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Synergy of Interleukin 3 and Tumor Necrosis Factor α in Stimulating Clonal Growth of Acute Myelogenous Leukemia Blasts Is the Result of Induction of Secondary Hematopoietic Cytokines by Tumor Necrosis Factor α[1]

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ABSTRACT

Colony growth of leukemic colony-forming units (L-CFU) obtained from patients with primary acute myelogenous leukemia stimulated with recombinant human interleukin 3 (rh IL-3) is significantly potentiated when recombinant human tumor necrosis factor α (rh TNF-α) is present in cultures. The costimulatory activity of tumor necrosis factor (TNF) α is dose dependent and maximum at TNF-α concentrations of 10 ng/ml. At high density, L-CFU proliferatively respond to TNF-α stimulation in the absence of exogenous rh IL-3. Studies of the mechanism of action of rh TNF-α on acute myelogenous leukemia L-CFU growth suggest that TNF-α acts by inducing release of growth stimulatory hematopoietic cytokines by the leukemic cells themselves, including IL-1α, IL-1β, Granulocyte-macrophage colony-stimulating factor (CSF), granulocyte CSF, and IL-6. Treatment of L-CFU cultures, with neutralizing antibodies to IL-1α, IL-1β, granulocyte-macrophage CSF, granulocyte CSF, and IL-6 to eliminate the endogenous source of these factors is associated with significant inhibition of the synergistic interplay of TNF-α and IL-3.

INTRODUCTION

It has been shown previously that TNF-α[3] synergizes with hematopoietic growth factors such as IL-3 to stimulate growth of human myeloid leukemia progenitor cells (1, 2), while it inhibits growth of normal hematopoietic progenitor cells under identical experimental conditions (3, 4). The mechanism of the synergistic interplay of TNF-α with IL-3 is, however, poorly understood. Recent work by Elbaz *et al.* (5) has suggested that TNF-α may act on leukemic blasts by augmenting number and affinity of IL-3 receptors. It has also been shown that TNF-α is a potent inducer of secretion of hematopoietic growth factors by accessory bone marrow cells including monocytes/macrophages (6, 7) and mesenchymal cells (8, 9). Given the ability of blast cells from AML patients to produce hematopoietic growth factors either constitutively (10–12) or upon induction with phorbol compounds (13), the goal of the present study was to determine whether synergy of TNF-α and IL-3 was associated with induction of autocrine production of hematopoietins, such as GM-CSF, G-CSF, IL-1α, IL-1β, and IL-6, by AML blasts. To this end, three complementary approaches were undertaken: (*a*) the expression of GM-CSF, G-CSF, IL-1α, IL-1β, and IL-6 transcripts was analyzed in cytoplasmic RNA obtained from AML blasts by Northern blot analysis upon exposure to TNF-α, IL-3, or a combination of both factors; (*b*) the corresponding proteins were assessed in culture supernatants of AML cells treated in the same fashion; and (*c*) the effects of neutralizing antibodies on GM-CSF, G-CSF, IL-1α, IL-1β, and IL-6 upon clonogenic growth of TNF-α- and IL-3-stimulated AML cells were studied in soft agar cultures. We show that TNF-α enhances production of hematopoietic cytokines by AML cells and that these cytokines are used by the leukemia cells to augment their clonal growth in an autocrine fashion.

MATERIALS AND METHODS

AML Cells. Peripheral blood specimens of 11 different AML patients with highly leukemic blood cell counts were sampled at the time of diagnosis. After contaminating T-cells and monocytes/macrophages were depleted by rosetting with sheep erythrocytes and repeated adherence to plastic surfaces, blast cell-enriched fractions were frozen in the vapor phase of liquid nitrogen until assayed. All samples contained at least 97% blasts by morphology. These 11 leukemias were selected from 85 consecutive AML specimens based on previously published experiments demonstrating a proliferative response of the respective leukemias to exogenous rh IL-3 (14) and absence of constitutive gene expression of TNF-α, GM-CSF, G-CSF, IL-1α, IL-1β, and IL-6 (15, 16).

Antibodies to Hematopoietic Cytokines. A neutralizing sheep antiserum to rh GM-CSF was kindly provided by G. Wong, Genetics Institute, Cambridge, MA. A neutralizing moAb to rh G-CSF was obtained through L. Souza, Amgen, Thousand Oaks, CA. A neutralizing moAb to rh IL-6 (17) was kindly provided by U. Schwulera, Biotest, Dreieich, Federal Republic of Germany. MoAbs to rh IL-1α and rh IL-1β were purchased from Genzyme, Boston, MA. Anti-TNF-α moAb was provided by G. Adolf, Bender KG, Vienna, Austria.

Soft Agar Colony Assay for Leukemic Colony-forming Units. L-CFU were assayed in a double-layer agar (Agar Noble; Difco Laboratories, Detroit, MI) (18). Underlayers (0.5 ml) were composed of 0.5% agar in Iscove's modified Dulbecco's medium supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin (GIBCO, Grand Island, NY), and 20% low-endotoxin fetal calf serum (Hazelton, Vienna, UT). As a colony-stimulating activity, rh IL-3 (50 ng/ml; kindly provided by Immunex, Seattle, WA, through D. Krumwieh, Behringwerke AG, Marburg, Federal Republic of Germany) was added in the presence or absence of rh TNF-α (0.1–25 ng/ml; kindly provided by G. Adolf, Bender KG, Vienna, Austria). The overlayers were composed of 0.3% agar in the same medium and contained 5×10^3-1×10^5 AML blasts/ml. In selected experiments, neutralizing antibodies to rh GM-CSF, G-CSF, IL-1α, IL-1β, and IL-6 were added to the overlayers either alone or in combinations in concentrations sufficient to neutralize >50 ng/ml of the respective cytokine. The cultures were set up in triplicates in 24-well plastic culture plates (Corning Glass Works, New York, NY) and were incubated at 37°C in 5% CO_2 and humidified air. After 8–10 days of culture, overlayers were removed from underlayers by agitation and were dried onto glass slides under filter paper. L-CFU containing >20 cells were enumerated on Wright-Giemsa-stained slides.

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[3] The abbreviations use are: TNF, tumor necrosis factor; IL, interleukin; AML, acute myelogenous leukemia; GM, granulocyte-macrophage; CSF, colony-stimulating factor; G-CSF, granulocyte CSF; rh, recombinant human; moAb, monoclonal antibody; L-CFU, leukemic colony-forming units; ELISA, enzyme-linked immunosorbent assay; cDNA, complementary DNA; SDS, sodium dodecyl sulfate; SSC, standard sodium citrate.

Assessment of Growth Factor Production in Supernatants of AML Cultures. In order to determine growth factor production by AML cells, cells were cultured at 10^6/ml in Iscove's modified Dulbecco's medium (as above). After 24 h (37°C, 5% CO_2 in air), cell-free culture supernatants were collected and stored at 4°C until tested by double-sandwich ELISA.

ELISA for GM-CSF, G-CSF, IL-1α, IL-1β, IL-3, and IL-6. GM-CSF, G-CSF, IL-1α, IL-1β, IL-3, and IL-6 were determined in cell-free supernatants of AML cultures using double-sandwich ELISA kits that were purchased from R&D Systems, Minneapolis, MN (for IL-1α, IL-1β, IL-3, and IL-6), from Medical Resources Ltd., Darlinghurst, Australia (for GM-CSF), and from Amgen, Thousand Oaks, CA (for G-CSF). ELISA protocols were followed according to the guidelines of the manufacturers.

cDNA Probes. For probing of GM-CSF, the 1.0-kilobase *PstI* fragment of pBR322-hGM-CSF (kindly provided by P. Habermann, Hoechst, Frankfurt, Federal Republic of Germany) was used. G-CSF was probed with the 0.6-kilobase *EcoRI/HindIII* fragment of pUC8-hG-CSF (kindly provided by L. Souza, Amgen). IL-1β was probed with a 0.5-kilobase fragment of YEpsec 1-hIL-1β (American Type Culture Collection, Rockville, MD). IL-1α mRNA was detected with an oligonucleotide as described before (19). For probing of IL-6, the 0.44-kilobase *TaqI/BanII* fragment of pGEM4-hIL-6 (kindly provided by T. Hirano, Osaka University, Osaka, Japan) was used. IL-3 was probed with the 1.6-kilobase *HpaI/XhoI* fragment of pGembl-hIL-3 (kindly provided by P. Habermann).

Isolation of Total Cellular RNA and Northern Blot Analysis. AML blasts were harvested by centrifugation and were resuspended in 0.1 M sodium acetate-1 mM EDTA, pH 5.2, and then lysed with 0.5% SDS and extracted with an equal volume of acetate/EDTA-equilibrated phenol (60°C). This mixture was incubated at 60°C for 25 min with frequent vortexing. The aqueous phase was recovered after centrifugation and extracted once with an equal volume of phenol/chloroform and twice with chloroform. The resulting RNA was precipitated overnight at −20°C with 2.5 volumes of ethanol. The total RNA from each sample was then electrophoresed in a 1% agarose gel containing 20 mM sodium borate, pH 8.3-0.5 mM EDTA-3% formaldehyde. The RNA was then transferred to nitrocellulose paper (Schleicher and Schuell, Dassel, Federal Republic of Germany) in 10 × SSC (1.5 M sodium chloride and 150 mM sodium citrate) using capillary blotting overnight. The blots were then backed and prehybridized at 65°C in 7% SDS, 10 × Denhardt's (1× Denhardt's = 0.2% Ficoll, 0.2% bovine serum albumin, and 0.02% polyvinylpyrolidine), 5 × SSC, and 20 mM salmon sperm DNA (Sigma). The blots were probed with specific cDNA probes that were radiolabeled by random priming with a [α-^{32}P]CTP (>6000 Ci/mmol) (Amersham, Arlington, Heights, IL) (20). The blots were washed at 55°C in 1% SDS-1 × SSC and were autoradiographed with Kodak XAR film (Eastman Kodak, Rochester, NY) at −70°C with an intensifying screen.

RESULTS

Effects of TNF-α on Expression of Hematopoietic Growth Factor Genes by AML Blasts. Given previous findings by others (1, 2) demonstrating synergy of TNF-α and IL-3 in enhancing clonogenic *in vitro* growth of AML cells, the objective of our first experiments was to investigate TNF-α-induced AML-conditioned media for the presence of hematopoietic growth factor activity by specific ELISA.

As indicated in Fig. 1, blast cells from two representative AML patients that failed to secrete IL-1α, IL-1β, GM-CSF, G-CSF, or IL-6 upon culture without TNF-α released these factors into their culture supernatants when stimulated with TNF-α. The stimulatory effect of TNF-α was dose dependent, and maximum growth factor release was observed with 10 ng/ml of TNF-α. In nine additional AML samples that also failed to constitutively produce these factors, treatment with 10 ng/ml rh TNF-α resulted in secretion of IL-1α (344–4204 pg/ml), IL-1β (386–4883 pg/ml), GM-CSF (127–2204 pg/ml), G-CSF (421–2221 pg/ml), and IL-6 (400–9948 pg/ml) but not IL-3 (Table 1). Addition of rh IL-3 (50 ng/ml) to TNF-stimulated cultures did not cause a further increase in transcript levels of the various growth factors or their corresponding proteins detectable in culture supernatants obtained from any of the 11 AML samples (not shown). To explore whether increased growth factor production was associated with increased growth factor mRNA accumulation, AML blasts were exposed to rh TNF-α (10 ng/(ml) for various times (1–16 h). As shown in Fig. 2 for a representative experiment with two different AML samples, IL-1α, IL-1β, GM-CSF, and G-CSF transcripts were rapidly (within 1 h) induced. Levels of IL-1 mRNA were stable over the entire culture period or even increased, while GM-

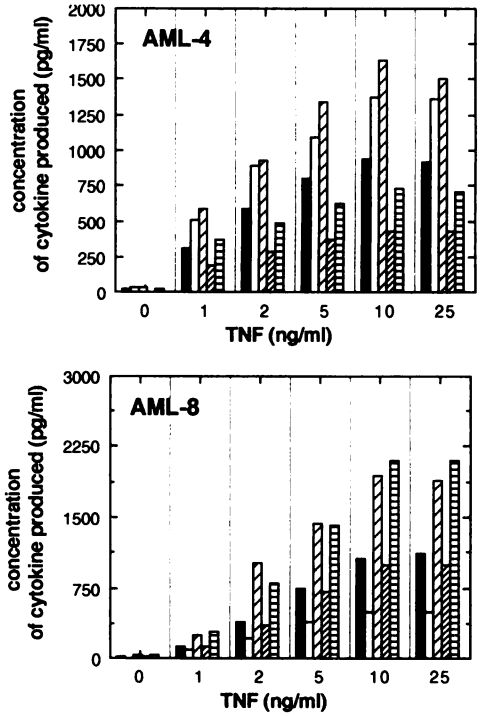

Fig. 1. Dose response of rh TNF-α on IL-1α (■), IL-1β (□), GM-CSF (▨), G-CSF (▨), and IL-6 (■) secretion by AML cells. Nonadherent and T-cell-depleted blasts from samples AML 4 and AML 8 were cultured at 10^6 cells/ml in the presence of various concentrations of rh TNF-α as indicated. After 24 h, cell-free culture supernatants were harvested, and cytokines were determined by ELISA. Shown are mean values of triplicate determinations with SD being <10%.

Table 1 *Production of hematopoietic cytokines by AML blasts upon exposure to rh TNF-α*

Nonadherent and T-cell-depleted blast cells from patients with AML were cultured at 10^6 cells/ml in the presence or absence of rh TNF-α (10 ng/ml). After 24 h cell-free supernatants were harvested and subjected to cytokine determination by ELISA. Shown are mean values of triplicate cultures with SD being always <10%. In the absence of rh TNF-α cytokines were always below 20 pg/ml (not shown).

Sample	IL-1α	IL-1β	GM-CSF	G-CSF	IL-6	IL-3
AML 1	344[a]	386	224	1990	400	<20
AML 2	1201	744	590	2221	1390	<20
AML 3	1144	1270	1541	6900	9948	<20
AML 4	944	1372	1640	421	729	<20
AML 5	4204	4883	1107	2004	2944	<20
AML 6	2701	1904	127	2112	923	<20
AML 7	401	1447	394	2704	1776	<20
AML 8	1062	492	1939	990	2101	<20
AML 9	3444	4007	2204	2008	4204	<20
AML 10	2009	3074	949	924	4277	<20
AML 11	1732	1741	722	494	3901	<20

[a] pg/ml.

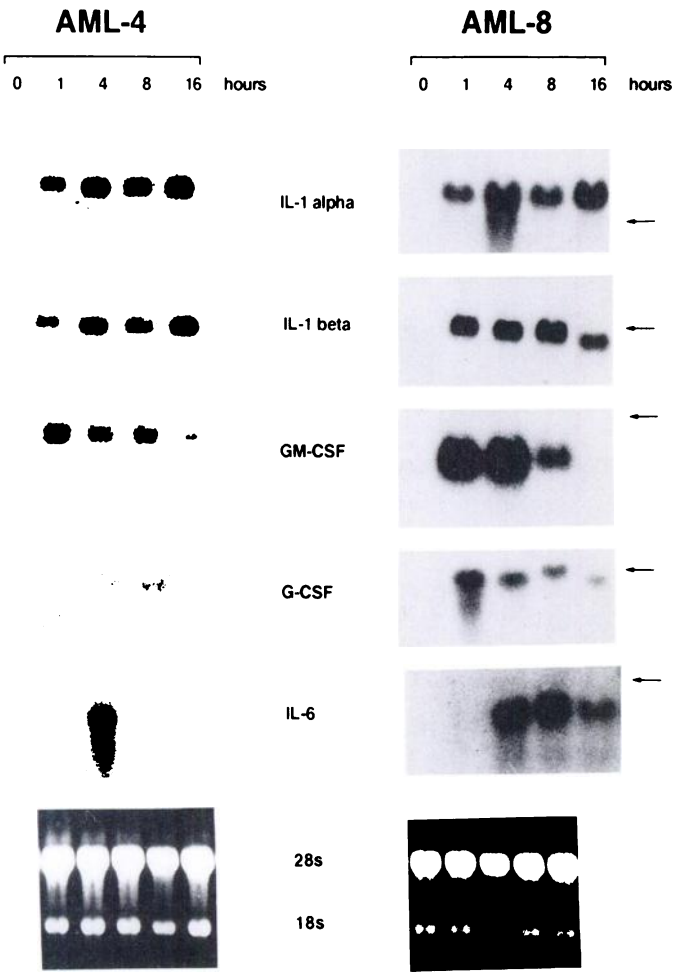

Fig. 2. Time kinetics of expression of IL-1α, IL-1β, GM-CSF, G-CSF, and IL-6 transcripts upon exposure of AML blasts to rh TNF-α. Blast cells of AML samples 4 and 8 (previously depleted from adherent cells and T-cells) were exposed to rh TNF-α (10 ng/ml) for the indicated times. Total cytoplasmic RNA was collected (15 μg RNA/lane) and subjected to Northern blot analysis. The blots were sequentially hybridized with IL-1α-, IL-1β-, GM-CSF-, G-CSF-, and IL-6-specific cDNA probes. *Arrows*, 18S ribosomal RNA. Staining of 28S and 18S rRNA in the ethidium bromide gel confirms integrity of RNA and demonstrates comparable RNA loading in single lanes. Treatment of AML cells with medium only or rh IL-3 (25 ng/ml) failed to induce transcripts for IL-1α, IL-1β, GM-CSF, G-CSF, and IL-6 (not shown).

CSF and G-CSF transcripts tended to return to almost undetectable levels after 8 h of culture. IL-6 transcripts, although undetectable after 1 h of TNF treatment, were expressed at the highest level after 4–8 h and decreased thereafter. Probing of mRNA of the remaining nine other TNF-α-stimulated AML samples with IL-1α-, IL-1β-, GM-CSF-, G-CSF-, and IL-6-specific cDNA yielded almost identical results (data not shown). Treatment of AML samples with medium only or rh IL-3 did not result in synthesis of growth factor transcripts by AML blasts (not shown), ruling out the possibility that the synergy of TNF-α and IL-3 was due to synergistic stimulation of growth factor production.

Effect of TNF-α on Clonogenic AML Growth. In order to determine a role of the various growth factors induced by TNF-α in mediating a proliferative effect of TNF-α on clonogenic AML growth, enriched blast cells obtained from four different AML samples were subjected to colony assays in agar cultures. To this end, AML blasts were plated at high density (10^5 cells/ml) in agar in the presence or absence of rh TNF-α (10 ng/ml). After 8–10 days of culture, growth of L-CFU was assessed. As shown in Table 2, stimulation of AML cells resulted in induction of colony growth by L-CFU of all 4 AML samples, which could be prevented when neutralizing antibodies to IL-1α, IL-1β, GM-CSF, G-CSF, and IL-6 were added to the cultures.

Effect of Neutralizing Antibodies to IL-1α, IL-1β, GM-CSF, G-CSF, and IL-6 on Synergistic Growth Stimulation of AML Blasts by TNF-α and IL-3. Stimulation of AML blasts with rh TNF-α (10 ng/ml) also enhanced L-CFU growth when blasts had been plated at low density (5×10^3 cells/ml). Stimulation with rh IL-3 (50 ng/ml) was 2.3- to 7.0-fold more effective in eliciting L-CFU growth of AML blasts than TNF-α alone (Table 3). Synergy of TNF-α and IL-3 resulted in a further increase of L-CFU growth in all 11 AML samples by 1.6- to 4.3-fold. Upon addition of an excess of a combination of antibodies directed against IL-1α, IL-1β, GM-CSF, G-CSF, and IL-6, synergy of TNF-α and IL-3 was abolished, supporting the notion that this synergy was the result of induction of release of these growth factors by TNF-α. To study the relative contribution of individual factors induced by TNF-α on the growth-enhancing effect of TNF-α in IL-3-stimulated L-CFU, antibodies to single factors were added at the start of the cultures. As shown in Table 3, antibody to IL-1α (αIL-1α) significantly reduced synergy between TNF-α and IL-3 in 7 of 11 AML samples, αIL-1β in 8 of 11 samples, αG-CSF in 10 of 11 samples, αIL-6 in 2 of 11 samples. The most effective abrogation of TNF-α/IL-3 synergy was observed when cultures had received αGM-CSF ($P < 0.05$ in 3 cases; $P < 0.01$ in 8 cases). These results suggest that the induction of release of several hematopoietic growth factors by AML cells in response to TNF-α mediates the synergistic interplay of TNF-α and IL-3 and that GM-CSF is the most effective intermediate product in this regard.

DISCUSSION

TNF-α exerts growth inhibitory effects upon a variety of different tissues including normal hematopoietic progenitor cells. However, TNF-α also confers growth stimulation on certain cell types such as diploid fibroblasts (21) and normal and neoplastic B-lymphocytes (22, 23) by an unknown mechanism. Recent reports have indicated that TNF-α also enhances proliferation of AML cells when synergizing with other growth factors such as IL-3 and GM-CSF (1, 2). We report here that the enhancing effect of TNF-α on IL-3-stimulated clonogenic AML proliferation may be the result of induction of growth stimulatory hematopoietic cytokines. Treatment of AML blasts with rh TNF-α is associated with increased production of IL-1α, IL-1β, GM-CSF, G-CSF, and IL-6 by AML cells that use these growth stimulatory molecules to augment their growth in an autocrine fashion. Increased production of IL-1α, IL-1β,

Table 2 *Effect of TNF-α on clonogenic AML growth*

Nonadherent and T-cell-depleted AML blasts were plated at high density (10^5/ml) in agar cultures. Culture medium contained no cytokine (medium), rh TNF-α (10 ng/ml), or rh TNF-α and neutralizing concentrations of a combination of antibodies to rh IL-1α, rh IL-1β, rh GM-CSF, G-CSF, and rh IL-6. Shown are means of triplicate cultures. The experiment was repeated twice and gave identical results. Antibodies were used in nontoxic concentrations that neutralized >50 ng/ml of the respective growth factor in pilot experiments.

Samples	Medium	TNF-α	TNF-α + antibodies
AML 1	11 ± 1[a]	123 ± 7	14 ± 2
AML 3	8 ± 2	203 ± 23	8 ± 2
AML 4	12 ± 1	198 ± 13	10 ± 1
AML 7	11 ± 4	275 ± 20	13 ± 4

[a] Colony growth/10^5 AML blasts/ml.

Table 3 *Effect of neutralizing antibodies against various hematopoietic cytokines on L-CFU growth stimulated wtih rh IL-3 and rh TNF-α*

Nonadherent and T-cell-depleted AML blasts were plated at low density (5×10^3 cells/well) in the presence or absence of rh TNF-α (10 ng/ml), rh IL-3 (50 ng/ml), or both factors. Cultures stimulated with IL-3 plus TNF-α received antibodies to rh IL-1α, IL-1β, GM-CSF, IL-6, or all five antibodies at concentrations that were nontoxic and that neutralized 50 ng/ml of the respective growth factor in pilot experiments. Results are expressed as percentages of control cultures (= 100%) in which IL-3 and TNF was added in the absence of antibodies. Cultures were performed in triplicates and mean values were used for data calculation. SD was always <10%. *P* values were determined between control cultures (see above) and cultures containing antibodies. To ensure that the neutralizing antibodies were not blocking TNF (±IL-3)-induced L-CFU growth nonspecifically, TNF and IL-3 were also added to the cultures in the presence of boiled antibodies. In these experiments (not shown), L-CFU growth was always >98% of control cultures.

Sample	Medium	TNF-α	IL-3	+αIL-1α	+αIL-1β	+αGM-CSF	+αG-CSF	+αIL-6	All	Medium
AML 1	10	7	25	72*	75*	62**	79	97	22**	100
AML 2	2	5	20	73*	79*	54**	70*	90	22**	100
AML 3	1	14	40	61**	70*	59**	79*	100	39**	100
AML 4	1	9	42	84*	85*	81*	49**	98	44**	100
AML 5	2	10	23	94	90*	29**	50**	100	19**	100
AML 6	2	6	42	89*	89*	54**	43**	96	38**	100
AML 7	3	10	62	89*	92	72*	70	100	60**	100
AML 8	5	8	24	77*	70*	50**	54**	88*	30**	100
AML 9	3	6	34	69**	79*	76*	70*	99	31**	
AML 10	1	9	25	100	100	51**	60**	79*	21**	100
AML 11	3	7	25	89*	94	70**	64**	100	26**	100

[a] $P < 0.05$.
[b] $P < 0.01$.

GM-CSF, G-CSF, and IL-6 is detectable at the mRNA and protein levels. Neutralizing antibodies to these factors in cultures of AML-derived L-CFU abrogate the synergistic effects of TNF-α on IL-3-stimulated clonogenic growth, and GM-CSF is the most potent intermediate in this regard.

It has been shown that exogenously added IL-1, GM-CSF, G-CSF, and IL-6 upregulates IL-3-dependent AML growth[4] (23–26). In line with these findings, the present study demonstrates that endogenously produced IL-1α, IL-1β, GM-CSF, G-CSF, and IL-6 induced by TNF-α can also enhance L-CFU growth of AML blasts. Given previous reports by others (3, 4) demonstrating that TNF-α exerts antiproliferative effects on normal hematopoietic progenitor cells, endogenous production by TNF in the bone marrow of AML patients, which may occur as the result of the interaction of leukemia cells with their surrounding stroma (16), may spur leukemia growth through induction of endogenous growth factors but may also confer a proliferative advantage to the leukemia cells through suppression of normal hematopoiesis. Although TNF-α induces secretion of hematopoietic growth factors by normal bone marrow bystander cells as well (25, 26), this effect may not be sufficient to stimulate normal hematopoiesis in a paracrine fashion because of the relative low abundance of accessory cells with the potential for growth factor production in bone marrow cultures. Future experiments will be needed to address the question of whether TNF-α also stimulates growth factor production by normal hematopoietic progenitor cells.

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