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c-*myc* Down-regulation in Suramin-treated HL60 Cells Precedes Growth Inhibition but Does Not Trigger Differentiation

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SUMMARY

Down-regulation of c-*myc* mRNA expression is linked to growth arrest and the state of differentiation of hematopoietic cells. We showed that treatment of HL60 cells with suramin results in a rapid reduction of c-*myc* expression followed by an inhibition of cell growth. Both c-*myc* mRNA and protein levels decreased by day 1 of treatment, and, by day 4, only 10% of control c-*myc* protein levels were detected. In contrast to retinoic acid, dimethyl sulfoxide, and 12-*O*-tetradecanoylphorbol-13-acetate treatment, however, exposure of HL60 cells to suramin did not result in the induction of differentiation. These results demonstrate that suramin modulates c-*myc* levels in HL60 cells and that the down-regulation of c-*myc* is not sufficient to trigger differentiation toward either granulocytic or monocytic lineages.

The human myeloid leukemia cell line HL60 and the murine Friend erythroleukemia (MEL) cell line are model systems for studying the effects of various pharmacological agents on cell growth and differentiation (1, 2). Treatment of MEL cells with a variety of agents, including dimethyl sulfoxide (Me_2SO), hexamethylenebisacetamide, and butyric acid, results in terminal differentiation to the erythroid lineage (2). In addition, treatment of HL60 cells with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) induces terminal differentiation toward the monocytic/macrophage lineages, and treatment with retinoic acid or Me_2SO results in differentiation toward the granulocytic phenotype (1). Based on these and other observations, retinoic acid has been tested as a therapeutic modality in patients with acute promyelocytic leukemia, and complete remissions in the absence of marrow aplasia have been reported (3, 4). The mechanism underlying terminal differentiation, however, is unclear, and the HL60 and MEL model systems have been extensively used to study the expression of various cellular oncogenes in relationship to specific hematopoietic differentiation pathways.

Substantial evidence indicates that the c-*myc* oncogene is linked closely to growth arrest and to the state of differentiation of hematopoietic cells. When HL60 cells are induced to differentiate, the steady state level of c-*myc* RNA decreases and remains at low levels (5). A reduction in c-*myc* expression also occurs in normal bone marrow progenitor cells undergoing terminal differentiation (6). To test the causal relationship of c-*myc* expression and differentiation, the c-*myc* oncogene was constitutively expressed in MEL cells prior to induction with the differentiating agent, and high levels of c-*myc* expression were associated with a block in differentiation (7, 8). Additional evidence for the role of the c-*myc* gene in differentiation came from the observation that HL60 cells and F9 embryonal carcinoma cells can be induced to differentiate by either the stable transfection of c-*myc* antisense constructs (9, 10) or by direct incubation with antisense oligonucleotides (11), both of which result in down-regulation of c-*myc* protein expression. Analysis of the growth rate and cell cycle distribution of Me_2SO-treated cells, however, showed no difference between mock or c-*myc* transfected cells (12). This observation conflicts with an earlier hypothesis proposing that c-*myc* blocks differentiation by blocking early withdrawal from the cell cycle (13). In addition, other reports suggested that the expression of the c-*myc* gene was not linked to the differentiation process (14–16). For example, keratinocytes retain high c-*myc* levels after calcium-induced differentiation (14). HL60 cells maintained at low cell densities are induced to undergo functional maturation in response to γ interferon without change in cellular proliferation or c-*myc* expression. In contrast, HL60 cells grown under the same conditions but with addition of retinoic acid exhibit differentiation, growth suppression, and reduced c-*myc* expression (15). Moreover, HL60 cells treated with inositol derivatives showed a decline in c-*myc* expression with no evidence for

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ABBREVIATIONS: MEL, murine Friend erythroleukemia; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; Me_2SO, dimethyl sulfoxide.

monocytic or granulocytic differentiation (16). Therefore, it is unclear whether down-regulating the expression of c-*myc* is a critical step to trigger a cellular shift into a differentiating pathway.

We investigated the mechanism of growth inhibition of suramin, a polysulfonated naphthylurea used as a antiparasitic (17) drug and more recently investigated as an antiviral (18) and antineoplastic agent (19). Because suramin exhibits antiproliferative properties in multiple cell lines by preventing growth factors from binding to their receptors (20–22), we examined whether this agent regulates cell growth and differentiation of HL60 cells by modulating c-*myc* expression in a manner similar to that of retinoic acid, Me_2SO, or TPA. We observed that treatment of HL60 cells with suramin caused a decline in c-*myc* expression followed by inhibition of cell growth. Suramin treatment, however, was not associated with any morphological or functional changes associated with cellular differentiation. These results demonstrate that suramin treatment is associated with a rapid reduction in c-*myc* levels and that the down-regulation of c-*myc* expression was not sufficient to trigger HL60 cells to undergo differentiation.

Materials and Methods

Cell culture and treatments. HL60 human promyelocytic leukemia cells were grown in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum supplemented with 100 μg/ml penicillin and streptomycin. Cell cultures were maintained in a humidified incubator containing 5% CO_2 at 37° and were passaged by a 1:3 dilution every two to three days. On day 0, exponentially growing cells were diluted to 5×10^5 cells/ml and treated with 100 μg/ml suramin (FBA Pharmaceuticals, CT) or left untreated for up to 5 days. Granulocytic differentiation was induced by treatment of HL60 cells with 1 μM retinoic acid (Sigma), or 1.3% Me_2SO (Sigma), and monocytic differentiation was induced by treatment with 50 ng/ml TPA (Life Technologies, Inc.). Growth curves were obtained by Trypan blue staining followed by cell counting by hemocytometer.

Cell differentiation assays. Cytospins and smears of control and treated HL60 cells were stained with a modified Wright/Giemsa stain (Sigma), and a histological evaluation of the degree of differentiation was performed. Cytospins were prepared on day 5 from 1×10^5 control, retinoic acid, Me_2SO, or suramin-treated cells. TPA-treated cells were washed once with phosphate-buffered saline, scraped, and smeared directly onto a slide. To assess the degree of granulocytic differentiation, the nitroblue tetrazolium reduction test was performed as previously described (23) on untreated, suramin-treated, and retinoic acid-treated cells. To assess macrophage/monocyte differentiation, esterase staining was performed with Sigma kit 91-A as described by the manufacturer on untreated, suramin-, retinoic acid-, and TPA-treated cells. Phagocytic activity was determined after exposing cells for 5 days to 100 μg/ml suramin, 1 μM retinoic acid, 1.3% Me_2SO, or 50 ng/ml TPA. Treated cells were washed, and fresh medium (1×10^6/ml) containing 2×10^7 fluorescent microspheres/ml (1.38 μm; Fluoresbrite Carboxylate Microspheres, Polysciences Inc.) was added (24). Following a 24-hr incubation, the cells were separated from the free microspheres by washing twice with phosphate-buffered saline ($300 \times g$ for 5 min) and were analyzed by flow cytometry (FACStar; Becton Dickinson Immunocytometry Systems, Mountain View, CA), using the 488-nm line of a 5 W argon laser.

RNA isolation and Northern blot analysis. RNA was isolated daily from controls and from suramin-treated HL60 cells by the guanidine isothiocyanate method (25). 15 μg of RNA was fractionated on a 1% agarose, 2% formaldehyde gel, and Northern blot analysis was performed as described (25). The c-*myc* probe (5PP) consisted of *Pst*I-*Pst*I fragments spanning exon I, intron I, and part of exon II (26). The c-*fms* probe consisted of a 1.2-kilobase *Eco*RI fragment purified from pcfms 104 vector from ATCC (27). The EGR-1 probe consisted of a 3.3-kilobase *Bam*HI fragment purified from TIS8H7 vector from ATCC (28). The c-*myb* probe consisted of a 2.6-kilobase *Eco*RI fragment purified from pHM 2.6 vector from ATCC (29). The actin probe consisted of a 2-kilobase *Bam*HI insert purified from the LK220 vector (30). Probes were ^{32}P labeled by nick translation and hybridized as described (25). Following hybridization, the filter was washed with 0.5 × SSC and 0.1% SDS at 65° and autoradiographed.

Protein extracts and Western blot analysis. Cellular proteins were extracted daily from control and suramin-treated HL60 cells. The washed cell pellets were resuspended in ELB buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM NaF, 0.5 mM Na orthovanadate) containing 10 μg/ml each of leupeptin and aprotinin and incubated for 15 min at 4°. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. Protein concentrations were determined using Bio-Rad protein assay reagent as described by the manufacturer. 200 μg of cellular proteins from treated and untreated cell extracts were electroblotted onto nitrocellulose filter following separation on a 7.5% SDS-polyacrylamide gel electrophoresis and incubated overnight with a 1:100 dilution of monoclonal anti-Myc (Ab-1) antibody (Oncogene Science). The immunoblot was then incubated for 4 hr with a 1:300 dilution of polyclonal rabbit anti-mouse antibody (PharMingen), followed by a 2-hr incubation with 1×10^7 cpm of ^{125}I-labeled protein A. Following autoradiography, the nitrocellulose filter was incubated in a stripping buffer (0.2 M glycine, pH 2.8) for 2 hr and then washed 3–4 times with 0.1% Triton in phosphate-buffered saline for a total of 30 min. Immunoblotting was then performed with actin Ab-1 antisera (Oncogene Science) as described above. The amount of radioactivity in the bands was quantitated by PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Clonogenic assay in semi-solid culture. 1×10^3 HL-60 cells were plated in 35-mm Petri dishes in α-modified Eagle's medium (Life Technologies, Inc., Gaithersburg, MD), 0.8% methylcellulose (4,000 centipoise; Fisher Scientific Co., Fair Lawn, NJ), and 15% fetal calf serum in the presence or absence of 100 μg/ml suramin (23). Colonies were scored at 7 and 14 days, and the results were expressed as the percentage of clonal growth. Single colonies were picked with a capillary tube on day 14, cytospined onto glass slide, stained with Wright/Giemsa, and examined by phase microscopy.

Results

Because suramin has been shown to exhibit antiproliferative activity in a wide variety of cell types (20, 21, 31), we examined its effect on the proliferation of HL60 cells. To determine a suramin concentration that would inhibit cell growth without having a significant effect on cell viability, we treated HL60 cells with increasing concentrations of suramin. The dose-dependent inhibition of cell growth is presented in Fig. 1. To study the effect of suramin on cell growth and differentiation, we chose 100 μg/ml suramin, a concentration that was cytostatic but not cytocidal to HL60 cells. At this concentration, inhibition of cell growth becomes apparent 3 days following exposure to suramin and reaches 50% growth inhibition by 4 days after treatment (Fig. 1).

To examine whether the suramin-related growth inhibition was accompanied by modulation of the growth-related oncogene c-*myc*, we collected RNA daily over 4 days from untreated and suramin-treated cells. Northern blot analysis indicated that c-*myc* expression significantly decreased by the first day of suramin treatment as compared with untreated controls and remained low over the 4-day period (Fig. 2). Therefore, the decrease of c-*myc* mRNA levels preceded the suramin-induced growth inhibition of HL60 cells (Fig. 1).

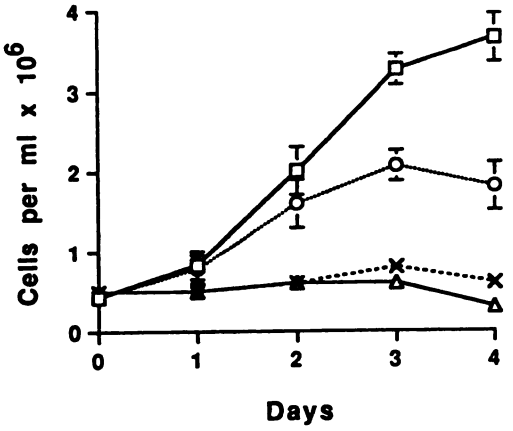


Fig. 1. The effect of suramin on growth of HL60 cells. Cells were treated with increasing concentrations of suramin (100–200 μg) or were left untreated as controls for 4 days. Cells were counted daily using Trypan blue and hemocytometer. The *bars* for control and 100 μg/ml suramin-treated cells represent the mean and SD of four independent experiments. □, control; ○, 100 μg/ml; ×, 150 μg/ml; △, 200 μg/ml.

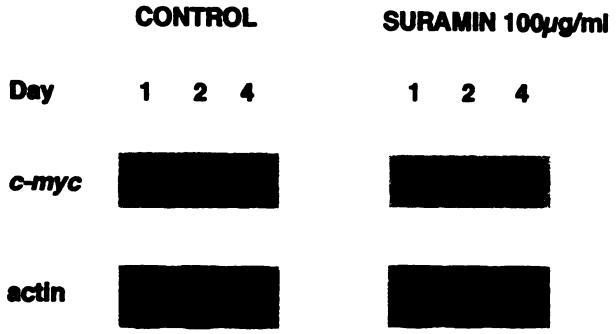


Fig. 2. c-*myc* mRNA levels in suramin-treated HL60 cells. Total RNA was collected daily from cells treated with 100 μg/ml suramin and from untreated controls. Fifteen μg of total RNA were used for each sample. Northern blots were probed first with ^{32}P-labeled c-*myc* (exons 1 and 2), and then the blots were stripped and hybridized to a ^{32}P-labeled actin probe.

To examine whether the reduced c-*myc* mRNA expression correlated with a decrease in c-Myc, we collected protein extracts daily from cells treated with 100 μg/ml suramin and control cells over 5 days. Immunoblot analysis indicated a biphasic decline of c-Myc following suramin treatment as compared with untreated control (Fig. 3). Incubation of cells with suramin showed an initial reduction of c-Myc levels (35 and 65% c-Myc reduction on days 1 and 3, respectively), followed by a second decline to 10% of the original levels on day 4 (Fig. 3). The reduction of c-Myc levels in HL60 cells treated with suramin for 4 or 5 days was not due to prolonged incubation of cells in culture, because the levels of c-Myc in untreated cells remained unchanged throughout the 5 days examined (Fig. 3). Moreover, actin levels remained constant in untreated and suramin-treated cells (Fig. 3).

Because growth inhibition and c-*myc* down-regulation are frequently seen in cells that are undergoing terminal differentiation, we examined whether suramin-treated HL60 cells undergo differentiation along a granulocytic or monocytic pathway. Morphologic evaluation of Wright/Giemsa-stained cytospin preparations indicated that suramin-treated cells remain morphologically undifferentiated with the typical appearance of promyelocytes up to 5 days in culture (Fig. 4). In contrast,

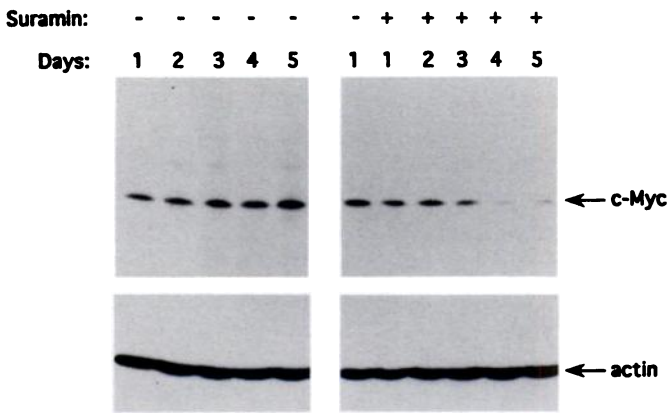


Fig. 3. c-*myc* protein levels in suramin-treated HL60 cells. Total cellular extracts were prepared daily from cells treated with 100 μg/ml suramin and from untreated controls. 200 μg of cellular proteins from treated and untreated cell extracts were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted using c-Myc and actin antisera as described under Materials and Methods.

retinoic acid-treated cells, used as a control for granulocytic differentiation, showed progressive maturation toward the granulocytic lineage, with up to 70% of cells having the appearance of metamyelocytes and banded neutrophils after 5 days of exposure (Fig. 4). To confirm that suramin-treated HL60 cells did not undergo the differentiation process, we performed the nitroblue tetrazolium test for the detection of granulocytes and monocytes and esterase staining for the presence of monocytic cells. Both tests demonstrate that suramin did not induce granulocytic or monocytic differentiation of HL60 cells. In contrast, 85% of retinoic acid-treated cells (used as controls for granulocytic differentiation) reduced nitroblue tetrazolium to blue formazan as determined by light microscopy. TPA-treated cells (used as controls for monocytic differentiation) were positive for nonspecific esterase activity. In addition, phagocytic activity, an indicator of granulocytic and monocytic differentiation, was measured by flow cytometric analysis of control and treated cells following incubation with fluorescent microspheres. As shown in Fig. 5, *panels A–E*, there was a significant increase in phagocytic activity of HL60 cells following granulocytic (retinoic acid and Me_2SO) or monocytic (TPA) differentiation, in contrast to the effect of suramin treatment. Quantitation of the percent of the cell population demonstrating phagocytic activity, calculated by integrating the peaks representing cells that associated with or ingested at least one latex particle, is given in Fig. 5, *panel F*. Morphological staining and phagocytic activity measurement by flow cytometry was also examined 7 days after suramin exposure, and no monocytic or granulocytic differentiation of the treated HL60 cells was observed (data not shown).

We analyzed the relationship between the differentiation of HL-60 cells and the loss of self-renewal and commitment (32) after exposure to 100 μg/ml suramin. Treatment of HL60 cells with suramin for 7 and 14 days reduced, by 50%, the ability of the cells to develop colonies in 0.8% of methyl cellulose (Fig. 6*C*). In addition, the size of colonies in suramin-treated cells were significantly smaller as shown in Fig. 6, *A* and *B*. To determine whether the reduction in size and number of the colony formation in semi-solid medium was due to inhibition of cell growth or to induction of the differentiation process, single colonies were picked on day 14 from the methyl cellulose,

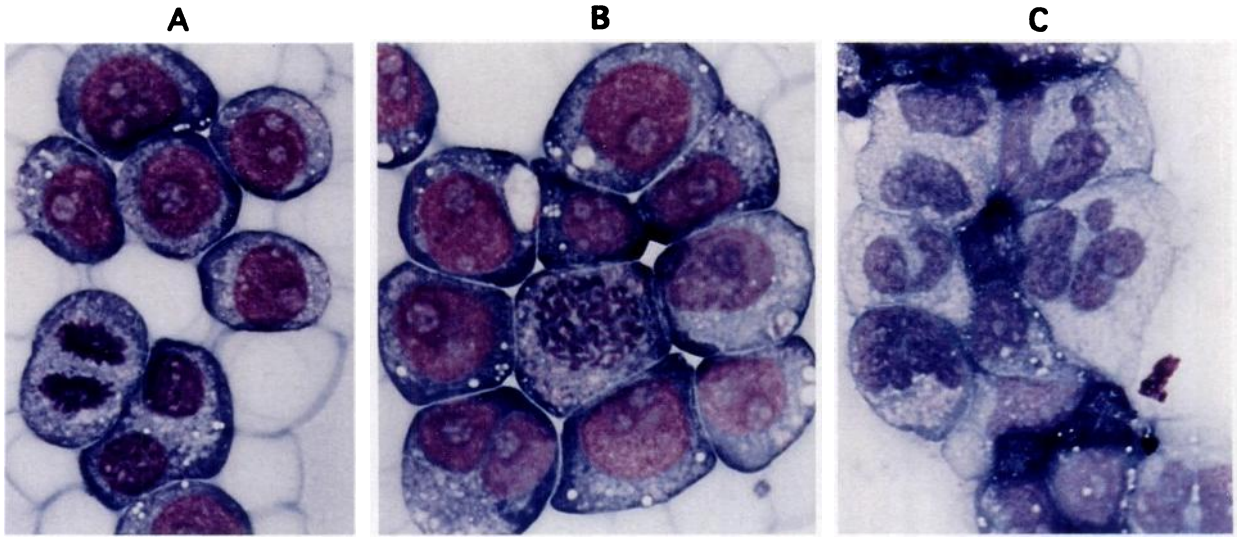


Fig. 4. Histological evaluation of differentiation. Cells were treated with 100 μg/ml suramin, with 1 μM retinoic acid, or were left untreated (control) for 5 days. Cytospins were visualized with a modified Wright/Giemsa stain. Control (*A*) and suramin-treated (*B*) cells remained morphologically at the promyelocytic stage. Retinoic acid-treated cells (*C*) exhibited differentiation toward the granulocytic lineage with the appearance of metamyelocytes and banded neutrophils.

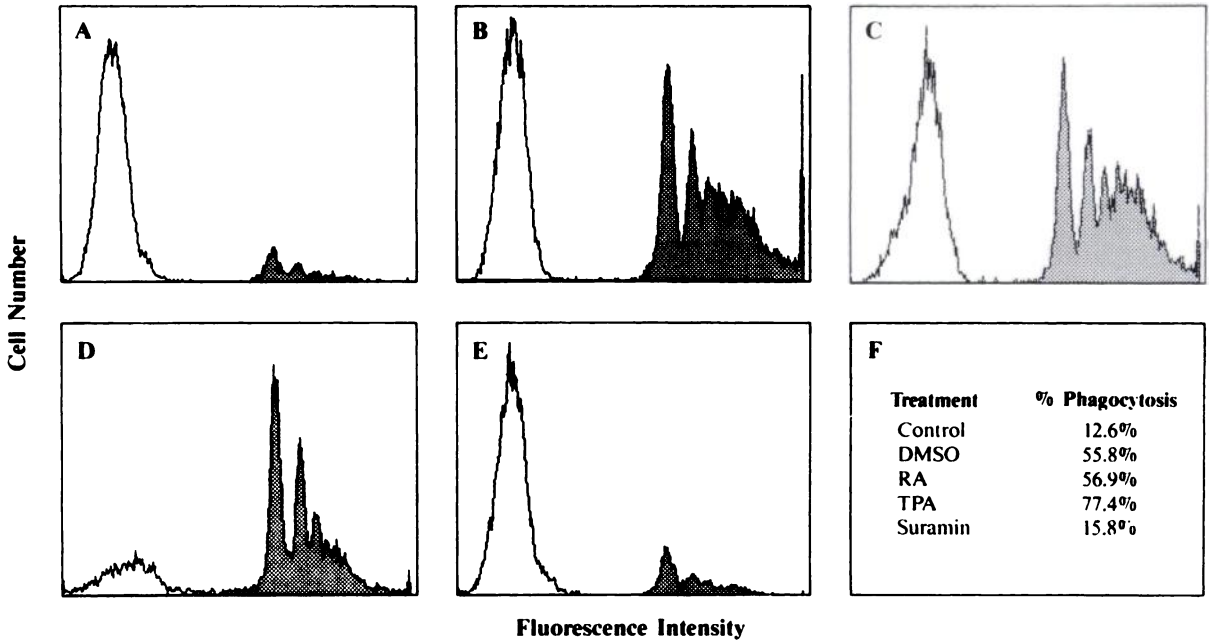
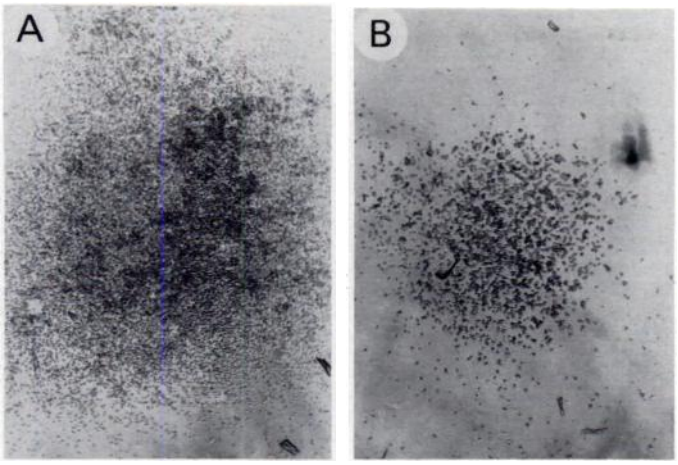


Fig. 5. Functional assays for differentiation. Fluorescence histograms of control (*A*), Me_2SO- (*B*), retinoic acid- (*C*), TPA- (*D*), and suramin- (*E*) treated HL60 cells following incubation with fluorescent microspheres, as described under Materials and Methods, are shown. The *unstippled peak* represents nonphagocytic cells. The *stippled peaks* represent cells that have phagocytized one, two, three, four, and more than four fluorescent microspheres/cell. The percentage of phagocytic cells for each treatment is detailed in *panel F*.

cytospined onto a glass slide, stained with Wright/Giemsa, and observed by phase microscopy. No morphologic features of differentiation were observed (data not shown).

To determine whether suramin affects the expression of other oncogenes involved in hematopoietic cell differentiation, we examined mRNA levels of c-*fms*, c-*fos*, Egr-1, c-*jun*, and c-*myb* in suramin-treated HL60 cells. Previous studies have demonstrated that the expression of c-*fms*, c-*fos*, Egr-1, and c-*jun* is induced, and c-*myb* and c-*myc* expression is down-regulated during the differentiation process (1, 33–35). We found that the expression of c-*fms* and Egr-1 (Fig. 7, *A* and *B*) was not affected by suramin treatment, although induction of these markers was observed following retinoic acid treatment used as a control for differentiation (Fig. 7, *A* and *B*). In addition, we observed no induction of c-*jun* or c-*fos* expression with suramin treatment (data not shown), again consistent with absence of differentiation following suramin exposure of HL60 cells.

Because the c-*myb* oncogene has been implicated in the regulation of hematopoietic cell growth and differentiation (35), we also studied the effect of suramin exposure on c-*myb* mRNA levels in HL60 cells. We observed that suramin reduced c-*myb* expression by 25% (relative to an actin control) after 4 days of treatment (Fig. 7*C*), and retinoic acid reduced c-*myb* expression by 90% during differentiation of these cells (Fig. 7*C*). Thus,



C

Days	Suramin	Numbers of colonies	Clonogenic efficiency
7	-	620	62%
7	+	348	35%
14	-	568	57%
14	+	280	28%

Fig. 6. Effect of suramin on clonogenic growth of HL-60 cells. 1×10^3 cells were cultured in semi-solid medium in the absence (*A*) or presence of 100 μg/ml suramin (*B*) as described under Materials and Methods. Photographs were taken on day 14. (Original magnification × 24). Clonogenic efficiency on days 7 and 14 of incubation (*C*).

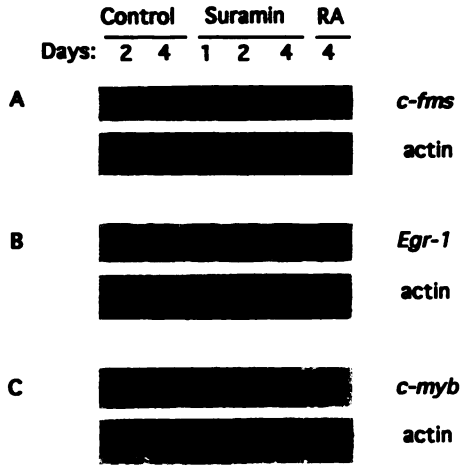


Fig. 7. Effect of suramin on the mRNA expression of differentiation and growth-related oncogenes in HL60 cells. 15 μg of total RNA was subjected to Northern blot analysis as described under Materials and Methods. Blots were probed first with ^{32}P-labeled c-*fms*, EGR-1, c-*jun*, c-*myb* as indicated and then were stripped and hybridized to a ^{32}P-labeled actin probe.

our data suggest that treatment of HL60 cells with suramin results in a modest decrease in c-*myb* levels that contrast with the marked down-regulation observed for c-*myc*.

Discussion

In this study, we demonstrated that treatment of HL60 cells with 100 μg/ml suramin caused a decrease in c-*myc* mRNA expression that was detected 48 hr before any changes in cellular proliferation were evident. In addition, we observed that despite the down-regulation of c-*myc* and inhibition of cell growth, a 5–7-day course of suramin treatment was not sufficient to induce terminal differentiation of HL60 cells toward granulocytic or monocytic lineages. Therefore, in contrast to pharmacologic agents, such as retinoic acid or Me_2SO, the reduction in c-*myc* mRNA and protein levels was not directly coupled to differentiation of HL60 cells.

Suramin is a polysulfonated naphthylurea that has been reported to bind to several different growth factors, such as platelet-derived growth factor, epidermal growth factor, transforming growth factor β, fibroblast growth factor, and insulin-like growth factor (21, 22, 36, 37). These observations suggested that suramin treatment might interrupt growth factor-receptor interactions, resulting in growth inhibition by antagonizing the ability of these soluble factors to stimulate the growth of tumor cells in tissue culture (21, 22, 36). For example, human fibroblast cells infected with Simian sarcoma virus (SSV) acquired a transformed phenotype due to the expression of the viral oncogene v-*sis*, which encodes one chain of the platelet-derived growth factor molecule. Treatment of SSV-transformed cells with suramin reverts the transformed phenotype of these cells consistent with the dissociation or neutralization of receptor-bound platelet-derived growth factor (36).

Growth factors binding to their receptors are also known to stimulate rapid elevation of the c-*myc* levels in quiescent cells, and several groups have examined the link between c-*myc* expression and growth factor stimulation of cell proliferation (38, 39). For example, it has been observed that the overexpression of c-*myc* in a lung fibroblast cell line abolished growth factor requirements, allowing the proliferation of these cells in serum-free medium. The subsequent addition of 100 μg/ml suramin restored growth inhibition, and these others have speculated that the overexpression of c-*myc* may lead to the production of autostimulatory factors that can be blocked by suramin (40). The steady state levels of c-*myc* expression, however, were not measured following suramin treatment. An alternate explanation for these findings, therefore, might be the suppression of c-*myc* expression by suramin. Consistent with this model was the observation that the suramin-like molecule heparin can suppress c-*myc* induction following serum stimulation (41). In addition, a recent report demonstrated that ^{3}H-labeled suramin enters the cell, localizes predominantly to the nucleus, which may affect gene expression, and modulates topoisomerase II activity (42).

Our studies on HL60 cells showed that suramin induced a rapid fall in c-*myc* expression that resembles the pattern previously reported for the differentiating agents, retinoic acid, Me_2SO, and TPA. In contrast to these agents, however, the down-regulation of c-*myc* expression following treatment with suramin did not trigger differentiation along monocytic and granulocytic lineages in HL60 cells. These results confirm that suramin induces growth inhibition by a distinct mechanism and suggests that changes in c-*myc* levels are not sufficient to trigger the differentiation cascade.

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