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Effects of 1-β-D-Arabinofuranosylcytosine and Phorbol Ester on Differentiation of Human K562 Erythroleukemia Cells

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SUMMARY

We have previously demonstrated that 1-β-D-arabinofuranosylcytosine (ara-C) induces hemoglobin synthesis in human K562 erythroleukemia cells. The present study extends these findings by demonstrating that ara-C treatment of K562 cells results in both increased heme synthesis and accumulation of α-, γ-, ε-, and ζ-globin RNA. The results also demonstrate that ara-C enhances K562 cell surface expression of glycophorin. Furthermore, we demonstrate that phorbol ester (12-*O*-tetradecanoylphorbol-13-acetate; TPA) inhibits the effects of ara-C on heme production, accumulation of globin RNA, and glycophorin expression. The inhibitory effect occurs maximally when K562 cells are treated with TPA before undergoing ara-C-induced commitment to erythroid differentiation. These findings suggest that TPA inhibits an early step in the process required for ara-C to enhance expression of genes involved in the erythroid program.

INTRODUCTION

The K562 human leukemic cell line was established from a patient with chronic myelogenous leukemia in blast crisis (1). Single K562 cells express markers of the erythroid (1), myeloid (1, 2), and megakaryocytic (3) lineages. K562 cells can be induced by hemin to form embryonic and fetal hemoglobins (4–6). The induction of K562 cells by hemin is mediated at a transcriptional level and results in increases of α-, γ-, ε-, and ζ-globin messenger RNA (8). Hemin also increases the expression of myeloid markers, but does not affect markers of the megakaryocytic lineage (3). In contrast, TPA[1] partially inhibits expression of erythroid and myeloid phenotypes, while this agent induces expression of megakaryocytic markers (3). Thus, certain agents alter the K562 cell phenotype in a selective manner.

We have recently demonstrated that ara-C induces hemoglobin synthesis in K562 cells (9). ara-C incorporates into DNA (10, 11) and inhibits DNA synthesis by acting as a relative chain terminator (12, 13). The induction of K562 hemoglobin synthesis by ara-C occurred maximally at a cytostatic concentration. K562 hemoglobin synthesis was similarly induced by aphidicolin and hydroxyurea, other inhibitors of S-phase DNA synthesis, but not by vinblastine, an inhibitor of mitosis. Previous studies with hydroxyurea had demonstrated an increase in the rate of K562 ε- and ζ-globin gene transcription (8). In contrast to the reversible effects of hemin and hydroxyurea on globin synthesis, we found that the induction of K562 hemoglobin synthesis by ara-C was irreversible. Further, ara-C induction of the differentiated K562 phenotype was accompanied by loss of self-renewal capacity.

The present report extends our previous findings by monitoring the effects of ara-C on multiple markers of the K562 erythroid phenotype. The results demonstrate that ara-C increases 1) K562 heme synthesis, 2) the production of α-, γ-, ε-, and ζ-globin mRNA, and 3) cell surface glycophorin expression. Furthermore, the results demonstrate that TPA inhibits the effects of ara-C on the expression of an erythroid phenotype. The inhibitory effect, however, occurred maximally with TPA treatment before K562 cells had been committed by ara-C to undergo erythroid differentiation. These findings suggest that the combination of maturational agents and their sequence of administration may result in the expression of a phenotype consistent with the effects of only a single inducer.

MATERIALS AND METHODS

Cell culture. K562 cells (obtained from the Institute for Medical Research, Camden, NJ) were maintained at a density of 1–2 × 10^5 cells/ml in RPMI 1640 medium (Flow Laboratories, Inc., McLean, VA) containing 2 mM L-glutamine, 1% penicillin/streptomycin (GIBCO Laboratories, Grand Island, NY), and 10% heat-inactivated fetal bovine serum (Flow Laboratories) in a 5% CO_2 humidified atmosphere at 37°. The K562 cells were grown in suspension culture in the presence of 0.5 μM ara-C (Sigma Chemical Company, St. Louis, MO), 33 nM 12-*O*-

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[1] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ara-C: 1-β-D-arabinofuranosylcytosine; SDS, sodium dodecyl sulfate; MOPS, 4-morpholinepropanesulfonic acid.

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tetradecanoylphorbol-13-acetate (Sigma), or 20 μM hemin (Sigma). Cultures were scored for benzidine-positive cells by the wet benzidine method (14). Heme concentration was determined fluorometrically (15). Viability was monitored by trypan blue exclusion.

Northern blot analysis for human globin RNAs. K562 total cellular RNA was purified by the guanidinium thiocyanate-cesium chloride method (16). The RNA was dissolved in 40% formamide, 2.2 M formaldehyde, 40 mM MOPS, 10 mM sodium acetate, and 1 mM EDTA. Samples (15 μg) were heated to 55° for 15 min and then quickly chilled at 0°. Gel electrophoresis was performed in 1% agarose containing 2.2 M formaldehyde, 40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA, and 0.01% ethidium bromide at 40 V for 16 hr. The gel was then washed and transferred onto nitrocellulose filters (17).

The filters were prehybridized at 42° for 8–12 hr in buffer consisting of 50% formamide, 5× SSC (0.15 M sodium chloride, 0.015 M sodium citrate), 0.1% SDS, 1× Denhardt's solution, and salmon sperm DNA (200 μg/ml). The RNA blots were then hybridized at 42° for 24 hr in the same buffer containing nick-translated ^{32}P-labeled globin cDNA probes as follows: 1) plasmid JW101 carrying a human α_1-globin cDNA (18); 2) plasmid JW151 carrying a human γ_2-globin cDNA (18); 3) plasmid ϵ0.7 carrying the 0.7-kilobase *Bam*HI restriction fragment from the human ϵ-globin gene (19); and 4) plasmid pHPζ carrying the 399-base pair *Pst*I-*Hinc*II restriction fragment from the human ψζ-globin gene (20). The probes were kindly provided by Dr. Thomas Maniatis, Harvard University, Cambridge, MA. The filters were then washed twice with 2× SSC, 0.1% SDS at room temperature and then twice with 0.1× SSC, 0.1% SDS at 50°. The filters were exposed to X-ray film for 24 to 48 hr at −70° using an intensifying screen.

Monoclonal antibodies. The monoclonal antibodies were prepared and used to monitor cellular differentiation as previously described (21). A monoclonal antibody reactive with glycophorin A (clone 39)[2] has been used to monitor expression of this red cell sialoglycoprotein known to be synthesized by K562 cells (22). Mo1 antigen is expressed by monocytes, granulocytes, null lymphocytes in the peripheral blood, and immature myeloid cells in the bone marrow. Monoclonal antibody MY-4 reacts with the cell surface of monocytes. Antigen expression was detected by an indirect immunofluorescence assay using an Epics C flow cytometer (Coulter Electronics, Hialeah, FL). The per cent positive cells and mean fluorescence intensity was recorded after analyzing 10,000 cells per sample. Background fluorescence was determined using a nonreactive IgG antibody.

RESULTS

Our previous results demonstrated a maximal increase in benzidine-positive cells with 0.5 μM ara-C. Fig. 1 illustrates the effects of 0.5 μM ara-C on K562 heme synthesis and hemoglobin production. In this experiment, the ara-C-treated cells achieved a heme concentration of over 500 pmol/5 × 10^5 cells by 120 hr. This increase in heme production was associated with nearly 45% benzidine-positive cells. In contrast, there was no detectable heme or hemoglobin in the TPA-treated cells. Further, the exposure of K562 cells to both TPA and ara-C completely inhibited heme synthesis and hemoglobin production.

Our previous results had also demonstrated that shorter exposures of 48 hr were sufficient for commitment to erythroid differentiation (9). K562 cells were thus exposed to 0.5 μM ara-C for 48 hr, washed, and resuspended in drug-free medium. The results obtained are illustrated in Fig. 2. Approximately 70% of the ara-C-treated cells were benzidine positive at 72 and 120 hr. In contrast, K562 cells exposed to both TPA and ara-C for 48 hr had no detectable increase in hemoglobin production by 120 hr. Furthermore, exposure of cells to ara-C for 48 hr, followed by exposure to TPA alone for 48 hr, demonstrated only a partial but significant decline in both heme and hemoglobin production. These studies indicate that pretreatment with TPA or concurrent TPA/ara-C exposure results in complete inhibition of the differentiated erythroid phenotype, but that TPA has only a limited effect on hemoglobin production in committed K562 cells.

It was of further interest to determine whether TPA inhibited the production of globin mRNA. K562 cells were exposed to 0.5 μM ara-C for 48 hr, washed, resuspended in drug-free medium for an additional 24 hr, and monitored for accumulation of α-, γ-, ϵ-, and ζ-globin mRNA. Northern blot analysis of K562 cellular RNA using cloned globin cDNA probes is shown in Fig. 3. Treatment with ara-C for 72 hr resulted in an increase of α-, γ-, ϵ-, and ζ-globin RNA. In contrast, TPA alone resulted in a detectable decrease in uninduced globin RNA accumulation (Fig. 3B). More importantly, TPA inhibited the increases in globin RNA production associated with ara-C treatment. Thus, the effect of TPA on ara-C-induced hemoglobin production is attributable to decreases in the accumulation of both heme and globin mRNA.

We have also monitored K562 cell surface expression of glycophorin. Fig. 4 illustrates patterns of reactivity obtained after 120 hr of exposure to both ara-C and hemin. The cell surface expression of glycophorin was significantly increased following exposure to ara-C (Fig. 4B). Mean fluorescence intensity (a measure of antigen density) increased from channel 95 to channel 155 after ara-C treatment, while hemin had little, if any, effect (Fig. 4C) on expression of this antigen. In contrast, exposure of K562 cells to TPA was associated with inhibition of glycophorin expression on cells treated with this agent alone (Fig. 4D), TPA/ara-C (Fig. 4E), or TPA/hemin (Fig. 4F). Although over 50% of the TPA-treated cells became adherent to the tissue culture flask, there was no detectable cell surface expression of monocyte/macrophage antigens, Mo1 or MY-4, for the adherent or nonadherent K562 populations. Furthermore, there was no detectable expression of these antigens on ara-C-treated K562 cells.

DISCUSSION

ara-C incorporates into cellular DNA and not RNA (10, 11). The extent of ara-C incorporation in DNA correlates significantly with inhibition of DNA synthesis (12). Furthermore, the incorporated ara-C residue provides a poor primer terminus for further chain elongation (12, 13). The inhibition of DNA synthesis by ara-C causes DNA fragmentation (23). An inhibition of DNA synthesis by sublethal doses of ara-C is also associated with the induction of differentiation in HL-60 leukemic cells (21). Similar phenotypic changes were observed with aphidicolin, an inhibitor of DNA polymerase α, which is not incorporated into DNA. Induction of differentiation by both of these agents was accompanied by loss of clonogenic survival, suggesting that terminal dif-

[2] K. Sabbath and J. Griffin, unpublished data.

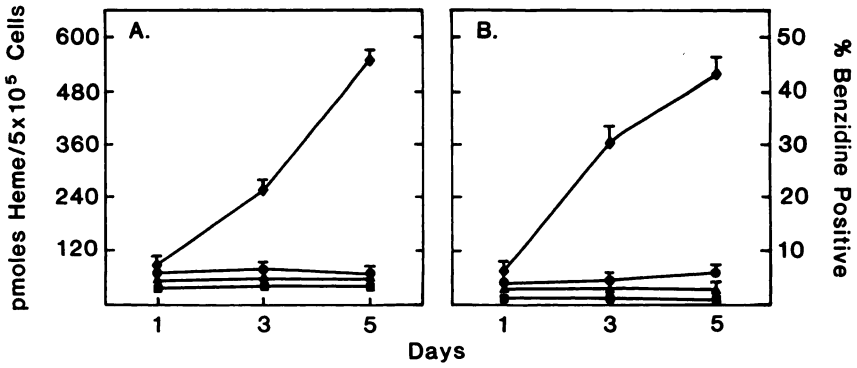

FIG. 1. *Effects of ara-C and TPA on K562 heme production and per cent benzidine-positive cells*

K562 cells in logarithmic growth phase (2×10^5 cells/ml) were treated with ara-C (0.5 μM) and/or TPA (33 nM) for 5 days. Cells were monitored for heme production (A) and per cent benzidine-positive cells (B) at the indicated times. Results are expressed as the mean ± standard deviation of three replicates within one experiment. Three separate experiments were performed, each with at least two replicates. The per cent benzidine-positive cells (mean ± standard deviation) obtained for day 3 were: ara-C, 46.0 ± 15.4; TPA, 2.38 ± 1.4; and TPA/ara-C, 1.5 ± 1.5. Control (▲), ara-C (♦), TPA (●), and TPA/ara-C (■).

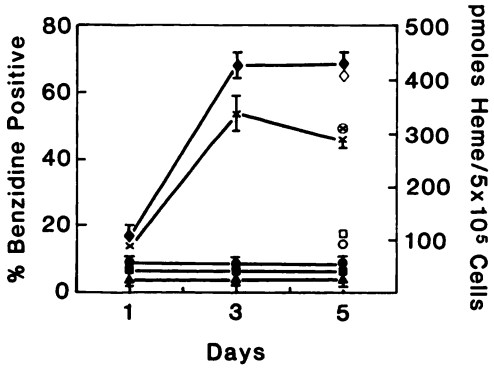

FIG. 2. *Effects of ara-C and TPA on commitment of K562 erythroid differentiation*

K562 cells in logarithmic growth phase were treated on day 0 with ara-C (0.5 μM) and/or TPA (33 nM) for 48 hr. The cells were then washed and seeded in drug-free medium. K562 cells were also treated on day 0 with ara-C for 48 hr, washed, and treated on day 2 with TPA alone for an additional 48 hr. Per cent benzidine-positive cells were monitored at the indicated times. Results are expressed as the mean ± standard deviation of three replicates. Three separate experiments were performed, each with at least two replicates. The per cent benzidine-positive cells (mean ± standard deviation) obtained for day 5 were: ara-C, 58.9 ± 10.5; TPA, 11.2 ± 1.3, and TPA/ara-C, 11.0 ± 1.4. Control (▲), ara-C (♦), TPA (●), TPA/ara-C (■), and ara-C followed by TPA (×). Heme production (mean of three determinations) was monitored on day 5. Control: 25 pmol/5 × 10^5 cells, ara-C (◇), TPA (○), TPA/ara-C (□), and ara-C followed by TPA (⊗). Cell counts on day 5 were as follows: control, 12.0×10^5; ara-C, 2.4×10^5; TPA, 3.2×10^5; and TPA/ara-C, 1.5×10^5. Viability was over 95% for each treatment group.

ferentiation of these cells could be induced by drugs known to inhibit DNA synthesis.

These findings were extended by demonstrating that ara-C induces hemoglobin synthesis in human K562 erythroleukemia cells (9). In contrast to the reversible effects of hemin and hydroxyurea on hemoglobin synthesis in this cell line (24), we found that induction of K562 hemoglobin synthesis by ara-C was irreversible. K562 hemoglobin production was also increased by other inhibitors of S-phase DNA synthesis, such as aphidicolin. The results of the present study confirm and extend these findings by demonstrating that ara-C treatment of K562 cells increases heme synthesis, as well as accumulation of α-, γ-, ε-, and ζ-globin RNA. Furthermore, ara-C treatment increases cell surface expression of glycophorin, a red cell sialoglycoprotein previously shown to be synthesized by K562 cells (22). Thus, ara-C increases the expression of multiple genes involved in erythroid differentiation.

The specific effect of ara-C on heme synthesis, globin RNA accumulation, and glycophorin expression remains unclear. Hydroxyurea, another S-phase-specific inhibitor, had previously been shown to increase the transcription rate of ε- and ζ-globin genes (8). Other studies have demonstrated that the β-globin gene is not expressed in the K562 cell line (6–8). The effect of ara-C on accumulation of α-, γ-, ε-, and ζ-globin RNA may also occur at the transcriptional level. ara-C inhibits DNA replication and results in an aberrant form of DNA synthesis with certain segments of DNA being replicated more than once in a single cell cycle (25). The additional copies of certain segments of DNA could be responsible for the accumulation of DNA fragments (21) and altered gene expression (9, 22). Since ara-C slows the rate of chain elongation without altering the site at which DNA replication is initiated within replicons (26), potentially expressed genes which replicate early could undergo a commitment to transcriptional competence (27). A change in the erythroid differentiation program might delay replication timing and prevent transcription of the multiple genes responsible for that phenotype (27).

The results of the present study also demonstrate that pretreatment or concurrent treatment with TPA inhibits the ara-C-associated increases in heme synthesis, globin mRNA production, and glycophorin expression. These findings are in concert with previous work which had demonstrated that TPA treatment decreased glycophorin expression and inhibited the effects of hemin on K562 hemoglobin synthesis (28). However, the appearance of the erythroid phenotype in K562 cells committed to differentiate by 48 hr of exposure to ara-C was only partially inhibited by a subsequent exposure to TPA.

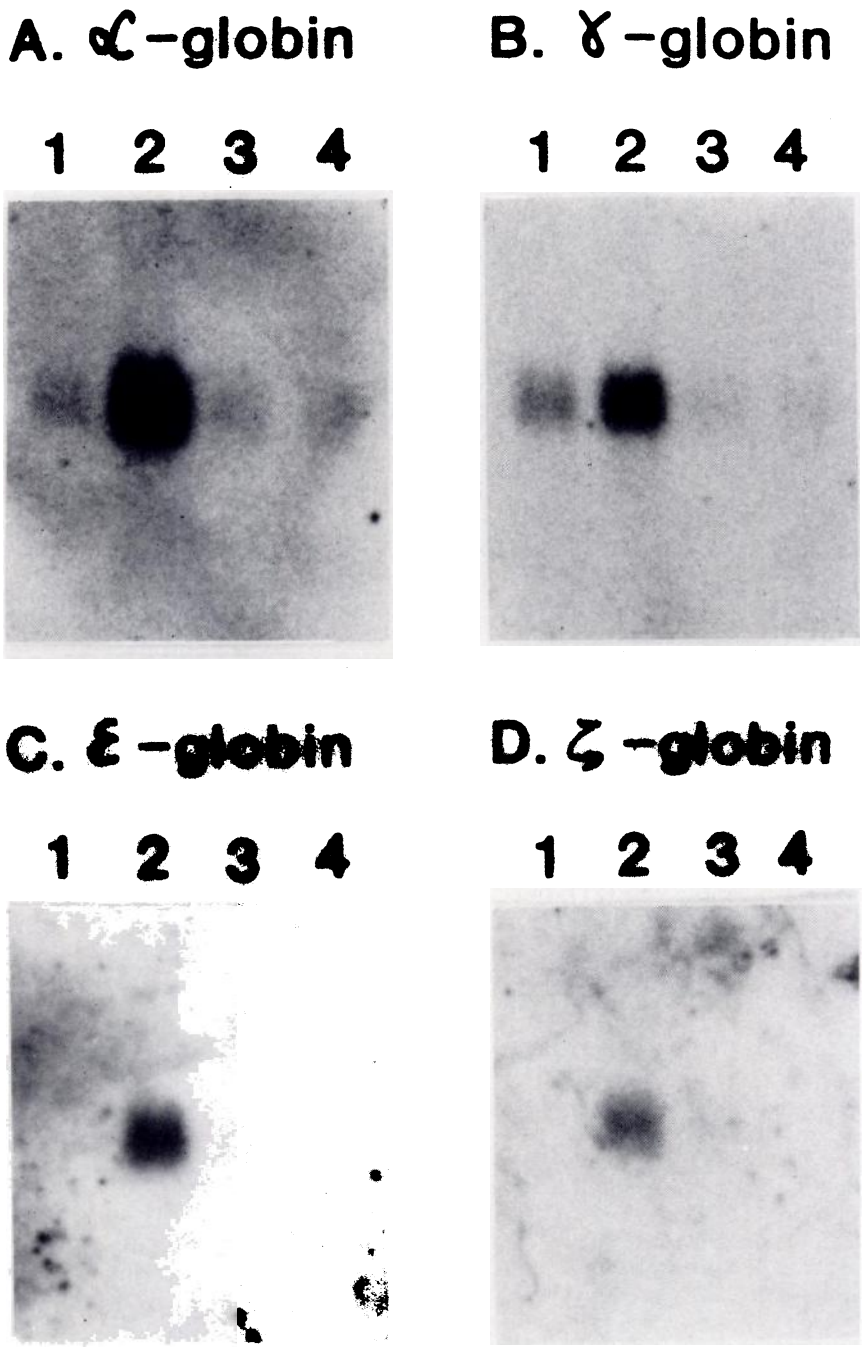

FIG. 3. *Effects of ara-C and TPA on K562 globin RNA production*

K562 cells in logarithmic growth phase were treated with ara-C (0.5 μM), TPA (33 nM), and TPA/ara-C. The cells were treated for 48 hr, washed, resuspended in drug-free medium for 24 hr, and then harvested for preparation of total cellular RNA. Samples (15 μg) were analyzed by Northern blots using ^{32}P-labeled α-globin cDNA (A), γ-globin cDNA (B), ε-globin cDNA (C), and ζ-globin cDNA (D). Control (*lane 1*), ara-C (*lane 2*), TPA (*lane 3*), and TPA/ara-C (*lane 4*). The globin RNAs are identified at 9–10 S.

These findings would suggest that TPA inhibits an early process required for ara-C-induced erythroid differentiation. The inhibition of proliferation by TPA, as compared to ara-C, clearly has different effects on K562 cells since TPA inhibits, rather than enhances, expression of globin RNA and glycophorin. Thus, the inhibition of proliferation by TPA could prevent the molecular events associated with the effects of ara-C on DNA replication.

TPA initiates multiple cellular changes that affect proliferation and differentiation (29). The molecular mechanisms responsible for these effects remain unidentified, although phorbol esters activate protein kinase C (30), and stimulate the phosphorylation of specific cell surface receptors (31) and cytosolic proteins (32). Similar events in K562 cells exposed to TPA may change the differentiation program and prevent the transcription of multiple genes involved in the expression of an erythroid phenotype. In this regard, TPA treatment of K562 cells increases the expression of megakaryocytic markers (3). In the present study, adherence was also observed with

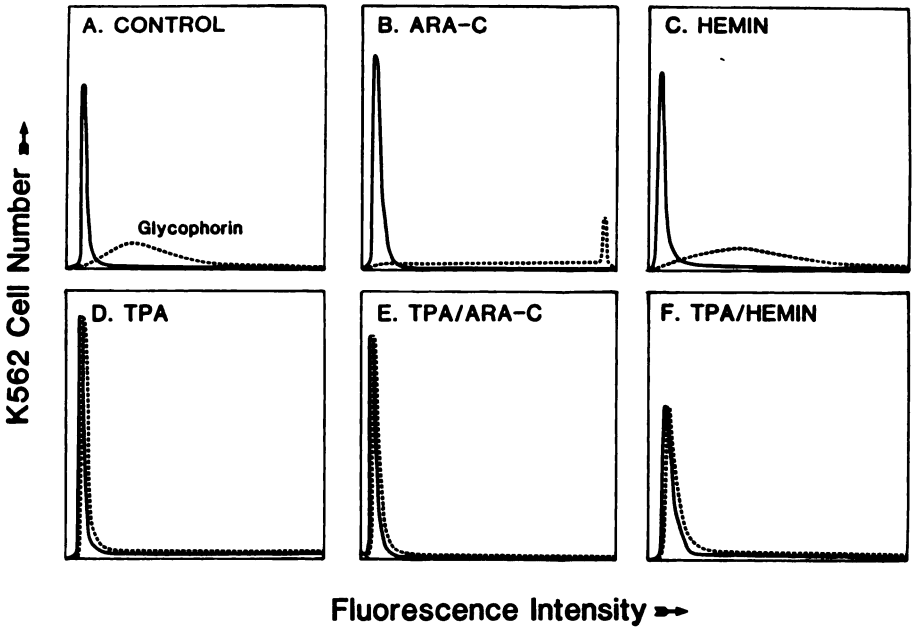

FIG. 4. *Effects of ara-C, hemin, and TPA on K562 cell surface expression of glycophorin*

K562 cells in logarithmic phase were monitored for cell surface antigen expression using a monoclonal antibody reactive with glycophorin A (- - -). Reactivity of an isotype-identical control antibody (———). The cells were exposed to no drug (A), 0.5 μM ara-C (B), 20 μM hemin (C), 33 nM TPA (D), TPA/ara-C (E), or TPA/hemin (F) for 5 days. Viability was over 95% in each treatment group.

the TPA-treated K562 cells, but there was no detectable increase in the cell surface expression of the monocyte/macrophage antigens, Mo1 and MY-4. In HL-60 cells, ara-C induces the appearance of monocyte (21), but not erythroid,[3] markers. In contrast, in K562 cells, ara-C increases the expression of erythroid markers in the absence of detectable increases in Mo1 or MY-4 antigens. Taken together, these findings might suggest that ara-C treatment increases expression of gene sequences already "committed" to transcription and that TPA is capable of changing this program.

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[3] Unpublished data.

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