61]

Introduction

Fms-like tyrosine kinase 3 (FLT3) belongs to the receptor tyrosine kinase class III family and plays an important role in an early stage of hematopoiesis (1–3). In normal bone marrow, FLT3 is expressed predominantly in hematopoietic stem/progenitor cells (2, 4) and FLT3-ligand (FL) shows a strong synergy with other cytokines in their proliferation (5, 6). FLT3 is also expressed at considerable levels in most clinical samples from acute myelo-
were incubated with biotinylated FL for 1h at 4°C. MAPK, phosphorylated p44/42 MAPK (Thr202/Tyr204), Akt, and phosphotyrosine, CDK6, p21, c-Myc, and cyclin B, cyclin D3, and STAT5 were purchased from Transduction Laboratories; phosphotyrosine, CDK6, p21, c-Myc, and cyclin A were from Santa Cruz Biotechnology. mAbs and p27 were from Upstate Biotechnology, Inc.; CDK2 was from polyclonal antibodies against cyclin-dependent kinase 4 (CDK4), cyclin E, poly-1-D-arabinofuranosylcytosine (AraC) were obtained from Meiji Seika (Japan). Immunohistochemistry was performed as previously described. Immunoprecipitation was performed with anti-FL (20 ng/mL) for 3 days. These cells were pulsed with [3H]thymidine (1 Ci/well) for 4 h, and the signals generated by FITC and propidium iodide were analyzed using a flow cytometer.

**Flow cytometric analysis.** For FLT3 expression, 5 × 10^5 leukemia cells were incubated with biotinylated FL for 1 h at 4°C followed by incubation with avidin-FITC for 30 min at 4°C. As a negative staining control, anti-FL blocking antibody was mixed with FL-biotin. For FL expression, cells were incubated with biotinylated anti-FL antibody followed by incubation with avidin-FITC. IgG-biotin was used for a negative staining control. These cells were washed and analyzed using a flow cytometer (FACS Calibur, Becton Dickinson).

Table 1. Characteristics of B-precursor leukemic cell lines and their FLT3 expression

<table>
<thead>
<tr>
<th>Surface antigens</th>
<th>Karyotype</th>
<th>FLT3 expression</th>
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<tbody>
<tr>
<td></td>
<td>CD 10</td>
<td>19</td>
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<tr>
<td><strong>MLL rearrangement</strong></td>
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<tr>
<td>KOCL-35</td>
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<tr>
<td><strong>MLL germ line</strong></td>
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<tr>
<td>KOPN-30bi</td>
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<tr>
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<tr>
<td>KOPN-70</td>
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Abbreviation: MFI, mean fluorescence intensity.

Minor bcr-abl.

Major bcr-abl.

[^H]thymidine uptake analysis. Leukemia cells (2.5 × 10^5 to 5 × 10^5 per well) were cultured in RPMI 1640 supplemented with 10% FCS in a 96-well flat-bottomed culture plate in triplicate in the presence or absence of various concentrations of FL at 37°C for the indicated periods. Subsequently, wells were pulsed with [^H]thymidine (1 μCi/well) for 4 h, after which the cells were harvested onto glass-fiber filters. In some experiments, a FLT3 kinase inhibitor, PKC412, was included at various concentrations. [^H]thymidine incorporated to DNA was measured using a liquid scintillation counter. Percentage stimulation was calculated as follows: [(cpm of treated) / (cpm of untreated)] – 1) × 100, Percentage thymidine uptake was calculated as follows: cpm of treated / (cpm of untreated) × 100.

Cell cycle analysis. Leukemia cells (5 × 10^4/mL) were cultured in the presence or absence of FL (20 ng/mL) for 3 days. These cells were pulsed with BrdUrd for 30 min at 37°C and harvested. After fixation in 70% ethanol on ice, cells were treated with RNase (Funakoshi) for 15 min at 37°C, and subsequently washed with 4 N HCl, 0.1 mol/L Na2B4O7, and 0.5% Tween 20. Cells were stained with FITC-conjugated anti-BrdUrd antibody for 20 min at 37°C and then treated with propidium iodide (50 μg/mL) for 20 min on ice. The signals generated by FITC and propidium iodide were analyzed using a flow cytometer.

Apoptosis analysis. Leukemia cells (5 × 10^4/mL) were incubated for 24 h in the presence or absence of FL (20 ng/mL) and then irradiated (4 Gy) followed by culture for 48 h, or exposed to daunorubicin (10 ng/mL) or AraC (200 mmol/L) for 48 h. Cells were then harvested, and stained with FITC-conjugated Annexin V and propidium iodide (MEBCYTO Apoptosis Detection Kit; MBL) at 37°C for 15 min in dark. Ten thousand events were analyzed using a flow cytometer.

Western blot analysis. Leukemia cells were solubilized in lysis buffer [50 mmol/L Tris-HCl (pH 7.5), containing 150 mmol/L NaCl, 1% NP40, 5 mmol/L EDTA, 0.05% NaDodecyl sulfate, 1 μg/mL aprotinin, 1 μg/mL pepstatin A, 10 mmol/L iodoacetamide, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium vanadate] SDS was added to the lysis buffer.
at a final concentration of 0.1% for analysis of nuclear proteins. The lysates were separated on a 6% to 15% SDS-polyacrylamide gel under reducing conditions and transferred to nitrocellulose membranes, which were incubated with various primary antibodies at 4°C overnight, and then with horseradish peroxidase–conjugated second antibody for 1 h at room temperature. The detection of the bands was done using an enhanced chemiluminescence kit (Amersham Japan).

RNAse protection assays. Leukemia cells (5 × 10⁵/mL) were cultured in the presence or absence of FL (20 ng/mL) for 24, 48, and 72 h, and their total RNAs were extracted. The RNase protection assay was done using [32P]UTP-labeled multiprobe template hCC-1 and the RiboQuant Multi-Probe RNAse Protection Assay system (PharMingen).

Dye-exclusion test. Leukemic cell lines (4 × 10⁵/well) were precultured in the presence or absence of FL (20 ng/mL) for 24 h, then exposed to daunorubicin (10–20 ng/mL) or AraC (200–400 nmol/L) for the indicated hours. In some experiments, the human bone marrow–derived stromal cell line KM-104 was used as the feeder. In analysis of primary samples, leukemia cells (1 × 10⁶/well) were precultured in the presence or absence of FL (40 ng/mL) or KM-104 cells with or without anti-FL antibody for 24 h, and then exposed to AraC (100 nmol/L) for 24 h. The numbers of living and dead cells were counted by dye-exclusion test in triplicate after each (except for culture conditions, and viability (%) and ∆viability (treated viability – control viability) were calculated.

Statistics. The Mann-Whitney’s test was used for the comparison of the differences in FLT3 expression among leukemia cell lines and fresh leukemia cells, and unpaired t test for the comparison of the differences in [3H]thymidine uptake and dye-exclusion analyses. Differences in the ∆viabilities of primary leukemia cells between culture conditions were analyzed using the matched paired Wilcoxon’s test. A P value of <0.05 was considered significant.

Results

Surface expression of FLT3 in leukemic cell lines. Surface expression of FLT3 in leukemic cell lines used in this study was first checked. B-precursor cell lines (n = 18) expressed a considerable amount of FLT3 on their surfaces (median 94.5%, range 22–99%) as shown in Table 1, whereas T-lineage cell lines (n = 2) did not (<10%; data not shown). There were no significant differences in the FLT3-positive cells between B-precursor cell lines with (n = 10) and without (n = 8) MLL rearrangement (median of positive population; 96.5% versus 89.0%). There were also no significant differences in the FLT3 expression levels between B-precursor cell lines with and without MLL rearrangement (median of mean fluorescence intensity: 165.6 versus 170.7).

FL stimulation inhibits proliferation of MLL-rearranged leukemia cells by induction of cell cycle arrest. To investigate the biological effect of FL, 18 B-precursor leukemic cell lines were incubated in the presence or absence of various concentrations of FL for 72 h, and their [3H]thymidine uptakes were assayed in the final 4-h incubation. As shown in Fig. 1A, three of four Ph1-positive leukemic cell lines showed stimulatory responses to FL in a dose-dependent manner. Two of other four B-precursor cell lines without MLL rearrangement also showed a marked stimulative response to FL. Unexpectedly, however, all of the MLL-rearranged cell lines with wild-type FLT3 (n = 9), irrespective of the types of translocation, showed inhibitory responses to FL in a dose-dependent manner as shown in Fig. 1B. The MLL-rearranged cell line with a D835 mutation, KOCL-33, was not affected by the addition of FL. The kinetics of [3H]thymidine uptakes after FL stimulation (20 ng/mL) was next analyzed using representative cell lines. The FL-induced inhibition in MLL-rearranged KOCL-51 and KOCL-58 reached maximal levels between 48 and 96 h of culture, whereas the FL-induced stimulation in KOPN-55bi (Ph1), KOPN-36 with t(1;19), and KOPN-70 (other B-precursor) peaked between 96 and 144 h of culture (data not shown). These results indicate that,

![Figure 1](https://example.com/fig1.png)

**Figure 1.** Effect of FL on [3H]thymidine uptakes and cell cycle progression in B-precursor leukemic cell lines. In [3H]thymidine uptake analysis, leukemic cell lines were cultured in triplicate in the presence or absence of FL for 72 h, pulsed, and harvested. Data are representative of three separate experiments and are shown as the mean. In cell cycle analysis, MLL-rearranged KOCL-58 and KOCL-51 cells were cultured in the presence or absence of FL (20 ng/mL) for 72 h, and stained with FITC-conjugated BrdUrd and propidium iodide. **A**, % stimulation of [3H]thymidine uptakes by various concentrations of FL in Ph1-positive (n = 4, open symbols) and other B-precursor cell lines without MLL rearrangement (n = 10, closed symbols). Bars, SE >3%. **B**, % stimulation of [3H]thymidine uptakes by various concentrations of FL in MLL-rearranged leukemic cell lines (n = 10). KOCL-33 is a special cell line with a D835 mutation of FLT3. Bars, SE >3%. **C**, effect of the FLT3 inhibitor (PKC412) on % thymidine uptake. MLL-rearranged KOCL-58 cells were cultured in the presence or absence of FL (20 ng/mL) with or without the addition of various concentrations of PKC412 (50–400 nmol/L), and % thymidine uptake was determined in each of culture conditions. *, P < 0.01, significant difference by t test. **D**, flow cytometric analysis of cell cycle progression. Vertical and horizontal axes, log fluorescence intensities of FITC and propidium iodide (PI), respectively.
in contrast to other types of leukemia cells, proliferation of \textit{MLL}-rearranged leukemia cells with wild-type FLT3 is specifically suppressed by ligand activation of FLT3.

To determine whether the inhibitory effect of FL is dependent on the kinase activity of FLT3, \textit{MLL}-rearranged KOCL-58 cells were cultured with or without FL (20 ng/mL) for 72 h in the presence of various concentrations of FL; top, changes in expression of p27 were examined in leukemic cell lines with (\(n = 4\)) or without (\(n = 2\)) \textit{MLL} rearrangement. A, Western blot analysis of changes in expression of cell cycle–related proteins after FL stimulation in \textit{MLL}-rearranged leukemia cells. Left, changes in expression of p27, p21, cyclins A, B, D3, and E; CDK2, CDK4, and CDK6; and c-Myc were examined in KOCL-58 cells. Right, top, changes in expression of p27 were examined in KOCL-58 cells after culture with increasing concentrations of FL; bottom, changes in expression of p27 were examined in leukemic cell lines with (\(n = 4\)) or without (\(n = 2\)) \textit{MLL} rearrangement.

To investigate the mechanism of the FL-induced growth inhibition seen in \textit{MLL}-rearranged cell lines, changes in phosphorylation of STAT5, MAPK, and Akt in KOCL-58 cells were examined by Western blot using antibodies against the phosphorylated (active) form of these molecules. FLT3 phosphorylation was examined using anti-phosphotyrosine antibody after FLT3 immunoprecipitation. FLT3, STAT5, MAPK, and Akt were constitutively phosphorylated before FL stimulation as were reported previously (20), and their phosphorylation was further up-regulated within 1 min after FL stimulation (100 ng/mL) and returned to the prestimulated level at 15 min (Supplementary Fig. S2). A similar pattern of changes in phosphorylation of these molecules after FL stimulation was observed in KOPN-70, which showed a stimulative response to FL in the \([\text{H}]\)thymidine uptake assay (data not shown), suggesting that an opposite biological

Figure 2. Changes in expression of cell cycle–related proteins and phosphorylation of FLT3-mediating signal transducing molecules after FL stimulation in \textit{MLL}-rearranged leukemia cells. 

A, Western blot analysis of changes in expression of cell cycle–related proteins at 48 h after culture in the presence or absence of FL (20 ng/mL). B, half-life of p27 after FL stimulation. KOCL-58 cells were incubated for 48 h in the presence or absence of FL (20 ng/mL), further incubated in the presence of cycloheximide, and harvested at indicated time points. Changes in the p27 expression were measured by densitometry and half-life of p27 was calculated. Data are representative of three separate experiments. C, Western blot analysis of changes in phosphorylation of STAT5, MAPK, and Akt after FL stimulation. KOCL-58 cells were incubated for 48 h in the presence or absence of FL (20 ng/mL). D, Western blot analysis of changes in STAT5 phosphorylation after FL stimulation. KOCL-58 cells were incubated for 1 or 3 h in the presence or absence of FL (20 ng/mL).
effect of FL, that is, stimulation or suppression of cell growth, is not simply due to differences in an early event of signaling after the FL/FLT3 interaction.

**FL stimulation markedly up-regulates p27 expression in MLL-rearranged leukemia cells.** It is known that cell cycle progression is controlled primarily by activities of CDKs that are up-regulated or down-regulated by cyclins and CDK inhibitors (CDKIs), including p16, p21, and p27, respectively. To examine the expression of these cell cycle-associated proteins after FL stimulation, KOCL-58 cells (5 x 10⁴/mL) were cultured 48 h in the presence or absence of FL (20 ng/mL). As shown in Fig. 2A (left), expression levels of cyclins A, B, D3, and E, and CDK2, CDK4, and CDK6, were completely unchanged. Expression of p16 was not detected as reported previously (23). Although expression of p21 was modestly up-regulated by FL, this was not seen consistently in repeated experiments. Of interest, expression of p27 was consistently and markedly up-regulated in the presence of FL. This p27 up-regulation was observed in a dose-dependent manner in response to FL, and reached a maximum level at 100 ng/mL (Fig. 2A, top right). In other experiments, it was shown that p27 was maximally up-regulated at FL concentrations between 20 and 40 ng/mL. Moreover, the FL-induced p27 up-regulation 48 h after FL stimulation was also observed to varying degrees in other MLL-rearranged cell lines with wild-type FLT3, but not in KOCL-33 with a D835 mutation (Fig. 2A, bottom right). Importantly, the FL-induced up-regulation of p27 was more profoundly observed in KOCL-58 and KOCL-50, which showed a marked suppression of [³H]thymidine uptake by FL than in KOCL-45 and KOPN-1, which showed a modest suppression of [³H]thymidine uptake by FL. In KOPN-55bi, KOPN-70 without MLL rearrangement that showed stimulative responses to FL, the p27 expression was somewhat down-regulated after FL stimulation.

To determine the mechanism of the FL-induced p27 up-regulation, changes in p27 mRNA expression after FL stimulation were examined in KOCL-58 by RNase protection assay. The level of p27 mRNA was completely unchanged after FL stimulation (Supplementary Fig. S3), suggesting that p27 up-regulation might be mediated by a posttranscriptional mechanism. To determine p27 stability, KOCL-58 cells were cultured for 48 h in the presence or absence of FL (20 ng/mL), further cultured after the addition of cycloheximide (150 µg/mL), and harvested 3, 6, and 9 h later. As shown in Fig. 2B, the estimated half-life of p27 was elongated from 2.6 h in the absence of FL to 3.9 h in the presence of FL. These results suggest that up-regulation of p27 induced by FL is due to suppression of its protein degradation.

**Phosphorylation of STAT5 is specifically abrogated after FL stimulation in MLL-rearranged leukemia cells.** To determine the activation status of FLT3-mediating signaling pathways at the time point where p27 is up-regulated by FL stimulation, KOCL-58 cells were cultured in the presence or absence of FL (20 ng/mL) for 48 h, and phosphorylation of STAT5, MAPK, and Akt was examined. As shown in Fig. 2C, in contrast to marked phosphorylation of STAT5 seen after 48 h of culture without FL, the addition of FL to the culture completely abrogated its phosphorylation. In contrast, phosphorylation of MAPK was up-regulated in the presence of FL at this time point, whereas phosphorylation of Akt showed no difference in the two culture conditions. This STAT5 dephosphorylation was observed in KOCL-58 within 1 h after FL stimulation (Fig. 2D). These results suggest that, among FLT3-mediating signaling pathways, the STAT5 pathway is specifically suppressed after FL stimulation.

**FL stimulation renders MLL-rearranged leukemic cells resistant to anti-leukemic agent–induced apoptosis.** It is thought that sensitivity of leukemia cells to irradiation and chemotherapeutic agents is reduced in “dormant” cells whose cell cycle progression is kept in sustained suppression. To assess whether the FL-induced cell cycle arrest affects sensitivity to irradiation-induced apoptosis, MLL-rearranged KOCL-58 and KOCL-51 cells (both possessing wild-type p53) were precultured for 24 h in the presence or absence of FL (20 ng/mL) and then irradiated (4 Gy). Induction of apoptosis was examined after 48 h of culture in the presence or absence of FL using FITC-conjugated Annexin V and propidium iodide. As shown in Fig. 3 (left), the Annexin V–positive apoptotic population decreased by FL from 45.3% to 24.5% in KOCL-58 and from 43.0% to 26.0% in KOCL-51. The propidium iodide–positive late apoptotic population also decreased in the presence of FL in both cell lines, suggesting that irradiation-induced apoptosis is effectively suppressed by pretreatment with FL followed by subsequent stimulation with FL. Similarly, to assess whether the FL-induced cell cycle arrest affects sensitivity to chemotherapeutic agent–induced apoptosis, KOCL-58 cells were precultured in the presence or absence of FL (20 ng/mL) for 24 h, and then exposed to daunorubicin (10 ng/mL) or AraC (200 nmol/L) for 48 h in the presence or absence of FL. As shown in Fig. 3A (right), the Annexin V–positive population decreased by FL from 38.1% to 20.5% in daunorubicin-treated cells and from 64.3% to 47.3% in AraC-treated cells. These results suggest that ligand activation of FLT3 in MLL-rearranged leukemia cells renders them resistant to irradiation- and chemotherapeutic agent–induced apoptosis.

To further evaluate the antiapoptotic activity of FL against chemotherapeutic agents in MLL-rearranged leukemia cells, KOCL-58 cells (4 x 10⁴/well) were precultured for 24 h in the presence or absence of FL (20 ng/mL) and then cultured for 3 days with or without the addition of daunorubicin (10 ng/mL) or AraC (200 nmol/L). Numbers of living and dead cells were determined by the dye-exclusion method at days 2, 3, and 4 after the start of preculture (Fig. 3B). In the culture without the addition of daunorubicin or AraC (top), cell proliferation was gradually inhibited (~50% inhibition at day 4) in the presence of FL. Of note, in the culture to which either daunorubicin (middle) or AraC (bottom) was added, an increase in the dead cell population was markedly suppressed in the presence of FL (~50% suppression at days 3 and 4). The FL-mediating suppression of cell death was similarly observed at a higher concentration of daunorubicin or AraC (Fig. 3C). Thus, it was largely estimated that IC₅₀ of daunorubicin and AraC was shifted by FL from 10 to 20 ng/mL and from 200 to 400 nmol/L, respectively. Of importance, the FL-induced inhibition of proliferation and resistance to AraC were specifically canceled by the addition of neutralizing anti-FL antibody in the culture medium (Fig. 3D, left). This FL effect was not observed in KOCL-33 with a D835 mutation (data not shown). These results indicate that the chemotherapeutic agent–induced apoptosis is suppressed in vitro via the interaction of FL with wild-type FLT3 in MLL-rearranged leukemia cells.

**Coculture with bone marrow stromal cells renders MLL-rearranged leukemia cells chemoresistant, which is canceled by anti-FL antibody.** Because FL is reported to be expressed at high levels as a soluble or membrane-bound form by bone marrow stromal cells (29), MLL-rearranged leukemia cells adhering to bone marrow stromal cells might be induced to cell cycle arrest via the FL/FLT3 interaction, resulting in acquisition of resistance to
Figure 3. FL-induced resistance to antileukemic agents in MLL-rearranged leukemia cells. A, flow cytometric analysis of the FL effect on irradiation- and chemotherapeutic agent–induced apoptosis. KOCL-58 and KOCL51 cells were precultured for 24 h in the presence or absence of FL (20 ng/mL), and then irradiated (4 Gy) or exposed to daunorubicin (10 ng/mL) or AraC (200 nmol/L). Flow cytometric analysis was done 48 h later using FITC-conjugated Annexin V and propidium iodide. Vertical and horizontal axes, log fluorescence intensities of propidium iodide and FITC, respectively. Data are representative of three separate experiments. B, analysis of FL-induced resistance to chemotherapeutic agents in MLL-rearranged leukemia cells by dye-exclusion test. KOCL-58 cells (4 × 10^4/well) were precultured in the presence or absence of FL (20 ng/mL) for 24 h, and then cultured with or without daunorubicin (10 ng/mL) or AraC (200 nmol/L) for 3 d. Columns, mean numbers of living (open columns) and dead (filled columns) cells at days 2, 3, and 4 after the start of preculture. Data are representative of three separate experiments. C, FL-induced resistance to different concentrations of chemotherapeutic agents. KOCL-58 cells (4 × 10^4/well) were precultured in the presence or absence of FL (20 ng/mL) for 24 h, and then cultured with or without different concentrations of daunorubicin (10 and 20 ng/mL) or AraC (200 and 400 nmol/L) for 48 h. Viability was calculated by dye-exclusion test. Points, mean; bars, SE. *, P < 0.05, significant difference by t test. D, effect of anti-FL antibody on soluble FL or bone marrow stromal cell–induced resistance to chemotherapeutic agents. Left, KOCL-58 cells (4 × 10^4/well) were precultured in the presence (filled columns) or absence (open columns) of FL (20 ng/mL) for 48 h with or without the addition of anti-FL antibody (2 μg/mL), and then cultured with or without AraC (200 nmol/L) for 48 h. Right, KOCL-58 cells (4 × 10^4/well) were precultured with (filled columns) or without (open columns) KM-104 cells for 48 h in the presence or absence of anti-FL antibody (2 μg/mL), and then cultured with or without AraC (200 nmol/L) for 48 h. Living and dead cell numbers were counted in triplicate by dye-exclusion tests. Columns, representative mean from three separate experiments; bars, SE.
antileukemic agents. Using the bone marrow stromal cell line KM-104 expressing FL at high levels as the membrane form (Supplementary Fig. S4), we thus did the in vitro model study. KOCL-58 cells (4 × 10⁵/well) were precultured for 2 days with or without KM-104 cells growing confluent on the bottom of the plate in the presence or absence of neutralizing anti-FL antibody (4 μg/ml), and then cultured in the presence or absence of AraC (200 nmol/L) for 2 days. As shown in Fig. 3D (right), the AraC-induced cell death was markedly (P < 0.01) suppressed when cocultured with stromal cells. Of importance, this stromal cell effect was partially but significantly canceled by anti-FL antibody in the culture medium, indicating that the FL/FLT3 interaction between MLL-rearranged leukemia cells and bone marrow stromal cells contributes, at least in part, to induction of cell cycle arrest of leukemia cells showing resistance to chemotherapeutic agents.

FL stimulation renders primary MLL-rearranged leukemic cells resistant to chemotherapeutic agent–induced cell death. To examine whether FL effect is also observed in primary MLL-rearranged leukemia cells, peripheral or bone marrow mononuclear cells (blasts >90%) stored in liquid nitrogen were thawed and cellscontributes,atleastinpart,toinductionofcellcyclearrestof
chemotherapeutic agent–induced cell death.

MLL-rearranged primary leukemia cells resistant to AraC and FLT3 expression

To examine whether FL effect is also observed in primary MLL-rearranged leukemia cells, peripheral or bone marrow mononuclear cells (blasts >90%) stored in liquid nitrogen were thawed and used for experiments. Characteristics of primary leukemia samples with (n = 9) or without (n = 5) MLL rearrangement are summarized in Table 2. MLL-rearranged primary leukemia cells expressed FLT3 at significantly (P < 0.01) higher levels than did those without MLL rearrangement (median of positive population; 76% versus 25%; median of mean fluorescence intensity; 64.5 versus 30.4). Primary leukemia cells (1 × 10⁵/well) were precultured for 24 h in the presence or absence of FL (40 ng/ml), further cultured for 24 h with AraC (100 nmol/L), and harvested. As representedively depicted in Fig. 4 (case 1), the addition of FL rendered MLL-rearranged primary leukemia cells resistant to AraC, which was partially but significantly (P < 0.05) canceled by anti-FL antibody. The viabilities (％) after 48 h culture in 14 cases with or without MLL rearrangement are summarized in Table 2. Of note, the viabilities after AraC exposure significantly (P < 0.05) increased by the addition of FL in five of seven primary leukemia cells with MLL rearrangement, but not in five of five primary leukemia cells without MLL rearrangement. This FL effect was specifically canceled by anti-FL antibody in all of the cases tested. Statistically, the Δviabilities (treated viabilities – control viabilities) after AraC exposure significantly (P < 0.05) increased by the addition of FL, which was canceled by the addition of anti-FL antibody (Fig. 4B).

In six cases with MLL rearrangement, leukemia cells (1 × 10⁵/well) were precultured for 24 h in the presence or absence of KM-104 cells with or without anti-FL antibody and further cultured for 24 h with AraC (100 nmol/L). As representedively depicted in Fig. 4A (case 8), coculture with bone marrow stromal cells rendered MLL-rearranged primary leukemia cells resistant to AraC, which was partially but significantly canceled by anti-FL antibody. The Δviabilities after AraC exposure significantly (P < 0.05) increased in the presence of stromal cells, which was canceled by the addition of anti-FL antibody (Fig. 4C). These results suggest that stimulation by FL, irrespective of its soluble or membrane form, specifically renders primary MLL-rearranged leukemia cells resistant to chemotherapeutic agent–induced cell death.

Discussion

MLL-rearranged infant ALL is known to have an especially poor prognosis (30), although its prognosis has gradually improved by intensified chemotherapy and hematopoietic stem cell transplantation (31–33). Nowadays, the complete remission rate in MLL-rearranged ALL after the induction chemotherapy has improved to

Table 2. Characteristics of B-precursor primary leukemia cells and their sensitivity to AraC and FLT3 expression

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Age (mo)/sex</th>
<th>WBC (×10⁹/L)</th>
<th>Sample</th>
<th>Karyotype/fusion gene</th>
<th>Viability (%) after 48 h culture*</th>
<th>FLT3 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Control AraC AraC + FL AraC + FL + Ab % MFI</td>
<td></td>
</tr>
<tr>
<td>MLL rearrangement</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1</td>
<td>4/M</td>
<td>271</td>
<td>Onset</td>
<td>PB</td>
<td>65 ± 4 46 ± 4 64 ± 2 † 53 ± 2 †</td>
<td>91 64.5</td>
</tr>
<tr>
<td>2</td>
<td>1/M</td>
<td>42</td>
<td>Onset</td>
<td>PB</td>
<td>57 ± 1 38 ± 1 50 ± 1 40 ± 2 †</td>
<td>91 139.3</td>
</tr>
<tr>
<td>3</td>
<td>0/M</td>
<td>182</td>
<td>Relapse</td>
<td>BM</td>
<td>61 ± 2 53 ± 2 59 ± 3 51 ± 3</td>
<td>70 76.5</td>
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<tr>
<td>4</td>
<td>5/M</td>
<td>440</td>
<td>Onset</td>
<td>PB</td>
<td>55 ± 5 32 ± 3 45 ± 1 † 34 ± 2 †</td>
<td>93 38.5</td>
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<td>5</td>
<td>6/M</td>
<td>1,059</td>
<td>Onset</td>
<td>PB</td>
<td>61 ± 1 54 ± 2 64 ± 2 † 53 ± 3</td>
<td>92 74.8</td>
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<tr>
<td>6</td>
<td>3/F</td>
<td>40</td>
<td>Onset</td>
<td>PB</td>
<td>57 ± 3 54 ± 1 58 ± 5 56 ± 2</td>
<td>43 43.3</td>
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<td>7</td>
<td>0/M</td>
<td>350</td>
<td>Onset</td>
<td>PB</td>
<td>51 ± 2 41 ± 2 56 ± 2 47 ± 2 †</td>
<td>68 94.9</td>
</tr>
<tr>
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<td>Relapse</td>
<td>BM</td>
<td>66 ± 2 46 ± 3 NT NT</td>
<td>70 33.4</td>
</tr>
<tr>
<td>9</td>
<td>5/F</td>
<td>225</td>
<td>Onset</td>
<td>PB</td>
<td>63 ± 1 39 ± 4 NT NT</td>
<td>76 41.3</td>
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<td>MLL germ line</td>
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<tr>
<td>10</td>
<td>56/M</td>
<td>88</td>
<td>Onset</td>
<td>PB</td>
<td>40 ± 2 26 ± 6 36 ± 6 33 ± 4</td>
<td>13 39.2</td>
</tr>
<tr>
<td>11</td>
<td>14/M</td>
<td>90</td>
<td>Onset</td>
<td>BM</td>
<td>46 ± 1 33 ± 4 33 ± 4 NT</td>
<td>40 20.5</td>
</tr>
<tr>
<td>12</td>
<td>28/M</td>
<td>59</td>
<td>Onset</td>
<td>PB</td>
<td>66 ± 3 58 ± 6 53 ± 3 51 ± 3</td>
<td>7 19.4</td>
</tr>
<tr>
<td>13</td>
<td>34/F</td>
<td>9</td>
<td>Onset</td>
<td>PB</td>
<td>66 ± 5 43 ± 1 51 ± 4 52 ± 6</td>
<td>39 32.2</td>
</tr>
<tr>
<td>14</td>
<td>18/F</td>
<td>51</td>
<td>Onset</td>
<td>PB</td>
<td>55 ± 5 49 ± 2 47 ± 3 NT</td>
<td>25 30.4</td>
</tr>
</tbody>
</table>

Abbreviations: F, female; M, male; PB, peripheral blood; BM, bone marrow; Ab, neutralizing anti-FL antibody; NT, not tested.

*Data are shown as mean ± SE of triplicate wells.
† Significant increase (P < 0.05, t test) compared with AraC+.
‡ Significant decrease (P < 0.05, t test) compared with AraC+FL+.
a greater than 90%; the most dismal clinical issue in this disease is an early relapse that frequently occurs during the first 6 months of chemotherapy and before hematopoietic stem cell transplantation (34), resulting in shorter event-free survival and overall survival. Recently, the prognostic value of MRD after induction chemotherapy has been emphasized in childhood ALL (35, 36). Although the importance of MRD is not fully characterized yet in MLL-rearranged ALL, persistence of high levels of MRD in bone marrow should be associated with a high and early relapse rate in this disease.

We found that MLL-rearranged leukemia cells with wild-type FLT3 showed an inhibitory response to FL. This FL-induced inhibition was due to the induction of cell cycle arrest, in the process of which up-regulation of p27 and dephosphorylation of STAT5 might be implicated profoundly. Importantly, these arrested leukemia cells, not only established lines but also primary samples, showed resistance to apoptosis after exposure to irradiation or chemotherapeutic drugs. Because FL is reported to be expressed at high levels as a soluble or membrane-bound form by bone marrow stromal cells (29), it is postulated that MLL-rearranged leukemia cells not adhering to bone marrow stromal cells are entering cell cycle and considerably sensitive to chemotherapy, but those adhering bone marrow cells are quiescent and resistant to chemotherapy, at least in part via the interaction of FL/FLT3, which results in persistent MRD. The FLT3 inhibitor might be effective by two mechanisms in vivo: one directly induces apoptosis of leukemia cells and another awakens quiescent leukemia cells adhering to bone marrow stromal cells to enter cell cycle.

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**Figure 4.** Effect of FL as a soluble or membrane form on chemotherapeutic agent–induced cell death of MLL-rearranged primary leukemia cells. MLL-rearranged primary leukemia cells (1 x 10^5/well) were precultured for 24 h in the presence or absence of FL (40 ng/mL) or bone marrow stromal (KM-104) cells with or without the addition of anti-FL antibody (4 μg/mL), and then cultured with or without AraC (100 nmol/L) for 24 h. Numbers of living and dead cells were counted in triplicate by dye-exclusion test and viabilities were calculated. The Δ viability (treated viability – control viability) was also calculated in each culture condition. A, representative data in cases 1 and 8. Columns, mean; bars, SE. *, P < 0.05, significant difference by t test. B, FL-mediating changes in the Δ viabilities in seven primary samples. *, P < 0.05, significant difference by the Wilcoxon’s test. C, bone marrow stromal cell–mediating changes in the Δ viabilities in six primary samples. *, P < 0.05, significant difference by the Wilcoxon’s test. D, schema of the hypothesis illustrating a possible role of the FL/FLT3 interaction for persistent MRD in MLL-rearranged leukemia. MLL-rearranged leukemia cells not adhering to bone marrow stromal cells are entering cell cycle and considerably sensitive to chemotherapy, but those adhering bone marrow cells are quiescent and resistant to chemotherapy, at least in part via the interaction of FL/FLT3, which results in persistent MRD. The FLT3 inhibitor might be effective by two mechanisms in vivo: one directly induces apoptosis of leukemia cells and another awakens quiescent leukemia cells adhering to bone marrow stromal cells to enter cell cycle.
in patients after intensified chemotherapy are speculated to be exposed to high level of FL not only in bone marrow but also in the periphery. We showed in the in vitro model study that MLL-rearranged leukemia cells adhering to the stroma cell line partially restored sensitivity to antileukemic agents in the presence of anti-FL antibody. In AML, it has been reported that leukemia cells acquire resistance to AraC and daunorubicin via the interaction of VLA4 expressed on AML cells with fibronectin expressed on bone marrow stromal cells (38). This VLA4/fibronectin interaction was confirmed to play a pivotal role in MLL-rearranged leukemia cells. This VLA4/fibronectin interaction was acquired to arrest AraC and daunorubicin via the interaction of FL antibody. In AML, it has been reported that leukemia cells restored sensitivity to antileukemic agents in the presence of anti-FL antibody. According to this scenario, the FLT3 kinase inhibitors, such as PKC412, should be effective in vivo in the treatment of MLL-rearranged leukemia because they can exert their inhibitory action through two mechanisms: one directly induces apoptosis of leukemia cells via blockade of the kinase activity required for their survival as recently reported (21, 22, 39) and another awakens “dormant” leukemia cells and induces them to enter a chemosensitive cell cycling state via blockade of the signal through the FL/FLT3 interaction that occurs on the surfaces of leukemia cells and bone marrow stromal cells. The cell surface adhesion molecule VCAM-1 or asparagine synthetase, expressed on or secreted from bone marrow stromal cells, respectively, have also been reported to be involved in resistance to chemotherapy in all cells (40, 41).

The precise molecular mechanism of the FL-induced cell cycle arrest in MLL-rearranged leukemia cells remains elusive. We found that p27 is markedly up-regulated after FL stimulation, and this was presumably due to prevention of degradation of this protein in MLL-rearranged leukemia. As a key member of the KIP/CIP family of CDKIs, p27 blocks cell cycle progression at G1 phase, primarily by inhibiting the cyclin E/CDK2 complex (42, 43). It is known that p27 is degraded by the ubiquitin-proteasome pathway and that quiescent cells exhibit a smaller amount of ubiquitinating activity (44), which might account for prolongation of the p27 half-life in FL-treated MLL-rearranged leukemia cells. Therefore, it is not clear at present whether up-regulation of p27 is the primary molecular event in FL-induced cell cycle arrest, or it is a secondary event in quiescent (“dormant”) cells occurring after cell cycle arrest has been induced by other molecular mechanism(s). We also found that phosphorylation of STAT5, but not p44/42 MAPK and Akt, was almost abolished in arrested MLL-rearranged leukemia cells after FL stimulation. Because selective activation of STAT5 is shown to play a pivotal role in the self-renewal of leukemia cells as well as in normal hematopoiesis (45), the specific inactivation of STAT5 after FL stimulation might be critical to the induction of cell cycle arrest. The molecular mechanism of FL-induced STAT5 inactivation is still elusive and will be the subject for the future study.

The most important issue to be addressed is why MLL-rearranged ALL cells, unlike other B-precursor ALL cells, show an inhibitory response in proliferation after FL stimulation. Analyses of gene expression in leukemia have provided direct insights into the pathogenesis of leukemias and their responses to therapy. Armstrong et al. (19) reported that MLL-rearranged ALL has a distinct gene expression profile, including high FLT3 expression compared with other types of ALL. Thus, specific genes and their products that are uniquely activated in MLL-rearranged leukemia might be associated with a unique inhibitory response to FL.

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Fms-like Tyrosine Kinase 3 Ligand Stimulation Induces MLL-Rearranged Leukemia Cells into Quiescence Resistant to Antileukemic Agents

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