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INHIBITION OF INDUCED MURINE ERYTHROLEUKEMIA CELL DIFFERENTIATION BY TUMOR PROMOTERS: RELATION TO THE CELL CYCLE

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SUMMARY

The tumor promoter, 12-0-tetradecanoyl-phorbol-13-acetate (TPA), inhibits induced differentiation of murine erythroleukemia cells (MELC). Employing MELC synchronized in the G_1/S phase of the cell cycle and placed in culture with hexamethylene bisacetamide, it is shown that TPA blocks the accumulation of globin mRNA and globin synthesis. The TPA-sensitive period for inhibition of globin synthesis persists for up to 9 hrs, a time corresponding to the transit of cells through the first S, G_2, M and into G_1. If addition of TPA is delayed for 11 hrs or more, there is induction of accumulation of globin mRNA and globin synthesis.

Murine erythroleukemia cells (MELC), originally isolated from spleens of Friend virus-infected mice (1) can be induced to express a program of erythroid differentiation when cultured with various compounds, including dimethylsulfoxide (2) and hexamethylene bisacetamide (HMBA) (3). The program of induced differentiation includes the expression of such erythroid characteristics as the synthesis of alpha and beta globin mRNAs (4,5), globins and hemoglobins (6,7). Recently we reported that in a MELC population synchronized with respect to the cell division cycle by culture with 2mM thymidine followed by 0.5mM hydroxyurea (which causes the cells to accumulate in late G_1/early S), HMBA does not alter the kinetics of progression of the cells from

Abbreviations - MELC, murine erythroleukemia cells
HMBA, hexamethylene bisacetamide
TPA, 12-0-tetradecanoyl-phorbol-13-acetate

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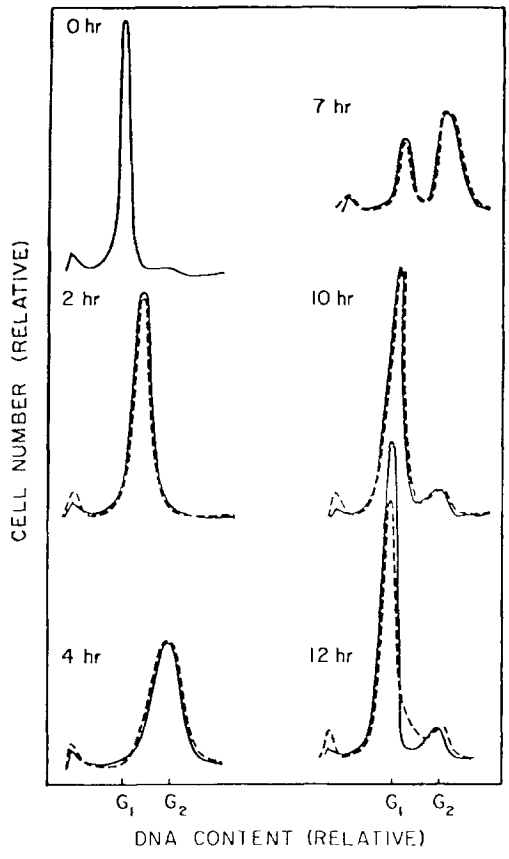

Figure 1. Distribution of synchronized MELC with respect to the cell cycle, during culture with 3mM HMBA or with 3mM HMBA plus 100 ng/ml TPA, determined by measuring the relative DNA content per cell (stained with propidium iodide) by flow microfluorometry at 0, 2, 4, 7, 10 and 12 hrs after removal of the cells from culture with hydroxyurea. For details of methods see Gambari et al (8). Cells were synchronized by serial culture with 2mM thymidine (9 hrs), no addition (5 hrs), 2mM thymidine (9 hrs), no addition (5 hrs) and then 0.5mM hydroxyurea (6 hrs). HMBA was added at the same time as hydroxyurea and, after 6 hrs, the cells were transferred to fresh medium with HMBA (without hydroxyurea) either without or with TPA. G_1 corresponds to 2C in DNA content, and G_2, to 4C in DNA content. Cells cultured with HMBA (---) and with HMBA plus TPA (——).

the G_1/S boundary through S, G_2 and M, but does cause prolongation of the subsequent G_1 phase (8). Under these conditions, the first detectable HMBA-induced accumulation of newly synthesized globin mRNA occurs during that prolonged G_1 phase which follows the first cell cycle (9). Certain tumor promoters,

among them 12-0-tetradecanoyl-phorbol-13-acetate (TPA), are potent, yet reversible, inhibitors of both spontaneous (10,11) and induced (11) differentiation in MELC. In the present study we have examined the action of TPA in inhibiting HMBA-induced MELC differentiation by determining the effect of this agent on accumulation of newly synthesized globin mRNA and globin synthesis in cells synchronized with respect to the cell cycle.

MELC were synchronized with respect to the cell cycle (at the G_1/S boundary) by serial exposure to 2mM thymidine and 0.5mM hydroxyurea (12), then transferred to culture with fresh medium containing 3mM HMBA or 3mM HMBA plus 100ng/ml TPA. Cells cultured with HMBA without or with TPA proceed through S, G_2 and M with similar kinetics (Figure 1). In accord with previously published observations (9) cells cultured with HMBA showed an initial increase in accumulation of newly synthesized globin mRNA at about 11 hrs, corresponding to the G_1 phase following transit of cells through S, G_2 and M. In MELC cultured with inducer plus TPA, there is a block in accumulation of newly synthesized globin mRNA compared to cells in culture with inducer alone (Table I). In 3 separate experiments, in cultures containing both HMBA and TPA, accumulation of newly synthesized globin mRNA was only 20 to 40% of that in cells cultured with inducer alone (Table I).

In experiments using unsynchronized MELC, globin synthesis was measured by determining ^{35}S-methionine incorporation into purified globin (10) and expressed as a percentage of ^{35}S-methionine incorporated into total protein. In cells cultured with HMBA for 56 hrs, 15% of the total protein synthesis, on the average, is globin. In cells cultured with HMBA plus TPA for 56 hrs, only 2.8%, on the average, of total protein synthesis is globin. After 100 hrs, 90% of MELC in culture with HMBA are

TABLE I

INHIBITION OF INITIATION OF GLOBIN mRNA ACCUMULATION BY TPA IN SYNCHRONIZED MELC

	HMBA	HMBA + TPA
Time in culture (hr)*	12	12
Cell-cycle stage**	G_1	G_1
Total cytoplasmic ^{3}H-RNA (cpm/10^6 cells)	156,400	81,000
Poly(A) containing ^{3}H-RNA (% of total cytoplasmic ^{3}H-RNA)	16.4	23.5
Globin ^{3}H-mRNA*** (% of total cytoplasmic ^{3}H-RNA)	0.12	0.05
(% of poly(A)-^{3}H-RNA)	0.7	0.19

* Cells were synchronized as described in the legend for Figure 1 and then cultured with 3mM HMBA or 3mM HMBA plus 100 ng/ml TPA for 12 hrs. At 10 hrs, an aliquot of cells was removed to label RNA by culture at 5 x 10^6 cells/ml with 0.1 ml/ml ^{3}H uridine 40 Ci/mM, 0.05 mCi/ml for 2 hrs.

** Cell cycle stage was determined by analysis of the relative DNA content per cell (stained with propidium iodide) by flow microfluorometry (8).

*** Accumulation of newly synthesized globin mRNA was assayed by oligo(dT)-globin cDNA-cellulose chromatography as previously described (8).

benzidine reactive, while fewer than 10% are benzidine-reactive in cultures with HMBA plus TPA.

To evaluate whether there is a relationship between the TPA-sensitive period for inhibition of globin mRNA synthesis and the cell cycle, the following experiment was performed. HMBA was added to cultures of MELC, synchronized at the G_1/S boundary as described above. At 0, 5, 7, 9, 11, 13, 15 and 17 hrs after release from cell cycle block, an aliquot of the culture was removed, TPA added to a final concentration of 100ng/ml and the culture continued. At 54 hrs, to measure the rate of synthesis of total proteins and globins, ^{35}S-methionine was added to each culture and the culture continued for 2 hrs. The proportion of benzidine-reactive cells was measured after 60 hrs of treatment. In cultures to which TPA was added at 0, 5, 7 and 9 hrs (a period corresponding to transit through the first S, G_2, M and into G_1), there was marked inhibition of globin synthesis and

appearance of benzidine-reactive cells (Table II). In cultures to which TPA was added at 11, 13, 15 and 17 hrs after release from cell cycle block, its effect in blocking both globin synthesis and the appearance of benzidine-reactive cells was less than observed in cultures to which tumor promoter was added prior to 11 hrs (Table II). In cells cultured for 56 hrs with HMBA and TPA, compared with HMBA alone, TPA caused an approximately 66% inhibition in accumulation of globin mRNA and an 80% inhibition in the synthesis of globin.

Our present findings indicate that the TPA-mediated inhibition of HMBA-induced globin synthesis is due, in part at least, to an early effect on blocking accumulation of newly synthesized globin mRNA. We have previously reported that the tumor promoter suppresses the expression of differentiated characteristics in cells "primed" or committed to erythroid differentiation by culture with HMBA (14). Taken together, the data are consistent with the hypothesis that TPA is acting at an early step related to induction of commitment to erythroid differentiation of MELC. In addition, the finding that there is inhibition of globin synthesis in populations of cells in which accumulation of these specialized proteins has already been initiated raises the possibility that TPA may act in cells expressing erythroid characteristics. The present findings are consistent with observations with a variety of other cell lines that tumor promoters inhibit terminal cell differentiation (14-17)

There are several reports that implicate cell-cycle related events in the process of induced differentiation of MELC (8,9,12, 20-23). In MELC (9,12), as well as in several other cell types (20-23), it appears that a portion of the genome which replicates during early S plays a critical role in regulation of growth rate

TABLE II

TPA MEDIATED INHIBITION OF HMBA-INDUCED ERYTHROID DIFFERENTIATION IN SYNCHRONIZED MELC: Relationship between cell cycle, time of addition of TPA and inhibition of globin synthesis and development of benzidine reactive cell.

Time (hr) of addition of TPA to culture with HMBA*	0	5	7	9	11	13	15	17
Globin mRNA synthesis**** (% of total cytoplasmic ^{3}H-RNA)	0.024	0.037	-	-	0.12	-	0.18	-
Globin synthesis** (% of total protein synthesis)	1.4	-	1.8	1.1	2.8	4.5	8.8	9.5
Benzidine reactive MELC***	2	4	12	18	15	39	38	49
CELL CYCLE*	G_1S	S+G_2	S+G_2	G_1	G_1	G_1	G_2+S	S

* MELC were synchronized as described in the legend to Figure 1. 3mM HMBA was added with the 0.5mM hydroxyurea. Time of addition of 100 ng/ml TPA is from the removal of cells from medium with hydroxyurea plus HMBA to fresh medium with HMBA. The stage of the cell cycle of the cell population was determined as indicated in footnote to Table I. At 5 hrs, cells are predominately in late S and beginning to enter G_2; at 7 hrs, cells have largely progressed into G_2 plus M.

** Globin synthesis was assayed after 56 hrs of culture and expressed as % of total proteins synthesized. To assay for total protein synthesis and for globin synthesis, ^{35}S-methionine was added to cultures 2 hrs prior to termination of culture at 56 hrs, and the amount of radioactivity incorporated in total proteins and into purified globins determined as described elsewhere (24). Globin synthesis is expressed as a percent of radioactivity incorporated into total protein.

*** Proportion of cells which are benzidine reactive were quantitated after 60 hrs.

**** Globin mRNA synthesis was assayed as previously described (8).

and expression of differentiated characteristics. With these previous findings, the present data are consistent with the interpretation that tumor promoters block a critical event caused by inducer during S phase of the cell cycle required for expression of globin genes.

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