Selective Expression of *fos* Proto-oncogene in Human Acute Myelomonocytic and Monocytic Leukemias: A Molecular Marker of Terminal Differentiation

By F. Mavilio, U. Testa, N. M. Sposi, M. Petrini, E. Pelosi, C. Bordignon, S. Amadori, F. Mandelli, and C. Peschle

Expression of human *fos* proto-oncogene (c-fos) was analyzed in primary cells from 50 untreated acute lymphocytic (ALL) and myeloblastic (AML) leukemias. c-fos mRNA, analyzed by blot hybridization, was detected virtually only in myelomonocytic (M4) and monocytic (M5) AML. Both M4 and M5 samples show a strong positive correlation between the amount of c-fos transcripts and the percentage of leukemic cells expressing surface antigens specific for mature monocytes and macrophages. Normal mature monocytes exhibit a detectable level of c-fos RNA, which is virtually unaltered on activation to macrophage differentiation, but is always below that observed in M4 through M5 monocytic-like cells. These data provide evidence that c-fos expression is linked to terminal monocyte and macrophage differentiation in normal and leukemic hemopoiesis.

METHODS

Patients and cells. We selected 50 hematology—oncology patients with acute leukemia of either myelogenous or lymphocytic type. Differential diagnosis of ALL in pre-B, B or T type and of AML in M1 through M5 type was carried out by both morphological evaluation of bone marrow smears according to French-American-British (FAB) criteria and analysis of surface antigen pattern of leukemic cells. All patients were advised of all procedures and attendant risks, and gave informed consent.

Leukemic cells were obtained immediately after diagnosis and before initiation of therapy from peripheral blood or bone marrow aspirates, and enriched up to 90% by centrifugation on Ficoll-Hypaque cushions. Binding of monoclonal antibodies (OKT3, T4, T6, T8, T9, T10, T11, M5, B1, and BA-2 from Ortho Diagnostics, Raritan, NJ; MO2 from Coulter Immunology, Hialeah, FL; Techno-Leu-DR, M1, and M7 from Tecnogenetics, Turin, Italy; Leu-1 and Leu-9 from Becton Dickinson, Mountain View, CA; R-10, which specifically recognizes human glycoprotein A, generously sent by Dr. P.A. Edwards; AN-S1 which recognizes human platelet glycoprotein I, kindly provided by Dr. A.J. McMichael) was determined by indirect immunofluorescence. Leukemic cells (2 × 10⁶), isolated by centrifugation on a Ficoll-Hypaque cushion, were washed three times in Hank's saline solution and incubated for 60 minutes at 4°C with 200 μL of an appropriated dilution in Hank's saline solution containing 1 mg/mL of bovine serum albumin (BSA) of the monoclonal antibody. The cells, washed at 4°C in Hank's saline solution, were then incubated (60 minutes at 4°C) with fluorescein isothiocyanate (FITC)-labeled F(ab') fragments of immunoadsorbent-purified sheep antibodies against mouse IgGs. After three additional washes, the cells were mounted on slides in 50% glycerol in phosphate-buffered saline (PBS). The proportion of fluorescent cells was scored in incident light on a Leitz standard universal fluorescent microscope, equipped with a set of filters for narrow-band fluorescence.

Mature resting monocytes were obtained from peripheral blood by buffy coat of healthy donors by enrichment on a Ficoll-Hypaque cushion and subsequent purification (>90%) by centrifugation and isolation by centrifugation on a Ficoll-Hypaque cushion, and enriched up to 90% by centrifugation on Ficoll-Hypaque cushions. Binding of monoclonal antibodies (OKT3, T4, T6, T8, T9, T10, T11, M5, B1, and BA-2 from Ortho Diagnostics, Raritan, NJ; MO2 from Coulter Immunology, Hialeah, FL; Techno-Leu-DR, M1, and M7 from Tecnogenetics, Turin, Italy; Leu-1 and Leu-9 from Becton Dickinson, Mountain View, CA; R-10, which specifically recognizes human glycoprotein A, generously sent by Dr. P.A. Edwards; AN-S1 which recognizes human platelet glycoprotein I, kindly provided by Dr. A.J. McMichael) was determined by indirect immunofluorescence. Leukemic cells (2 × 10⁶), isolated by centrifugation on a Ficoll-Hypaque cushion, were washed three times in Hank's saline solution and incubated for 60 minutes at 4°C with 200 μL of an appropriated dilution in Hank's saline solution containing 1 mg/mL of bovine serum albumin (BSA) of the monoclonal antibody. The cells, washed at 4°C in Hank's saline solution, were then incubated (60 minutes at 4°C) with fluorescein isothiocyanate (FITC)-labeled F(ab') fragments of immunoadsorbent-purified sheep antibodies against mouse IgGs. After three additional washes, the cells were mounted on slides in 50% glycerol in phosphate-buffered saline (PBS). The proportion of fluorescent cells was scored in incident light on a Leitz standard universal fluorescent microscope, equipped with a set of filters for narrow-band fluorescence.

Mature resting monocytes were obtained from peripheral blood by buffy coat of healthy donors by enrichment on a Ficoll-Hypaque cushion and subsequent purification (>90%) by centrifugation on a Percoll density gradient or standard adherence on tissue culture flasks.

RNA analysis. Total cellular RNA was extracted from 10⁶ cells by the guanidine thiocyanate technique, dotted in 8.0 to 0.5 μg aliquots onto nitrocellulose paper (BA-85, Schleicher & Schull, Keene, NH), and hybridized to 10⁶ cpm of the 1.1-kilobase (kb) PstI/PstI fragment containing most of v-fos, labeled by nick-translation up to a specific activity of 4 to 9 × 10⁶ dpm/μg. Stringency of washing (30 mmol/L of NaCl, 5 mmol/L of Na-citrate at 65°C) was accurately tested on appropriate negative blood monocytes, although at a level approximately threefold lower than in leukemic cells.

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controls to avoid nonspecific binding or cross-hybridization of the viral probe. Hybridization to a chicken β-actin probe was carried out to allow normalization of RNA levels. Poly-A+ RNAs were obtained by one passage on oligo-dT cellulose columns, run in 5-μg aliquots on 1% agarose-formaldehyde gels, transferred onto nitrocellulose paper by capillary blot, and hybridized as described for dot blots.

**DNA analysis.** High mol wt DNA was extracted from 10⁶ cells, digested with restriction endonucleases, run on 1% agarose gels, and aliquots on 1% agarose-formaldehyde gels, transferred onto nitrocellulose paper, and hybridized to 2 x 10⁶ cpm of radiolabeled probe as described.

**RESULTS**

We analyzed a sample of 20 ALL (8 T ALL, 1 B cell precursor, 8 pre-B, and 3 B ALL) and 30 AML (3 M1, 6 M2, 5 M3, 8 M4 and 8 M5 AML). The expression of c-fos in purified leukemic cells was quantitatively evaluated by filter hybridization of total RNA to a fos-specific probe (Fig 1). These transcripts were barely detectable or undetectable in all leukemic samples, with the exception of myelomonocytic (M4) and monocytic (M5) AML cells, which showed variable levels of fos RNA (Fig 1). Analysis of poly-A+ RNA by Northern blot (Fig 2) indicates that c-fos is transcribed into a predominant 2.3-kb mRNA band and a minor 3.6-kb RNA species, probably a precursor. A faint band of 1.9 kb is also detectable, which apparently corresponds to fos-related sequences (r-fos), transcribed together with c-fos after platelet-derived growth factor (PDGF) stimulation of cultured fibroblasts. c-fos is thus specifically expressed in M4 and M5 AML, both characterized by presence of a variable proportion of neoplastic cells, more or less differentiated along the monocytic-macrophagic pathway. In none of these cases did we observe amplification of the fos gene, as evaluated by Southern blot hybridization of genomic DNA (results not shown).

We further investigated whether in M4 and M5 AML samples expression of c-fos was related to the number of monocytic-like cells and/or their stage of differentiation. The former parameter was evaluated on the basis of morphologic and cytochemical features (ie, inhibition of α-naphthyl acetate esterase staining after treatment with sodium fluoride, which is specific for monocyes): these also allowed a more precise classification of the M5 AML in M5A and M5B subtypes according to FAB criteria. On the other hand, the stage of differentiation of leukemic cells within the monocytic pathway was evaluated by immunofluorescence studies with a panel of ten monoclonal antibodies. In particular, we used OKM5, MO2, and Tec-M1, which recognize surface antigens selectively expressed on mature monocytes and macrophages, as well as on terminally differentiated leukemic lines induced in vitro by various agents. All morphological, cytochemical, and immunologic data are summarized in Table 1.

The levels of c-fos RNA, showing a virtually equivalent spread in M4 and M5 AML, were not correlated with the number of monocytic-like cells in each sample, as evaluated by standard cytochemical criteria (Fig 1). Conversely, a strong positive correlation was observed between the abundance of c-fos transcripts and the number of cells recognized by either OKM5, MO2, or Tec-M1 antibodies, in both M4 and M5 samples (Fig 3). Among M5 AML, c-fos transcripts were more abundant in the M5B subtype (Table 1), which is characterized by a more elevated number of cells morphologically resembling mature monocytes, according to the FAB criteria.

We further investigated c-fos expression in normal monocytes. c-fos transcripts were clearly detected in nonadherent resting monocytes isolated by centrifugation on density gra-
We studied the expression of c-fos in primary cells from both lymphocytic and myeloblastic acute leukemia patients. Our data indicate that accumulation of c-fos transcripts is limited virtually to the myelomonocytic and monocytic subtypes. The amount of c-fos transcripts appears to be strongly correlated with the number of cells expressing surface markers specific for mature monocytes and macrophages, whereas no correlation exists with the number of monocyticlike cells recognized by conventional cytochemical analysis. We interpret these findings to suggest that leukemic cells expressing surface antigens specific for terminal monocytic differentiation selectively accumulate c-fos transcripts (ie, the amount of fos RNA detected by blot hybridization in the overall sample depends essentially on the relative number of these cells). Therefore, expression of c-fos does not generally occur in leukemic cells belonging to the monocytic lineage (eg, monoblastic or promonocyticlike elements), but only in the more differentiated ones, as defined by expression of specific surface markers. Accordingly, c-fos transcripts in M5 AML are particularly abundant in M5B samples, which are characterized by the presence of a more elevated number of cells resembling mature monocytes at morphological and cytochemical level (Table 1).

Interestingly, a single case of M2-AML abundantly expressed c-fos RNA. These cells were strongly positive to one of the monocytic-specific antibodies (ie, MO2), even though no other monocytic feature could be demonstrated by morphological, cytochemical, and surface antigen criteria.

**DISCUSSION**

Growing evidence accumulated in the last years suggests a role for "activated" cellular proto-oncogenes (c-onc) in establishment, maintenance and progression of neoplastic growth, particularly in the hemolymphopoietic system. In this regard, abnormal c-onc expression, at both quantitative and qualitative levels is significantly associated to different types of human leukemias and lymphomas; however, cause-effect relationship(s) between these abnormalities and oncogenesis of hematopoietic tumors have not been conclusively established so far.

Previous studies on c-onc expression in leukemias have met with various limitations. A crucial aspect is that most observations have been carried out on leukemic lines, which do not often represent faithfully both the genotype and phenotype of the original neoplastic clone(s). On the other hand, interpretation of the limited number of studies carried out on primary cells is hampered by both the small number of examined samples and the poor classification of leukemic cells.

We studied the expression of c-fos in primary cells from both lymphocytic and myeloblastic acute leukemia patients. Our data indicate that accumulation of c-fos transcripts is limited virtually to the myelomonocytic and monocytic subtypes. The amount of c-fos transcripts appears to be strongly correlated with the number of cells expressing surface markers specific for mature monocytes and macrophages, whereas no correlation exists with the number of monocyticlike cells recognized by conventional cytochemical analysis. We interpret these findings to suggest that leukemic cells expressing surface antigens specific for terminal monocytic differentiation selectively accumulate c-fos transcripts (ie, the amount of fos RNA detected by blot hybridization in the overall sample depends essentially on the relative number of these cells). Therefore, expression of c-fos does not generally occur in leukemic cells belonging to the monocytic lineage (eg, monoblastic or promonocyticlike elements), but only in the more differentiated ones, as defined by expression of specific surface markers. Accordingly, c-fos transcripts in M5 AML are particularly abundant in M5B samples, which are characterized by the presence of a more elevated number of cells resembling mature monocytes at morphological and cytochemical level (Table 1).

Interrestingly, a single case of M2-AML abundantly expressed c-fos RNA. These cells were strongly positive to one of the monocytic-specific antibodies (ie, MO2), even though no other monocytic feature could be demonstrated by morphological, cytochemical, or surface antigen criteria. This confirms that c-fos expression is linked to the genetic program of terminal macrocyte differentiation: occasionally, this program may be partially expressed by leukemic cells otherwise differentiated into a nonmonocytic pathway, as a typical example of lineage infidelity.

Our results also reflect on previous studies on HL-60 (promyelocytic) and U937 (monoblastic) human leukemic lines, which indicated that addition of 12-O-tetradecanoylphorbol-13-acetate (TPA) triggers early c-fos transcription and differentiation to adherent macrophages. Interpretation of these studies is rendered uncertain by observations reporting that TPA (as well as other activators of protein kinase C) also induces c-fos transcription in various hemo-

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**Table 1. Surface Antigen Pattern, Cytochemistry, and Expression of c-fos in Leukemic Cells From M4 and M5 AML Patients**

<table>
<thead>
<tr>
<th>Case</th>
<th>FAB</th>
<th>HLA-DR</th>
<th>MG1</th>
<th>OKM5</th>
<th>MO2</th>
<th>M1</th>
<th>OKT9</th>
<th>OKT10</th>
<th>R-10</th>
<th>AN-51</th>
<th>c-fos Expression (% Positive Cells)</th>
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<tr>
<td>CR-2</td>
<td>M4</td>
<td>21</td>
<td>81</td>
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<td>ND</td>
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</table>

FAB, classification according to the French-American-British criteria. ND, not determined; Neg, negative; NASA, α-naphthyl acetate esterase activity; NASA-F, NASA after Na fluoride inhibition.
EXPRESSION OF c-fos IN M4 AND M5 LEUKEMIAS

**Fig 1.** Evaluation of c-fos expression in M4 through MS AML cases. The amount of monoclonal antibodies was assessed by indirect immunofluorescence through MS AML elements are mostly blocked in the G0 phase of cell cycle. Indeed, expression of c-myc, generally correlated to proliferative activity, is barely detectable in M4 through M5 cells, whereas it is clearly demonstrable in other AML subtypes (M1, M2, M3) showing more intensive proliferation (results not shown here).

In conclusion, our studies provide evidence that c-fos transcripts are selectively accumulated in leukemic cells characterized by expression of antigen markers specific for normal mature monocytes and macrophages. In this respect, c-fos expression in M4 through M5 AML cells mimics that observed in their normal hemopoietic counterparts, but at a more elevated level. Therefore, expression of c-fos may be considered a molecular marker of monocytic differentiation in leukemic cells, possibly of diagnostic significance. Further studies are necessary to assess whether the apparently enhanced expression of fos gene in M4 through M5 cells plays a significant role in the leukemogenic process.

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