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Hexamethylenebisacetamide-induced erythroleukemia cell differentiation involves modulation of events required for cell cycle progression through G_1

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ABSTRACT **Hexamethylenebisacetamide (HMBA), a potent inducer of differentiation of transformed cells such as murine erythroleukemia cells, causes a prolongation of the G_1 phase of the cell cycle during which commitment to terminal differentiation is first detected. Removal of HMBA prior to the G_1 phase aborts commitment. To further define the relationship between the G_1 phase and commitment to differentiation, we used two inhibitors of cell cycle progression: aphidicolin, which blocks cells at the G_1/S interphase, and deferoxamine, which blocks cells at an earlier stage during G_1. HMBA-induced prolongation of G_1 is associated with the accumulation of underphosphorylated retinoblastoma protein, decrease in cyclin A protein levels, and commitment to differentiation. G_1 arrest of murine erythroleukemia cells induced by aphidicolin or deferoxamine is not associated with accumulation of underphosphorylated retinoblastoma protein, suppression of cyclin A protein, or commitment of cells to terminal differentiation. Neither of the cell cycle inhibitors alters the effect of HMBA in inducing the G_1-associated changes or commitment to differentiation. Taken together, the present findings indicate that the site of action of HMBA which leads to commitment is in a stage of the G_1 phase prior to the point of cell cycle block caused by deferoxamine or aphidicolin. HMBA appears to cause cell differentiation with suppression of cell cycle progression by an action that affects events required for cell progression through G_1, including accumulation of underphosphorylated retinoblastoma protein and changes in regulation of cyclin levels.**

Several studies indicate that the retinoblastoma-susceptibility gene product, the RB protein, is an important regulator of cell cycle progression (1–6). RB is a nuclear protein whose phosphorylation is regulated during progression of cells through the cell division cycle. The phosphorylation of RB appears necessary for the transition from G_1 to S phase and the underphosphorylated form of RB is associated with arrest of cells in G_1. It has been suggested that RB regulates transcription of growth-regulatory genes through its interaction with transcription factor E2F and possibly other factors (7–9). Recent evidence suggests that RB is a substrate of cyclin-dependent kinases (10–12). A series of steps must occur during the G_1 phase for mammalian cells irreversibly to enter the S phase, including expression of various cyclins (e.g., cyclins D, E, and A) which associate with one or another of the cyclin-dependent kinases (10, 11, 13–15). Regulation of expression of these cyclins and RB phosphorylation by extrinsic signals, such as growth factors (16) or chemical inducers of differentiation (6, 17), are involved in the pathway determining cell proliferation or differentiation.

Hexamethylenebisacetamide (HMBA) induces murine erythroleukemia cell (MELC) commitment to terminal erythroid differentiation. Commitment is defined as the irreversible ability of the cell to express the differentiated phenotype, including globin gene expression, and cease DNA synthesis, despite removal of the inducer. HMBA-mediated induction is characterized by a series of changes in gene expression which occur within 1–4 hr of exposure to the inducer, including cessation of transcription of c-*myb* and c-*myc* genes and down-regulation of p53 protein levels (18). Although these changes occur in most, and perhaps all, cells and appear to be required for subsequent commitment, they are not in themselves sufficient since commitment, which can first be detected after 11–12 hr in culture, can be aborted if the inducer is removed prior to this time (19). These observations suggested that another inducer-mediated step (or steps) is required to recruit cells to terminal differentiation. We have shown that HMBA causes a prolongation of G_1 (20) during which there is an increase in the amount of underphosphorylated RB (6) and a suppression of cyclin A expression and a marked decrease in histone H1 kinase activity (17). HMBA-induced commitment to terminal differentiation is a stochastic process. Thus, during the first inducer-mediated prolonged G_1, only 15–20% of the cells become committed. It may require two to four subsequent cell cycles for essentially 100% of the cells to be recruited to terminal differentiation. We have suggested that the level of a factor regulating cell cycle progression, possibly underphosphorylated RB, plays an important role in determining whether MELCs become committed to terminal differentiation (6). Consistent with this hypothesis is the finding that during HMBA-induced terminal cell divisions, the increase in the proportion of cells committed to terminal differentiation is accompanied by an increase in the amount of RB (6).

The present study further defines the relationship between the G_1 phase of the cell cycle and commitment to terminal differentiation. In these studies, we blocked cell cycle progression from G_1 to S with two different agents, aphidicolin or deferoxamine, which arrest cells at different stages of the cell cycle. We analyzed cells arrested in G_1 by each of these inhibitors and by the inducer, HMBA, with respect to accumulation of underphosphorylated RB, expression of cyclin A protein, prolongation of G_1 phase, and commitment of cells to terminal cell division.

MATERIALS AND METHODS

Cell Culture. MELC line DS19/Sc9, derived from 745A cells (19), was maintained in α modified minimal essential medium supplemented with 10% (vol/vol) fetal bovine se-

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Abbreviations: HMBA, hexamethylenebisacetamide; MELC, murine erythroleukemia cell.

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rum. Suspension cultures were initiated with cells in logarithmic growth phase at a density of 10^5 cells per ml. Commitment to terminal differentiation was assayed as described (19). Aphidicolin (Sigma) was dissolved at a concentration of 10 mM in dimethyl sulfoxide. Deferoxamine (CIBA–Geigy) was prepared as a 200 mM stock solution in water. In preliminary experiments, we determined the concentrations of aphidicolin and of deferoxamine which inhibited $G_1 \rightarrow S$ progression with minimal cell death. In these studies, it was also determined at what time after initiation of culture with cells synchronized in G_1 the inhibitors should be added to result in a G_1 arrest following passage through S, G_2, and M phases of the cell cycle. By these criteria, the final concentrations used in cell cultures were 2 μM and 50 μM for aphidicolin and deferoxamine, respectively. Aphidicolin was added at 10 hr and deferoxamine at 7 hr after initiation of the culture.

Cell Cycle Synchronization. After 36 hr in culture, 6×10^8 MELCs in logarithmic growth were loaded onto a Beckman JE-6B elutriation rotor as described (21), and fractions were elutriated by stepwise increase in the rate of pump flow. Homogeneity of the cell size was determined with a Coulter Counter, and fractions of cells predominantly in G_1 were pooled. Cellular DNA content was analyzed by flow cytometry after cells were stained with propidium iodide (50 μg/ml; Sigma) (21). The percentage of cells in each phase of the cell cycle was determined with a CELL FIT program (Becton Dickinson).

Assay for RB and Cyclin A Proteins. Cells were lysed in 50 mM Tris·HCl, pH 8.0/250 mM NaCl/1% (vol/vol) Nonidet P-40/0.5% (wt/vol) sodium deoxycholate/0.1% (wt/vol) SDS/5 mM EDTA/50 mM NaF/0.5 mM Na_3VO_4/0.5 mM phenylmethylsulfonyl fluoride containing aprotinin (10 μg/ml), leupeptin (10 μg/ml), and L-1-tosylamido-2-phenylethyl chloromethyl ketone (10 μg/ml), and the lysate was clarified by centrifugation. Protein was assayed by using the Bradford reagent (22) with bovine serum albumin as a standard. Anti-RB monoclonal antibody pMG3-245.11 (PharMingen, San Diego) and anti-cyclin A polyclonal antiserum, a gift from J. Pines (15), were used to assay for RB and cyclin A, respectively. Fifty micrograms of protein was mixed with the SDS sample buffer, boiled, and subjected to SDS/PAGE. Acrylamide concentrations of the gels were 7.5% for RB and 12.5% for cyclin A. Electrophoretic transfer of proteins to nitrocellulose (0.45-μm pore, Schleicher & Schuell) was performed for 14 hr at 45 V (23). Filters were blocked in 10 mM Tris·HCl, pH 7.5/150 mM NaCl/0.1% (vol/vol) Tween 20 supplemented with 5% (vol/vol) skimmed milk. All antibody binding reactions were performed in 4% skimmed milk/25 mM Tris·HCl, pH 7.5/125 mM NaCl/0.1% Tween 20/0.1% NaN_3 for 1 hr with shaking at room temperature. Anti-RB antibody was used at 10 μg/ml. To detect the specific binding, biotinylated anti-mouse IgG antibody (Vector Laboratories) was used at 3 μg/ml, followed by a wash with 10 mM Tris·HCl, pH 7.5/125 mM NaCl/0.05% Nonidet P-40 and incubation with ^{125}I-labeled streptavidin (40 μCi/μg, Amersham) at 1 μCi/ml (1 μCi = 37 kBq). The dried filter was exposed to Kodak XAR film. Cyclin A was detected with the anti-cyclin A polyclonal antiserum at 1:1000 dilution, and horseradish peroxidase-linked anti-rabbit immunoglobulin antibody (Amersham) was used at 1:1000 dilution as the secondary antibody. Specific proteins were visualized with an enhanced chemiluminescence system (Amersham). The films were scanned with an LKB 2202 Ultrascan densitometer.

RESULTS

Effect of Aphidicolin on MELCs Cultured Without and With HMBA. *Cell cycle progression.* Aphidicolin is a potent inhibitor of DNA polymerases and blocks cells at the G_1/S interphase (24, 25). Its effects can be reversed by removal of the inhibitor. Populations of MELCs synchronized in the G_1 phase of the cell cycle were suspended in culture medium without or with 5 mM HMBA (Fig. 1). By 10 hr in culture without inducer, cells were distributed predominantly between the G_2/M and G_1 phases; by 12 hr they showed further progression from G_2/M to G_1, and by 15 hr they entered S phase (Fig. 1, column 1). By comparison, cells in culture with HMBA remained predominantly in G_1 through at least 19 hr (Fig. 1, column 3). Addition of 2 μM aphidicolin to cultures without HMBA (Fig. 1, column 2) at 10 hr after initiation of the culture resulted in arrest of cells in G_1 through 19 hr. Similar results were obtained when aphidicolin was added to cultures with HMBA (Fig. 1, column 4). At 19 hr, cells from each of the four sets of cultures were recovered by centrifugation and suspended in fresh culture medium without HMBA (Fig. 1, columns 1 and 3) or with HMBA (columns 2 and 4), without aphidicolin. The cells resumed progression through the cell division cycle. Thus, aphidicolin inhibited transition of cells from G_1 to S phase, and the effect was reversed on removal of the inhibitor.

Phosphorylation of RB, level of cyclin A, and commitment to terminal differentiation. We then determined whether,

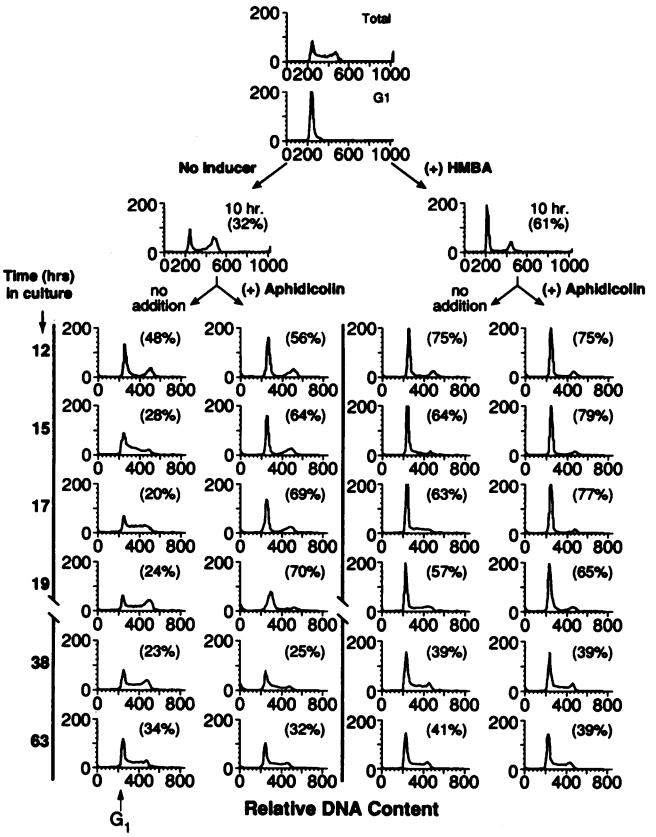

FIG. 1. Effects of HMBA and aphidicolin on the cell cycle progression of MELCs. A synchronized population of cells predominantly in G_1 (prepared from the total population of MELCs by elutriation) was placed in culture without or with 5 mM HMBA. Ten hours after initiation of these cultures, each was divided into equal portions and aphidicolin (final concentration, 2 μM) was added to one of each of the two pairs of cultures. At 19 hr, each of the four cultures was centrifuged, and the cells were recovered and resuspended in fresh medium without (columns 1 and 3) or with (columns 2 and 4) HMBA, without aphidicolin. Aliquots of the cultures were removed at the times indicated on the left and the distribution of cells in the cell cycle was determined by flow cytometry. Percentages indicate the proportion of cells in G_1. Abscissa, relative DNA content; ordinate, relative number of cells.

during prolongation of G_1 caused by aphidicolin, there was an accumulation of underphosphorylated RB, suppression of cyclin A protein, and commitment to terminal cell differentiation, as observed with HMBA. Underphosphorylated RB, which migrates more rapidly in SDS/PAGE than the hyperphosphorylated form, accumulated in HMBA-induced MELCs during the prolonged G_1 (Fig. 2*A*, lane H). No accumulation of underphosphorylated RB was detected in cells cultured with aphidicolin without HMBA (Fig. 2*A*, lane A). Cells in culture with HMBA and aphidicolin accumulated underphosphorylated RB to a similar extent as those treated with HMBA alone (Fig. 2*A*, lane A+H). There was a marked decrease in cyclin A protein in HMBA-induced MELCs during the prolonged G_1 (Fig. 2*B*, lane H). By comparison, aphidicolin-mediated G_1 prolongation was not associated with suppression of cyclin A expression. Rather, aphidicolin appeared to increase the cyclin A protein level of G_1 cells compared with cells in culture without the inhibitor. MELCs in culture with aphidicolin and HMBA had a level of cyclin A during G_1 comparable to that of cells cultured without inducer or inhibitor (Fig. 2*B*, lane A+H).

HMBA induced commitment to terminal cell division in a stochastic manner (Fig. 2*C*) (19); 8% of cells were committed after 12 hr, 33% after 38 hr, and 65% after 63 hr in culture.

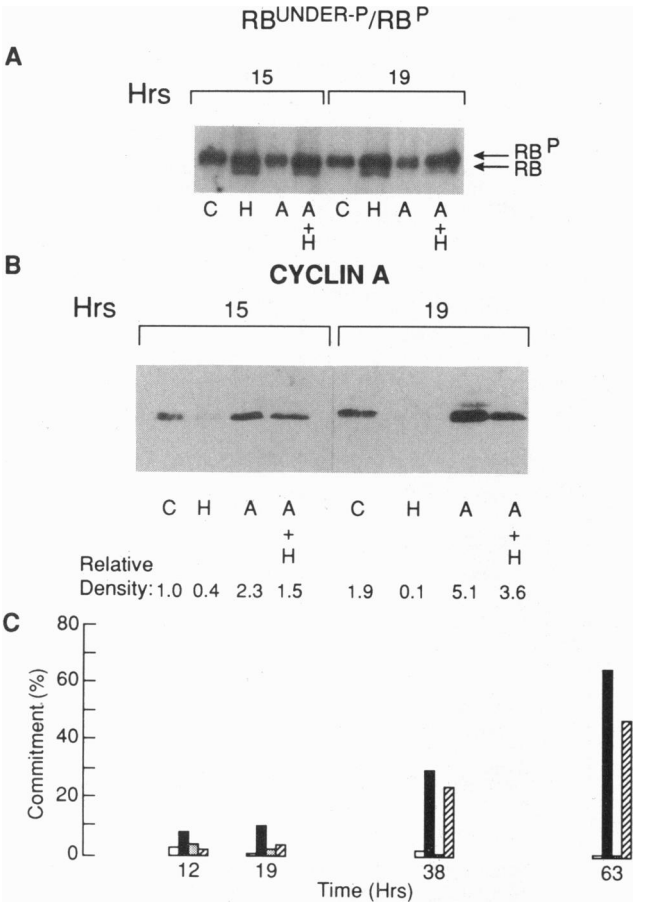

FIG. 2. Effects of HMBA and aphidicolin on phosphorylation of RB, level of cyclin A, and commitment to terminal differentiation in MELCs. Cell extracts were prepared at the times indicated from the cells in the study illustrated in Fig. 1. C, control; H, HMBA; A, aphidicolin. (*A*) Immunoblot of RB. (*B*) Immunoblot of cyclin A. Relative density of cyclin A signal, assayed by densitometric scanning, is indicated. (*C*) Commitment of MELCs in culture without addition (open bars), with 5 mM HMBA (solid bars), with 2 μM aphidicolin (added at 10 hr and removed at 19 hr) (stippled bars), and with 5 mM HMBA plus 2 μM aphidicolin (aphidicolin added at 10 hr and removed at 19 hr) (hatched bars).

Aphidicolin did not induce MELC commitment, and the addition of aphidicolin to cells in culture with HMBA did not alter the extent of HMBA-induced commitment.

Effect of Deferoxamine on MELCs Cultured Without or With HMBA. *Cell cycle progression.* Deferoxamine is an iron-chelating agent which causes cell cycle arrest in G_1. The major effect of this agent appears to be inhibition of ribonucleotide reductase (26). The deferoxamine-induced block in G_1 occurs at an earlier stage of the cell cycle than the aphidicolin block (27). Effects of deferoxamine on progression of MELCs through the cell cycle were examined with an experimental design similar to that used in the study with aphidicolin (Fig. 3). Deferoxamine was added 7 hr after initiation of the culture, with cells synchronized in G_1. At 16 hr after initiation of the culture (9 hr after addition of deferoxamine), the proportion of cells in G_1 phase was substantially higher than that of cells in culture without the inhibitor (Fig. 3, compare columns 1 and 2). By 20 hr, without removal of deferoxamine from the culture, most of the cells had progressed from G_1 to S (Fig. 3, column 2). Cells in culture with HMBA and deferoxamine progressed through the cell cycle with kinetics similar to those cells in culture with HMBA alone (Fig. 3, columns 3 and 4).

Phosphorylation of RB, level of cyclin A, and commitment to terminal cell differentiation. Deferoxamine alone caused no detectable accumulation of underphosphorylated RB during the prolonged G_1 phase (Fig. 4*A*, lane D). HMBA induced a similar level of accumulation of underphosphorylated RB in cells in culture without or with addition of deferoxamine (Fig. 4*A*, lanes H and D+H). HMBA-treated cells showed a marked suppression of cyclin A protein at 12 hr and reaccumulated the protein by 16 hr and 20 hr (Fig. 4*B*, lane H). In this study, a substantial proportion of cells had progressed to

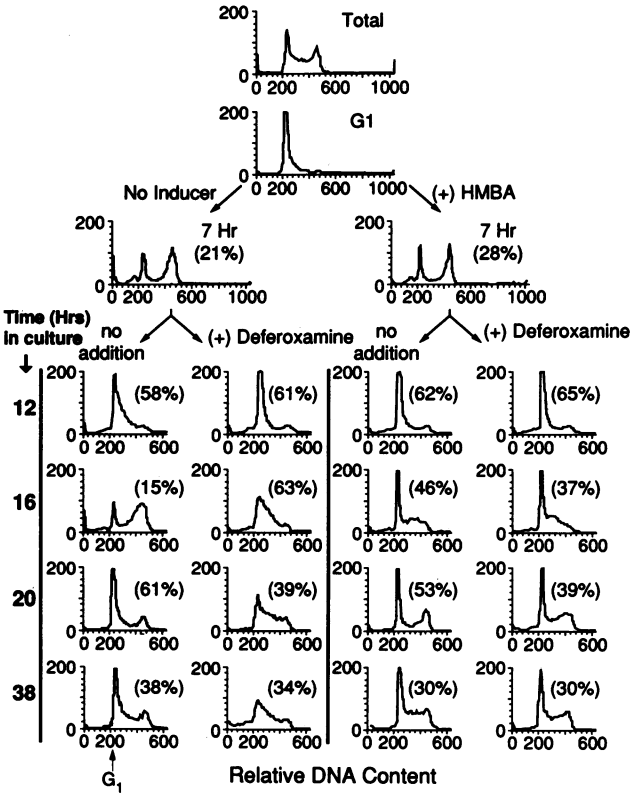

FIG. 3. Effects of HMBA and deferoxamine on the cell cycle progression of MELCs. These studies were performed as described in the legend to Fig. 1, save that 50 μM deferoxamine was added 7 hr after initiation of cultures and remained in the medium throughout the period of culture.

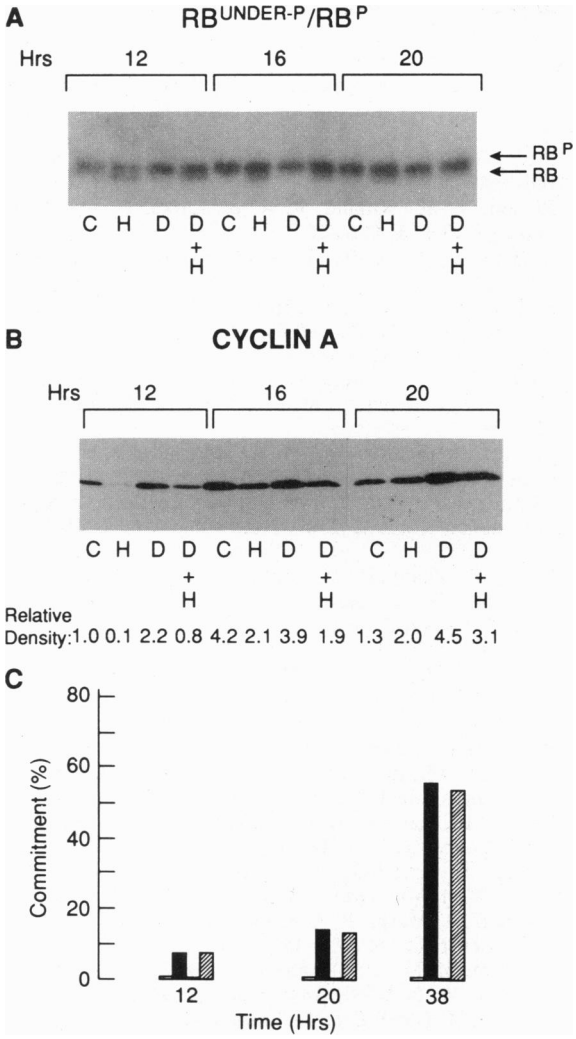

FIG. 4. Effects of HMBA and deferoxamine on phosphorylation of RB and commitment to terminal differentiation in MELCs. Extracts were prepared at the times indicated from the cells in the study illustrated in Fig. 3. (*A*) Immunoblot of RB. (*B*) Immunoblot of cyclin A. Relative density of cyclin A signal, assayed by densitometric scanning, is indicated. (*C*) Commitment of MELCs in culture without addition (open bars), with 5 mM HMBA (solid bars), with 50 μM deferoxamine (stippled bars), and with 5 mM HMBA plus 50 μM deferoxamine (hatched bars).

S and G_2/M by 16 and 20 hr, respectively (Fig. 3, column 3). The level of cyclin A has been shown to increase as MELCs transit from G_1 to S and to G_2/M (17). MELCs in culture with HMBA were transiently delayed in G_1 compared with cells in culture without inducer, but the prolongation of G_1 in the study illustrated in Fig. 3 was shorter than that illustrated in Fig. 1, a variation observed among these experiments. Deferoxamine caused an increase in the cyclin A level at 12–20 hr compared with that in cells cultured without inhibitor (Fig. 4*B*, lanes C and D). Addition of deferoxamine to cells in culture with HMBA results in higher levels of cyclin A at 12 hr compared with that in cells cultured with inducer alone (Fig. 4*B*, lanes H and H+D). Cells in culture without additions (Fig. 4*B*, lane C) had a lower level of cyclin A at 20 hr than at 16 hr and similar to that in cells at 12 hr, reflecting the fact that most of the cells were again in G_1.

Deferoxamine did not induce MELC commitment to differentiation (Fig. 4*B*) and did not alter the proportion of HMBA-induced MELCs committed to differentiate when compared with cells in culture with HMBA alone (Fig. 4*B*).

Effect of Delaying Addition of HMBA to Cultures of MELCs. We examined the effect of delaying for 7 hr addition of HMBA to cultures without or with deferoxamine. As controls, cells were cultured (*i*) without addition, (*ii*) with HMBA added at initiation of culture, and (*iii*) with inducer added at initiation of culture and deferoxamine at 7 hr of culture. The commitment to differentiation with HMBA added at 7 hr without or with deferoxamine was similar—10% and 12% by 20 hr, respectively. The commitment to differentiation with HMBA added at time of initiation of culture without or with deferoxamine added at 7 hr was also similar—22% and 24% by 20 hr, respectively. These studies indicate that prolongation of G_1 by deferoxamine had no effect on HMBA-induced MELC commitment, whether HMBA was added at initiation of culture or 7 hr later. Fewer cells were committed when addition of HMBA was delayed, presumably reflecting the shorter duration of culture with inducer—13 hr versus 20 hr.

In a further study, HMBA addition to cultures of G_1-synchronized cells was delayed until 7, 10, and 13 hr after initiation of culture. No inhibitor was added to these cultures. The commitment to differentiation determined 21 hr after onset of culture was 5% when HMBA was added at 10 hr, undetectable when HMBA was added at 13 hr, 19% when HMBA was added at zero time, and 7% when HMBA was added at 7 hr.

DISCUSSION

HMBA, a potent inducer of differentiation of transformed cells such as MELCs (28), causes a block to phosphorylation of RB and a suppression of cyclin A protein that are associated with prolongation of the G_1 phase of the cell cycle. On the other hand, G_1 arrest of MELCs induced by aphidicolin, which blocks at the G_1/S interphase, or by deferoxamine, which blocks at an earlier stage during the G_1 phase, is not associated with accumulation of underphosphorylated RB, suppression in expression of cyclin A, or commitment to terminal differentiation. Further, neither aphidicolin nor deferoxamine alters the effect of HMBA in inducing G_1-phase prolongation and commitment to differentiation. We previously showed that HMBA did not cause commitment if it was removed from culture prior to progression of MELCs to the G_1 phase (19). In those studies, addition of HMBA to MELCs enriched for cells in G_1 7 and 10 hr after onset of culture was associated with a small proportion of cells becoming committed following the ensuing G_1, but not if addition of inducer was delayed for 13 hr. Taken together, these findings suggest that the site of action of HMBA which leads to commitment is in an early stage of the G_1 phase and prior to the point at which cell cycle progression is blocked by deferoxamine or aphidicolin.

The underphosphorylated form of RB appears to suppress the progression of cells from G_1 to S phase, and this action can be abolished by phosphorylation of RB during G_1 (1–6). Strong evidence for a role for RB in cell cycle regulation was the demonstration that microinjection of purified RB into cells lacking wild-type RB caused a reversible G_1 arrest (29). In those studies it was shown that the cells did not undergo arrest if microinjection was after the middle of G_1. Several lines of evidence suggest that there is a critical point during the G_1 phase of mammalian cells prior to which external stimuli, such as growth factors, act to effect either continued cell proliferation or terminal differentiation (30). HMBA may also act prior to such a critical point in G_1 to cause those changes which initiate commitment to terminal differentiation.

Cells cultured with aphidicolin or with deferoxamine exhibited an increase in cyclin A. A possible explanation for this finding is that neither of these agents prevents accumulation

of cyclin A in late G_1 or early S, but either agent may inhibit cyclin A degradation (15).

Previous studies, using MELCs synchronized with respect to cell cycle phase, showed that HMBA must be present during G_1 for subsequent commitment to terminal differentiation (21). In molecular terms, HMBA causes a prompt cessation in c-*myc* and c-*myb* gene transcription and down-regulation of p53 protein (18). The subsequent G_1 is prolonged and underphosphorylated RB accumulates (6) and cyclin A protein is down-regulated and cyclin-dependent kinase activities decrease (17). During the initial prolonged G_1, only about 15% of the cells become committed to terminal differentiation (19) with increased transcription of globin genes (21). It requires two to four additional cell cycle generations to recruit virtually all cells to arrest in G_1-phase and commit to terminal differentiation. To become committed, a cell may have to achieve a critical level of some factor, such as underphosphorylated RB. According to this hypothesis, HMBA causes only about 15% of cells to achieve such a level during the initial prolonged G_1, and then, with each succeeding G_1 additional cells are induced to attain the critical level of the factor(s) regulating cell cycle progression and recruitment to differentiation.

It is clear from the present results that simply prolonging G_1 by exposure to either aphidicolin or deferoxamine does not cause an increase in underphosphorylated RB accumulation, suppression of cyclin A protein, or commitment to differentiation. Further, neither of these agents inhibits or augments the action of HMBA in inducing MELC differentiation. These agents presumably block the $G_1 \rightarrow S$ progression at a stage of the cell cycle subsequent to that at which HMBA affects cell cycle regulators.

Studies with transforming growth factor (31, 32) and colony-stimulating factor 1 (10, 16) indicate that a critical site of action of these agents in affecting cell proliferation and differentiation is in the G_1 phase. Transforming growth factor β has been shown to prevent RB phosphorylation and suppress progression into S phase. Colony-stimulating factor 1 and other growth factors cause expression of cyclins during the G_1 phase of the cell cycle; these cyclins appear to govern G_1 progression and/or entry into S phase. HMBA also appears to affect cell differentiation with suppression of cell cycle progression by causing alterations in RB, cyclins, and cyclin-related kinase activity during G_1 and to favor differentiation of MELCs.

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