Flt3 Ligand Stimulates Proliferation and Inhibits Apoptosis of Acute Myeloid Leukemia Cells: Regulation of Bcl-2 and Bax


Flt3/flk-2 ligand (flt3-L) is a potent costimulator of normal bone marrow (BM) myeloid progenitors. Flt3-L is produced by BM stromal cells and its receptor is expressed in the majority of acute myeloid leukemia (AML) cases. Therefore, flt3-L may play a role in the paracrine and/or autocrine loops sustaining leukemic cell growth. We evaluated the effects of recombinant human flt3-L on proliferation, apoptosis, and Bcl-2 and Bax expression in primary AML cells and compared them with those of stem cell factor (SCF). Mononuclear BM cells from patients with newly diagnosed AML were cultured in serum-free conditions with flt3-L, SCF, granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) alone and in combination. In 9 of 10 samples, flt3-L significantly increased [3H]thymidine uptake (geometric mean stimulation index, 7.5; range, 2.4 to 41.5). Flt3-L also increased the number of AML blast colonies by 128% (range, 61% to 181%). In these 9 samples, flt3-L significantly enhanced the proliferative response triggered by G-CSF or GM-CSF. Flt3-L prevented apoptosis in AML blasts. It reduced the number of apoptotic cells by 36% ± 3.9% compared with control cultures. Combining flt3-L with G-CSF or GM-CSF doubled the antiapoptotic effect. Cellular Bcl-2 and Bax levels were determined separately for apoptotic and nonapoptotic cells by flow cytometry. Cells undergoing spontaneous apoptosis had low Bcl-2 and high Bax levels, whereas nonapoptotic cells had high Bcl-2 and low Bax levels. Flt3-L alone or in combination with G-CSF or GM-CSF did not upregulate Bcl-2. However, Bax expression decreased in viable cells in the presence of these cytokines and the lowest level was achieved when a combination of flt3 and GM-CSF was used. Proliferative and viability effects of flt3-L were similar to those of SCF. Our results demonstrate that flt3-L acts as a stimulatory factor for primary AML cells. The antiapoptotic effects of flt3-L or its combinations with G-CSF or GM-CSF correlate with their ability to prevent upregulation of Bax.

R ECEPTOR-TYPE TYROSINE kinases are believed to play a major role in the development, proliferation, and differentiation of various cell systems.1 Two stimulatory cytokines, stem cell factor (SCF), also termed c-kit ligand, and macrophage colony-stimulating factor (M-CSF), are nonredundant in hematopoiesis2 and have class III tyrosine kinase receptors4 encoded by c-kit and c-fms, respectively. Recently, a newly discovered member of this class, flt3/flk-2 receptor (flt3R), was cloned from both mouse26 and human cells.7,8 In the hematopoietic system, flt3R is selectively expressed in progenitor cell-enriched populations.5,6 In the mouse, it was found on proliferating rather than quiescent stem cells9,10 and only within a subpopulation of c-kit-positive stem cells.11 Flt3R is not detected in human T and B cells,9 but in monocytes and granulocytes, its expression can be detected by reverse-transcriptase polymerase chain reaction (RT-PCR), although not by Northern blotting.12 The ligand to flt3R (flt3-L) is expressed in membrane-bound and soluble forms,13,14 which are controlled by alternative mRNA splicing,13 and is produced by bone marrow (BM) stromal cells.15 Flt3-L by itself supports sparse colony formation from granulocyte-macrophage colony-forming units (CFU-GM)13,17. In suspension cultures of purified progenitor cells, flt3-L alone induces differentiation along the monocytic and basophilic lineages.18 Flt3-L does not stimulate colony formation of purified primitive progenitors, however, it synergizes with other cytokines such as SCF, interleukin-3 (IL-3), IL-6, and granulocyte-colony-stimulating factor (G-CSF).19,20 While these properties of flt3-L are similar to those of SCF, flt3-L’s lack of effect either alone or together with erythropoietin on erythroid burst-forming units differentiates flt3-L from SCF.19,20 However, in one report, flt3-L was found to synergize with erythropoietin alone or in combination with IL-3 in stimulating BM and cord blood erythroid burst-forming units.17 Other features differentiating flt3-L from SCF are the expansion of long-term culture-initiating cells,18,21 and lack of mast cell stimulation.22

Scarce data are available on the effects of flt3-L on leukemic cell proliferation.23 Because flt3-L is produced by BM stromal cells, it may stimulate leukemic cells in a paracrine fashion. This hypothesis is supported by the finding of flt3R transcripts in blast cells of the majority of acute myeloid leukemia (AML), B-cell acute lymphoid leukemia (ALL), and T-cell ALL cases.12 Flt3R is also frequently expressed in pre-B and some monocytic and myeloid cell lines, but not in the erythroid TF-1 or the megakaryocytic MO7e cell lines.24,25 While some studies have addressed the effect of flt3-L on hematopoietic cell proliferation, little is known about its effect on apoptosis. Stimulatory cytokines, a group to which flt3-L belongs, are known to promote survival of hematopoietic cells by suppressing apoptosis.26,27 Expression of the family of Bcl-2-related proteins, of which Bcl-2, Bcl-x, and Bax are of major importance, define the susceptibility of cells to apoptosis.28,29 Changes in the expression of these proteins are of major importance, define the susceptibility of cells to apoptosis.28,29

From the Section of Molecular Hematology and Therapy, Department of Hematology and Department of Bioimmunotherapy, The University of Texas M.D. Anderson Cancer Center, Houston, TX; and the Department of Hematological Oncology and Bone Marrow Transplantation, Hematological Research Center, Moscow, Russia.

Submitted January 31, 1996; accepted July 16, 1996.

Supported in part by Grants No. CA55154 and CA57639 from the National Cancer Institute (Bethesda, MD) and by a grant from the Ladies’ Leukemia League (New Orleans, LA).

Address reprint requests to M. Andreeff MD, PhD, Section of Molecular Hematology and Therapy, Box 81, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX, 77030.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.


3987
could be responsible for antiapoptotic effects of the cytokines and flt3-L, in particular.

In the study presented here, we investigated the proliferative and antiapoptotic effects of flt3-L on primary AML cells. To better understand the mechanisms of the antiapoptotic effect of flt3-L, we studied Bcl-2 and Bax expression in AML cells separately for apoptotic and nonapoptotic cells by flow cytometry. We found that flt3-L stimulates AML cell proliferation similar to SCF. This effect was augmented by either granulocyte-macrophage colony-stimulating factor (GM-CSF) or G-CSF. Cells undergoing spontaneous apoptosis in serum-free cultures had low Bcl-2 and high Bax levels, whereas nonapoptotic cells had high Bcl-2 and low Bax levels. Flt3-L alone or in combination with GM-CSF or G-CSF did not affect Bcl-2 levels, but significantly downregulated Bax in viable cells. These data suggest that maintenance of a low Bax level is required for protection of primary AML cells from apoptosis by tested cytokines.

MATERIALS AND METHODS

Cells. BM aspirates were obtained from 13 patients with AML at the time of diagnosis. Clinical data on the patients are shown in Table 1. These studies were performed with the patients’ informed consent and were approved by the Human Institutional Review Board at M.D. Anderson Cancer Center. Leukemic blast cells were separated by using Ficoll-Hypaque (Sigma Chemical Co, St Louis, MO) density gradient centrifugation and low-density cells were used. When samples contained less than 90% blasts, they were depleted of T lymphocyte and monocyte by magnetic cell separation (Miltenyi Biotec, Auburn, CA), or by granulocyte-macrophage colony-stimulating factor (GM-CSF) or G-CSF. Cells undergoing spontaneous apoptosis in serum-free cultures had low Bcl-2 and high Bax levels, whereas nonapoptotic cells had high Bcl-2 and low Bax levels. Flt3-L alone or in combination with GM-CSF or G-CSF did not affect Bcl-2 levels, but significantly downregulated Bax in viable cells. These data suggest that maintenance of a low Bax level is required for protection of primary AML cells from apoptosis by tested cytokines.

Table 1. Clinical Data of AML Patients

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yrs)/Sex</th>
<th>Cytogenetic Abnormality</th>
<th>FAB Category</th>
<th>WBC (×10⁹/L)</th>
<th>Platelets (×10⁹/L)</th>
<th>Percent Blasts in BM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46/M</td>
<td>47 + 21</td>
<td>M3</td>
<td>0.6</td>
<td>125</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>44/M</td>
<td>45X, t(8;21)</td>
<td>M1</td>
<td>23.4</td>
<td>26</td>
<td>94</td>
</tr>
<tr>
<td>3</td>
<td>70/F</td>
<td>diploid</td>
<td>M1</td>
<td>10.4</td>
<td>130</td>
<td>85</td>
</tr>
<tr>
<td>4</td>
<td>54/F</td>
<td>t(7;17)</td>
<td>M2</td>
<td>0.8</td>
<td>31</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>42/F</td>
<td>diploid</td>
<td>M2</td>
<td>208.4</td>
<td>17</td>
<td>88</td>
</tr>
<tr>
<td>6</td>
<td>36/F</td>
<td>t(15;17)</td>
<td>M3</td>
<td>142.2</td>
<td>17</td>
<td>86</td>
</tr>
<tr>
<td>7</td>
<td>82/M</td>
<td>−11, −17, −20, −22</td>
<td>M4</td>
<td>18.9</td>
<td>62</td>
<td>51.3</td>
</tr>
<tr>
<td>8</td>
<td>80/F</td>
<td>t(8;21)</td>
<td>M2</td>
<td>111.5</td>
<td>317</td>
<td>80.6</td>
</tr>
<tr>
<td>9</td>
<td>70/F</td>
<td>diploid</td>
<td>M1</td>
<td>52.9</td>
<td>38</td>
<td>82</td>
</tr>
<tr>
<td>10</td>
<td>46/F</td>
<td>diploid</td>
<td>M3</td>
<td>3.9</td>
<td>89</td>
<td>98</td>
</tr>
<tr>
<td>11</td>
<td>54/F</td>
<td>+13</td>
<td>M2</td>
<td>78.8</td>
<td>62</td>
<td>93</td>
</tr>
<tr>
<td>12</td>
<td>47/M</td>
<td>−7</td>
<td>M1</td>
<td>112.4</td>
<td>28</td>
<td>89</td>
</tr>
<tr>
<td>13</td>
<td>68/F</td>
<td>diploid</td>
<td>M2</td>
<td>61.2</td>
<td>44</td>
<td>96</td>
</tr>
</tbody>
</table>

Abbreviations: FAB, French-American-British; WBC, white blood cells; BM, bone marrow.

For the detection of Bcl-2, we used mouse antihuman Bcl-2 monoclonal antibody conjugated to fluorescein isothiocyanate (FITC) from DAKO (Carpinteria, CA); for detection of Bax, we used rabbit polyclonal affinity-purified antihuman Bax antibody purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell proliferation assay. Cellular proliferation was assessed by using the tritiated thymidine [3H]Tdr uptake assay.33 Quadruplicate aliquots of 2 × 10⁶ cells, suspended in 100 μL of serum-free medium (AIM-V, cat. no. 12055; GIBCO-BRL, Grand Island, NY), were cultured in a humidified atmosphere of 5% CO2 in air at 37°C. After 2 days, 0.1 μCi of [3H]TdR (6.7 Ci per mmol; Du Pont, Wilmington, DE) was added to each well, and the cultures were incubated overnight. The cells were then deposited onto nitrocellulose filters using a cell harvester. Radioactivity was measured in a scintillation counter and expressed as counts per minute. For assessment of the level of background incorporation of radioactivity, leukemic cells were irradiated with 30 Gy 8 hours before addition of [3H]TdR and cultured in parallel. The stimulation index (SI) was calculated as the ratio between the radioactivity of the cytokine-treated sample and the radioactivity of the untreated control sample.

AML blast colony assay. A previously described method was used to detect AML blast colony formation.34,35 Briefly, 2 × 10⁶ low-density BM cells containing more than 90% blasts were plated in 0.8% methylcellulose in α-modified Eagle’s medium (GIBCO) supplemented with 10% fetal calf serum (FCS). Flt3-L and SCF as single agents or in combinations with G-CSF or GM-CSF were used to stimulate colony formation. The cultures were incubated in 35-mm Petri dishes (Lux, Nunc, Naperville, IL) in quadruplicate at 37°C in a humidified atmosphere of 5% CO2 in air. AML blast colonies were microscopically evaluated on day 7 of culture. A cluster of 40 or more cells was defined as a blast colony. Individual colonies were plucked, smeared on glass slides, and stained to confirm leukemic morphology. The leukemic origin of these colonies has been previously demonstrated by cytogenetic analysis.36

Detection of apoptosis in cultured cells. After 3 days of culture in serum-free medium (AIM-V; GIBCO) at 105/mL, in the presence or absence of cytokines, cells were stained for DNA with acridine orange. The method of cell staining is described in detail elsewhere and was used with some modifications.37 Aliquots (60 μL) of cell suspensions were mixed with 120 μL of a solution containing 0.05 mol/L HCl, 0.15 mol/L NaCl, and 0.1% Triton X-100 (Sigma). After mixing and equilibration for 30 seconds, 240 μL of a solution containing...
containing 0.2 mol/L Na₂HPO₄/0.1 mol/L citric acid buffer (pH 6.0), 1 mmol/L Na EDTA, 0.15 mol/L NaCl, and acridine orange at 8 µg/mL. (Polysciences, Warrington, PA) was added. The cell fluorescence was measured during the next 5 minutes. The entire procedure was performed on ice. Exposure of cells to Triton X-100 solution releases low molecular weight DNA, allowing apoptotic cells to be distinguished by their fractional DNA content. This treatment has no effect on nonapoptotic cells.

**Immunostaining procedure and flow cytometry.** After 48 hours of culture in serum-free medium at 10⁶ cells/mL, the cells were treated with FACS Lysing Solution (Becton Dickinson, San Jose, CA), which permitted fixation and permeabilization in one step. Cells were then washed twice with a solution of 0.1% bovine serum albumin and 0.1% NaN₃ in phosphate-buffered saline (PBS) and stained with directly conjugated or primary antibody in a solution of Alexa Fluor 488 (DAKO) and rabbit Ig G (Caltag Laboratories, San Francisco, CA) to enhance chemiluminescence (ECL) solution (Amersham, Buckinghamshire, UK). The chemiluminescence of the membrane was detected on X-OMAT AR5 x-ray film (Kodak, Rochester, NY).

**Statistical analysis.** The statistical significance of differences in measured qualities was determined by the two-tailed Student’s t-test. A log-transformation was applied to the SI data to stabilize the variances and permit application of the t-test. A P < .05 was considered statistically significant. Unless otherwise indicated, average values were expressed as means ± standard errors of mean.

**RESULTS**

**Flt3-L stimulates AML cell proliferation.** To evaluate the effect of flt3-L on AML cell proliferation, we first used the [³²P]Tdr uptake assay. Because cellular receptors for flt3-L and SCF belong to the same family and because SCF was reported to interact synergistically with various cytokines in AML, we compared the effects of flt3-L with those of SCF, both as single agents and in combination with other growth factors (Table 2). We found that flt3-L significantly stimulated AML cell proliferation, associated with unresponsiveness to all other cytokines tested and with an extremely high rate of spontaneous proliferation. We believe that this case repre-

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Mean Radioactivity of Control (cpm/min)*</th>
<th>flt3-L</th>
<th>GM-CSF</th>
<th>flt3-L + G-CSF</th>
<th>G-CSF</th>
<th>flt3-L + SCF</th>
<th>SCF</th>
<th>SCF + GM-CSF</th>
<th>SCF + G-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>107 ± 22</td>
<td>3.2</td>
<td>16.7</td>
<td>15.8</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>2</td>
<td>119 ± 14</td>
<td>4.5</td>
<td>2.8</td>
<td>12.1</td>
<td>28.5</td>
<td>95.1</td>
<td>4.5</td>
<td>10.9</td>
<td>57.3</td>
</tr>
<tr>
<td>3</td>
<td>368 ± 92</td>
<td>3.8</td>
<td>10.0</td>
<td>24.2</td>
<td>27.9</td>
<td>31.5</td>
<td>16.7</td>
<td>59.2</td>
<td>48.2</td>
</tr>
<tr>
<td>4</td>
<td>101 ± 6</td>
<td>5.9</td>
<td>1.4</td>
<td>14.3</td>
<td>1.9</td>
<td>16.5</td>
<td>1.1</td>
<td>4.5</td>
<td>6.3</td>
</tr>
<tr>
<td>5</td>
<td>117 ± 29</td>
<td>16.0</td>
<td>1.5</td>
<td>19.8</td>
<td>2.0</td>
<td>35.2</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>6</td>
<td>183 ± 48</td>
<td>41.5</td>
<td>8.1</td>
<td>70.3</td>
<td>1.2</td>
<td>43.8</td>
<td>1.2</td>
<td>12.6</td>
<td>3.9</td>
</tr>
<tr>
<td>7</td>
<td>1,808 ± 40</td>
<td>4.6</td>
<td>8.9</td>
<td>13.5</td>
<td>3.1</td>
<td>8.2</td>
<td>3.2</td>
<td>11.0</td>
<td>6.3</td>
</tr>
<tr>
<td>8</td>
<td>1,112 ± 12</td>
<td>6.1</td>
<td>7.3</td>
<td>20.7</td>
<td>4.0</td>
<td>11.1</td>
<td>10.9</td>
<td>24.0</td>
<td>15.5</td>
</tr>
<tr>
<td>9</td>
<td>212 ± 50</td>
<td>2.4</td>
<td>41.8</td>
<td>48.4</td>
<td>14.7</td>
<td>23.5</td>
<td>52.1</td>
<td>136.9</td>
<td>83.6</td>
</tr>
<tr>
<td>Geometric mean SI for patients 1 to 9</td>
<td>7.5</td>
<td>8.1</td>
<td>6.9</td>
<td>32.2</td>
<td>25.7</td>
<td>7.4</td>
<td>21.6</td>
<td>19.4</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10,150 ± 780</td>
<td>1.1</td>
<td>1.0</td>
<td>0.9</td>
<td>0.8</td>
<td>0.8</td>
<td>0.7</td>
<td>0.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>

A total of 2×10⁶ AML cells were cultured in the presence of flt3-L, G-CSF, GM-CSF and SCF, alone and in combination, in 96-well microtiter plates in quadruplicates. After 2 days, 0.1 µCi was added to each well overnight and then cells were harvested onto nitrocellulose filters. Radioactivity was measured by scintillation counter.

Abbreviation: NT, not tested.

* Mean radioactivity of background=50 ± 11 cpm/min.

† Stimulation index was calculated as the ratio between radioactivity of cytokine-treated sample and that of the untreated control sample.
sents a separate subgroup of AML, as previously described.35 Like flt3-L, both GM-CSF and G-CSF significantly upregulated \(^{[3]H} \text{TdR} \) incorporation into AML cells: GM-CSF, in 7 of 9 cases (gmSI, 8.1; range, 1.4 to 41.8); G-CSF, in 7 of 8 cases (gmSI, 6.9; range, 1.2 to 28.5). Incubation of AML cells in the presence of flt3-L and GM-CSF significantly increased gmSI to 23.2 (range, 12.1 to 70.3; \( P = .003 \)) in 8 of 9 samples. Similar results were obtained in 7 of 8 samples when flt3-L was combined with G-CSF: gmSI was increased to 25.7 (range, 6.2 to 95; \( P = .005 \)). SCF induced similar \(^{[3]H} \text{TdR} \) incorporation in 5 of 7 cases; gmSI was 7.4 (range, 1.1 to 52.8). SCF synergized with GM-CSF in 6 of 7 cases (gmSI, 21.6; range, 4.5 to 135.9) and with G-CSF in 7 of 7 cases (gmSI, 19.4; range, 3.9 to 83.6). There was no significant difference in \(^{[3]H} \text{TdR} \) uptake induced by combinations of flt3-L with GM-CSF or G-CSF and that induced by combinations of SCF with the same growth factors.

**Flt3-L stimulates AML blast colony formation.** We studied the effect of flt3-L on AML progenitor cells. When used as a single agent, flt3-L increased the number of AML blast colony-forming units (AML-CFU) by 61% to 181%, and enlarged colony size in 8 of 9 cases as compared with control cultures (Table 3). It was interesting that in patient 6, flt3-L was the only factor to induce colony formation. We then examined the interaction of flt3-L with other cytokines on AML-CFU proliferation (Table 3). Incubation of the cells with flt3-L and GM-CSF resulted in an increment in blast colony formation as compared with the effect observed with each cytokine alone (\( P < .02 \)). A similar effect was obvious when flt3-L was combined with G-CSF (\( P < .005 \)). In patient 6, the combination of flt3-L with G-CSF induced significantly higher colony formation than flt3-L alone, although neither GM-CSF nor G-CSF alone stimulated blast colony formation. AML cells from patient 10, which did not respond to flt3-L in \(^{[3]H} \text{TdR} \) uptake assay, formed leukemic colonies and responded to this cytokine. These results are not surprising, as the two assays detect different cell populations.

In three additional experiments we compared the AML-CFU-stimulating effects of flt3-L and SCF. We found that both cytokines had similar effects on AML blast colony formation (Table 4).

**Flt3-L protects AML cells from apoptosis.** Because various cytokines \(^{36,47} \) and SCF in particular, \(^{37,48} \) were found to promote cell survival, we tested the effect of flt3-L on AML cells undergoing spontaneous apoptosis under serum-free culture conditions. We incubated cells in the presence or absence of various cytokines for 3 days and then detected DNA fragmentation by acridine orange staining (Fig 1). Control cultures showed 49% ± 8.7% of apoptotic cells after 3 days for nine cases studied. Compared with control, flt3-L decreased the number of apoptotic cells by 37% ± 7.8% (\( P < .002 \); Fig 2). The antiapoptotic effects of GM-CSF, G-CSF, and SCF were not statistically different from that of flt3-L. However, when flt3-L was used in combination with GM-CSF or G-CSF, the antiapoptotic effect was more pronounced than the effect of flt3-L alone: the proportions of apoptotic cells were reduced by 63.3% ± 7.9% and 62.4%

### Table 3. Effects of flt3-L on AML Colony Formation

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Control</th>
<th>flt3-L</th>
<th>GM-CSF</th>
<th>flt3-L + GM-CSF</th>
<th>G-CSF</th>
<th>flt3-L + G-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>732</td>
<td>1,356</td>
<td>1,234</td>
<td>2,152</td>
<td>3,160</td>
<td>5,289</td>
</tr>
<tr>
<td>3</td>
<td>623</td>
<td>1,472</td>
<td>2,480</td>
<td>3,972</td>
<td>2,284</td>
<td>3,598</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>513</td>
<td>828</td>
<td>911</td>
<td>1,237</td>
<td>954</td>
<td>1,319</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>784</td>
<td>0</td>
<td>880</td>
<td>0</td>
<td>1,845</td>
</tr>
<tr>
<td>7</td>
<td>260</td>
<td>640</td>
<td>1,384</td>
<td>1,920</td>
<td>1,325</td>
<td>1,971</td>
</tr>
<tr>
<td>8</td>
<td>364</td>
<td>710</td>
<td>1,474</td>
<td>4,778</td>
<td>973</td>
<td>2,766</td>
</tr>
<tr>
<td>10</td>
<td>772</td>
<td>1,600</td>
<td>3,344</td>
<td>4,071</td>
<td>2,408</td>
<td>2,989</td>
</tr>
</tbody>
</table>

A total of 2 x 10^6 AML cells were cultured in the presence of flt3-L, G-CSF, and GM-CSF alone and in combination in 1 mL of 0.8% methylcellulose in α-MEM and 10% FCS. Colonies containing 40 cells or more were scored after 7 days. Data are means from quadruplicate cultures.

* Data are means from duplicate cultures.

### Table 4. Effects of flt3-L and SCF on AML Colony Formation

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Control</th>
<th>flt3-L</th>
<th>SCF</th>
<th>GM-CSF</th>
<th>flt3-L + GM-CSF</th>
<th>SCF + GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>0</td>
<td>322</td>
<td>211</td>
<td>320</td>
<td>1,025</td>
<td>410</td>
</tr>
<tr>
<td>12</td>
<td>133</td>
<td>1,704</td>
<td>1,955</td>
<td>1,537</td>
<td>2,462</td>
<td>2,403</td>
</tr>
<tr>
<td>13</td>
<td>132</td>
<td>276</td>
<td>282</td>
<td>280</td>
<td>487</td>
<td>526</td>
</tr>
</tbody>
</table>

A total of 2 x 10^6 AML cells were cultured in the presence of flt3-L, SCF, and GM-CSF, alone and in combination. Data are means from quadruplicate cultures.
PROLIFERATION AND APOPTOSIS IN AML

Fig 1. Detection of apoptotic cells in 3-day serum-free cultures of AML cells. A representative experiment is shown. Cells were permeabilized with Triton X-100 solution, and DNA was stained with acridine orange as indicated in Materials and Methods. Apoptotic cells are in the "sub G1 peak of the DNA histogram. The percentage of apoptotic cells is reduced from 30% in controls (A) to 20% in flt3-L supplemented cultures (B), and to 14.5% in cultures supplemented with GM-CSF (histogram not shown). Combination of flt3-L and GM-CSF resulted in a reduction of apoptotic cells to 5.6% (C). The logarithmic fluorescence of acridine orange is expressed in channel numbers.

± 7.4%, respectively (P < .05). Like flt3-L, SCF was more effective in combination with GM-CSF and G-CSF than alone: these combinations reduced the numbers of apoptotic cells to the same degree as combinations of flt3-L with GM-CSF and G-CSF (Fig 2).

The effect of flt3-L on Bcl-2 and Bax protein levels in AML cells. Different proteins of the Bcl-2 family have been implicated in triggering apoptosis or preventing it. Therefore, we evaluated whether Bcl-2 and Bax proteins, the two major members of the Bcl-2 family, are involved in the regulation of AML cell apoptosis and whether flt3-L can prevent apoptosis through modulation of Bcl-2 and Bax protein expression. To this end, we examined the levels of Bcl-2 and Bax proteins in these cells by flow cytometry. The separate determination of Bcl-2 and Bax in apoptotic and nonapoptotic cells was based on the previously reported changes in the physical properties of apoptotic cells: when analyzed by flow cytometry, apoptotic cells form a cluster separate from nonapoptotic cells because of lower forward and higher side scatter (Fig 3). We first evaluated the anti-Bcl-2 and anti-Bax antibodies by Western blotting. Both antibodies produced single bands on Western blots (Fig 4), indicating that they did not cross-react with other cellular antigens. We then tested Bcl-2 and Bax expression separately in apoptotic and viable cells (Fig 5). The Bcl-2 levels were significantly lower in apoptotic cells than in nonapoptotic cells (P < .0002) (Fig 6A). While the cytokines reduced the proportion of apoptotic cells, none of those tested upregulated Bcl-2 expression either in apoptotic or nonapoptotic AML cells (Fig 6A). This finding suggests that although Bcl-2 protein is downregulated during apoptosis, its level is stable in viable cells and is not regulated by any of the cytokines added to the cultures.

In contrast to Bcl-2, Bax levels were higher in apoptotic cells than in nonapoptotic cells (P < .03; Fig 6B). Incubation of AML cells with flt3-L or SCF, alone or in combination with either GM-CSF or G-CSF, downregulated Bax levels in both populations. The decrease reached statistical significance in nonapoptotic cells when combinations of flt3-L or SCF with GM-CSF were used (P < .05). It is interesting that the same combination of cytokines also had the strongest antiapoptotic effect in our experiments. Furthermore, Bax expression in viable cells correlated strongly and positively with the percentage of apoptotic cells observed in cultures, maintained with or without cytokines: the correlation coefficient between mean values of Bax expression and mean number of apoptotic cells for all experiments and all culture conditions was 0.92. These data suggest that Bax is responsive to stimulation by flt3-L and other tested cytokines and is involved in mechanisms of cytokine protection of primary AML cells from apoptosis.

Fig 2. The effects of flt3-L, SCF, GM-CSF, and G-CSF, as single agents and in combination, on apoptosis of AML cells after 3 days in serum-free culture. Apoptotic cells were detected as shown in Fig 1. Spontaneous apoptosis was determined in control cultures (not treated with cytokines). Control cultures contained 49% ± 8.7% apoptotic cells. The proportion of apoptotic cells in cytokine-supplemented cultures is expressed as percentages of control. Data from nine experiments are presented.
Although a subset of AML cells produce growth factors and proliferate in an autocrine fashion, in the majority of cases, these cells require hematopoietic growth factors for their proliferation and viability. The effects of the recently identified growth factor flt3-L on AML cells are still poorly characterized. However, high expression of flt3R on AML blast cells indicates their potential responsiveness to flt3-L.

We studied the effects of flt3-L on AML cells in two respects: on proliferation and on viability. Because AML cells are already known to be responsive to SCF and flt3-L is similar to SCF in many biological activities, we compared the effects exerted by flt3-L with those of SCF. We found that flt3-L significantly increased AML cell proliferation and blast colony formation, thus confirming the expression of functionally competent flt3R on primary AML cells. Combinations of flt3-L with G-CSF or GM-CSF had additive or more-than-additive effects on [3H]TdR incorporation and additive effects on colony formation. Similar results were recently reported by Piaccibello et al. The heterogeneity that we saw in the proliferative responses was also observed by these investigators and others when the effects of SCF, IL-3, GM-CSF, G-CSF, and M-CSF on [3H]TdR uptake by primary AML cells were investigated; it probably reflects heterogeneous biological properties of AML cells in different patients. In one patient (no. 10), AML cells had a very high rate of spontaneous proliferation and were unresponsive to any of the tested cytokines. Although we have no direct explanation for this finding, it may be related to maximal activation of proliferative signaling due to autocrine cytokine or oncogene stimulation.

The stimulation of AML cell proliferation and blast colony by SCF alone and in combination with G-CSF, GM-CSF, or IL-3 formation was previously reported. Our data support these previous findings and demonstrate that flt3-L is comparable to SCF in stimulating primary AML cells.

We also found that flt3-L had significant antiapoptotic effects on primary AML cells. The antiapoptotic effects of flt3-L alone or in combination with G-CSF or GM-CSF were again similar to those of SCF, indicating their comparable antiapoptotic potency. Promotion of survival has already been shown for different stimulatory cytokines in hematopoietic precursor and leukemic cell lines, normal progenitors, and mature cells. Our data show that primary AML cells are also responsive to the antiapoptotic effects of cytokines and that the antiapoptotic effect is increased when combinations of cytokines are used.
An expanding family of Bcl-2–related proteins regulates susceptibility to apoptosis. Bcl-2 and Bcl-xI act to prevent or delay cell death,\textsuperscript{29,30} while Bax and Bcl-xI act to induce it.\textsuperscript{28,29} New members of this family, Bad\textsuperscript{31} and Bak\textsuperscript{32,33} were recently shown to promote cell death, and Bag-1\textsuperscript{34} to prevent it. The prevention of apoptosis requires heterodimerization of Bax with the antiapoptotic molecules Bcl-2 and Bcl-xI, and homodimers of Bax have been shown to promote apoptosis.\textsuperscript{35,36,37} Thus, the ratios of Bcl-2 and Bcl-xI to Bax are believed to be important for determining a cell’s susceptibility to an apoptotic stimulus.\textsuperscript{28} Normal primitive hematopoietic progenitors express Bcl-x, but not Bcl-2\textsuperscript{28}; no data on Bax have been reported. Morphologically recognizable myeloid precursor cells contain Bcl-2 and Bax, and mature granulocytes have no detectable expression of either molecule.\textsuperscript{40,46,50,57} AML cells, whose differentiation is interrupted at the intermediate stage, are also positive for Bcl-2.\textsuperscript{40,47,71-73} To date, no reports are available describing Bax expression in AML cells. Our study confirms expression of Bcl-2 in these cells and demonstrates for the first time their expression of the Bax protein.

To define whether apoptosis induced by incubation of AML cells in serum-free conditions is associated with changes in the expression of Bcl-2 and Bax and whether cytokines have effects on the expression of these molecules, Bcl-2 and Bax proteins were detected by flow cytometry separately in apoptotic and nonapoptotic cells by gating on respective clusters characterized by differences in scattered light.\textsuperscript{41,43} Apoptotic cells had significantly lower expression of Bcl-2 than nonapoptotic cells, which is in concordance with the known role of Bcl-2 in protecting from apoptosis\textsuperscript{29} and its downregulation during apoptotic cell death.\textsuperscript{28} In contrast, Bax expression was significantly higher in apoptotic cells than in viable cells, which suggests the importance of Bax for development of apoptosis in AML cells and supports the suggested roles for Bcl-2 and Bax in the development of apoptosis.\textsuperscript{28}

Surprisingly, flt3-L and other cytokines did not upregulate Bcl-2 in apoptotic or nonapoptotic AML cells. Moreover, combinations of cytokines with stronger proliferative and antiapoptotic effects did not differ in their effects on Bcl-2. Several studies have reported upregulation of Bcl-2 in response to cytokines.\textsuperscript{57,75,76} One of them showed Bcl-2 upregulation in cultured AML cells in response to GM-CSF.\textsuperscript{57} The discrepancy between our results and previously reported data can be resolved by considering that we measured Bcl-2 separately in apoptotic and viable cells, whereas in the other studies, Bcl-2 was measured in bulk populations of cells.\textsuperscript{57,75,76} Upregulation of Bcl-2 in those studies could reflect an increase in the proportion of viable cells with higher Bcl-2 content, which indeed occurs in the presence of stimulatory cytokines.

In contrast, Bax expression decreased in cultured nonapoptotic AML cells in the presence of cytokines. The decrease reached statistical significance (\(P < .05\)) when combination of flt3-L and GM-CSF was used, which coincided with the best antiapoptotic effect achieved. Moreover, Bax levels were highly inversely correlated with the ability of cytokines to protect AML cells from apoptosis.

Taken together, our results indicate that Bax upregulation is an early event in the apoptosis of AML cells in our model, as it can be detected in viable cells. Bax is regulated by flt3-L and other tested cytokines and is maintained in viable cells at low level. Bcl-2 expression changes later than that of Bax. Our inability to detect downregulation of Bcl-2 in viable AML cells during cytokine deprivation implies that the downregulation happens later than “apoptotic” changes in the light scatter properties of the cells. The other, less likely, explanation of our findings is that any change in Bcl-2 expression after Bax upregulation renders AML cells apoptotic, thus not permitting detection of lower levels of Bcl-2 in viable cells. Our findings indicate that Bax is one of the primary targets for the antiapoptotic effects of tested cytokines and that maintenance of a low Bax level is one of the mechanisms of protection from apoptosis by flt3-L and other stimulatory hematopoietic cytokines in primary AML cells.

In conclusion, flt3-L acts as a stimulatory factor for primary AML cells, promoting their proliferation and viability. Apoptosis in these cells is associated with decreased Bcl-2 and increased Bax levels. The antiapoptotic effect of flt3-L, alone or in combination with G-SCF or GM-CSF, correlates with its ability to maintain low level of Bax. These findings

![Fig 4. Expression of Bcl-2 (A) and Bax (B) proteins in different samples of freshly isolated normal bone marrow mononuclear cells. Total cell lysates were subjected to SDS-PAGE, followed by Western blotting analysis using anti-Bcl-2 monoclonal antibody and an anti-Bax polyclonal antibody (see Material and Methods).](image-url)
Fig 5. Flow cytometric detection of Bcl-2 in apoptotic (A) and non-apoptotic (B) cells and of Bax in apoptotic (C) and non-apoptotic (D) cells. Isotypic controls define nonspecific fluorescence. The specificity of antibodies to Bcl-2 and Bax was confirmed by Western blot (see Fig 4). Data from a representative experiment is shown.

Fig 6. The effects of flt3-L, SCF, GM-CSF, and G-CSF, alone and in combination, on Bcl-2 (A) and Bax (B) protein expression in AML cells cultured in serum-free conditions for 2 days. Specific fluorescence was determined by flow cytometry separately within apoptotic and non-apoptotic cell populations defined by forward and side scatter dot plot histograms. (■), apoptotic cells; (□), non-apoptotic cells. *, P < 0.05 compared with control non-apoptotic cells. Data represent seven experiments.
may be relevant for the development of new strategies of cytokine-related therapies in AML.

REFERENCES

ervative blast cells circulating in myeloblastic leukemia. Lancet 1:862, 1977


73. Andreeff M, Jiang S, Consoli U, Brandes J, Sanchez-Williams G, Deisseroth A, Estey E: In vivo regulation of Bcl-2 ex-
pression in AML progenitors by granulocyte--colony stimulating factor (G-CSF) and direct evidence for selection of Bcl-2" cells by induction chemotherapy of AML. Blood 86:511a, 1995 (abstr, suppl 1)


Flt3 ligand stimulates proliferation and inhibits apoptosis of acute myeloid leukemia cells: regulation of Bcl-2 and Bax

M Lisovsky, Z Estrov, X Zhang, U Consoli, G Sanchez-Williams, V Snell, R Munker, A Goodacre, V Savchenko and M Andreeff