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Enhanced Expression of the Granulocyte-Macrophage Colony Stimulating Factor Gene in Acute Myelocytic Leukemia Cells Following in vitro Blast Cell Enrichment

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Expression of the granulocyte-macrophage colony-stimulating factor (GM-CSF) gene in acute myelocytic leukemia (AML) was assayed by Northern blot analysis. GM-CSF messenger RNA (mRNA) was detected in the freshly obtained mononuclear cells of only one of 48 cases of AML, in contrast with recent reports that GM-CSF mRNA might be detected in half of the cases of AML when RNA is prepared from T-cell- and monocyte-depleted leukemic cells. We did find, however, that expression of the GM-CSF gene was detectable in five of ten cases after in vitro T-cell and monocyte depletion steps. Additional studies suggest that expression of GM-CSF in the bone marrow of the one positive case, rather than being autonomous, was under exogenous control, possibly by a paracrine factor secreted by marrow stromal cells. These studies emphasize the potential for altering in vivo patterns of gene expression by in vitro cell manipulation.
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THE HYPOTHESIS THAT autocrine phenomena play a significant role in neoplastic disease, supported largely by in vitro studies, is intellectually attractive.[1] Recent reports that the leukemic cells of as many as half of patients with acute myelocytic leukemia (AML) contain the messenger RNA (mRNA) for granulocyte-macrophage colony-stimulating factor (GM-CSF) have been used in support of this hypothesis.[2-4] GM-CSF is an hematopoietic growth factor normally produced by activated T cells[5,6] and stimulated macrophages[7] capable of stimulating the in vitro growth of leukemic blasts from some patients with AML.[8-11] The studies described here demonstrate that GM-CSF transcripts are not detectable in the freshly obtained leukemic peripheral blood or bone marrow cells from most AML patients; rather, detectable mRNA levels appear only if the cells are manipulated in vitro to deplete the blasts of T cells and monocytes. The data suggest that in vivo autocrine production of GM-CSF is not common in unperturbed AML, but allow the possibility that either autocrine or paracrine GM-CSF activity could be induced in leukemic cells under certain stress conditions.

MATERIAL AND METHODS

Cell fractionation. Bone marrow aspirates or peripheral blood phlebotomy specimens from patients with AML were transported to the laboratory on ice. Informed consent was obtained prior to acquisition of the specimens. These specimens were immediately centrifuged over Ficoll-Hypaque (specific gravity [sp gr] = 1.077) to isolate mononuclear cells. The mononuclear cell fraction was depleted of T cells by rosetting with sheep erythrocytes and was depleted of monocytes, either by adherence to polystyrene at 37°C for two hours, or in some cases by magnetic attraction of phagocytized iron. For the latter procedure, cells were suspended in RPMI 1640 + 0.25% $Fe(CO)_6$ at a density of 5×10^6 cells/mL and were incubated at 37°C for 0.5 hour. A strong magnet was abutted against the flask for several seconds, then the cell suspension was aspirated. RNA was prepared as described below from mononuclear cells before processing and from the blast-enriched cells. In some cases, RNA was prepared from blast-enriched cells after an additional 24 hours of 37°C incubation in RPMI 1640 plus 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY) at 5×10^6 cells/mL. Cell morphology was determined on cytocentrifuge preparations of each specimen processed for RNA.

Sham processing. Mononuclear cells were carried through the processing steps described above but the nonblast cells were not removed. Peripheral blood mononuclear cells from patient no. 15, obtained at relapse, were incubated at 4°C overnight in RPMI 1640 plus heat-inactivated AB serum in the absence of sheep erythrocytes; the cells were then centrifuged over Ficoll-Hypaque (sp gr = 1.077), the interface was recovered, and the cells were washed twice in RPMI 1640. The cells were resuspended in RPMI 1640 plus 10% heat-inactivated fetal bovine serum, placed in a 50-mL polypropylene tube (Falcon 2070), and incubated at 37°C for two hours with agitation every ten minutes. No cells adhered to this surface. Half of the sham-processed cells were then placed in a culture flask at 5×10^6 cells/mL and incubated at 37°C for 24 hours and were then processed for RNA. The other half was cooled on ice, pelleted by centrifugation at 4°C, then processed for RNA.

Preparation of RNA. Sixty to 200×10^6 cells were lysed in 4 mol/L guanidinium thiocyanate and total cellular RNA was recovered by the method of Chirgwin et al.[12] The quality of the recovered RNA was ascertained by minidenaturing agarose gel electrophoresis and photography of the ethidium bromide-stained gel.

Northern blots. Five-microgram samples of total cellular RNA were denatured in 1 mol/L glyoxal and 50% dimethyl sulfoxide (DMSO) buffer, and RNA species were size-separated by gel electrophoresis in 1% agarose, 0.01 mol/L NaH_2PO_4 (pH 7.0). The RNA species were transferred to a nylon membrane (Zetabind, Cuno, Inc, Meriden, Conn) by elution with 20 × SSC (3 mol/L NaCl, 0.3 mol/L Na_3 citrate, pH 7.0). The quality of the RNA, the uniformity of loading, and the uniformity of transfer were ascertained by visualization of the membrane under short-wavelength ultraviolet light. Uniformity of loading was further documented by rehybridization with a probe for the glycolytic pathway gene triose phosphate isomerase (TPI) (data not shown).

Membranes were prehybridized, hybridized, and washed by the method of Church and Gilbert.[13] Prehybridization and hybridization buffer was 0.5 mol/L NaH_2PO_4, 1% bovine serum albumin (BSA), 1 mmol/L EDTA, and 7% sodium dodecyl sulfate (SDS) (pH 7.0). Membranes were hybridized overnight at 70°C with approximately 7×10^6 cpm/mL using DNA probes uniformly labeled with α-^{32}P

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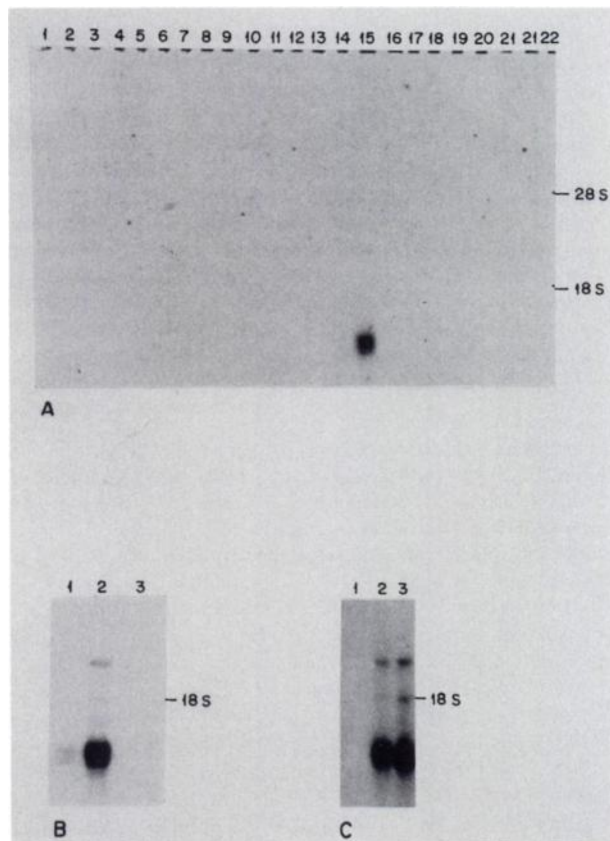

Fig 1. GM-CSF mRNA levels, determined by Northern blot analysis of total cellular RNA. (A) Mononuclear cell fraction, unprocessed case nos. 1 thorugh 22 (exposed for 20 hours at −70°C with intensifying screen). (B) Lane 1: phytohemagglutinin-stimulated lymphocytes; lane 2: case no. 15, bone marrow; lane 3: case no. 15, peripheral blood (exposed for 20 hours, at −70°C with screen). (C) Case no. 15, peripheral blood. Lane 1: mononuclear cell fraction; lane 2: blast enriched, immediately after processing; lane 3: blast enriched, 24-hour culture (exposed for ten days at −70°C with screen).

deoxycytosine triphosphate (dCTP) by the random-primer method[14] to a specific activity of 0.8 to 1.5×10^9 cpm/μg DNA.

Membranes were washed at 70°C, twice in 0.04 mol/L NaH_2PO_4 (pH 6.8), 0.05% BSA, 1 mmol/L EDTA, 5% SDS, then four times in 0.04 mol/L NaH_2PO_4 (pH 6.8), 1 mmol/L EDTA, 1% SDS. Blots were autoradiographed on Kodak XAR film at −70°C with an intensifying screen.

The GM-CSF probe was the *Eco*R1-*Aha*III fragment of cloned human GM-CSF cDNA,[5] provided by Drs G. Wong and S. Clark, Genetics Institute, Cambridge, Mass. The TPI probe was provided by Dr L. Maquatt, Roswell Park Memorial Institute, Buffalo.

RESULTS

Mononuclear cells from 49 AML patients were assayed by Northern blot analysis for expression of GM-CSF. One case (no. 13) was inevaluable due to RNA degradation. The distribution of French-American-British (FAB) types was M1, 14; M2, 9; M3, 0; M4, 18; M5, 4; M6, 1; RAEB-t, 1; Ph′(+) AML(M1), 1; Ph′(+) biphenotypic leukemia, 1. Bone marrow was the source of cells in 31 studies and peripheral blood in 18. Specimens were obtained at the time of first diagnosis in 37 cases and at first or subsequent relapse in 12. In 47 of the 48 evaluable cases, GM-CSF mRNA could not be detected (Fig 1A; case nos. 23 through 42, all GM-CSF negative, data not shown), with a detection limit estimated at <0.002 the level found in the one positive case (seven-day autoradiography was completely negative, as compared with readily detectable RNA in case no. 15 with four-hour exposure). Bone marrow mononuclear cells from patient no. 15, a newly diagnosed case of AML FAB M-1, expressed GM-CSF mRNA at high levels (Fig 1B, lane 2). However, GM-CSF mRNA could not be detected in this patient's peripheral blood mononuclear cells obtained almost simultaneously with the bone marrow aspirate (Fig 1B, lane 3). When the peripheral blood mononuclear cell fraction from patient no. 15 was processed to remove T cells and adherent cells, GM-CSF mRNA was detectable with steady state mRNA levels approximately one fifth those detected in the patient's unprocessed bone marrow cells (Fig 1C, lane 2). Mononuclear cells from peripheral blood in nine additional cases, in none of whom was GM-CSF mRNA detectable before processing (Fig 2, odd lanes), were similarly depleted of T cells and monocytes. Expression of GM-CSF was detectable in 2/4 of these cases following adherent-cell and T-cell depletion (Fig 2, lanes 2, 4, 6, and 8) and in 2/5 following T-cell depletion and magnetic attraction of phagocytized iron, followed by 24 hours of suspension culture at 37°C (lanes 10, 12, 16, 18 and 20). GM-CSF mRNA was detectable in one of one bone marrow specimen following processing and 24 hours of suspension culture (lane 14). These cases are summarized in Table 1.

A comparison of GM-CSF mRNA levels in cells processed to enrich for blast cells by E-rosetting and adherent-cell depletion with levels found in sham-processed cells, carried through the incubation steps but not depleted of the nonblast cells, was made as a preliminary effort to discover which

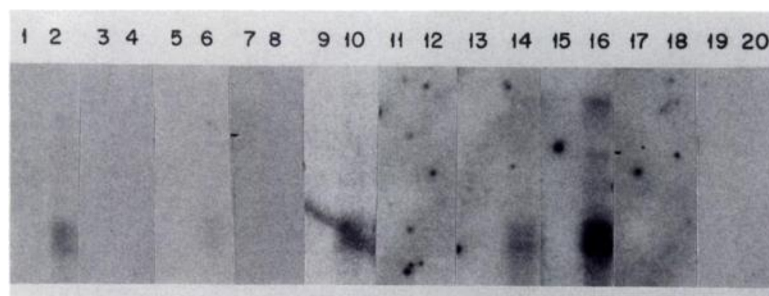

Fig 2. GM-CSF mRNA levels; comparison of mononuclear cell fraction with T-cell and monocyte-depleted fraction. Odd lanes: unprocessed mononuclear cell fraction. Lanes 2, 4, 6, 8: T-cell- and adherent-cell-depleted peripheral blood from case nos. 17, 43, 44, and 45, repectively. Lanes 10, 12, 16, 18, and 20: T-cell- and iron phagocytizing cell-depleted peripheral blood, cultured for 24 hours at 37°C from case nos. 46, 47, 18, 48, and 49, respectively. Lane 14: processed and cultured bone marrow specimen, case no. 18.

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Table 1. Effect of In Vitro Cell Processing on GM-CSF Expression

				Cell Differentials							
				Preprocessing			Postprocessing				
Case No.	FAB*	Disease Status	Cell Source	Blasts	Lymphs	Monos.	Blasts	Lymphs	Monos.	GM-CSF Preprocessing	mRNA Postprocessing
15	M1	Diagnosis	BM	92	3	5	—	—	—	+	ND
15		Diagnosis	PB	98	2	0	96†	4	0	−	+
15		Relapse	PB	71	23	7	85†	15	0	−	+
17	M4	Diagnosis	BM	96	0	0	—	—	—	−	ND
17		Diagnosis	PB	94	6	0	91†	6	0	−	+
18	Ph' (+)	Relapse	BM	94	7	0	96‡§	2	0	−	+
18	Biphenotypic	Relapse	PB	96	2	0	100‡§	0	0	−	+
43	M4	Diagnosis	PB	89	10	1	90†	4	0	−	−
44	M4	Relapse	PB	97	0	0	95†	2	0	−	+
45	M4	Diagnosis	PB	100	0	0	100†	0	0	−	−
46	M4	Diagnosis	PB	100	0	0	100‡§	0	0	−	+
47	M2	Diagnosis	PB	96	0	0	99‡§	1	0	−	−
48	M4	Diagnosis	PB	95	0	0	95‡§	0	0	−	−
49	M4	Diagnosis	PB	100	0	0	99.5‡§	0.5	0	−	−

Abbreviations: blasts, myeloblasts; lymphs, lymphocytes; monos, monocytes; BM, bone marrow; PB, peripheral blood.

*French-American-British classifications system.[22]

†Processed by E-rosetting and adherence to plastic.

‡Processed by E-rosetting and magnetic attraction of phagocytized iron.

§Cells incubated 24 hours in RPMI 1640 + 10% fetal bovine serum after processing.

aspects of processing might be important in allowing the enhanced gene expression—the removal of accessory cells per se or the incubations (Fig 3, lane 1). Both the sham-processed cells and the authentically processed cells expressed GM-CSF at similar levels (Fig 3, lanes 3 and 4). When unprocessed cells or sham-processed cells were placed in suspension culture at 37°C for an additional 24 hours, GM-CSF mRNA levels increased 20-fold (Fig 3, lanes 4 and 5, respectively), in contrast with the lack of further enhancement of GM-CSF mRNA levels by 24 hours of suspension culture of blast-enriched cells (Fig 1C, lanes 2 and 3).

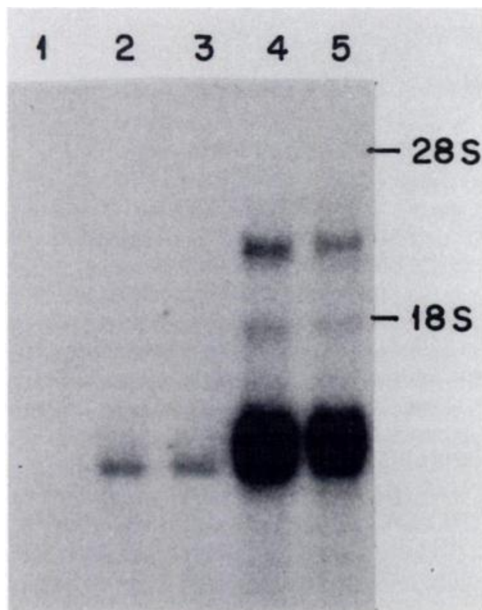

Fig 3. GM-CSF mRNA levels; comparison of authentically processed cells with sham-processed cells, case no. 15 peripheral blood at relapse. Lane 1: unprocessed mononuclear cell fraction; lane 2: T-cell- and adherent-cell depleted; lane 3: sham-processed cells; lane 4: mononuclear cell fraction, cultured for 24 hours at 37°C; lane 5: sham-processed cells, cultured for 24 hours.

DISCUSSION

Young et al[2] have demonstrated the presence of GM-CSF transcripts in AML leukemic cells and GM-CSF–like biologic activity in the conditioned media from cultured cells. T cells and adherent monocytes, respectively, were depleted from the population of assayed cells by sheep erythrocyte rosetting, then incubation at 37°C in plastic dishes. When total cell RNA from the resultant blast-enriched population was assayed by Northern blot analysis, GM-CSF transcripts were found in 11 of 22 cases. This group had previously demonstrated a correlation in a smaller number of cases between GM-CSF transcripts and the ability of cells to proliferate autonomously in vitro;[3] hence, their claim that GM-CSF is "constitutively" expressed in the leukemic cells from a large fraction of AML cases and the suggestion that this factor might be important for maintaining the abnormal proliferation of AML cells in vivo by autocrine action. Similar results have been obtained by others.[4]

It has been demonstrated that removal of either neoplastic or normal cells from their in vivo tissue and incubation in growth medium and (usually) fetal bovine serum, even for brief periods, can induce the expression of genes that are normally quiescent.[15] In order to minimize this potential for influencing gene expression by laboratory manipulations of cells, we assayed GM-CSF mRNA levels in AML bone marrow or peripheral blood mononuclear cell fraction without further enrichment steps. In contrast with the findings described above,[2] GM-CSF transcripts were detectable in only one of 48 evaluable cases of AML (case no. 15, Fig 1A). Moreover, in the one case in which GM-CSF expression was found in bone marrow mononuclear cells, no detectable mRNA was found in the preponderantly leukemic mononu-

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clear fraction of peripheral blood obtained on the same day. We did find, however, that GM-CSF expression was detectable in blasts of this patient's peripheral blood and in those of four of nine additional patients after in vitro monocyte and T-lymphocyte depletion steps similar to the processing used by Young et al.[2] Thus, a contribution of autocrine GM-CSF to AML cell proliferation appears to be unusual. In the one case in which GM-CSF mRNA was present in the bone marrow, an autocrine role is possible; however, even this case does not exemplify autonomous, unregulated expression of an autocrine factor, since the GM-CSF mRNA could not be detected in the peripheral blood leukemic cells.

At this time the mechanism of enhancement of GM-CSF transcript levels during in vitro processing of AML cells has not been elucidated. Although stabilization of the short half-life GM-CSF mRNA without transcriptional enhancement could increase steady-state mRNA levels above the detection limit,[7] a direct augmentation of transcription seems more likely. Enhanced GM-CSF expression could result from factors present in the serum-containing culture medium or released by accessory cells during the incubation steps. One possibility is the presence of endotoxin in the fetal bovine serum used in the depletion steps. Endotoxin levels are routinely quite high (>20 ng/mL) in the product we used, according to the supplier; however, endotoxin was not assayed in the lots of fetal bovine serum used for these experiments. A second possibility is the release of a paracrine factor by accessory cells during the processing steps. Since GM-CSF mRNA was present after processing the cells from one patient (no. 46) in whom only blasts were present prior to processing, an absolute dependence on accessory cells during incubation is excluded. However, a possible role for a paracrine factor was suggested by the finding in one patient (no. 15) that additional incubation of either sham-processed cells or unprocessed cells containing both monocytes and lymphocytes resulted in levels of GM-CSF mRNA that were 20-fold higher than before the incubation; whereas, incubation of authentically processed leukemic blasts resulted in no additional enhancement of GM-CSF mRNA. One might also postulate in vivo paracrine augmentation of leukemic cell gene expression[16-20] by factors released by marrow stromal cells in case no. 15. A dilution effect or loss of cell to cell contact[21] would release the leukemic cells from the stimulatory effect as they moved to the peripheral blood.

These studies emphasize the potential for altering in vivo patterns of gene expression by in vitro cell manipulations, with possible resultant erroneous hypotheses regarding the in vivo cell biology.

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