Effect of Tumor Necrosis Factor-α on the Proliferation of Leukemic Cells From Children With B-Cell Precursor-Acute Lymphoblastic Leukemia (BCP-ALL): Studies of Primary Leukemic Cells and BCP-ALL Cell Lines

By Muxiang Zhou, Harry W. Findley, Lanhua Ma, Sherif R. Zaki, Tim Hill, Melissa Harnid, W. Craig Hooper, and Abdelsalam H. Ragab

The effect of recombinant tumor necrosis factor-α (rTNF-α) on the primary leukemic blasts and leukemic cell lines derived from children with B-cell precursor acute lymphoblastic leukemia (BCP-ALL) was studied. The proliferation of leukemic cells from the bone marrow of 11 of 13 patients (seven at diagnosis, four in relapse) and from the 697 (BCP-ALL) cell line was significantly inhibited by rTNF-α at the lowest dose tested (0.1 ng/mL), as measured by [3H]-TdR uptake. The degree of inhibition was variable, ranging from 17% to 78%. Furthermore, a dose-dependent inhibitory effect was observed, with approximately 70% mean inhibition of DNA synthesis detected when cells from 12 of 13 patients were incubated with 100 ng/mL of rTNF-α for 3 days. In contrast, rTNF-α did not inhibit another BCP-ALL cell line (EU-1/ALL) established recently in our laboratory. Studies indicated that the TNF-α gene was expressed by the primary leukemic blasts of one TNF-resistant case in his third relapse and by EU-1 cells. Also, TNF-α protein was detected by Western blot analysis and enzyme-linked immunosorbent assay in the supernatant of EU-1 cells; this is the first report of TNF production by a BCP-ALL cell line. The production of TNF-α mRNA and protein was not detected in the 697 cell line and in the primary leukemic blasts from six patients (four at diagnosis, two in relapse) whose leukemic cells were inhibited by TNF. The partially purified TNF-α obtained from the EU-1 cell line also suppressed the proliferation of TNF-sensitive primary leukemic cells, and this inhibitory activity was abolished by an anti-TNF-α specific antibody. Our results demonstrate that TNF-α is an inhibitor of in vitro proliferation of BCP-ALL cells from most patients. The TNF-resistant leukemic cells from a few patients and the EU-1 cell line express TNF mRNA, suggesting that the induction of TNF gene expression is associated with the development of TNF resistance.

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MATERIALS AND METHODS

Source of cells. Following informed consent, primary leukemic BM cells were obtained directly from 13 children with ALL (eight in diagnosis and five in relapse) (Table 1). Mononuclear cells were isolated on Ficoll-Hypaque, washed twice in phosphate-buffered saline, and resuspended at 10^9 cells/mL in RPMI 1640 containing 5% fetal bovine serum and 15% bovine calf serum. The cells were incubated on plastic Petri dishes for 1 hour at 37°C. The nonadherent cells were obtained by gently washing the dishes. An immunomagnetic separation procedure for depletion of T cells from the nonadherent cells was used as described in detail previously. The procedure was performed on BM cells containing less than 90% blasts before Ficoll-Hypaque separation. This yielded greater than 95% blasts after these procedures.

The EU-1 cell line was established from BM cells obtained from a child with ALL during his second relapse. Purified leukemic BM cells were cultured in a clonal assay in methyl cellulose medium and low molecular weight B-cell growth factor (LMW-BCGF). Lymphoblast colonies were obtained by day 14, and the cell line was subsequently established by transfer of colony cells to culture flasks. This cell line has an early pre-B phenotype and is positive for a B-lineage associated marker including TdT, CD10 (CALLA), and CD19(B4); it is negative for ClgM and slg. It is also negative for EBV by DNA hybridization and for mycoplasma. The karyotype
Table 1. Phenotypes of Primary Leukemic Cells From BCP-ALL Patients and of the EU-1/ALL Cell Line

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Diagnosis</th>
<th>BM Blast (%)</th>
<th>HLA-Dr</th>
<th>CD10</th>
<th>CD19</th>
<th>CD3</th>
<th>Sig</th>
<th>TdT</th>
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<tr>
<td>1</td>
<td>c-ALL (R)</td>
<td>78</td>
<td>89</td>
<td>76</td>
<td>NT</td>
<td>5</td>
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<tr>
<td>2</td>
<td>c-ALL (I)</td>
<td>97</td>
<td>91</td>
<td>95</td>
<td>66</td>
<td>10</td>
<td>NT</td>
<td>+</td>
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<td>c-ALL (I)</td>
<td>84</td>
<td>80</td>
<td>72</td>
<td>77</td>
<td>14</td>
<td>19</td>
<td>+</td>
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<tr>
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<td>c-ALL (I)</td>
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<td>76</td>
<td>75</td>
<td>13</td>
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<td>-</td>
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<tr>
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<td>91</td>
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<td>75</td>
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<tr>
<td>6</td>
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<td>98</td>
<td>100</td>
<td>84</td>
<td>93</td>
<td>5</td>
<td>3</td>
<td>+</td>
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<td>97</td>
<td>93</td>
<td>96</td>
<td>10</td>
<td>6</td>
<td>+</td>
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<tr>
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<td>91</td>
<td>2</td>
<td>89</td>
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<td>7</td>
<td>3</td>
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<td>89</td>
<td>94</td>
<td>2</td>
<td>0</td>
<td>+</td>
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<tr>
<td>EU-1/ALL cell line</td>
<td></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

The immunologic surface markers of cells were detected by immunofluorescence and flow cytometry.

Abbreviations: c-ALL, common ALL; N-ALL, null ALL; R, relapse; I, initial; Blast, lymphoblast; NT, not tested.

Preparation of EU-1/ALL conditioned medium (EU-1/CM). EU-1/CM was prepared by incubating cloned EU-1 cells in RPMI 1640 media without serum or serum substitutes for 72 hours. Supernatant was harvested by centrifugation to remove cells. The supernatant (1 L) was concentrated to 4 mL by ultrafiltration using a 5,000 molecular-weight-cutoff membrane (YM5; Amicon, Danvers, MA). The concentrated supernatant was subjected to TNF-α immunoassay, Western blot analysis, and gel filtration.

Western blot analysis and TNF-α immunoassay. A 250-fold concentration of EU-1/CM was used for Western blot analysis. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using an SDS-Tris-glycine buffer. Protein bands were electrophoretically transferred onto nitrocellu-
Western analysis for immunoreactive TNF-α. Lane 1, culture supernatant of EU-1 ALL cell line; lane 2, rTNF-α. The molecular-weight markers are indicated at left.

RESULTS

Effect of rTNF-α on BCP-ALL cells. rTNF-α at 0.1 ng/mL was able to significantly \((P < .01\) to \(< .001)\) inhibit DNA synthesis of BM cells from 11 of 13 patients with a mean percent inhibition of 42% ± 18%. Considerable heterogeneity was observed in the TNF response of leukemic cells from individual BCP-ALL patients, with the
degree of inhibition ranging from 17% to 78%. Cells from patient 9 had less sensitivity to TNF; DNA synthesis was not significantly inhibited at 0.1 ng/mL (Fig 1). However, a dose-related inhibition was observed for 12 of 13 patients (including patient 9) at higher concentrations, with approximately 70% mean inhibition occurring at 100 ng/mL TNF. Cells from patient 13 did not respond to any dose of TNF (Fig 2). Similarly, rTNF-α did not show any effect on the EU-1 cell line (Fig 3). In contrast, the 697 cell line was sensitive to rTNF-α even at 0.1 ng/mL.

**Northern analysis for TNF-α mRNA.** TNF-α transcripts were undetectable in total RNA from primary BM cells of six patients (four at diagnosis, two in relapse) and the 697 cell line. In contrast, the TNF-α cDNA probe hybridized to a 1.4-kb transcript found in the cells from patient 13, from the EU-1/ALL cell line, and from the HL60 cell line incubated with TPA for 3 hours, as well as in normal peripheral blood lymphocytes cultured with PHA for 12 hours (Fig 4). The HL60 cell line and peripheral blood lymphocytes were used as positive controls for TNF production.

**Synthesis of TNF-α by EU-1 cells.** Because TNF-α mRNA was expressed in the EU-1 cell line, we examined the production of TNF-α protein by the cell line using Western blot analysis. Supernatant from 72-hour serum-free cultures contained a strong 17-Kd and two faint 34- and 52-Kd immunoreactive protein bands (Fig 5). Additionally, the TNF level in EU-1/CM was determined by ELISA to be 1.7 pg/mL in unconcentrated serum-free culture, and 22 pg/mL in culture with serum.

Because primary ALL cells from most cases were sensitive to rTNF-α, we examined the response of these cells to EU-1/CM. Fractions from FPLC gel filtration containing 30- to 60-Kd proteins, which include TNF-α dimers and trimers of a single 17-Kd chain, significantly inhibited DNA synthesis as measured by [3H]-TdR uptake in EU-1/CM or EU-1/CM plus anti-TNF-α antibody.

**Fig 6.** Separation of inhibitory and stimulatory activity for BCP-ALL proliferation contained in EU-1/ALL CM. EU-1/CM was concentrated to 250-fold and fractionated by FPLC using a Superdex-200 16/20 column. Molecular-weight standards are shown as a dotted line, and relative protein concentrations in the EU-1/CM are indicated by the solid line. Fractions were collected and assayed for their effects on the proliferation of primary BCP-ALL BM cells and EU-1 cells. DNA synthesis (indicated by vertical bars) was measured by [3H]-TdR uptake, and the results are expressed as percent of control for fractions 12, 15, 24, and 31 that contain protein peaks. The data for ALL BM cells represent the mean values for six patients.

**Fig 7.** The neutralization of inhibitory activity for BCP-ALL proliferation contained in EU-1/ALL CM. Fractions 12, 17, and 22 from Fig 5 were pooled (indicated as EU-1/CM) and tested on BCP-ALL BM cells from patient 10. Proliferation of cells was detected by the DNA synthesis assay in culture with EU-1/CM or EU-1/CM plus anti-TNF-α antibody.
proliferation of primary ALL cells from all six patients tested. DNA synthesis from these fractions was less than 20% of control. Other fractions tested did not inhibit the growth of primary ALL cells. In contrast, fraction 31 containing proteins of less than 6 Kd showed stimulatory activity for both EU-1 cells and primary ALL cells (Fig 6).

To detect whether the inhibition of primary ALL cells by EU-1/CM was due to TNF-α, a specific anti-TNF-α antibody was used to block TNF-α activity. The inhibitory effect of pooled fractions containing 30- to 60-Kd proteins on ALL BM cells was abolished by the neutralizing antibody (Fig 7).

**DISCUSSION**

TNF is currently under clinical trial as an antineoplastic agent. To our knowledge, no data about the effect of TNF-α on leukemic cells from children with ALL in vivo and in vitro have been reported, although TNF-α inhibited the proliferation of leukemic cells from acute myeloid leukemia patients \(^7^,\) \(^8^,\) \(^9^\) and from the erythroleukemia cell line K562. \(^10^\) In the present study, we report that rTNF-α inhibits the proliferation of leukemic cells from 12 of 13 children with ALL and a BCP-ALL cell line (697) in a dose-dependent manner, as measured by tritiated-thymidine incorporation.

TNF-sensitive leukemic cells from 6 of 12 patients and the 697 cell line were tested for production of TNF mRNA and proteins and were negative for both. However, leukemic cells from a child with ALL in his third relapse and a BCP-ALL cell line established in our laboratory (EU-1/ALL) were found to be resistant to exogenous TNF-α. Furthermore, we discovered that leukemic cells from this child and the EU-1 line expressed TNF-α mRNA, and EU-1 cells also secreted TNF-α. These findings are in agreement with earlier studies showing that expression of TNF transcripts and endogenous synthesis of TNF-α are associated with TNF resistance in breast carcinoma and fibrosarcoma cells.\(^2^,\) \(^3^,\) \(^11^,\) \(^12^\) However, one study has shown that TNF is an autocrine inhibitory factor in chronic myelogenous leukemia.\(^13^\) These investigators suggest that competition between autocrine growth-promoting and inhibiting factors may contribute to the prolongation of the stable, chronic phase in this disease.

Maximum inhibition of leukemic cell proliferation was observed with fractions containing 30- to 60-Kd proteins, indicating that a multimeric arrangement of a single 17-Kd chain TNF-α\(^2^\) is required for biologic activity. Furthermore, the inhibitory activity of the fractions could be neutralized by anti-TNF-α antibody. We have previously shown that the EU-1 cell line produces ALL autocrine growth factors, which are autostimulatory and which also stimulate the growth of primary BCP-ALL cells.\(^6^\) The stimulatory fractions contained protein(s) of less than 6 Kd molecular weight. As expected, none of the TNF fractions inhibited the EU-1 cell line. However, the less than 6-Kd fraction significantly stimulated the proliferation of the EU-1 cells.

The mechanism for the development of resistance to TNF is not known, but several studies have shown that resistance is associated with induction of the TNF gene after exposure to TNF. This induction is suppressed by neutralizing antibody against TNF, indicating a specific role for the cytokine. Inhibitors of RNA and protein synthesis prevent the development of resistance, suggesting that certain resistance-associated genes must be expressed.

A TNF-sensitive BCP-ALL cell line (697) should permit studies of the mechanism for induction of TNF resistance. Proliferation of this cell line is inhibited by concentrations of TNF as low as 0.1 ng/mL, and thus it resembles primary BCP-ALL cells in its response to this cytokine. Given the initial sensitivity of this BCP-ALL cell line to TNF, it will be important to determine if these cells can become refractory to TNF after exposure, and whether the occurrence of the resistant phenotype can be prevented or reversed.

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