Vincristine-resistant erythroleukemia cell line has marked increased sensitivity to hexamethylenebisacetamide-induced differentiation

(commitment/hemoglobin/dexamethasone/tumor promotor/protein kinase C)

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ABSTRACT Hexamethylenebisacetamide (HMBA)-induced murine erythroleukemia (MEL) differentiation is a multistep process. Commitment is the capacity to express terminal cell division and characteristics of the differentiated phenotype even after the cells are removed from culture with inducer. Culture of MEL cell line 745A-DS19 (DS19) with HMBA causes commitment to terminal differentiation after a latent period of about 10–12 hr. Previous studies have shown that during this latent period, HMBA causes a number of metabolic changes, including modulation in expression of certain protooncogenes. We now report the development of a MEL cell line (designated V3-17) derived from DS19 that is resistant to vincristine and is (i) markedly more sensitive to HMBA, (ii) induced to commitment without a detectable latent period, and (iii) resistant to the effects of phorbol ester and dexamethasone, which are potent inhibitors of HMBA-mediated DS19 differentiation. We suggest that this V3-17 MEL cell line may express a factor that circumvents HMBA-mediated early events, which prepare the cells for commitment to terminal differentiation.

Hexamethylenebisacetamide (HMBA)-mediated murine erythroleukemia (MEL) cell terminal differentiation is a multistep process (1, 2). Upon culture of MEL cell line 745A-DS19 (DS19) (3) with HMBA (4), there is a latent period of ≈ 10 to 12 hr during which commitment to terminal differentiation cannot be detected. Commitment is defined as the capacity to express characteristics of the erythroid differentiated phenotype, including loss of proliferative capacity, despite removal of the inducer (5, 6). This early, latent period is followed by a period during which an increasing proportion of the population expresses characteristics of terminal differentiation, including loss of proliferative capacity.

During the latent period, the inducer initiates a number of metabolic changes that precede irreversible commitment to differentiation. Among these are alterations in membrane permeability, which involve Na^+ , K^+ , and Ca^{2+} flux (7–9); changes in cell volume (10); a transient increase in cyclic AMP concentration (11); a prompt increase in membrane-associated protein kinase C activity (PKC); the appearance in the cytosol of a Ca^{2+} - and phospholipid-independent form of PKC, presumably generated by proteolytic cleavage of membrane-bound PKC (12); and the modulation in expression of a number of genes, including *c-myb*, *c-myc*, *c-fos*, and *p53* (13–16). Upon more prolonged culture with HMBA, DS19 cells become irreversibly committed (5, 6). Morphological and molecular changes occur that are similar to normal erythroid terminal cell differentiation, including the coordinated expression of genes for α^1 - and β^{maj} -globin, for the

heme synthetic enzymes, and for erythroid-specific membrane proteins, as well as suppression of DNA replication and rRNA synthesis (1, 17, 18).

In the present studies we describe the development of a MEL cell line derived from DS19 that is resistant to inhibition of cell growth by vincristine and is designated V3-17. This MEL cell line, V3-17, is markedly more sensitive to HMBA-induced terminal erythroid differentiation. Another striking characteristic of HMBA-induced V3-17 commitment is absence of the latent period characteristic of induced DS19 differentiation. In addition, the tumor promotor, phorbol 12-myristate 13-acetate (TPA) and the steroid, dexamethasone, both potent inhibitors of HMBA-mediated DS19 cell differentiation (2, 19), fail to inhibit differentiation of V3-17 cells.

MATERIALS AND METHODS

Cell Culture and Materials. MEL cell line DS19 and cell lines derived from it—namely, N23 and V3-17—were maintained in α minimal essential medium containing 10% (vol/vol) fetal calf serum (2). MEL cell line N23 is a subclone of DS19 (20) and, just as DS19, was used in these studies to compare with the vincristine-resistant MEL cell line, V3-17, which was derived as described below. Cultures were initiated with an inoculum of 10^5 cells per ml in Corning 75-cm² flasks; HMBA (4) was added to cultures at a final concentration of 5 mM unless otherwise indicated.

[³²P]ATP was obtained from Amersham. DEAE-cellulose (type DE 52) was purchased from Whatman. Dexamethasone, phosphatidylserine, 1,2-diolein, histone (type III-S), leupeptin, vincristine sulfate, and TPA were purchased from Sigma.

Cell density, benzidine-reactive cells, and hemoglobin content were assayed as detailed (2, 12).

Isolation of a MEL Cell Line Resistant to Vincristine. A MEL cell line resistant to vincristine was derived from cell line DS19 in the following manner. Cells were cultured with vincristine at 500 ng/ml for 3 days, and then dead cells were separated from viable cells by density gradient centrifugation on lymphocyte-separation medium (Flow 16-920-54) for 20 min at $1000 \times g$ and at room temperature. Cells at the interface were recovered, washed once in culture medium, resuspended at 10^5 cells per ml in medium containing vincristine at 200 ng/ml, and cultured for 4 days. Surviving cells were recovered by centrifugation, resuspended in fresh medium without vincristine, and cultured for 22 days. Cells again were recovered by centrifugation, resuspended in culture medium, and passaged at 2-day intervals in culture

FIG. 1. Effect of vincristine on MEL cell growth. MEL cell lines DS19 (●) and N23 (◆) were not selected for resistance to vincristine. V3-17 cell line (■) was selected for resistance to vincristine (see text for details). Cell density is expressed as a percentage of the cell density of each cell line grown for 48 hr in culture without vincristine.

with vincristine (5 ng/ml); the cells have been maintained continuously in this manner and were designated V3-17.

Commitment Assay. Commitment, characterized by the irreversible induction of differentiation and limited cell division (small colony size), was assayed as described (6). MEL cells were transferred from suspension culture with inducer to semisolid medium (1.8% methyl cellulose) without inducer and, after 5 days, the plates were stained for hemoglobin and scored for colony size (6).

RNA Preparation. Total RNA was prepared by the guanidium isothiocyanate method, and polyadenylated [poly-(A)⁺] RNA was isolated by oligo(dT)-cellulose chromatography (21, 22).

Globin mRNA Assay. The α^1 -, β^{maj} -, and β^{min} -globin RNA probes used in these studies have been described (23). RNase protection assays were carried out as described by Melton *et al.* (24). Globin probes were hybridized with 10 μ g of total cellular RNA. Protected RNA fragments were analyzed by electrophoresis on 8% polyacrylamide/8 M urea denaturing gels. Relative quantitation was by liquid scintillation of individual gel bands.

PKC. PKC activity was assayed as described (4).

RESULTS

Growth of MEL Cell Lines with Vincristine. MEL cell lines DS19, N23, and V3-17 were grown in culture without vincristine and with increasing amounts of vincristine (between 0.5 and 20 ng/ml). The vincristine-sensitive cell lines, DS19 and N23, showed marked growth inhibition at vincristine concentrations of 1.0 ng/ml or higher, while V3-17 cells, selected for their vincristine resistance, grew in concentra-

tions up to 5 ng/ml at rates similar to cell growth rates in the absence of vincristine (Fig. 1). The rate of increase in cell density of V3-17 cells grown in culture with vincristine at 5 ng/ml was similar to that of DS19 cells and N23 cells grown without vincristine (Fig. 2A).

Effect of HMBA on V3-17 MEL Cell Growth, Commitment, and Development of Benzidine-Reactive Cells. HMBA decreased the cell density of V3-17 cells by about 50% at 48 hr, which was similar to the decrease in cell density of DS19 in the presence of inducer (Fig. 2A). The V3-17 cell line displayed distinctly more (3–4%) spontaneously differentiating cells (benzidine-reactive without exposure to HMBA) than did DS19 cells (<1.0%). After 24 hr of exposure to HMBA, V3-17 cells were 15% benzidine-reactive, whereas there were <1% benzidine-reactive DS19 cells. By 48 hr, when DS19 cells were about 15% benzidine-reactive, 85% of V3-17 cells contained stainable hemoglobin (Fig. 2B).

V3-17 cells in culture with HMBA became committed to terminal differentiation and cessation of proliferation at a substantially faster rate than did DS19 cells (Fig. 2C). Furthermore, the characteristic latent period of about 12 hr prior to detectable commitment of cell line DS19 was eliminated for the V3-17 cells in culture with inducer. Whereas DS19 cells displayed little or no spontaneous commitment, 8–14% of the V3-17 cell population were committed in the absence of HMBA.

HMBA Effect on Hemoglobin Accumulation in V3-17 Cells. In the absence of HMBA, hemoglobin accumulation after 98 hr of culture was <0.3 μ g per 10^6 V3-17 cells, and <0.1 μ g per 10^6 DS19 or N23 cells. In culture with HMBA, hemoglobin accumulation was detectable earlier in V3-17 cells (by 24 hr) than in DS19 or N23 cells (by 48 hr) and by 98 hr reached

FIG. 2. HMBA effect on cell growth (A), benzidine-reactive cells (B), and commitment (C) of DS19 MEL cells grown without (●) or with HMBA (○) and of V3-17 MEL cells grown without (▲) or with HMBA (△).

FIG. 3. Effect of HMBA concentration on induction of MEL cell lines DS19 (●), V3-17 cultured without vincristine (○), and V3-17 cultured with vincristine (Δ). The effect of HMBA on these MEL cell lines was evaluated by determining the proportion of the cells induced to become benzidine-reactive (A) and committed (B).

higher concentrations—that is, $8.1 \mu\text{g}$ per 10^6 V3-17 cells compared to $4.0 \mu\text{g}$ per 10^6 DS19 or N23 cells.

Sensitivity of V3-17 Cells to HMBA. MEL cell lines DS19, N23, and V3-17 were assayed for sensitivity to induction by HMBA over a range of inducer concentration between 0.5 mM and 5 mM. The rates of cell growth of DS19, N23, and V3-17 (the last grown in culture without as well as with vincristine at 5 ng/ml) were similar and reached densities of $2.0\text{--}2.4 \times 10^6$ cells per ml after 5 days. V3-17 cells (cultured without or with vincristine at 5 ng/ml) were more sensitive to HMBA-mediated induction than were DS19 or N23 cells, by the dual criteria of the proportion of the population induced to hemoglobin accumulation (reactivity to benzidine) and commitment to terminal cell differentiation (Fig. 3). For example, 1.25 mM HMBA induced 85–88% of V3-17 cells to become benzidine-reactive and 78–84% of these cells to become committed, compared with 25%–30% benzidine-reactive and 15%–18% committed DS19 cells.

Globin mRNA Levels in V3-17 MEL Cells. As reported previously (25–27), in uninduced DS19 there are low levels of α^1 - and β^{min} -globin gene transcription and even less β^{maj} -globin gene transcription. By comparison, in uninduced V3-17 cells there were ≈ 10 -fold higher levels of α^1 -, β^{min} -, and β^{maj} -globin mRNA than in uninduced DS19 (Fig. 4). When cultured with HMBA, V3-17 cells showed no increase in accumulated α^1 -, β^{maj} -, or β^{min} -globin mRNA during the initial 12 hr of culture; by 24 hr an increase in the levels of both α^1 - and β^{maj} -globin mRNA (but not β^{min} -globin mRNA) was observed. By 24 hr in V3-17 cells in culture with HMBA, α^1 -globin mRNA had increased almost 6-fold and β^{maj} -globin mRNA had increased almost 2-fold, relative to uninduced cells. With respect to both the time of onset of HMBA-induced increased accumulation of globin mRNA and the relative increase in α^1 - and β^{maj} -globin mRNA, V3-17 cells were similar to DS19 cells (data not shown).

FIG. 4. Analysis of globin mRNAs in uninduced DS19 (vincristine sensitive) and V3-17 (vincristine resistant) cells. Total RNA was isolated from logarithmic-phase growing cells in culture without HMBA: DS19 cells (lanes 1, 2, and 3) and V3-17 cells (lanes 4, 5, and 6). Lanes: 1 and 4, α -globin; 2 and 5, β^{maj} -globin; 3 and 6, β^{min} -globin; M, size markers of pBR322 [^{32}P]-DNA cut with *Msp* I.

Effect of Phorbol Ester on V3-17 MEL Cells. The phorbol ester TPA is a potent inhibitor of HMBA-induced commitment of MEL cell line DS19, and it inhibited growth of these cells in concentrations as low as 10 ng/ml (19) (Fig. 5). In contrast, V3-17 cell growth was only mildly inhibited by TPA at 10 ng/ml. In culture with both TPA and HMBA, V3-17 cell growth was somewhat more inhibited, but not to the same degree as growth of DS19 cells (Fig. 5). TPA at 10 ng/ml inhibited relatively slightly HMBA-induced V3-17 cell commitment or accumulation of benzidine-reactive cells (Table 1). This is in striking contrast to the complete inhibition by TPA of HMBA-mediated induction of cell lines DS19 (Table 1) or N23 (data not shown).

Effect of Dexamethasone on V3-17 MEL Cells. Dexamethasone inhibits HMBA-induced expression of terminal differentiation in cell line DS19 (2). V3-17 cells in culture with dexamethasone without or with HMBA displayed growth characteristics similar to DS19 cells cultured under the same conditions (Fig. 5). HMBA-induced V3-17 commitment and accumulation of benzidine-reactive cells were much less inhibited by the steroid compared with DS19 cells (Table 1). Furthermore, it was consistently observed that V3-17 cells cultured with TPA or dexamethasone, without inducer, displayed a lower spontaneous level of benzidine-reactive cells (after 5 or 6 days in culture) and a somewhat lower level of spontaneous commitment than did V3-17 cells cultured without inhibitor (Table 1).

FIG. 5. Effect of TPA and dexamethasone on cell growth of MEL cells DS19 (vincristine sensitive) and V3-17 (vincristine resistant). (Left) DS19 cells. ♦, Grown without addition (control); ▲, grown with HMBA; ●, grown with dexamethasone (DEXA); ▼, grown with TPA; ■, grown with HMBA and dexamethasone (H + DEXA); ◆, grown with HMBA and TPA (H + TPA). (Right) V3-17 cells grown under similar conditions as indicated for DS19 cells.

PKC Activity in MEL Cells. Some of us have reported (12) that HMBA-mediated MEL cell DS19 differentiation involves a PKC-related mechanism (12). The evidence for this includes the observation that (i) TPA inhibits HMBA-induced MEL cell differentiation and causes depletion of total PKC activity, (ii) MEL cells depleted of PKC activity by exposure to TPA are resistant to induction by HMBA, and (iii) upon removal of TPA, restoration of sensitivity to HMBA accompanies reaccumulation of PKC activity. The PKC activity in V3-17 was found to be almost 2-fold higher in the V3-17 cells compared to N23 cells—7.0 units per 10^6 cells and 3.9 units per 10^6 cells, respectively, on average.

Earlier studies (12) found that HMBA induces the formation of a soluble, proteolytically activated form of PKC that is catalytically active in the absence of Ca^{2+} and phospholipid. The protease inhibitor, leupeptin, blocks formation of this activated form of PKC and inhibits HMBA-induced MEL cell (DS19 or N23) hemoglobin accumulation. Leupeptin (0.1 mM) added to cultures of V3-17 cells inhibited HMBA-mediated induction of benzidine-reactive cells to approximately the same degree as observed with the DS19 or N23 cell lines. This suggests that the V3-17 cells may share with DS19 a common target of leupeptin action, which lies on the pathway to induced differentiation.

DISCUSSION

These studies describe the characteristics of a new variant of MEL cells that was developed as a cell line resistant to vincristine inhibition of cell growth. The concentration of vincristine to which these cells are resistant is 5 ng/ml, a relatively low level of drug resistance, since the relative resistance of several established multidrug resistant cell lines is 20- to several-hundred-fold greater (28). This variant MEL cell line, V3-17, displays remarkable sensitivity to the induction of differentiation by HMBA. V3-17 MEL cells are (i) induced to commit with little or no latent-period characteristic of the induction of DS19 MEL cells; (ii) induced more rapidly to accumulate benzidine-reactive cells and to commit to terminal cell division than are the vincristine-sensitive cell lines; (iii) responsive to lower concentrations of HMBA—e.g., 1/10th the concentration of HMBA optimal for inducing vincristine-sensitive MEL cells will induce well over 50% of V3-17 cells; and (iv) resistant to both phorbol ester and dexamethasone inhibition of HMBA-induced differentiation.

HMBA induction of the DS19 MEL cell line is a multistep process (1, 17, 18) that entails an initial, latent period of ≈ 10 –12 hr before detectable commitment. In V3-17 cells, the time-dependent process involved in this latent period is

essentially eliminated. Although this study does not define the mechanism of the altered response to HMBA of V3-17 compared with DS19, the present evidence leads us to hypothesize that the lack of the latent period during HMBA-induced differentiation of V3-17 cells may be the result of constitutive expression of a factor that circumvents HMBA-mediated early events in the multistep pathway of induced differentiation.

The initiation of inducer-mediated V3-17 cell commitment with little or no latent period is similar to that seen in DS19 cells cultured with inducer in the presence of a calcium ionophore (9). Certain vincristine-resistant cell lines have been shown to amplify a genomic region that contains a small group of related genes designated *mdr* (29–32). Several lines of evidence suggest that the *mdr* protein acts as a drug efflux pump (28). Furthermore, the increased rate of drug removal from multidrug resistant cells is energy dependent (33–35), and calcium-channel blockers such as verapamil can overcome multidrug resistance *in vivo* and *in vitro* (36). Altered ion transport in V3-17 cells could be a factor determining the differences between DS19 and V3-17 cell response to HMBA. Other inducer-mediated events involved in the pathway leading to expression of the terminal erythroid phenotype may be sites of modification in vincristine-resistant MEL cells. There is a transient inhibition of DNA replication, accumulation of single-strand DNA breaks, and prolongation of G_1 during HMBA induction of DS19 cells (37, 38). Inhibition of DNA replication is associated with accumulation of a factor, probably a protein, which acts synergistically with a second factor, also induced by HMBA, to cause commitment (39–41). The latter activity is short-lived and inhibited by TPA. It will be interesting to test whether this TPA-sensitive factor is present in V3-17 cells.

Vincristine can prevent tubulin polymerization to microtubules and, thus, exert an anti-mitotic effect (42). In V3-17 MEL cells, a change in tubulin structure may account for the resistance of these cells to vincristine and an alteration in cell-cycle progression, which could be associated with the altered response of V3-17 cells to HMBA.

We previously reported that the tumor promoter TPA and the steroid dexamethasone are potent inhibitors of HMBA-induced DS19 cell differentiation (2, 19). The evidence from these studies indicated that HMBA in the presence of TPA or dexamethasone initiates changes that prepare MEL cells for commitment to terminal cell division and globin gene expression, since removing the inhibitors is associated with rapid commitment, without additional latent periods (2). V3-17 cells, which are not as sensitive to TPA or dexamethasone, may have established certain changes that are inducer-mediated in DS19 cells and are the target of the TPA or dexamethasone block to terminal differentiation.

We have recently found that PKC activity plays a role in HMBA-induced modulation of globin gene expression (12). PKC activity can be increased several fold in certain drug-resistant tumor cell lines (43, 44). We find an almost 2-fold greater PKC activity in vincristine-resistant MEL cells compared to vincristine-sensitive cells. The increased PKC activity in vincristine-resistant cells does not abolish the capacity of leupeptin, a protease inhibitor that blocks the conversion of PKC to the soluble Ca^{2+} - and phospholipid-independent but catalytically active form of the enzyme, to inhibit HMBA-mediated differentiation. The effect of leupeptin in blocking HMBA-induced hemoglobin accumulation was similar with V3-17 and DS19 or N23 cells. This provides further evidence of a role for proteolysis in MEL cell-induced differentiation and the possible involvement of a soluble Ca^{2+} - and phospholipid-independent form of the kinase.

The V3-17 MEL cell line provides a tool to dissect further the steps involved in HMBA-mediated MEL cell terminal erythroid differentiation. In particular, it will be important to

Table 1. Effect of TPA and of dexamethasone on HMBA-induced commitment and accumulation of benzidine-reactive cells

Addition*	DS19 cells, %		V3-17 cells,† %	
	Benzidine reactive‡	Commitment§	Benzidine reactive‡	Commitment§
None	0	0	7	9
HMBA	90	82	100	97
HMBA + DEXA	2	0	85	81
HMBA + TPA	0	0	80	74
DEXA	0	0	0	4
TPA	0	0	2	4

*HMBA was at 5 mM; dexamethasone (DEXA) at 4 μM ; and TPA at 10 ng/ml.

†V3-17 cultures included vincristine at 5 ng/ml and the additions indicated.

‡Benzidine-reactive cells were scored after 6 days in culture ($0 \leq 1.0\%$).

§Commitment was assayed after 2 days in culture.

characterize the differences between DS19 and V3-17 cells that make the latter inducible without a latent period and more sensitive to the action of HMBA.

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