

Spermine Acts as a Negative Regulator of Macrophage Differentiation in Human Myeloid Leukemia Cells

Igor M. Gavin, David Glesne, Yong Zhao, Cathryn Kubera, and Eliezer Huberman

Biochip Technology Center, Argonne National Laboratory, Argonne, Illinois

ABSTRACT

The role of putrescine, spermidine and spermine in phorbol 12-myristate-13-acetate (PMA)-induced macrophage differentiation was examined in human HL-60 and U-937 myeloid leukemia cells. Unlike other polyamines, spermine affected this differentiation by acting as a negative regulator. This negative regulation was established by showing that the PMA-induced macrophage phenotype, but not PMA-associated replication arrest, was abrogated (a) by replenishing the PMA-evoked decrease in cellular spermine levels with this polyamine from an exogenous source and (b) by blocking PMA-induced expression of the polyamine catabolic enzyme *N*¹-spermidine/spermine acetyltransferase (SSAT) with antisense oligonucleotides in the presence of low substrate level. The PMA-evoked reduction in cellular spermine appears to result from an increase in the activity of SSAT and a decrease in the activity of ornithine decarboxylase, the polyamine biosynthetic enzyme. To a degree, these changes are due to corresponding changes in the expression of the genes that code for these enzymes. When cell differentiation is initiated, SSAT expression is increased after PMA-evoked activation of protein kinase C- β . The present studies raise the possibility that agents able to reduce spermine levels in patients' myeloid leukemia cells may enhance the activity of differentiation therapy drugs for this type of leukemia.

INTRODUCTION

Phorbol 12-myristate 13-acetate (PMA), a protein kinase C activator (PKC) (1), is a known skin tumor promoter in the mouse (2). In cultured myelomonocytic leukemia cells, including human HL-60 and U-937 cells, PMA evokes an opposing effect, namely it causes such cells to undergo terminal differentiation into cells with a macrophage phenotype (3–6). This latter capability raised the notion that PMA may function as a differentiation therapy drug. Because of this possibility, PMA has received attention in a clinical setting; it was shown that its infusion alone or in combination with other drugs conferred remissions in some patients (7).

Previously, we have shown that macrophage differentiation of HL-60 cells is initiated as a result of PMA-evoked PKC- β activation (8), which in turn leads to the expression and interaction of a number of downstream signaling proteins that, among others, include protein kinase X, tumor necrosis factor α , fibronectin and $\alpha_5\beta_1$ integrin (9–11). There is, however, a need to identify additional signaling molecules, in particular those that can be easily modulated by differentiation therapy drugs, which hopefully may be devoid of tumor promoting activity.

A number of studies have shown that putrescine, spermidine, and spermine levels change during HL-60 cell differentiation (12–15). Because these polyamines are required for cell viability and various cellular processes, including gene expression, protein synthesis, and signal transduction events (16), their content is finely tuned at the level of biosynthesis, degradation, and transport (17). The rate-limit-

ing step in polyamine biosynthesis involves ornithine decarboxylase (ODC), which converts ornithine into putrescine, which in turn is metabolized into spermidine and then into spermine. The catabolism of the polyamines is achieved mainly by *N*¹-spermidine/spermidine acetyltransferase (SSAT), which acetylates spermine and spermidine and targets them for degradation (18).

The present studies were initiated to determine the potential involvement of specific polyamines in the regulation of PMA-induced macrophage differentiation of leukemia cells and to explore the mechanism of this regulation.

MATERIALS AND METHODS

Cells and Cell Culture. Human myeloid HL-60 leukemia cells were originally obtained from Robert C. Gallo (National Cancer Institute, Bethesda, MD) and human monocytic U-937 leukemia cells from American Type Culture Collection (Manassas, VA). Differentiation-resistant HL-60 cells, termed HL-525 cells (19), and their stable transfectants, HL-525/3–2 (HL-525/PKC- β A), HL-525/3–30 (HL-525/PKC- β B) and HL-525/*neo* (HL-525/vector; refs. 8, 10), were generated in our laboratory (19). Cells were cultured in plastic dishes (Falcon) or in 16-well Lab-Tek chamber slides at 4×10^4 to 1.5×10^5 cells/mL in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (HyCLone, Logan, UT), 100 units/mL penicillin, and 100 μ g/mL streptomycin (Life Technologies, Inc., Rockville, MD). The cells were incubated at 37°C in humidified air containing 8% CO₂.

Macrophage Differentiation. Macrophage differentiation was induced with 3 or 30 nmol/L PMA. The fraction of the attached and spread cells was determined by light microscopy 14 hours after initiating PMA treatment, and phagocytosis was assessed by the ability of the cells to ingest fluorescent beads at 21 hours after treatment initiation, as described previously (20). For testing the effect of polyamines on cellular replication and acquisition of the macrophage phenotype, the cells were initially incubated with putrescine, spermidine, or spermine for 6 hours in serum-free RPMI 1640 supplemented with 10 μ g/mL transferrin and 5 μ g/mL insulin, and then fresh fetal bovine serum was added to a final concentration of 10%. PMA was added 1 hour later, and the cells were incubated for 14 or 21 hours as needed. A total of 1 mmol/L aminoguanidine (AG) was added to the cells before adding serum when needed.

Apoptosis. Apoptosis was determined after treating 1.6×10^5 cells for 1 and 2 days with 3 nmol/L PMA and/or 4 μ mol/L spermine in the presence or absence of 1 mmol/L AG. After this treatment, the cells were collected by centrifugation, washed with PBS, and fixed for 10 minutes in 4% paraformaldehyde in PBS. After additional two washes with PBS, the cells were stained for 10 minutes in a solution containing 0.1 mg/mL *L*- α -lysophosphatidylcholine, 20 μ g/mL 4',6-diamidino-2-phenylindole (Boehringer-Mannheim, Indianapolis, IN), and 10 μ g/mL hydroethidine (Polysciences, Inc., Warrington, PA). Thereafter, the stained cells were washed again, spun onto microscope slides using a Cytospin 2 centrifuge (Shandon, Inc., Pittsburgh, PA), and examined by fluorescence microscopy for the appearance of nuclear fragmentation as described previously (21). The results are based on 200 cells per point in randomly chosen fields.

Polyamine Content and Enzyme Activity. To determine polyamine content and enzyme activity, $\sim 5 \times 10^7$ untreated or 3 or 30 nmol/L PMA-treated HL-60 cells were collected at indicated times and washed with PBS. ODC and SSAT activities were assayed as described previously (22). Intracellular polyamine pools were analyzed by high-performance liquid chromatography as described by Vujcic *et al.* (23).

Antisense Oligonucleotides. The antisense oligonucleotides used in the inhibition experiments were CCATTTTCGTC'TTTT, corresponding to bases

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Requests for reprints: Eliezer Huberman, Biochip Technology Center, Argonne National Laboratory, 9700 South Cass Avenue, Building 202, Argonne, IL 60439. Phone: (630) 252-3820; Fax: (630) 252-9155; E-mail: elih@anl.gov.

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+4 to -11 of the SSAT mRNA sequence (the position +1 denotes the A residue in the ATG start codon), and AATCCATGTCCTGCT, corresponded to bases +7 to -8 of the Nrf2 mRNA sequence. For the antisense experiments, cells were collected, washed in serum-free RPMI 1640 supplemented with 10 $\mu\text{g}/\text{mL}$ transferrin and 5 $\mu\text{g}/\text{mL}$ insulin and resuspended in the same medium at 2×10^4 cells/mL. Polyamines, 100 $\mu\text{mol}/\text{L}$ antisense oligonucleotides or a mixture of 15-mer oligonucleotides of random sequence, were added, and the cells were incubated for 6 hours. Thereafter, 10% fetal bovine serum was added, and the cells were incubated for another 1 hour after PMA treatment.

Northern Blotting. Total cellular RNA from 2×10^7 cells was isolated by centrifugation through a 5.7 mol/L CsCl gradient as described previously (24). Northern blot analyses were performed as previously described (25) using probes corresponding to bases +310 to +577 of the SSAT mRNA sequence, +28 to +538 of the ODC mRNA sequence, and +75 to +606 of the Nrf2 mRNA. A glyceraldehyde-3-phosphate dehydrogenase probe, which served as a reference, was used as described previously (26).

Immunostaining. Rabbit SSAT antibodies were kindly provided by Dr. Anthony E. Pegg (Pennsylvania State University, College of Medicine) and were used at a 1:100 dilution. Rabbit Nrf2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used at a 1:200 dilution. FITC-conjugated goat anti-rabbit antibodies (Coulter Immunology, Fullerton, CA) were used as the secondary antibody at a 1:200 to a 1:400 dilution. The cells were centrifuged onto the slides using a Cytospin 2 centrifuge at 770 rpm for 5 minutes and were fixed thereafter with either methanol for 5 minutes for SSAT immunostaining or with 4% formaldehyde for 15 minutes for Nrf2 immunostaining. After two washes with PBS, the formaldehyde-fixed cells were permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. Cells were then incubated for 1 hour in a PBS solution supplemented with 1 mg/mL BSA (PBS/BSA) and then reacted with the primary antibody in PBS/BSA for 30 to 60 minutes. After three additional washes with PBS/BSA, the cells were incubated with the secondary antibody in PBS/BSA for 30 to 60 minutes and then washed twice with PBS/BSA. Cellular DNA, which in the case of methanol fixed cells was stained for 5 minutes with 1 $\mu\text{g}/\text{mL}$ propidium iodide in PBS/BSA and in the case of formaldehyde-fixed cells for 15 minutes with 2 $\mu\text{g}/\text{mL}$ propidium iodide, was used as a reference for immunostaining. After this staining, the cells were washed twice with PBS/BSA and were mounted with 5% gelvatol and 30% glycerol in PBS. Images were acquired with a Leitz orthoplan fluorescent microscope equipped with dual excitation and emission filter wheels (Sutter Instrument Company, Novato, CA) and a Sedat quad pass beam splitter (Chroma Technology Corp., Brattleboro, VT) using a C2400 C-SIT camera (Hamamatsu Corp., Bridgewater, NJ). Image analysis was performed using Slidebook software (Intelligent Imaging Innovations, Denver, CO).

RESULTS

Decreased Polyamine Biosynthesis and Increased Spermine Degradation in PMA-treated HL-60 Cells. Previously, we have reported that PMA treatment causes in HL-60 cells a decrease in the cellular content of spermine (13), which implies that there is either a decrease in the synthesis of polyamines and/or an increase in their degradation. To test for these possibilities, we measured in these cells the activities of ODC, the polyamine biosynthetic enzyme (17), and SSAT, the rate-limiting enzyme in their degradation (18). The results indicated that PMA treatment caused a time-dependent decrease in ODC activity, which at 20 hours of treatment, was virtually undetectable (Fig. 1A). In contrast, PMA failed to exhibit a detectable increase in spermidine acetyltransferase activity (data not shown). The absence of the latter could be due to a masking effect by nuclear N^8 -spermidine acetyltransferases, which under basal conditions can comprise up to 70% of the total activity in the cell homogenates (22). An alternative indication of SSAT activity can be obtained by measuring intracellular levels of the enzyme products, namely N^1 -acetylspermidine and N^1 -acetylspermine. The acetylated spermine, which is rarely detected in cell homogenates (23), was also not observed in homogenates of untreated or PMA-treated HL-60 cells. Unlike this metabolite, N^1 -acetylspermidine was detected in the homogenates, and PMA treatment caused a time-dependent increase in its production (Fig.

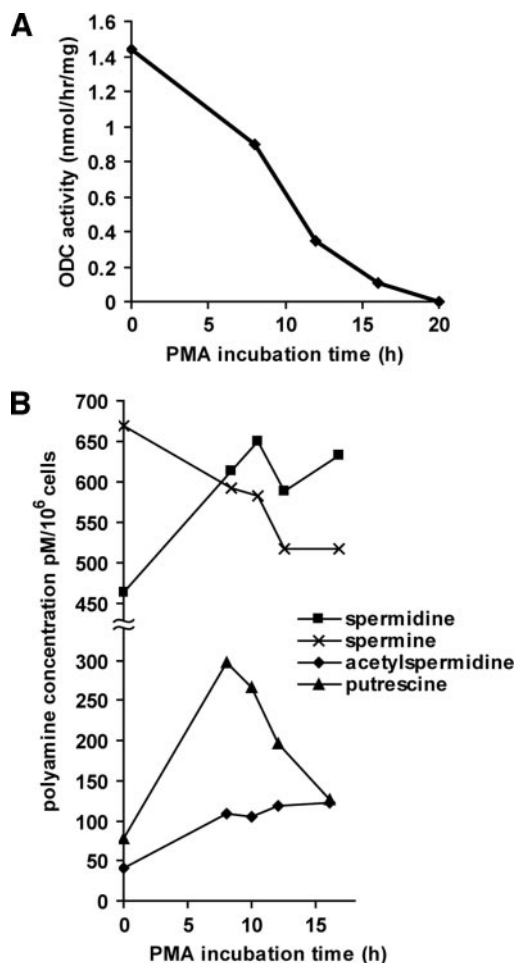


Fig. 1. ODC activity and polyamine acetylation in PMA-treated HL-60 cells. Ornithine decarboxylase activity (A) and polyamine content (B) are shown in HL-60 cells induced with 3 nmol/L PMA.

1B). In addition, PMA also caused a decrease in spermine levels and an increase in the levels of spermidine and putrescine. These changes as well as the observed decrease in ODC activity and increase in SSAT activity support the notion that in HL-60 cells PMA evokes a polyamine back-conversion, which involves the SSAT-dependent catabolic pathway (13, 27).

In addition to ODC and SSAT activities, we have by Northern blotting tested the ability of PMA to alter the expression of the genes that code for these enzymes. In accord with the time-dependent reduction in ODC activity (Fig. 1A), there was a concordant decrease in its gene expression (Fig. 2), and in agreement with the increase in N^1 -acetylspermidine concentration (Fig. 1B), there was a marked increase in *SSAT* gene expression at 8 hours after PMA treatment (Fig. 2).

These data indicate that the observed PMA-induced shift toward a decrease in polyamine biosynthesis and an increase in their degradation is, at least in part, due to inducer-evoked changes in the expression of *ODC* and *SSAT* genes.

PMA-induced Macrophage Differentiation Inhibited by Spermine. In the previous section, we found that PMA treatment of HL-60 cells results in a reduced cellular level of spermine, the end product of polyamine biosynthesis. To determine whether this reduction is required for PMA-induced macrophage differentiation of these cells, we queried whether circumventing this decrease by the addition of exogenous spermine would affect the acquisition of the mature phenotype. For comparison, we included U-937 cells, which can also be induced

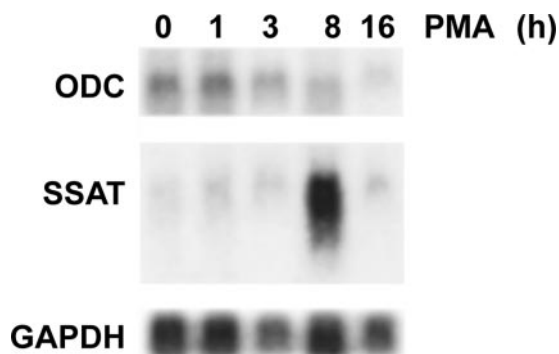


Fig. 2. *SSAT* and *ODC* gene expression in PMA-treated HL-60 cells determined by Northern blotting. RNA isolated from untreated or HL-60 cells treated with 3 nmol/L PMA was hybridized in a sequential order to *ODC*, *SSAT*, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) probes.

Table 1 Inhibition of PMA-induced macrophage differentiation of HL-60 and U-937 cells by exogenous spermine

	Attached and spread cells (%)	Phagocytic cells (%)
HL-60 cells treated with		
None	<5	<3
PMA	83	78
4 μ mol/L spermine	<5	<3
4 μ mol/L spermine + PMA	20	22
10 μ mol/L spermidine	<5	<3
10 μ mol/L spermidine + PMA	73	76
10 μ mol/L putrescine	<5	<3
10 μ mol/L putrescine + PMA	70	76
U937 cells treated with		
None	5	6
PMA	88	81
4 μ mol/L spermine	<5	<5
4 μ mol/L spermine + PMA	27	16
10 μ mol/L spermidine	<5	<5
10 μ mol/L spermidine + PMA	83	77
10 μ mol/L putrescine	<5	<5
10 μ mol/L putrescine + PMA	84	82

NOTE. The data from a representative experiment out of three are shown. The differentiation markers were determined at 21 hours after treatment with 3 nmol/L PMA.

to acquire a macrophage phenotype (28). Treatment of these two cell types with PMA caused a marked increase in the percentage of cells (78 to 88%) exhibiting cell attachment and spreading and phagocytosis (Table 1), hallmarks of the macrophage phenotype (20). The addition of 4 μ mol/L spermine, which in itself failed to affect cell attachment and spreading or phagocytosis, reduced the percentage of cells that display these macrophage markers in PMA-treated cells by 50 to 60% (Table 1). Unlike these results, exogenous spermidine and putrescine, even at 10 μ mol/L, had little to no effect on cell attach-

ment and spreading or phagocytosis in either untreated or PMA-treated cells (Table 1).

Serum amine oxidases can convert exogenous polyamines into cytotoxic products (29). To determine whether these products have an impact on the spermine-evoked inhibition of PMA-induced macrophage phenotype, we repeated the previous experiments in the absence and presence of 1 mmol/L AG, an effective polyamine oxidase inhibitor (29). The results indicated that AG, which reduced the cell number by \sim 20%, had little to no effect on the expression of the macrophage markers in either untreated or PMA-treated cells (Table 2). It did, however, abolish the inhibitory effect of spermine on HL-60 and U-937 cell replication. Yet, AG failed to prevent the spermine-evoked inhibition of PMA-induced macrophage differentiation.

These results indicate the ability of exogenous spermine to abrogate PMA-induced macrophage differentiation in HL-60 and U-937 cells, most likely by way of circumventing the inducer-evoked reduction in the level of this polyamine.

PMA- and/or Spermine-induced Apoptosis Inhibited by Aminoguanidine. To establish the potential involvement of apoptosis in the observed inhibitory effect of exogenous spermine, we examined in HL-60 cells PMA- and/or spermine-induced nuclear fragmentation, a useful apoptotic marker (21, 30). The results indicated that PMA treatment increased the percentage of cells with fragmented nuclei from 3 to 15%, whereas spermine to about twice as much. These increases were, however, to a large degree prevented by the presence of AG (Table 3). These results indicate that PMA- and/or spermine-induced apoptosis, which to a degree may be due to spermine oxidative products, has at best a limited impact on the inhibitory effect of exogenous spermine on PMA-induced macrophage differentiation.

Induction of *SSAT* Gene Expression in Differentiation-susceptible and -resistant HL-60 Cells. Because *SSAT* gene expression can be induced by a variety of toxic agents (18), one can argue that our observed increase in *SSAT* gene expression (Fig. 2) is a consequence of cytotoxicity and not necessarily differentiation. To distinguish between these possibilities, we included in our experiments the HL-60 cell variant HL-525, which unlike the parental cells, is resistant to differentiation induction by either PMA or the topoisomerase II inhibitor novobiocin (31). Helpful to our case is the fact that novobiocin induces a similar cytotoxic effect in both the HL-60 and HL-525 cells but differentiation only in the parental cells (31). Untreated HL-60 or HL-525 cells exhibit a similar low level of *SSAT* gene expression (Fig. 3A). Treatment with novobiocin for 8 hours resulted in an increase in *SSAT* mRNA levels in the differentiation-susceptible HL-60 cells but not in the differentiation-resistant HL-525 cells (Fig. 3A). Similar results were also observed in PMA-treated cells (Fig. 3B)

The results with differentiation-resistant HL-525 cells support the notion that induction of *SSAT* gene expression in the HL-60 system is

Table 2 Inhibition of PMA-induced macrophage differentiation of HL-60 and U-937 cells by exogenous spermine in presence or absence of AG

	Cells concentration ($\times 10^3$ cells/mL)		Attached and spread cells (%)		Phagocytic cells (%)	
	Control	AG	Control	AG	Control	AG
HL-60 cells treated with						
None	38	31	<5	<5	<3	<3
PMA	24	23	77	73	74	68
4 μ mol/L spermine	18	31	<5	<5	<3	<3
4 μ mol/L spermine + PMA	14	22	28	17	22	15
U-937 cells treated with						
None	42	34	<5	<5	<5	<5
PMA	22	22	86	84	80	79
4 μ mol/L spermine	20	35	<5	<5	<5	<5
4 μ mol/L spermine + PMA	15	23	25	21	18	17

NOTE. The data from a representative experiment out of two are shown. Cell concentration was determined at 36 hours after treatment with or without 3 nmol/L PMA while cell attachment and spreading and phagocytosis at 21 hours after this treatment. The final concentration of AG was 1 mmol/L.

Table 3 PMA- and spermine-induced apoptosis in the presence or absence of AG

	Cells with fragmented nuclei (%)	
	Control	AG
HL-60 cells treated with		
None	3	3
PMA	15	3
4 $\mu\text{mol/L}$ spermine	32	7
4 $\mu\text{mol/L}$ spermine + PMA	35	4

NOTE. The data from a representative experiment out of two are shown. Cells were treated for 21 hours with 3 nmol/L PMA and 1 mmol/L AG.



Fig. 3. SSAT gene expression in PMA- or novobiocin-treated HL-60 and HL-525 cells determined by Northern blotting. RNA from HL-60 and HL-525 cells untreated or treated for 8 hours with either 200 $\mu\text{mol/L}$ novobiocin (A) or PMA (B) was hybridized first to a SSAT probe and then to a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe. HL-60 cells were treated with 3 nmol/L PMA and HL-525 cells—with 30 nmol/L PMA.

associated with differentiation induction and not merely with cytotoxicity. In addition, the results indicate that two unrelated differentiation-inducing agents, novobiocin and PMA, can evoke SSAT gene expression.

Inhibition of SSAT Expression Abrogates PMA-induced Macrophage Differentiation in HL-60 Cells. Our previous experiment with exogenous spermine (Tables 1 and 2) support the notion that this polyamine acts as a negative regulator of PMA-induced macrophage differentiation, namely spermine has to be reduced to allow such a differentiation. To further substantiate this concept, we have by specific antisense oligonucleotides examined the effect of repressing SSAT expression on PMA-induced macrophage differentiation of HL-60 cells. Initially, we tested the ability of these oligonucleotides to inhibit SSAT expression as assessed by immunostaining with specific antibodies. In agreement with the Northern blot analysis (Fig. 2), the immunofluorescence intensity in PMA-treated HL-60 cells was markedly higher than that of control cells (Fig. 4), and the presence of SSAT antisense oligonucleotides reduces this intensity, thus indicating that the antisense oligonucleotides were effective in suppressing SSAT expression. Unlike these results, the mixture of random sequence oligonucleotides, which served as a control, had little to no effect on PMA-evoked increase in SSAT immunostaining (Fig. 4).

To determine the effect of repressing SSAT expression on macrophage differentiation, we have incubated HL-60 cells with PMA in the presence and absence of either antisense or control oligonucleotides. The results indicated that these oligonucleotides had little to no effect on PMA-induced macrophage differentiation (data not shown). This negative outcome could be due to depletion of the SSAT substrate spermine, which may result from either a residual SSAT activity that reduces intracellular spermine content and/or from a decreased biosynthesis of its precursor putrescine by ODC after 15 hours of incubation with PMA (Fig. 1). To overcome these potential hurdles, we repeated the experiments in the presence of 2 $\mu\text{mol/L}$ exogenous spermine, which at this concentration showed little to no effect on PMA-induced differentiation (Table 4). This time the SSAT antisense oligonucleotides thwarted the acquisition of the PMA-induced macrophage differentiation, although the control oligonucleotides had little

to no effect (Table 4). These results indicate that blocking SSAT expression in the presence of a low level of its substrate spermine inhibits PMA-induced macrophage differentiation of HL-60 cells, thus further substantiating the concept that spermine acts as a negative regulator of such a differentiation.

PMA-activated PKC- β Is Involved in the Regulation of SSAT Expression. Previous studies have indicated that HL-60 cells, which express PKC- β , are susceptible to PMA-induced differentiation, whereas HL-525 cells, which are PKC- β deficient, are resistant to such a differentiation. Moreover, transfecting HL-525 cells with a PKC- β expression vector restored their susceptibility to PMA-induced macrophage differentiation (8). These studies and our experiments, which indicated that SSAT gene expression is induced in HL-60 but not in HL-525 cells (Fig. 3B), suggest that this PKC isozyme is involved in the regulation of SSAT expression. To substantiate this premise, we have, by a SSAT antibody, tested the impact of PKC- β restoration in HL-525 cells on SSAT expression. The results indicated that PMA treatment of two independent HL-525 cell clones stably transfected with an expression vector containing PKC- β cDNA, and the neomycin resistance gene (*neo*) resulted in an increase in SSAT immunostaining (Fig. 5A). No such increase was observed in a HL-525 cell clone transfected with an expression vector containing *neo* but not PKC- β cDNA. A corresponding increase in PMA-induced

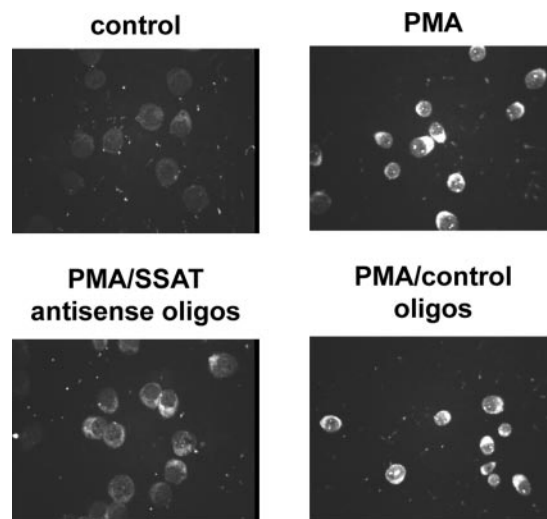


Fig. 4. Inhibition of PMA-induced SSAT expression by its antisense oligonucleotides (oligos); SSAT expression was determined by immunostaining with a specific antibody. Immunostaining was performed after 8 hours of treatment of HL-60 cells with 3 nmol/L PMA in the presence of either SSAT antisense oligos or a mixture of oligos of random sequence, which served as the control. After treatment, the cells were fixed with methanol and reacted for 30 minutes with the primary antibodies at a 1:100 dilution and then with the secondary antibodies at a 1:200 dilution in the presence of 1 $\mu\text{g/mL}$ propidium iodide. The fluorescent intensity of propidium iodide was used as a reference.

Table 4 Inhibition of PMA-induced macrophage differentiation of HL-60 cells by SSAT antisense oligonucleotides

	Attached and spread cells (%)	Phagocytic cells (%)
HL-60 cells treated with		
None	<5	<5
PMA	79	87
2 $\mu\text{mol/L}$ spermine	<5	<5
PMA + 2 $\mu\text{mol/L}$ spermine	81	78
PMA + 2 $\mu\text{mol/L}$ spermine + antisense oligonucleotides	9	21
PMA + 2 $\mu\text{mol/L}$ spermine + control oligonucleotides	72	86

NOTE. The data from a representative experiment out of three are shown. The concentration of PMA and oligonucleotides were 3 nmol/L and 100 $\mu\text{mol/L}$, respectively. Differentiation markers were determined at 21 hours after PMA treatment.

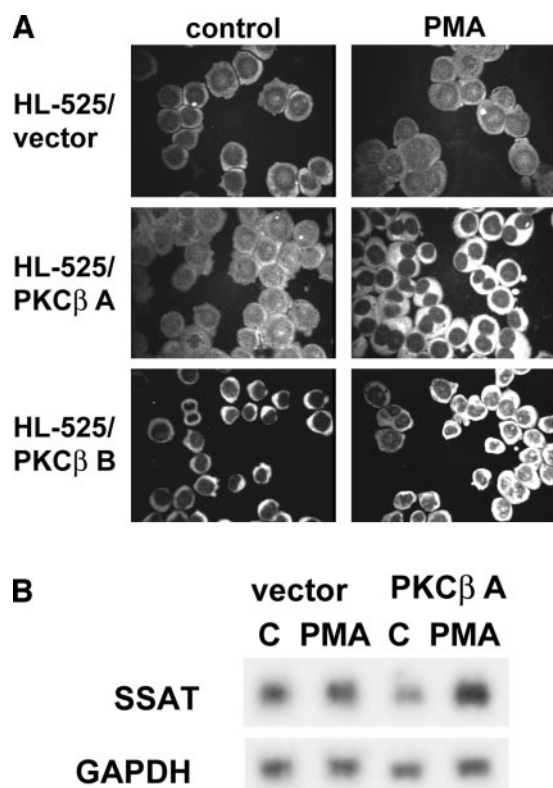


Fig. 5. PMA-induced SSAT expression in HL-525 cells transfected with a PKC- β expression vector determined by immunostaining with SSAT antibodies (A) and expression of corresponding gene determined by Northern blotting (B). The HL-525 cells were either transfected with a PKC- β cDNA *neo* gene expression vector (HL-525/PKC- β) or with an expression vector containing only the *neo* gene (HL-525/vector). HL-525 cells were treated with 30 nmol/L PMA for 8 hours. The immunostaining with the specific antibody was as described in the legend to Fig. 3. For Northern blotting, RNA was hybridized first to the SSAT probe and then to the GAPDH probe.

SSAT gene expression was also observed by Northern blotting (Fig. 5B). These results demonstrate that reintroduction of PKC- β into the deficient HL-525 cells restores their susceptibility to PMA-evoked induction of SSAT expression. Because PMA is a PKC activator (1), the results implicate the activation of this PKC in the control of SSAT expression in the HL-60 cell system.

Nrf2 Transcription Factor Is Involved in the Regulation of PMA-induced SSAT Expression. To further study the mechanism of PKC- β -dependent SSAT expression, we searched for a pertinent transcription factor. We focused our attention on Nrf2, a NF-E2-related transcription factor, because it binds to the polyamine responsive element in the promoter of the *SSAT* gene (32) and it has PKC phosphorylation sites (33). Treatment of HL-60 cells with PMA resulted in an induction of *Nrf2* gene expression, which peaked at 3 hours and decreased thereafter (Fig. 6). The same Northern blotting was also hybridized to a *SSAT* probe. The results indicated that the PMA-evoked expression of the *Nrf2* gene preceded that of the *SSAT* gene (Fig. 6). PMA also induced *Nrf2* gene expression in the PKC- β -deficient HL-525 cells but at a lower level than in the parental HL-60 cells (Fig. 6). In contrast, little to no PMA-evoked *SSAT* gene expression was detected in HL-525 cells (Fig. 6). These results indicate that unlike in the case of *SSAT* gene expression, PKC- β is responsible only in part for PMA-induced *Nrf2* gene expression. Most likely, another PMA-dependent PKC enzyme(s) is also required.

To demonstrate the direct involvement of Nrf2 in PMA-induced SSAT expression, we have examined the effect of Nrf2 antisense oligonucleotides on such an induction in the HL-60 cells. Initially, by immunostaining with specific antibodies, we have confirmed the

ability of these oligonucleotides to block Nrf2 expression thus determining the effectiveness of the antisense oligonucleotides (Fig. 7). Next, we tested the ability of the Nrf2 antisense oligonucleotides to suppress PMA-induced SSAT expression. An 8-hour treatment of HL-60 cells with PMA resulted in an increased SSAT immunostaining, which was reduced by the Nrf2 antisense but not by the control oligonucleotides (Fig. 8).

These results indicate that Nrf2 expression and PKC- β -dependent activation are critical for PMA-induced SSAT expression. Nrf2 production seems to involve PKC- β and another isozyme(s) because it is generated, albeit at a lower level, in PMA-treated HL-525 cells, which are PKC- β deficient. The inability of PMA to induce SSAT expression in the HL-525 cells, despite the Nrf2 presence, raised the possibility that this induction may necessitate Nrf2 phosphorylation by PKC- β . This notion is supported by the observation that Nrf2 is phosphorylated by PKC to promote induction of antioxidative stress genes (33).

DISCUSSION

A central conclusion of the present studies is that the observed PMA-evoked reduction in spermine levels during macrophage differ-

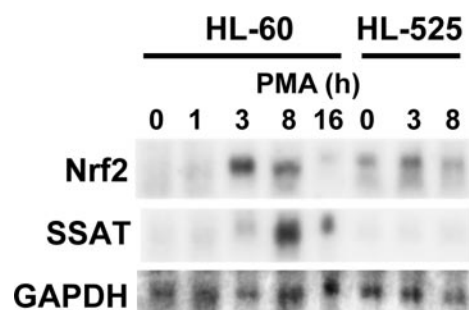


Fig. 6. *Nrf2* and *SSAT* gene expression in HL-60 and HL-525 cells treated with PMA determined by Northern blotting. RNA from untreated or PMA-treated cells was hybridized in a sequential order to the *Nrf2*, *SSAT*, and GAPDH probes. HL-60 cells were treated with 3 nmol/L PMA and HL-525 cells—with 30 nmol/L PMA.

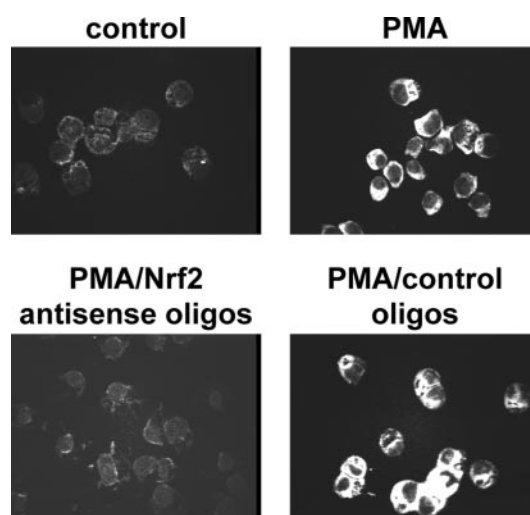


Fig. 7. Inhibition of PMA-induced Nrf2 expression in HL-60 cells by its antisense oligonucleotides (oligos) determined by immunostaining with specific antibodies. Nrf2 expression was assessed by immunostaining with Nrf2 antibodies. The cells were treated for 4 hours with 3 nmol/L PMA in the presence or absence of Nrf2 antisense oligos or a mixture of oligos of random sequence, which served as a control. After treatment, the cells were fixed with formaldehyde and permeabilized with Triton X-100 as described in Materials and Methods. The primary and secondary antibodies were incubated with the cells for 1 hour at a 1:200 and 1:400 dilution, respectively.

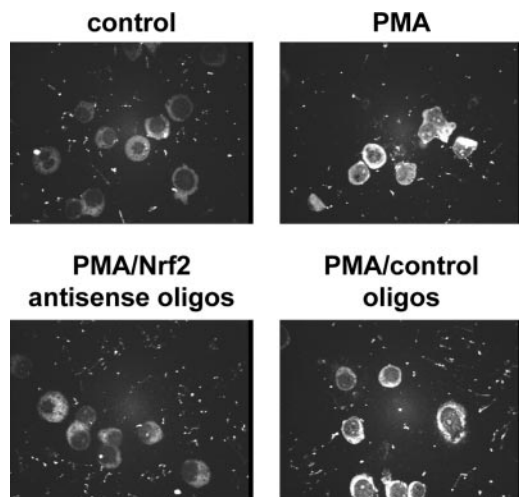


Fig. 8. Inhibition of PMA-induced SSAT expression in HL-60 cells by Nrf2 antisense oligonucleotides (oligos). SSAT expression was assessed by immunostaining with SSAT antibodies. The cells were treated for 8 hours with 3 nmol/L PMA in the presence or absence of Nrf2 antisense oligos or a mixture of oligos of random sequence, which served as a control. After treatment, the cells were fixed with methanol as described in Materials and Methods. The primary and secondary antibodies were incubated with the cells for 30 minutes after 1:100 and 1:200 dilutions, respectively.

entiation is not just associated with this differentiation but is in fact critical for its induction; spermine appears to act as a negative regulator. This conclusion was based on the observations that PMA-induced differentiation, but not replication arrest, was inhibited either by (a) replenishing the depleted spermine with this polyamine from an exogenous source or (b) by blocking SSAT expression with antisense oligonucleotides in the presence of low level of its substrate. It is interesting to note that, unlike in our studies, another polyamine spermidine was reported to be actually required for leukocyte-induced granulocytic differentiation (15). These and the studies reported here suggest that different polyamines may participate as dissimilar regulators of differentiation of distinct myelomonocytic cell lineages.

The present studies also implicate a PMA-evoked decline in the activity of the polyamine synthetic enzyme ODC and an increase in the activity of the polyamine catabolic enzyme SSAT in the reduction of spermine, which acts as a negative regulator of macrophage differentiation. These changes are at least in part due to the alterations in the expression of the genes that code for these enzymes. In addition, our results indicate that SSAT expression is induced by PMA-activated PKC- β and the Nrf2 transcription factor, which acts downstream of the kinase. In this context, it is interesting to note that PKC- β is an initial and an essential element in the signal transduction pathway that leads to such a differentiation (8, 11) and that Nrf2 is involved in regulating the expression of antioxidative stress genes (33, 34). The initial indication for PKC- β involvement was derived from the observation that the PKC- β -deficient HL-525 cells (8) failed to exhibit the PMA-evoked increase in SSAT expression. More direct evidence was obtained by showing that restoring the missing PKC- β in HL-525 cells reestablished their susceptibility to PMA-induced SSAT expression. The involvement of Nrf2 was demonstrated by showing that antisense oligonucleotides to this transcription factor abrogated PMA-evoked SSAT expression. As expected for a transcription factor, its gene expression preceded that of its target SSAT gene. In this context, it is interesting to note that induced Nrf2 gene expression was observed during megakaryocytic differentiation of human cord blood cells (35) and osteoblast differentiation of mouse MC3T3-E1 cells (36), whereas induced SSAT gene expression was found during PMA-induced macrophage differentiation of a number

of mouse and human myelomonocytic leukemia cells, including U-937 cells (37, 38).

Our studies did, however, indicate that the presence of Nrf2 is not sufficient for generating SSAT because HL-525 cells, which exhibited PMA-evoked low level Nrf2 gene expression, failed to display PMA-induced SSAT expression. To explain this discrepancy, we have to invoke the possibility that SSAT expression induced by PMA, which is a PKC activator (1), requires both the presence of Nrf2 and its activation (phosphorylation) by PKC- β . PMA-evoked Nrf2 production seems to involve PKC- β and another PKC isozyme(s) because it is produced, albeit at a lower level, in the PKC- β -deficient HL-525 cells.

Irrespective of these possibilities, PKC- β and Nrf2 appear to be critical for the production and action of SSAT, whose function is most likely to reduce the cellular levels of the negative regulator spermine and thereby allow the manifestation of the macrophage phenotype. This action of spermine and the ability of PMA to evoke remissions in myeloid leukemia patients (7) raises the possibility that agents that have the ability to additionally reduce spermine levels in patients' myeloid leukemia cells may enhance the effectiveness of PMA as a potential leukemia differentiation therapy drug. It is also interesting to note that the spermine analogue N^1,N^{11} -diethylnorspermine, which reduces spermine content by increasing SSAT activity, has been recommended for testing as an antitumor drug "in combination with other cytotoxic chemotherapeutic agents" (39).

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