Increased Erythroid Potentiating Activity/Tissue Inhibitor of Metalloproteinases and jun/fos Transcription Factor Complex Characterize Tumor Promoter-Induced Megakaryoblastic Differentiation of K562 Leukemia Cells

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Molecular cloning has revealed that erythroid potentiating activity (EPA) and tissue inhibitor of metalloproteinases (TIMP) represent two distinct activities of a single protein. We have studied the expression of the EPA/TIMP gene at the mRNA and protein levels during 12-O-tetradecanoyl-phorbol-13-acetate (TPA)-induced megakaryoblastic differentiation of K562 human chronic myeloid leukemia cells. Northern hybridization analysis showed that the EPA/TIMP mRNA was increased within 3 hours of TPA-induction and reached maximal levels (about 50-fold induction) during the first day of treatment. The expression of mRNAs for two major metalloproteinases, collagenase-1 and stromelysin, were activated in parallel in the differentiation-induced K562 cells. The increase of EPA/TIMP mRNA correlated with increased EPA/TIMP protein biosynthesis and secretion: the TPA-induced cells secreted substantially enhanced amounts of metabolically labeled proteins, of which EPA/TIMP represented up to 50% after the first day of treatment (over 100-fold induction). The induction of EPA/TIMP mRNA was associated with its increased transcription. EPA/TIMP induction required continuous protein synthesis, being completely inhibited by addition of the protein synthesis inhibitor cycloheximide simultaneously with TPA, but only partially inhibited in a time-dependent manner if cycloheximide was added after TPA. Unlike in other cells tested, the jun and c-fos transcription factor mRNAs showed a prolonged biphasic induction response in K562 cells during TPA treatment. This response was associated with enhanced activity of a transfected recombinant reporter plasmid containing binding sites for the jun/fos transcription factor complex AP-1 similar to the TPA-responsive element (TRE) sequence we found in the EPA/TIMP gene promoter. We suggest that the induction of EPA/TIMP and several other genes specific for the differentiating K562 cells may be a consequence of the sustained activation of immediate early genes encoding transcription factors, such as jun and c-fos.

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Submitted July 3, 1989; accepted January 30, 1990.

Supported by the Finnish Medical Foundation, the Finnish Cultural Foundation, the Sigrid Juselius Foundation, the Academy of Finland, and the Finnish Cancer Organizations.

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activator proteins (AP). The transcription factor AP-1 is a protein complex that contains the c-jun oncoprotein with affinity for the TRE sequence. The c-jun and related genes have been identified as immediate early genes responding to serum stimulation, and have also been shown to be induced by stimulation with several growth factors, including epidermal growth factor (EGF) and TGF-β.

The c-jun protein has been shown to bind to the TRE in a complex with proteins of the fos proto-oncogene family. This complex formation is essential for AP-1 enhancer function. In addition to the AP-1 binding site, several other TRE sequences have been identified, and the TPA-activated signal transduction network seems to involve at least five different DNA-binding proteins.

We report here that TPA-treated K562 cells, unlike other leukemia cells, secrete over 100-fold enhanced amounts of erythroid potentiating activity/tissue inhibitor of metalloproteinases (EPA/TIMP), a major inhibitor of matrix metalloproteinases (EPA/TIMP), which constitutes over half of the total radiolabeled protein secreted by the differentiating cells. In contrast to other cells, the K562 cells also show a prolonged induction of the c-fos and jun transcription factor mRNAs after TPA treatment. Differences in the induction of early transcription factor responses may be of significance for the specificity of the effects of TPA in cell differentiation models.

MATERIALS AND METHODS

Cell culture and reagents. K562 leukemia cells, U937 (histiocytic lymphoma), HL-60 (promyelocytic leukemia), HEL (human erythroleukemia), and RD rhabdomyosarcoma cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, antibiotics, and L-glutamine. The four sublines of K562 studied included the original line obtained from Dr C. Lozzio (Chicago, IL), K562 clone 4 obtained from Dr K. Nilsson (Uppsala, Sweden), a clone obtained from Dr O. Colamonici (National Institutes of Health, Bethesda, MD), and K562 cells obtained through the American Type Culture Collection (ATCC; Rockville, MD). Induction of differentiation was started in a logarithmic growth phase at a density of 3 x 10^5 cells/mL. TPA was dissolved in dimethyl sulfoxide (DMSO) or ethanol and used at a final concentration of 1.6 to 3.2 mmol/L.

Cell culture media and fetal calf serum were from GIBCO (Paisley, Scotland, UK). TPA and cycloheximide were from Sigma Chemical Co (St Louis, MO); proteinase K and DMSO were from Merck (Darmstadt, FRG); protein A sepharose and polyacrylamide gels were from Pharmacia (Uppsala, Sweden). Oligo-dT-cellulose was from Collaborative Research (Bedford, MA); and all radioisotopes were from Pharmacia (Uppsala, Sweden), a clone obtained from Dr C. Lozzio (Chicago, IL), K562 clone 4 obtained from Dr K. Nilsson (Uppsala, Sweden), a clone obtained from Dr O. Colamonici (National Institutes of Health, Bethesda, MD), and K562 cells obtained through the American Type Culture Collection (ATCC; Rockville, MD). Induction of differentiation was started in a logarithmic growth phase at a density of 3 x 10^5 cells/mL. TPA was dissolved in dimethyl sulfoxide (DMSO) or ethanol and used at a final concentration of 1.6 to 3.2 mmol/L.

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Analysis of proteins by metabolic labeling and immunoprecipitation. For analysis of secreted proteins, cell cultures were labeled for 2 hours. Labeled, cell-free culture media were used in the analysis. Supernatant (5 mL) was mixed with 95 mL Laemmli sample buffer containing 10% mercaptoethanol. For immunoprecipitation, 2 mL of sheep anti-human TIMP antiserum (a kind gift from Dr J.J. Reynolds, Strangways Research Laboratory, Cambridge, UK) was added to 800 mL aliquots of supernatants and incubated at 4°C for 2 to 12 hours. The immune complexes were collected by adsorption to protein A-Sepharose in an end-over mixer for 1 hour. Immuno precipitates were washed several times with immunoprecipitation buffer, twice with phosphate-buffered saline (PBS), and finally with 20 mmol/L Tris-HCl, pH 7.5. Immune complexes were dissolved by boiling in electrophoresis sample buffer and subjected to electrophoresis in 10% sodium dodecyl sulfate (SDS) polyacrylamide gels under reducing and nonreducing conditions. Fixed gels were treated with Amplify (Amersham) and exposed to Kodak XAR-5 film for 1 to 5 days at -70°C.

RNA extraction and analysis. Polyadenylated RNA was extracted from 10^4 to 10^6 cells at various time points after TPA induction. Where indicated, cycloheximide was used at a final concentration of 10 μg/mL. Poly(A)+ mRNA was bound to oligo-DT cellulose directly from cell lysates and eluted as described. RNA (4 μg) was dissolved in sample buffer, size-fractionated in formaldehyde-agarose (0.6%) gels, and transferred to Biodyne membranes (Pall Corp, Glen Cove, NY) in 20 x SSC (1 x SSC is 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.0) for 20 hours. Hybridizations with cDNA probes were carried out at 42°C for 18 to 24 hours in a hybridization mixture containing Denhardt's solution (2% each of Ficoll, polyvinylpyrrolidone, and bovine serum albumin [BSA]), 3 x SSC, 5% formamide, 50 mmol/L 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, 200 μg/mL salmon sperm DNA, and 150 μg/mL yeast tRNA. Posthybridization washes were done for 2 x 15 minutes at room temperature with 1 x SSC and for 2 x 30 minutes with 0.1 x SSC, 0.1% SDS at 65°C. Oligonucleotide hybridizations were carried out for 16 to 20 hours at 42°C in 5 x SSC, 20 mmol/L sodium phosphate (pH 7.0), 10 x Denhardt's solution, 7% SDS, and 100 μg/mL salmon sperm DNA. After oligonucleotide hybridization, the filters were washed at 65°C for 2 x 15 minutes with 3 x SSC, 10 mmol/L sodium phosphate (pH 7.0), 5 x Denhardt's solution, and 5% SDS, and then for 2 x 15 minutes with 1 x SSC, 1% SDS. The filters were exposed to Kodak XAR-5 film at -70°C for 6 to 48 hours. Radioactive signals were quantitated from the autoradiograms with a densitometric scanner (Helena Laboratories, Beaumont, TX).

Molecular probes. The EPA/TIMP cDNA was a gift from Dr Steven Clark (Genetics Institute, Boston, MA). The rat transferrin cDNA has been described. The human collagenase cDNA c-p1 was a gift from ATCC. The junB cDNA clone 465.20 was a gift from Dr Peter Angel, University of California, San Diego, CA, and a high-performance liquid chromatography-purified 30-mer antisense oligonucleotide (corresponding to nucleotides 1321 to 1350 in the published human c-jun sequence) labeled at its 5'-end with [32P]-adenosine triphosphate (ATP) using T4 polynucleotide kinase. The human c-fos genomic clone p21A1 was a 3.25 kb Nco I-Halo fragment in the pGEM2 vector, given by Dr Rolf Müller (University of Marburg, Marburg, FRG). The glyceraldehyde-3-phosphate-dehydrogenase cDNA plasmid pGAPDH-13 was a gift from Dr Philippe Fort (University of Montpellier, Montpellier, France), and the cDNA clone of the retinoblastoma gene (RB) was from Dr Yuen-Kai Fung (Children's Hospital of Los Angeles, Los Angeles, CA). Nick translations were carried out according to the manufacturer's instructions (Amersham).

Nuclear run-on transcription assay. Isolation of nuclei and the nuclear run-on transcription assays were done following the method of Greenberg and Ziff as previously described.

Transfection and CAT assay. 5xTREb2CAT plasmid DNA (5 μg; a gift from Dr Peter Angel, UCSD, La Jolla, CA) was transfected into K562 cells (4 x 10^5) by the DEAE-dextran method with minor modifications. The cells were washed twice with serum-free RPMI 1640 culture medium, suspended in 2 mL of transfection buffer (50 mmol/L Tris-HCl, pH 7.4 in RPMI 1640 medium) containing plasmid DNA and DEAE-dextran (final concentration, 250 μg/mL; Pharmacia). After incubation for 30 minutes at 37°C, cells were washed once with serum-free medium containing 10 U/mL heparin (Sigma) and once with serum-free medium after 18
hours, the transfected cells were divided into two aliquots. One aliquot was treated with 5 ng/mL TPA, and the other received the same amount of solution used to dissolve TPA. The cells were lysed 24 hours later for the determination of CAT activity. The CAT assay was done as described by Gorman et al.

Sequencing of EPA/TIMP promoter region. Nucleic acid sequence in the promoter region of the TIMP gene was determined from a λEPA3B DNA clone (a gift from Dr Judith Gasson, UCLA School of Medicine, Los Angeles, CA) by the dideoxy chain termination method of Sanger et al. starting with a primer deduced from the 5' end of the cDNA clone.

RESULTS

Increased secretion of EPA/TIMP by K562 cells undergoing TPA-induced megakaryoblastic differentiation. K562 cells were induced with TPA and labeled with [35S]methionine for 2 hours after various times of TPA-treatment. The biosynthesis and secretion of proteins was followed by electrophoresis of aliquots of culture media. Within 24 hours of TPA treatment, the secretion of two major and several minor polypeptide species into the media of labeled cultures was seen (Fig 1A). The major secreted polypeptide of molecular weight (mol wt) 28,000 was identified by the use of specific antibodies as EPA/TIMP. Quantitative radiomunoprecipitation of EPA/TIMP using specific antibodies is shown in Fig 1B. Densitometric scanning of the EPA/TIMP polypeptide from optimally exposed gels indicated that EPA/TIMP secretion is induced 2-, 5-, 40-, and 60-fold during 2, 4, 8, and 24 hours of treatment. The induced cells also secreted an inhibitor of plasminogen activators (PAI-1). Comparison of metabolically labeled secreted proteins from cultures of TPA-treated K562 cells and U937 monoblastic leukemia cells and HEL erythroleukemia cells is shown in Fig 2. Although comparable amounts of EPA/TIMP are secreted by four different sublines of K562 cells, very little radioactivity from cultures of the U937 or HL-60 cells comigrated with the EPA/TIMP polypeptide band (Fig 2 and data not shown). No EPA/TIMP was found in culture media of HEL cells.

Accumulation of EPA/TIMP and matrix metalloproteinase mRNAs. In order to see whether EPA/TIMP was induced at the mRNA level, polyadenylated RNA was isolated from the cells at various times after the addition of TPA, followed by gel electrophoresis, Northern blotting, and hybridization with EPA/TIMP cDNA probe. As has been reported by Gasson et al., a 0.9 kb mRNA signal was obtained from samples of uninduced cells (Fig 3A). In addition, weaker hybridizing bands and some nonspecific background signal were seen above the specific EPA/TIMP signal. Only the specific signal was elevated in cells treated with TPA for 3 hours or longer. Densitometric scanning revealed that the increase of EPA/TIMP mRNA roughly corresponded to the stimulation of EPA/TIMP protein secretion: 3-, 5-, 50- and 50-fold increased amounts of mRNA were measured at 3, 6, 12, and 24 hours of treatment, respectively. A dose-response experiment with various concentrations of TPA showed that the minimal amount of TPA

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1}
\caption{Analysis of metabolically labeled proteins secreted by the K562 cells during TPA-induction. Aliquots of serum free media of K562 cultures treated with TPA for the indicated periods of time and labeled with [35S]-methionine for the last 2 hours of culture were subjected to SDS-polyacrylamide gel electrophoresis in a 10% gel (A). TIMP was immunoprecipitated from aliquots of culture media, and the samples were analyzed in a 12.5% gel (B). PI indicates preimmune serum; O, untreated cells.}
\end{figure}
Fig 2. Comparison of the secreted proteins of four different K562 sublines and U937 and HEL leukemia cells. Note the weak polypeptide band in labeled U937 culture media, which comigrates with the 28,000 mol wt EPA/TIMP polypeptide from the K562 cells.

needed for enhancement EPA/TIMP mRNA was between 0.1 and 0.5 ng/mL (Fig 3B).

EPA/TIMP is an efficient inhibitor of the collagenase family of matrix metalloproteinases. Therefore, we examined the kinetics of TPA-induction of collagenase mRNA in the K562 cells. The 2.2 kb collagenase mRNA was first detected at about 12 hours of treatment (Fig 4), simultaneously with the expression of transin (stromelysin), another TPA-regulated metalloproteinase (data not shown). Type IV collagenase mRNA was not detected in either induced or uninduced K562 cells. Thus, the stimulation of the expression of a collagenase inhibitor precedes the stimulation of collagenases by at least 9 hours in the K562 model.

EPA/TIMP mRNA induction is transcriptional and requires de novo protein synthesis. The 3-hour lag before the increase of EPA/TIMP mRNA after the administration of TPA suggested that the effect of TPA was indirect and required the biosynthesis or activation of factor(s) mediating the response. Therefore, we studied the possibility that ongoing protein synthesis was required for the induction of the EPA/TIMP mRNA by carrying out the TPA treatments in the presence of the protein synthesis inhibitor, cycloheximide (CHX). As shown in Fig 5, CHX completely abolished the TPA-induced increase of EPA/TIMP mRNA. Figure 6 shows that the inhibition caused by CHX was dependent on the time of its administration after TPA. A clear correlation was obtained between the amount of EPA/TIMP steady state mRNA measured at 24 hours of treatment and the length of TPA treatment time before protein synthesis was inhibited (Fig 6). In the absence of TPA, CHX had no effect on the basal level of EPA/TIMP mRNA expression. These results strongly suggested that TPA caused the biosynthesis of (a) protein(s) responsible for the subsequent induction of EPA/TIMP at the mRNA level. The relative stability of EPA/TIMP mRNA both in noninduced and in induced cells (see below) suggested that these protein(s) did not act by stabilizing EPA/TIMP mRNA, but possibly by increasing the transcription of the EPA/TIMP gene. This was confirmed by nuclear run-on transcription experiments carried out with nuclei from uninduced and TPA-induced cells. The EPA/TIMP transcription rate increased steadily with time of TPA treatment of the cells (2, 4, and 6 hours) and was about 10-fold stimulated after 6 hours (as measured by densitometric scanning of the autoradiogram shown in Fig 7).

Expression of the c-jun, junB, and c-fos mRNAs and the AP-1 transcription factor in the differentiating K562 cells.

We were then interested in the possibility that the stimulation of EPA/TIMP transcription was associated with the induction of the biosynthesis of the c-jun/AP-1 protein, which binds to the TRE of the collagenase gene and other genes containing a motif similar to the consensus binding sequence (TGACTCA). Consistent with such a possibility, our nucleotide sequence analysis (data not shown)
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mealed the DNA sequence element TGAGTCA about 250 bp upstream of a TAAT element homologous to the suggested rudimentary TATA box of the mouse TIMP gene, 24 nt upstream of its 5'-most cap site. In order to study the induction of the expression of TPA-responsive transcription factors binding to the TRE element, the RNA blots from TPA-treated K562 cells were hybridized with the c-jun cDNA probe and autoradiographed. As expected on the basis of previously published results using different cells, the 3.2 and 2.6 kb c-jun mRNAs were induced about fivefold already 30 minutes after TPA stimulation (Fig 8). However, unlike in other cells where the stimulation is transient (data not shown), the increase of c-jun mRNA in the K562 cells was biphasic in the continuous presence of TPA. At least part of the second increase of c-jun mRNA may occur by messenger stabilization, since the decay of the c-jun mRNA signal after actinomycin D treatment was substantially decreased at 24 hours of induction (data not shown). The biphasic response was even more evident for junB mRNA (Fig 8), which encodes a transcription factor closely related to the c-jun factor. The c-fos mRNA, which represents another immediate early mRNA induced by TPA, was undetectable in uninduced cells, but showed a rapid, transient increase to very high levels after TPA stimulation, after which time lower expression levels were maintained for a prolonged period (Fig 8).

Fig 3. Induction of EPA/TIMP mRNA: time (A) and dose-dependence (B). (A) Polyadenylated RNA was extracted from 10^7 K562 cells at several time points after addition of TPA to the cultures, as indicated at top of the lanes. After gel electrophoresis and Northern blotting of equal amounts of RNA samples, the filter was hybridized with the EPA/TIMP cDNA probe and autoradiographed. (B) K562 cultures were treated with different concentrations of TPA for 24 hours. Polyadenylated RNA was then extracted and analyzed as above. Only the relevant portion of the autoradiogram is shown.

Fig 4. Induction of collagenase mRNA during TPA-induced differentiation of the K562 cells. The experiment and its analysis with the human collagenase cDNA probe were carried out essentially as in Fig 3A. Hybridization analysis with the glyceraldehyde-phosphate-dehydrogenase cDNA (GAPDH) was done as an internal control of RNA loading (lower panel).
REGULATION OF EPA/TIMP IN K562 CELLS

TPA

1 h 2 h 3 h 6 h

- 0.9 kb

CHX

- + - + - + - +

The functional role of a TRE on gene transcription in K562 cells was tested with a recombinant reporter plasmid, having a fivefold repeated oligonucleotide TRE consensus element in front of the thymidine kinase promoter and the cloramphenicol acetyltransferase gene (5xTRE-tk-CAT plasmid). This reporter plasmid or a control plasmid lacking the TRE sequence element (pT4 CAT) was transfected into K562 cells, and half of the transfected cells were treated with TPA as detailed in Materials and Methods. Virtual quantitative conversion of cloramphenicol into the acetylated forms was obtained by treatment with extracts from 5xTRE-tk-CAT-transfected, TPA-induced K562 cells, whereas very little CAT activity was obtained from similarly transfected, but untreated cells or cells transfected with the control plasmid. The CAT activity obtained from other types of cells similarly tested was about 10-fold less (data not shown).

The protein synthesis-dependent induction of EPA/TIMP and prolonged induction of the jun/fos complex are characteristic for the differentiating K562 cells. The human A549 lung adenocarcinoma cells and RD rhabdomyosarcoma cells showed much weaker and transient inductions of the immediate early mRNAs in response to TPA (data for A549 cells shown by shaded circles in Fig 8). Only the junB mRNA was still about twofold induced at 24 hours of treatment in the A549 cells. Unlike in the K562 cells, no increase of EPA/TIMP expression was seen at 5 hours or at 12 hours of treatment in either A549 cells or RD cells (shown for the latter in Fig 9) CHX did not modify EPA/TIMP expression by the RD cells, although it strongly superinduced jun and fos mRNAs (Fig 9 and data not shown). These
results indicate that the protein synthesis-dependent induction of EPA/TIMP, and the biphasic response and prolonged maintenance of these immediate early mRNAs are characteristic for K562 cells.

**DISCUSSION**

Our results reveal that the K562 leukemia cells respond to TPA in a cell-specific manner. Their major secreted protein after TPA treatment is the matrix metalloprotease inhibitor EPA/TIMP, which, in comparison, accounts for only less than 1% of secreted protein in TPA-stimulated human fibroblasts. Induction of EPA/TIMP secretion is relatively specific among polypeptides produced by K562 cells. EPA/TIMP mRNA, biosynthesis, and secretion were induced nearly 100-fold in the K562 cells during the first day of TPA treatment. Thus, the EPA/TIMP response can be accounted for by the accumulation of steady-state EPA/TIMP mRNA, and it probably occurs secondary to the biosynthesis of TPA-stimulated transcription factor(s), which increase EPA/TIMP mRNA transcription. We found that EPA/TIMP mRNA accumulation was dependent on de novo protein synthesis: CHX, when added simultaneously with TPA, completely inhibited the accumulation of EPA/TIMP mRNA, but when added 6 to 12 hours after TPA, it had only a very slight effect. Moreover, CHX alone did not affect EPA/TIMP mRNA amounts in K562 cells. Thus, the induction of EPA/TIMP may be dependent on the synthesis of transcription factors, which enhance EPA/TIMP gene expression. In addition to the increased rate of transcription, the accumulation of the EPA/TIMP mRNA to very high levels may also reflect its stability: the EPA/TIMP mRNA of K562 cells was not detectably degraded during 2 hours of actinomycin D treatment under any of the conditions we used in the present experiments.

Preceding increased EPA/TIMP expression, the *jun* and *c-fos* transcription factor mRNAs and AP-1 activity undergo marked stimulation. The *jun* and *c-fos* mRNAs are induced in a biphasic manner and remain at an elevated level for at least 3 days of treatment, unlike in other cells. It is tempting to speculate that the *jun/c-fos* transcription factor complex is involved in the induction of EPA/TIMP in the K562 cells, though other transcriptional activators may also play a role. We cannot definitively establish this conclusion, since we have not analyzed the induction of EPA/TIMP promoter by transfection experiments. However, sequence analysis of the 5′-promoter region of EPA/TIMP revealed the presence of a consensus AP-1 binding site. Regarding the possible involvement of the AP-1 transcription factor in EPA/TIMP induction, it is interesting to note that TGF-β also induces both the
c-jun, junB, and c-fos proteins, as well as EPA/TIMP in many cells.\textsuperscript{20,48}

In most cells, the c-jun and c-fos mRNAs and the AP-1 transcription factor are induced rapidly and transiently by TPA.\textsuperscript{41} At least two mechanisms contribute to the rapid downregulation of the c-jun and c-fos responses: (1) These mRNAs are highly unstable (like the junB mRNA), having a half-life of less than 90 to 120 minutes and (2) their promoters are silenced soon after the initial stimulation. The shut-off of the c-fos promoter may occur by an autoregulatory mechanism.\textsuperscript{49} In K562 cells, however, the c-jun mRNA is greatly stabilized at 24 hours of TPA induction, which may explain the second, less prominent increase of the c-jun steady-state mRNA. Colamonici et al\textsuperscript{50} have reported that the c-fos mRNA remains elevated throughout the TPA-induced differentiation of K562 cells, a finding that we have confirmed in this study. It is known that the endodermal differentiation of embryonal carcinoma cells is associated with the appearance of transcription factor activity, which binds to and activates a typical AP-1 binding site.\textsuperscript{51} The differentiation of cells may thus be coupled to long-term modulation of the activities of some transcription factors. It is also of interest that prolonged activation of jun and collagenase genes has been reported to occur after tumor necrosis factor α-treatment of fibroblasts.\textsuperscript{52}

The proteins secreted by the differentiating K562 cells include two major proteinase inhibitors: PAI-1, which inhibits serine proteinases, and EPA/TIMP, which inhibits all four known matrix metalloproteinases. The phenotype of the differentiating K562 cells favors a nonproliferative pericellular environment, which may also be relevant for the functions of platelets. However, although PAI-1 has been demonstrated in platelets, it is not known whether platelets contain EPA/TIMP. In addition to its role as a proteinase inhibitor, EPA/TIMP has erythroid potentiating growth factor activity, first purified from the conditioned medium of human T-lymphotropic virus type II (HTLV-II)-infected Mo T lymphoblast line.\textsuperscript{33,34} K562 cells have surface receptors for recombinant EPA/TIMP, which also enhances their colony formation in semisolid medium.\textsuperscript{59} According to Fraser et al,\textsuperscript{60} the increased expression of erythropoietin receptors on the surface of K562 cells treated with conditioned medium from U937 cells is due to EPA/TIMP secreted by the latter cells. Also, Niskanen et al\textsuperscript{61} showed that the administration of EPA/TIMP to anemic mice led to a significant increase in the number of reticulocytes in the peripheral blood and erythroid precursors in the spleen. From these studies, it can be concluded that EPA/TIMP may be a significant modulator of erythroid differentiation. On the basis of our studies, it may then be speculated that EPA/TIMP may also play a role as a cytokine possibly produced by bone marrow megakaryoblast precursors.

ACKNOWLEDGMENT

We are grateful for the technical assistance of Hilika Toivonen, Elina Roina, and Anne Aronta. We also thank several colleagues for the donation of molecular probes, Dr J.J. Reynolds for the donation of TIMP antisera, Dr Judith Gasson for the genomic EPA clone, and Dr Steven Clark for the EPA/TIMP cDNA.

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