Expression of Leukocyte Alkaline Phosphatase Gene in Normal and Leukemic Cells: Regulation of the Transcript by Granulocyte Colony-Stimulating Factor

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The levels of leukocyte alkaline phosphatase (LAP) messenger RNA (mRNA) are evaluated in B and T lymphocytes, monocytes, and polymorphonuclear cells (PMNs), and this transcript is found to be present only in PMNs. Precursors of the myelomonocytic pathway, represented by leukemic cells isolated from several cases of chronic myelogenous leukemia (CML) in its stable and blastic phase and acute myelogenous leukemia (AML), are devoid of LAP transcript. These data support the notion that LAP is a marker of the granulocyte terminal differentiation. Despite the absence of LAP mRNA in both the myeloid and the lymphoid precursors, nuclear run-on experiments show constitutive transcription of the LAP gene in leukemic cells obtained from AML, CML, as well as acute lymphoblastic leukemia (ALL) and B-cell chronic lymphocytic leukemia (B-CLL). In CML and in chronic myelomonocytic leukemia (CMML) PMNs, granulocyte colony-stimulating factor (G-CSF) specifically accumulates LAP mRNA without showing a substantial increase in the rate of transcription of the LAP gene. Once increased by G-CSF, LAP mRNA is very stable, showing a half-life of more than 4 hours in the presence of actinomycin-D. G-CSF is suggested to play a pivotal role in the modulation of LAP transcript in PMNs.

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ALP mRNA expression in neutrophils isolated from CML and CMML: human interleukin-8 (IL-1β) (generously provided by Dr D. Boraschi, Sclavo, Siena, Italy), specific activity 1 × 10⁷ U/mg of protein; human tumor necrosis factor-α (TNF-α) (a kind gift of Dr G. Adolf, Ernst Behring Institute, Vienna, Austria), specific activity 1 × 10⁷ U/mg of protein; human granulocyte monocyte CSF (GM-CSF) (kindly provided by Dr S. Gillis, Immunex, Seattle WA), specific activity 1 × 10⁷ U/mg of protein; human G-CSF (kindly provided by Dr L. Souza, AMgene, Thousand Oaks, CA), specific activity 8 × 10⁶ U/mg of protein; and human interferon-γ (IFN-γ) (kindly provided by Dr S. Reich, Biogen, Cambridge, MA). IFN-α 2A is for clinical use (Roferon; Hoffmann-La Roche, Basel, Switzerland). At the end of the incubation, the cells were harvested, washed, and used for total cellular RNA or for nuclei preparation.

**RNA preparation, Northern blot analysis, and measurement of mRNA half life.** Northern blot analysis was performed as previously described. Briefly, cells were lysed in 4 mol/L guanidinium isothiocyanate and total cellular RNA was recovered by centrifugation through a cesium chloride gradient. RNA preparation. Northern blot analysis, and measurement of each of the cDNA plasmids immobilized to the nylon membranes (Gene Screen Plus; New England Nuclear, Boston, MA). These membranes were hybridized to the different probes labeled at specific activity of 1 to 2 × 10⁶ cpm/pg by using hexanucleotide primers and ¹²⁵P-dCTP. Hybridization was performed at 60°C overnight in a solution containing 1 mol/L sodium chloride, 1% sodium dodecyl sulphate (SDS), 10% dextran sulphate (Sigma), 100 μg/mL salmon sperm DNA (Boehringer, Mannheim, West Germany) and 1 to 2 × 10⁶ cpm/mL labeled probe. The membranes were washed twice with 2X SSC/1% SDS (1X SSC being 0.15 mol/L sodium chloride, 0.015 mol/L sodium citrate, pH 7.0) for 1 hour at 65°C and 0.1X SSC for 30 minutes at room temperature. The membranes were dried and exposed to Kodak X-Omat X-ray films (Eastman Kodak Company, Rochester, NY) with two intensifying screens (Dupont Cronex, Dupont Co, Boston, MA) at −70°C.

The half-life of LAP mRNA was measured by adding actinomycin-D (Sigma) at 1 mg/mL to cultured cells at designated times, and analyzed by Northern blotting.

**Transcriptional run-on assay.** Nuclear transcription run-on assays were performed as described with some modifications. Briefly, nuclei were prepared by lysing cells with 4 mL of 0.5% Nonidet P-40 lysis buffer (10 mmol/L Tris pH 7.4, 10 mmol/L sodium chloride, 3 mmol/L magnesium chloride). After washing with ice-cold lysis buffer, nuclei were resuspended in glycerol buffer (50 mmol/L Tris pH 8.0, 40% glycerol, 5 mmol/L magnesium chloride, 0.1 mmol/L EDTA) and incubated at 30°C for 30 minutes in run-on buffer containing 5 mmol/L Tris pH 8.0; 2.5 mmol/L magnesium chloride; 150 mmol/L potassium chloride; 1.25 mmol/L each of ATP, CTP, and GTP (Pharmacia, Uppsala, Sweden); and 100 μCi of ³²P-UTP (Amersham). Nuclei were then resuspended in 4 mol/L guanidium isothiocyanate followed by recovery of nascent RNA by centrifugation through cesium chloride and ethanol precipitation. Labeled elongated RNAs (minimum 1 × 10⁶ cpm/mL) were hybridized to 5 μg of each of the cDNA plasmids immobilized on nitrocellulose membranes after denaturation by heat and alkaline treatment. The filters were washed at a final stringency of 2X SSC at 55°C for 1 hour and then treated with RNAase A (Sigma) 10 μg/mL in 2X SSC for 30 minutes at 37°C. The two CML cases in nuclear run-on experiments presented 56% and 60% mature neutrophils in their PMN fraction, as determined by flow cytometric analysis of CD-16 antigen expression.

**DNA probes.** The probe used for the detection of LAP mRNA, by Northern blot analysis, is an EcoR1-Smal fragment derived from the human L/B/K-type ALP cDNA and contains part of the 3'-untranslated sequence as well as the translated sequence from nucleotide 1136. The plasmids used for run-on assays are the human L/B/K-type ALP cDNA containing the same translated sequence as above, as well as the whole 3'-untranslated sequence.

The placent ALP mRNA was studied with its full-length cDNA. Expression of the protooncogene c-fos mRNA was investigated using the plasmid pc-fos 3. The TNF-α cDNA probe (plasmid pE4) was kindly provided by Drs D. Mark and P. Ralph (Cetus Corp, Emeryville, CA). Actin mRNA was detected using a mouse α-actin cDNA. Densitometric analysis of the autoradiogram was performed using a laser beam densitometer (300 A computing densitometer Fast Scan; Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

**LAP mRNA is expressed selectively in mature PMNs.** The expression of LAP mRNA was studied in various hematopoietic cell lineages by Northern blotting analysis. Figure 1 shows that the transcript is absent in peripheral blood T lymphocytes, in tonsillar B lymphocytes, and in peripheral blood monocytes, whereas high levels of LAP mRNA are expressed in blood PMN. The amount of LAP mRNA correlates with the levels of LAP enzymatic activity determined in parallel on the various cell populations (data not shown).

The presence of LAP mRNA in the PMN prompted us to study the expression of this transcript in various precursors of...
the myelomonocytic lineage using freshly isolated leukemic cells. Figure 2A shows that RNA preparations from leukemic cells isolated from the blast crisis of CML are negative for the LAP transcript. Similarly, AML (M1 to M5, according to the French-American-British [FAB] classification) are all negative for the LAP transcript, except for an M4 case where a low level of LAP mRNA is detectable (see Fig 2B, lane 2). The absence of LAP mRNA is not due to degradation because the actin transcript, used as a control of the quality and quantity of RNA, constantly appears. Similar experiments performed on cells derived from cases of ALL of either B- and T-cell origin as well as B-CLL show that LAP mRNA expression is absent also during the early and late stages of both lymphoid differentiation pathways (data not shown).

Constitutive transcription of LAP gene does not result in LAP mRNA accumulation. To better understand the molecular mechanisms underlying the regulation of the expression of LAP mRNA in hematopoietic cells, nuclear run-on experiments on purified nuclei isolated from cases of AML, ALL, and B-CLL were performed. Figure 3 shows that all three cell types have significant nuclear transcription of the LAP gene, albeit with quantitative variations. The intensity of the signal is comparable with that of α-actin, even though it is generally lower than that of TNF-α used as a positive control. No transcription of the gene coding for the placental alkaline phosphatase isozyme is observed (compare placental-ALP with LAP), showing that the hybridization is specific for one of the members of the alkaline phosphatase multigene family.

G-CSF increases LAP mRNA in CMML and CML PMNs. CML and CMML belong to the family of chronic myeloid leukemias. Both pathologic conditions are associated with low levels of LAP enzymatic activity in PMNs, which can be increased by treatment with crude supernatants from tumor cells and activated monocytes as well as by exposure to human G-CSF. To elucidate the mechanism underlying this phenomenon, we tested the effect of G-CSF on LAP mRNA steady state levels by Northern blotting analysis. As shown in Fig 4A, after G-CSF treatment (lane 4), the amount of LAP transcript is significantly increased with respect to control conditions (lane 1). Figure 4B shows the accumulation of LAP mRNA by G-CSF also in two representative cases of CML during the stable phase of the disease (compare lanes 1 and 4 with lanes 2 and 5). The slight difference in mobility of both LAP and actin transcripts in lane 3 is probably an artifact due to partial degradation of RNA.

The specificity of G-CSF on LAP mRNA induction is...
Fig 4. (A) Effect of various modulators of the myeloid differentiation on LAP mRNA in CMML PMNs. RNA was extracted from PMNs isolated from a representative case of CMML, cultured for 4 hours either with medium alone (lane 1) or with the following cytokines: IFN-α 10^3 U/mL (lane 2); IFN-γ 10^3 U/mL (lane 3); G-CSF 10^3 U/mL (lane 4); GM-CSF 10^3 U/mL (lane 5); IL-1β 10^3 U/mL (lane 6); G-CSF 10^3 U/mL + IFN-α 10^3 U/mL (lane 7); and from untreated PMNs of a healthy volunteer (lane 8). (B) G-CSF induction of LAP transcript in two representative cases of CML during the stable phase of the disease. RNA was extracted from CML PMNs cultured for 2 hours with medium alone (lanes 1 and 4) or with G-CSF 10^3 U/mL (lanes 2 and 5), and from PMNs of healthy volunteers (lanes 3 and 6). Actin signal was detected as described in the legend to Fig 1.

Fig 5. Effect of G-CSF on transcription in CML PMNs during the stable phase of the disease. Radiolabeled RNA was prepared from nuclei isolated from resting (lane 1) or G-CSF activated (10^3 U/mL) CML PMNs (lane 2). As indicated on the left of the figure, the plasmids used in slot-bLOTS are LAP, c-fos, and actin as positive controls for transcription, pUC18 and PL-ALP as negative controls. Independent experiments performed on nuclei from PMNs of two distinct cases of CML are shown in (A) and (B).
The data presented in this report demonstrate that LAP mRNA is expressed only in the late phases of the myeloid differentiation. The specific transcript is, in fact, absent in all the precursors of the myelomonocytic maturation pathway. To verify this point, the model of AML and CML, during its blastic phase, was used. These cells represent frozen stages of the established steps along the myeloid differentiation pathway, allowing us to study the expression of the LAP gene.

The LAP mRNA is absent in the precursors of granulocytes represented by AML blast cells and in cells during the blast crisis of CML, which are usually considered as representatives of the most immature steps along the myeloid differentiation. These data also expand and complement our previous studies, showing that LAP mRNA is absent in PMNs of CML patients during the stable phase of the disease, and strongly support the notion that LAP is a marker of terminal differentiation of the human neutrophil.

Despite the lack of accumulation of mature LAP transcripts in the precursors of the myelomonocytic as well as the B- and T-lymphocytic pathways, nuclear run-on experiments show significant levels of transcription of the LAP gene in these cells. This situation is very similar to that observed for the mRNA of some cytokines secreted by hematopoietic cells, where high levels of transcription do not result in accumulation of the messages. The molecular basis for this phenomenon is not yet clear for the LAP gene. Intrinsic instability or defective maturation of the nuclear message could explain it.

To clarify this point and to define the molecules physiologically regulating LAP expression during the final steps of the granulocyte maturation pathway, a series of known functional modulators of the myeloid differentiation were tested for their ability to increase the steady state levels of LAP mRNA in CML as well as CMML PMNs. Among the various cytokines used, only G-CSF proved to be capable of inducing LAP mRNA. This effect is very specific because even GM-CSF, which is often overlapping G-CSF in its ability to stimulate the function of the mature neutrophil, is inactive in this respect. Although nuclear run-on experiments are technically very challenging in PMNs, the ability of G-CSF to elevate LAP mRNA levels does not seem to be primarily related to transcription itself, because G-CSF fails to demonstrate a substantial increase in the rate of transcription of the LAP gene. More likely, LAP mRNA levels are regulated posttranscriptionally by G-CSF, perhaps by inducing the proper maturation of nuclear RNA or by stabilizing the mature cytoplasmic mRNA. To address this second possibility, experiments were performed to determine the stability of LAP mRNA after G-CSF treatment of CML PMNs. In resting CML PMNs, the amount of LAP mRNA is too low to reliably measure the half-life with actinomycin-D. However, following stimulation with G-CSF, the half-life of LAP mRNA is shown to be longer than 4 hours. The extreme stability of the message is the same as that observed in normal PMNs (data not presented) and it is longer than the in vivo median half-life of the human circulating mature neutrophil. It is tempting to speculate that G-CSF controls the metabolic rate of LAP nuclear pre-mRNA, by increasing its steady state level, which results in the accumulation of very stable mature mRNA in the circulating PMN. The lack of induction of LAP mRNA by the protein-inhibitor cycloheximide rules out the possibility
that the stability of this transcript is under the control of labile proteins, as observed for other mRNAs. Moreover, the L/B/K-type ALP transcript does not contain the AUUUA consensus sequence, which is generally (though not always) present in mRNAs under the control of labile proteins, in its 3′-untranslated region.\textsuperscript{47,48}

Collectively our results suggest that the regulation of the LAP gene expression by G-CSF in the neutrophil is mainly posttranscriptional. However, we cannot rule out the possibility that a small increase in the transcriptional rate of the gene (barely detectable with nuclear run-on experiments) may lead to a major increase in mRNA accumulation, due to the long half-life of LAP mRNA. Studies are ongoing to clarify this point.

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