Enhanced Expression of Transforming Growth Factor β During Megakaryoblastic Differentiation of K562 Leukemia Cells

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Platelet granules contain several growth factors such as the transforming growth factor β (TGF-β) that are released during blood clotting and are thought to participate in the repair of tissue injury; however, the site of synthesis of platelet TGF-β has not been demonstrated. We studied TGF-β expression during megakaryoblastic differentiation of the chronic myeloid leukemia cell line K562 in vitro. These cells have mainly erythroid characteristics but acquire several megakaryoblastic properties when treated with the phorbol diester 12-O-tetradecanoyl-13-phorbol-acetate (TPA). During four subsequent days of megakaryoblastic differentiation the amount of the 2.5-kilobase (kb) TGF-β mRNA increased about eightfold, and a novel 2.3-kb mRNA species was induced in the K562 cells. This occurred concomitantly with distinct induction patterns of platelet-derived growth factor A (PDGF-A) and c-sis (PDGF-B chain) RNAs and several platelet antigens. The expression of erythroid markers such as glycophorin A decreased. Culture media of TPA-differentiated K562 cells also contained TGF-β polypeptides as shown by a sensitive radioreceptor assay and by immunoprecipitation after metabolic labeling of the cells. These polypeptides were not seen in culture media from dimethyl sulfoxide- or sodium butyrate-treated cells. Unlike in several other cells, exogenously added TGF-β 1 or 2 affected neither TGF-β nor PDGF RNA expression in K562 cells.

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Glen Cove, NY) in 20× SSC for 16 hours. After baking for two
ten minutes at 60°C and electrophoresed in formaldehyde-agarose
gels. The electrophoresis buffer was the same as the sample buffer.
The position of rRNA was visualized by ethidium bromide staining,
and the RNA was transferred to Biodyne membranes (Pall Corp,
Glen Cove, NY) in 20× SSC for 16 hours. After baking for two
hours at 80°C, prehybridizations and hybridizations were performed
in Denhardt's mixture (0.02% each of bovine serum albumin, Ficoll,
and polyvinylpyrrolidone), 3× SSC, 50% formamide, 50 mmol/L
4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (pH 7.2),
salmon sperm DNA (200 μg/mL), and yeast tRNA (150 μg/mL)
for 24 hours at 42°C. Approximately 10⁷ cpm was applied to each
filter of 150 to 200 cm² in 15 mL hybridization mixture. The filters
were first washed at room temperature with 1× SSC and then at
65°C with 0.1× SSC and 0.1% sodium dodecyl sulfate (SDS) twice
for 30 minutes. The filters were then exposed to Kodak XAR-5 film
with an intensifying screen at –70°C. RNAs were also quantitated
by serial dilutions and dot blot analyses. Signal intensities from the
autoradiograms were measured and compared in competition with
the signals from the invariant glyceraldehyde-3-phosphate-dehydro-
genase (GAPDH) RNA by using a laser densitometer (Ultraphot,
LKB, Bromma, Sweden). The time courses of the changes in RNA
expression were calculated as means of three separate experiments.

Independent estimates for the amounts of c-sis and TGF-β-
specific RNAs were obtained by the ribonuclease protection analysis
of labeled, in vitro--synthesized RNA transcripts. For c-sis RNA
protection, a 1,510-base pair Bam H1-Bglll fragment of the plasmid
pSM-17 was subcloned between the SP6 and T7 promoters in the
pGEM4 vector (Promega Biotec, Madison, WI). Restriction endo-
nuclease-digested plasmids were used as templates for the synthesis
of single-stranded antisense RNA probes, which were hybridized to
5 μg of polyadenylated RNA from K562 cells. Digestion with RNase
A and RNase T1 and subsequent analysis were done essentially as
described by Melton et al.24

Molecular probes. The cDNA clones for TGF-β (pBC1)3 and
GAPDH7 were kind gifts from Drs Rik Derynck and Philip Fort,
respectively. The PDGF-A cDNA was a kind gift from Drs Christen
Betscholtz and Carl-Henrik Heldin25 and the c-fos probe was from
Amersham Corp (Buckinghamshire, UK). Nick-translations of
cDNA by using dCTP (3,000 Ci/mmol, Amersham) were carried
out according to manufacturer’s instructions (Amersham).

Radiohoreceptor binding analysis of TGF-β. The amount of
active TGF-β in cell-free supernatants of TPA- or DMSO-treated
K562 cells was kindly measured by Drs Jorma Keski-Oja and
Harold Moses (Nashville, TN). This was done by using the TGF-β
radiohoreceptor binding inhibition assay and AKR 2B (clone 84A)
cells as described by Tucker et al.26 For collection of the samples, the cells were induced with TPA or DMSO
as described earlier and transferred to serum-free culture medium
after 48 hours. Media were collected after another 48 hours. The
supernatants were lyophilized, dissolved in H₂O, and treated with
HCl to achieve a pH of 2 for two hours and then renaturized before
the radiohoreceptor assay.

Metabolic labeling and immunoprecipitation. K562 cells
induced for three days with TPA, DMSO, or sodium butyrate were
labelled with 35S-cysteine as described elsewhere.28 Briefly, 2 to 3 ×
10⁶ cells were transferred for 12 hours to 5 mL cysteine-free medium
containing 200 μCi/mL. 35S-cysteine (600 Ci/mmol, Amersham).
Cell-free, labeled media were first acidified and renaturized as for
the radiohoreceptor assay and then sequentially precipitated with a
control rabbit serum and a polyclonal rabbit antiserum against
purified human platelet TGF-β (a kind gift from Drs Jorma
Keski-Oja and Harold Moses) in the absence or in the presence of
100 ng competing TGF-β, purified from platelets (TGF-β₁,
and TGF-β₂ were from R&D Systems, Minneapolis). Immunoprecipi-
tates were adsorbed to protein A-Sepharose (Pharmacia Fine
Chemicals, Uppsala, Sweden) in an end-over mixer for one hour at
+4°C, washed, and analyzed in a 13% to 18% gradient SDS-
polyacrylamide gel under reducing conditions.

Glycophorin A immunoprecipitation (antisera was a kind gift
from Dr Carl G. Gahrnberg) was carried out after a 30-minute
starvation in methionine-free medium followed by a 140-minute
labeling with 35S-methionine (400 μCi/mL >800 Ci/mmol; Amer-
sham). The labelled cells were collected, lysed in 10 mmol/L
Tris-HCl (pH 7.5), 50 mmol/L NaCl, 0.5% sodium deoxycholate,
0.5% NP-40, 0.1% SDS, and 100 U/mL aprotinin (Apronin, Medi-
ca, Helsinki, Finland) as a protease inhibitor. Immunocomplexes
adsorbed to protein A-Sepharose were washed several times with the
immunoprecipitation buffer and analyzed in a 10% SDS–polyacryl-
amide gel run under reducing conditions. Fixed gels were treated
with Ampliti (Amersham), dried, and exposed to Kodak XAR-5
film at –70°C.

RESULTS
Increase in TGF-β RNA during megakaryoblastic differ-
etiation. Polyadenylated RNAs from K562 cells induced for
four hours and one and two days with TPA were analyzed by
Northern blotting and hybridization with the TGF-β
probe (Fig 1). To confirm that all autoradiographic signals
were derived from similar amounts of cellular RNA the gels
were stained with ethidium bromide (not shown), and also
the blots were hybridized with the probe for GAPDH, which

Fig 1. Hybridization analysis of TGF-β, c-sis (PDGF-B) and
GAPDH RNAs during megakaryoblastic differentiation of K562
cells. Shown is an autoradiogram of Northern RNA analysis from
K562 cells treated for four hours or one or two days with 1.6
mmol/L TPA or (lane C) for two days with a corresponding volume
of DMSO used to dissolve TPA. The numbers below each lane
represent the relative value of the TGF-β signal intensity com-
pared with the GAPDH signal measured with laser scanning
densitometry. The relative TGF-β signal from DMSO-treated cells
equals 1.

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is a widely used probe thought to represent a relatively ubiquitous and stably expressed RNA species within a given cell lineage. As shown, there is a marked accumulation of the 2.5-kb TGF-β RNA during two consecutive days of TPA treatment (3.1- and 5.2-fold, respectively) when compared with DMSO-treated control cells (Fig 1, lane C). A 1.3-fold increase in TGF-β RNA was observed four hours after the addition of TPA when the autoradiographic signal was normalized to the invariant 1.3-kb GAPDH RNA signal, which was measured by densitometric scanning. Expression of c-sis (Fig 1, 3.4-kb RNA band) and PDGF-A RNA was also induced as previously reported. Using the RNA protection assay, we could also identify small amounts of c-sis RNA in uninduced K562 cells (data not shown). In comparison, the amount of the 1.3-kb GAPDH RNA was not changed relative to the total RNA content of the cells.

The increase in the TGF-β signal was also estimated by serial dilution and dot blotting of RNA followed by hybridization with the TGF-β and GAPDH probes (Fig 2). The total increase in TGF-β RNA as judged from this experiment is four- to sixfold after three days of differentiation (Fig 2, lanes T). A comparison was also made with TGF-β RNA in butyrate-treated K562 cells (lanes B), which had acquired further erythroblastic properties as judged by a 70% to 100% increase in the benzidine reaction to measure hemoglobin. The TGF-β RNA in these cells was less than twofold enhanced during the three days of induction.

It was also observed from the experiment of Fig 1 that there is an apparent broadening of the TGF-β RNA band in the TPA-treated cells, with an enhancement of a lower molecular weight (mol wt) component. To analyze this phenomenon with a better resolution, the RNA from TPA- and butyrate-differentiated cells was then diluted to a similar TGF-β signal intensity and reanalyzed in Northern blotting along with RNA from DMSO-treated cells. The results, in Fig 3, show that RNA from TPA-differentiated cells contains a novel TGF-β band at 2.3 kb in addition to the normal band at 2.5 kb. Only very small amounts of this lower-mol wt component can be discerned in RNA isolated from cells treated with sodium butyrate or DMSO.

**TGF-β RNA expression is not acutely affected by TPA or cycloheximide.** We next compared the enhancement of TGF-β RNA with PDGF and c-fos oncogene RNAs that are also induced by TPA in K562 cells. Careful blotting and hybridization analysis during the first 14 hours of TPA treatment indicated that the expression of c-fos and PDGF RNAs were already markedly enhanced after one and two hours of TPA treatment, respectively (Fig 4). Comparison of dose responses with 1.6 and 16 nmol/L concentrations of TPA showed that the induction of PDGF chain RNAs was delayed but stronger with the lower concentration of TPA. In contrast, the TGF-β RNAs accumulated more slowly, being only about twofold enhanced after 14 hours of treatment.

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**Fig 2.** Dot blot analysis of TGF-β and GAPDH RNAs in TPA (T), DMSO (D), and butyrate (B)-treated cells. K562 cells were treated with the different agents for three days. One microgram of polyadenylated RNA isolated from the cells was spotted on nitrocellulose; this was followed by serial twofold dilutions. After baking, the filter was hybridized with TGF-β and GAPDH probes.

**Fig 3.** High-resolution analysis of TGF-β RNAs in K562 cells. The RNA samples from cells treated for three days with DMSO, TPA, or sodium butyrate were diluted on basis of dot blot analysis to similar TGF-β signal intensities. The RNAs were then analyzed by Northern blotting and TGF-β hybridization. Here one can clearly distinguish the lower-mol wt (2.3 kb) RNA component present predominantly in TPA-treated K562 cells.
regardless of the concentration of TPA used (Fig 4, see also Fig 5). Also, although the c-fos RNA was superinduced and PDGF RNA expression inhibited in the presence of 10 μg/mL of the protein synthesis inhibitor cycloheximide (Fig 4, lane CHX), the TGF-β RNAs were not affected by the drug. Taken together, these results show that in K562 cells both the kinetics and mechanism of TPA induction of c-fos, PDGF, and TGF-β RNAs are strikingly different.

**Quantitation and specificity of TGF-β induction.** To obtain a quantitative time course of the increase in TGF-β RNA expression, the Northern and dot blot autoradiograms were scanned with a laser densitometer and the results expressed as the amount of enhancement of the hybridization signal in comparison with the GAPDH signal (Fig 5). As can be seen from the Figure, there is a gradual increase in TGF-β RNA during TPA treatment; however, the enhancement of the minor component (2.3 kb) is more pronounced, eight- to 12-fold over four days (Fig 5) as compared with the major component (2.5 kb), which increases about six- to eightfold over the same period but which is already abundantly expressed in the noninduced cells. In contrast, butyrate-induced erythroblastic differentiation is not accompanied by significantly elevated TGF-β RNA levels.

Throughout the four days of TPA-induced differentiation the percentage of cells positive for the megakaryocyte antigens C17 and FMC 27 progressively increased from 5% to
95% and from 7% to 70%, respectively. The factor VIII-related antigen was not detected in untreated control cells but appeared in 50% of the TPA-induced cells by day 4.

As previously reported by Siebert and Fukuda, we also observed a remarkable decrease in glycophorin A expression during TPA treatment: the amount of immunoprecipitated polypeptides decreased to 10% of that of untreated control cells 48 hours after TPA induction, whereas during butyrate treatment glycophorin A levels remained unchanged (Fig. 6). Also, the RNA level for this erythroid marker decreased to 50% of the control at three hours and was undetectable 24 hours after TPA induction (data not shown).

TPA-differentiated K562 cells secrete TGF-β. The amount of active TGF-β measured by a radioreceptor binding inhibition assay from serum-free, acid-treated culture media conditioned by 10⁶ TPA-treated cells for two days was 90 ± 10 and 18 ± 4 ng for 10⁴ DMSO-treated cells as judged from three separate experiments. TGF-β polypeptides were also immunoprecipitated from labeled culture media of TPA-, DMSO- and butyrate-treated cells. Acidification and reneutralization of the culture media was carried out before the radioreceptor assay and before the addition of antibodies for immunoprecipitation because TGF-β secreted by cultured cells does not bind to the TGF-β receptor or to the antibodies we used, probably because it exists in an as yet poorly characterized inactive form activated by acid and some proteinases. Results of gel electrophoresis are shown in Fig. 7. There is a polypeptide band of 12,500 in samples from TPA-treated K562 cultures that was not precipitated with normal rabbit serum (not shown) or in the presence of 100 ng/mL purified TGF-β. This polypeptide was not present in immunoprecipitates from either DMSO- or butyrate-treated cells (Fig 7).

TPA does not induce PDGF or TGF-β expression in K562 cells. TGF-β is known to induce the expression of c-sis in fibroblastic cells and PDGF-A in several cell types. These effects are similar to those seen with TPA in K562 cells. Unlike in monocytic leukemia cells, however, the addition of 5 ng/mL TGF-β₁ or -β₂ purified from platelets had no effect on the expression of PDGF or TGF-β RNAs in K562 cells when analyzed at two and 20 hours of treatment. Similarly, the presence of 5 ng/mL purified PDGF under serum-free conditions had no effect on TGF-β RNA expression or on the TPA response of K562 cells. We therefore consider it unlikely that TGF-β or PDGF would have an autocrine role in the secretion of growth factors in these cells.

DISCUSSION

We show in this report that TPA-induced megakaryoblastic differentiation of K562 human erythroleukemia cells is associated with about an eightfold enhancement of TGF-β RNA expression and with at least a fivefold increased secretion of TGF-β to the culture medium of cells differentiated for four days. Though the K562 cell line is an aneuploid leukemia cell line and its incomplete differentiation must be interpreted with caution, these events may reflect the changes in gene expression during normal differentiation of megakaryocyte precursors.

The TPA-induced differentiation of the K562 cells seems to provide a fruitful model for molecular studies of megakaryoblastic differentiation because most of the megakaryocyte markers that we and others have studied thus far are increased in the differentiated cells. These include the glycoprotein Ib/IIa, platelet peroxidase, the FMC 27 antigen, the factor VIII-related antigen, and PDGF. Our experiments confirm that the cells lose erythroid features
such as the expression of glycophorin A in parallel with the increase in TGF-β expression. The decline of glycophorin A protein levels was slower in our studies than that reported by Siebert and Fukuda; this difference may be due to the lower concentration of TPA used in our studies (1.6 compared with 60 nmol/L).

The slight enhancement of TGF-β expression during butyrate treatment may be related to the fact that some megakaryoblasts are also formed under these conditions as reported by Vainchenker et al. The majority of all K562 cells from different sources that we have used, however, reproducibly yield an erythroid phenotype upon butyrate induction as confirmed by glycophorin A expression and positive staining by benzidine.

The slow accumulation of TGF-β RNA in comparison with PDGF and c-fos RNAs after the addition of TPA suggests that its transcription or RNA stability is not immediately affected by the inducer. The lack of effect of cycloheximide is also in line with these deductions and suggests that indirect, secondary effects of TPA possibly associated with the megakaryoblastic differentiation process are responsible for the slow increase in the TGF-β messenger RNA levels. This effect is specific for TGF-β because, for example, the expression of the acidic and basic fibroblast growth factors is not altered in this model (our unpublished data).

The nature of the novel 2.3-kb TGF-β messenger RNA is also a subject of further investigation. The preliminary DNA-RNA duplex melting experiments show closely similar melting temperatures for both the 2.5- and 2.3-kb hybridization signals. It therefore seems likely that the 2.3-kb RNA shares significant segments of its nucleic acid sequence with the 2.5-kb messenger and differs from it only, for example, in its splicing or the site of polyadenylation; however, it cannot yet be excluded that the 2.3-kb RNA is transcribed from another, related gene. A second, less abundant type of TGF-β (TGF-β2) that has greater than 80% homology to TGF-β1 and that appears to interact differently with a family of receptors on target cells has recently been characterized from bovine and porcine as well as from human sources.

Aasio et al. have noted that blood platelets are a rich source of TGF-β and Ellingsworth et al. detected enhanced amounts of TGF-β antigen in bone marrow megakaryoblastic cells and in hematopoietic stem cells in the fetal liver by immunohistochemistry. TGF-β may be stored in blood platelets as a latent high-mol wt component that is processed to an active form during isolation and acid treatment. Although serum contains relatively large quantities of TGF-β released from platelets during blood clotting, most of the platelet-derived TGF-β in serum is in an inactive form. Therefore, studies of the biosynthesis of TGF-β are difficult because the native, latent form of the growth factor is not recognized by antibodies currently used against active TGF-β.

We used a sensitive radioreceptor assay and immunoprecipitation of culture media after acid treatment to demonstrate TGF-β in K562 cell cultures. The estimates obtained from three separate experiments are consistent with at least a fivefold enhancement of TGF-β secretion during the differentiation process. In fact, our failure to recover TGF-β polypeptides from butyrate-treated and control cell cultures by immunoprecipitation suggests that the differences in the production of TGF-β are even greater between the megakaryoblastlike and erythroblastlike K562 cells. It should also be noted that the induction of TGF-β RNA in activated T cells was followed by the secretion of TGF-β protein only after a considerable lag period, and thus the kinetics of steady-state RNA accumulation may not accurately correlate with the amount of TGF-β secretion.

Thus far we have been unable to show TGF-β or significant amounts of PDGF in K562 cells. This suggests that the mechanism for growth factor retention or concentration in intracellular stores that operates in megakaryocytes and platelets is missing even in the differentiated K562 cells but becomes active at a stage of normal differentiation not seen in this model. Alternatively, the negative results may reflect difficulties in recognizing the TGF-β precursor by antibodies.

The major role of TGF-β in platelets may be to regulate, in concert with other growth factors, connective tissue activation as well as inflammatory and angiogenic reactions during wound healing. Although TGF-β did not affect K562 cells in our assay, it is also particularly interesting to us that purified TGF-β inhibits the growth of primary cultures of human megakaryocytes and platelets is missing even in the differentiated K562 cells but becomes active at a stage of normal differentiation not seen in this model. Alternatively, the negative results may reflect difficulties in recognizing the TGF-β precursor by antibodies.

NOTE ADDED IN PROOF

Our preliminary experiments suggest that TGF-β2 is not expressed by the K562 cells consistent with its absence from human platelets.

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