Calcium Regulates the Commitment of Murine Erythroleukemia Cells to Terminal Erythroid Differentiation

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ABSTRACT An alteration in the rate of calcium transport appears to be the rate-limiting event for the commitment of murine erythroleukemia (MEL) cells to initiate a program of terminal erythroid differentiation. The dimethyl sulfoxide (DMSO)-induced commitment of MEL cells to erythroid differentiation can be inhibited by treatment of cells with the calcium-chelating agent EGTA. Upon removal of EGTA, cells initiate commitment without the 12-h lag normally observed after treatment with DMSO alone. Treatment of cells with DMSO in the presence of calcium ionophore A23187 causes cells to initiate commitment from time zero with no lag. These results suggest that the lag is the time required for DMSO to alter the calcium transport properties of the cell.

After exposure to dimethyl sulfoxide (DMSO) or a wide variety of other agents, murine erythroleukemia (MEL) cells undergo a program of terminal erythroid differentiation (3). MEL cell differentiation closely resembles the process of normal murine erythropoiesis (4). The differentiation of MEL cells thus represents a useful model system in which the regulation of erythropoiesis can be studied at both the cellular and molecular levels.

A central question in MEL cell differentiation concerns the mechanism of cellular response to inducer treatment. We have approached this problem by examining the kinetics with which individual cells become committed to the program of terminal erythroid differentiation. This method has shown that (*a*) a lag of at least 9 h occurs between the addition of inducer and the appearance of a significant proportion of committed cells in culture (6), (*b*) once a cell is committed, it no longer requires the presence of inducer to execute the differentiation program (6), and (*c*) the kinetics of commitment of individual cells are consistent with a stochastic model for the commitment event (2).

The identification of a specific reprogramming step that initiates a coordinated differentiation program raises the issue, "What is the molecular basis for the commitment process?" Previous studies (7, 8) have shown that an increase in the intracellular level of calcium ions occurs during the lag period. Inhibition of calcium transport prevents commitment, suggesting that an influx of calcium ions plays a central role in the commitment process. Here we have focused directly on the relationship between calcium transport and the initiation of commitment. Treatment of cells with DMSO in the presence of the calcium ionophore A23187 causes the initiation of commitment with no lag. This suggests that the lag is the time required for DMSO to alter the calcium transport properties of the cell.

MATERIALS AND METHODS

Cell line 745 was originally obtained from Dr. C. Friend (Mt. Sinai School of Medicine, N. Y.). 745-PC-4 is a subclone of this cell line. Cultures were maintained in α medium lacking nucleosides and supplemented with 13% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.). Cell density was maintained at between 1×10^4 and 5×10^5/ml to maintain continuous logarithmic growth. Cell counts were performed with an automatic cell counter (Coulter Counter model ZBI, Coulter Electronics Inc., Hialeah, Fla.)

Plasma culture was performed as described by McLeod et al. (9) and Gusella et al. (2). Briefly, 100–200 cells were plated in 0.1-ml clots in microtiter wells (Linbro Scientific Co., Hamden, Conn.) that had been sterilized by ultraviolet radiation. The clots were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 90–100 h of culture, clots were transferred to precleaned microscope slides, dehydrated with filter paper, fixed in glutaraldehyde, stained in benzidine, counterstained with hematoxylin, and covered with Permount (Fisher Scientific Co., Pittsburgh, Pa.) and a cover slip. Scoring of colony type was performed at a magnification of × 100. Ionophore A23187 was a gift from Eli Lilly Co. (Indianapolis, Ind.).

RESULTS

The Assay System

We have analyzed the occurrence of an irreversible commitment event in individual MEL cells by cloning cells in the plasma culture system originally adapted for erythroid culture by McLeod and co-workers (9). Cells exposed to an inducer in

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liquid culture are cloned in plasma culture in the absence of inducer. The phenotype of the colony that each cell gives rise to indicates for this individual cell whether exposure to inducer has altered its programming with respect to the expression of a differentiated function.

We have determined colony phenotype with respect to heme content using benzidine stain. Cells containing high levels of heme react strongly with benzidine reagent, giving a bright orange color. Cells with a basal level of heme do not react with benzidine reagent and are counterstained blue with hematoxylin. Because our experiments are designed to evaluate the state of programming of the cell giving rise to the colony with respect to heme synthesis, cells of an intermediate phenotype are almost never observed. This is because the 96-h incubation period is apparently sufficient for cells to fully execute a program leading to a high level of heme synthesis. Using these criteria, we have demonstrated that an uncommitted cell gives rise to an undifferentiated colony that will be large (>32 cells) and blue. A committed cell gives rise to a differentiated colony. Such a colony is small (<32 cells) and stains bright orange.

It is important to note that in the experiments reported here, cells derived from both drug-treated and control cultures give rise to colonies with an efficiency of >95% in plasma culture (data not shown). This indicates that the cells do not suffer cytotoxic or irreversible damage due to drug treatment or an adverse ionic environment during the course of an experiment (data not shown).

Alteration in Calcium Transport Is Required for the Initiation of Commitment

Initial experiments to analyze the relationship between the commitment process and calcium transport were carried out employing the calcium-chelating agent EGTA. We have previously shown that at an EGTA concentration of 2.7 mM, the free extra cellular level of calcium ions in the culture medium is reduced to submicromolar levels while MEL cell growth is only slightly (<10%) inhibited (7). As shown in Fig. 1, treatment of MEL cells with EGTA in the presence of 1.5% DMSO caused complete inhibition of commitment. This inhibition could be reversed by the removal of EGTA or the addition of excess calcium to the culture medium of EGTA-treated cells. Upon reversal of EGTA inhibition, commitment was initiated immediately, i.e., without the 12-h lag shown by a control culture treated with DMSO only. This result suggests that the lag observed before the initiation of commitment may reflect the time required for DMSO to alter the calcium transport properties of individual cells.

To test this idea directly, we treated MEL cells with the calcium ionophore A23187 to rapidly increase calcium transport rates. As shown in Fig. 2, a culture treated with 1.5% DMSO only attained a level of 37% committed cells by 42 h. The lag time of this culture was >12 h. A second culture was treated simultaneously with A23187 + 1.5% DMSO. By 42 h, this culture had reached a level of 64% committed cells. A third culture was treated with A23187 for 1 h, then was shifted into medium containing 1.5% DMSO only. This culture attained a level of 60% committed cells by 42 h. Both ionophore-treated cultures initiated commitment from zero time (time of DMSO addition) with no lag. These results suggest that making cells selectively permeable to calcium effectively circumvents the lag.

DISCUSSION

The results reported in the present study suggest that the intracellular level of calcium ions may act as a signal in the initiation of the commitment of MEL cells to terminal erythroid differentiation. That A23187 alone is not sufficient to induce commitment, as shown in Fig. 2, implies that a change in the rate of calcium transport alone is not a sufficient signal for the initiation of commitment. Alteration in at least one additional component must also be involved in the commitment process. This view is supported by the results shown in Fig. 1. The initiation of commitment with no lag upon reversal of EGTA

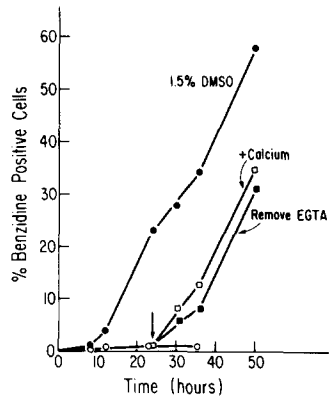

FIGURE 1 Effect of EGTA on MEL cell commitment. Logarithmically growing cells were grown in liquid culture in the presence of 1.5% DMSO and 2.7 mM EGTA for 24 h (○). At this time, cells were subcultured and growth continued in either 1.5% DMSO + 2.7 mM EGTA (○), 1.5% DMSO only (■), or 1.5% DMSO + 2.7 mM EGTA + 3 mM $CaCl_2$ (□). A control culture was grown continuously in the presence of 1.5% DMSO alone (●). At the times indicated aliquots of cells were plated in plasma culture in the absence of any drugs as described in Materials and Methods.

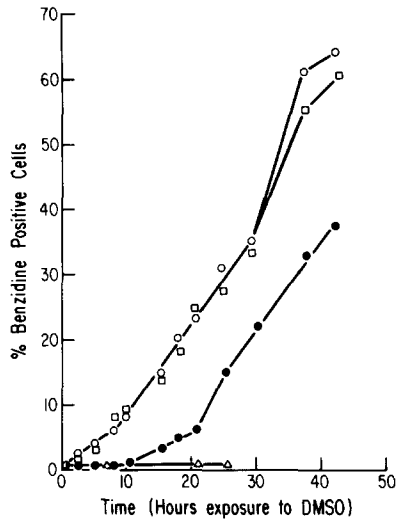

FIGURE 2 Effect of A23187 on lag. MEL cells were grown in liquid culture under the following conditions: (*a*) 1.5% DMSO alone (●). (*b*) A23187 (1 μg/ml) alone (Δ). (*c*) 1.5% DMSO + 1 μg/ml A23187 for 6 h. A23187 was removed and cells were grown in 1.5% DMSO only for the remainder of the experiment (○). (*d*) A23187 for 1 h. A23187 was removed and cells were grown in 1.5% DMSO for the remainder of the experiment (□). At the times indicated, aliquots of cells were removed and plated in plasma culture in the absence of DMSO or A23187. Conditions for plasma culture, harvesting of cells and scoring of colony type were as described in Fig. 1.

inhibition suggests that a cellular component necessary for commitment rapidly builds up in the presence of DMSO and is not inhibited by EGTA. Commitment can be initiated in such cells when EGTA is removed or calcium is made available. The lag must thus represent the time required for the slow accumulation of a second component required for commitment. Our results employing A23187 as well as our previous data employing the drug amiloride (8) suggest that the lag is the time required for a slow change in the calcium transport rate that allows for a slow buildup of calcium ions above a threshold level.

Recent data from several systems indicate that alterations in calcium transport may play a crucial role in initiating a variety of developmental processes. The activation of echinoderm and fish eggs after fertilization is accompanied by dramatic increases in the intracellular levels of calcium (11, 12). A high extracellular Ca^{2+} concentration is required for terminal differentiation of mouse epidermal cells (5). The course of MEL cell induction has also been shown to be sensitive to both extracellular and intracellular calcium levels (1). Interestingly, the erythropoietin-sensitive formation of normal murine erythroid colonies in vitro is stimulated by A23187 treatment and is inhibited by EGTA (10). These observations and the data presented in the current study suggest that calcium may play a key role not only in the process of MEL cell differentiation but in normal murine erythropoiesis as well.

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