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Role of calcium in differentiation of murine erythroleukemia cells

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ABSTRACT

Calcium plays a crucial role in the normal and abnormal cell metabolism. The role of calcium in the differentiation process of murine erythroleukemia cells(MELC) remains controversial. Here, based upon quantitative measurement of fluorescence in single cells, a method was developed to investigate the intracellular free calcium $[Ca^{2+}]i$ concentration and DNA contents simultaneously, by employing the fluorescent probe, fluo-3 acetoxymethyl ester and DNA dye Hoechst 33342. During MELC differentiation, $[Ca^{2+}]i$ concentration incresed. We also demonstrated that calcium ionophore, A23187, enhanced the HMBA-induced MELC differentiation, while verapamil, an inhibitor of calcuim uptake, slightly reduced differentiation. These results suggested that an increase in the $[Ca^{2+}]i$ level was an essential step in HMBA-induced MELC differentiation.

Key words: *calcium, fluo-3 AM, microphotometry, murine erythroleukemia cells.*

INTRODUCTION

After exposure to hexamethylene bisacetamide (HMBA) [1,2] or a wide variety of other agents, murine erythroleukemia cells (MELC) may undergo a program of terminal erythroid differentiation. The differentiation of MELC thus represents a useful model system in which the regulation of differentiation can be studied at both the cellular and molecular levels.

Calcium, a great signaler, is at the core of cell proliferation, differentiation and

neoplastic transformation. There are controversial reports regarding the role of calcium in the process of differentiation. Support for the importance of increased Ca^{2+} transport activity in the process was offered by Smith et al.[3], through studying the commitment to differentiation of MELC. Similarly, Sawyer et al. [4] have demonstrated an increased rate of calcium influx, efflux and steady-state level in differentiating FVA (anemia strain of Friend virus)-infected erythroid cells. Faletto and Macara[5] have presented data that disputed the correlation between increased calcium uptake and differentiation of MELC. Recently Housman et al. [6] demonstrated that an increase in cytosolic calcium can trigger the commitment to differentiation of MELC. To confirm that cytoplasmic Ca^{2+} is involed in the regulation of cell differentiation, we critically tested the role of calcium in MELC differentiation by direct measurement of $[Ca^{2+}]i$ and DNA contents, using fluo-3 AM and DNA binding dye Hochest 33342. We found that the $[Ca^{2+}]i$ increased with the MELC differentiation. We also presented that A23187 enhanced the HMBA induced MELC differentiation, while verapamil slightly reduced this process.

MATERIALS AND METHODS

Cell culture and the selection of highly differentiation clones

MELC were maintained in suspension cultures at 37 °C in humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle medium (DMEM), supplemented with 15% fetal calf serum. Cells were kept at densities between 1×10^4 and 1×10^6 cells /ml.

In order to obtain a homogeneous MELC population with high differentiation potential, cells were inoculated in 96-well plates at a final density of 1-10/ml, then individual clones were collected and induced with 5 mM HMBA respectively for 96 h. The clone with the highest benzidine positive percentage was chosen for the following experiments.

Differentiation induction and determination

Exponentially growing cells were suspended in DMEM at a concentration of $1\text{-}2\times10^5$/ ml and induced with 5 m*M* HMBA. The presence of benzidine-positive cells were assayed at times specified with each experiment. The cells were stained for hemoglobin using the benzidine staining procedures[7]. A fresh solution of 0.4% of 30% H_2O_2 (V /V) in the benzidine stock solution (0.2% benzidine dihydrochloride in 0.5 *M* acetic acid) was prepared just prior to use. One tenth volume of the staining solution was added to the cell suspension. The percentage of benzidine-positive cells was determined by counting 200-500 cells.

Measurement of relative volume of cells

By using Coulter counter and multichannel pulse height analyzer, the relative volumes of cell populations during the HMBA-induced MELC differentiation were measured.

Measurement of $[Ca^{2+}]i$ and nuclear DNA contents

Simultaneous measurements of $[Ca^{2+}]i$ and nuclear DNA were performed by using the Ca^{2+} sensitive fluorescence emission of fluo-3 and DNA binding dye Hoechst 33342.

The cells were loaded with 10 μM Hoechst 33342 and 10 μM fluo-3 AM in DMEM at 37 °C for 60 min. Generally, 1 μl of 25% (W /W) pluronic acid was added to disperse the mixture into aqueous solution. After 3 washes with DMEM, the fluorescence emission intensity of Hoechst 33342 and fluo-3 was measured in individual cells using a Leitz MPV2 microspectrofluorometer[14].

The calibration of the fluorescence emission of fluo-3 to calcium ion concentration was perfomed using ionomycin and heavy metal ion according to Kao et al.[8]. Simultaneously, the fluorescence intensity of Hoechst 33342 showed the relative contents of nuclear DNA.

Assay of the effect of A23187 or Vp on MELC differentiation

Exponentially growing cells were collected and inoculated in 24-well plates at a final density of 3-4$\times 10^4$ cells/ml. After the drugs (A23187 or verapamil) were added, the cultures were incubated at 37℃ for 3 d. Cell number was determined with a Coulter counter (Coulter Electronic Ltd, Model D). The results were represented as the ratio of cell numbers of test groups to those of control groups. We chose the dose with 20%-30% growth inhibition for the following experiment.

Exponentially growing cells were collected and incubated at a concentration of 1-2 x 10^5/ml with HMBA (5 m*M*) and A23187 (0.2 μg/ml) or verapamil (10 μ*M*). The benzidine positive cell percentage was determined at 24, 48, 72 and 96 h respectively.

RESULTS

1. Induction of MELC differentiation by HMBA

When MELC were cultured with 5 m*M* HMBA, cells were induced to differentiate. After 48 h exposure, the percentage of benzidine positive-differentiated cells was 35%, and this percentage reached its highest peak (90%) in 96 h (Fig 1). Therefore, HMBA was effective in the induction of MELC differentiation.

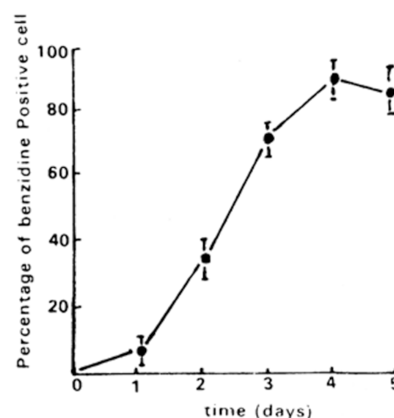

Fig 1. Effect of HMBA (5 m*M*) on MELC differentiation

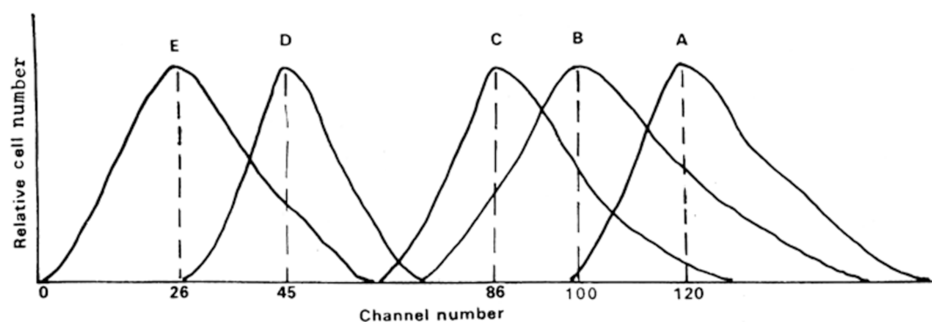

Fig 2.. Relative volume of MELC (A) and the cells treated with 5 mM HMBA for 24 h (B), 48 h (C), 72 h (D) and 96 h (E)

2. Change of relative volume of cells

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During the MELC differentiation induced by HMBA, the relative volume of cells decreased (Fig 2). When treated with 5 m*M* HMBA for 24, 48, 72 and 96 h, the percentage of differentiated cells was 8%, 34%, 72% and 90% respectively. The results showed that the relative volume of cell population decreased from 120 to 26 (the peak channel number) during this process.

3. *Change of [Ca^{2+}]i during MELC differentiation*

Analysis of the [Ca^{2+}]i with respect to the phase of the cell cycle based on the DNA contents for these cells, G_1 (2C), S(2C-4C) and G_2 (4C) revealed a characteristic pattern of changes during the MELC differentiation. The distribution of DNA and [Ca^{2+}]i were shown in Fig 3 and the statistical data for all phases in the cell cycle were sketched in Tab 1.

[Ca^{2+}]i was accumulated in all phases. We found that the [Ca^{2+}]i in S cells was never lower than the [Ca^{2+}]i in G_1 cells. These results implied that G_1 cells must have accumulated the [Ca^{2+}]i to some critical concentration before they could enter into the S phase.

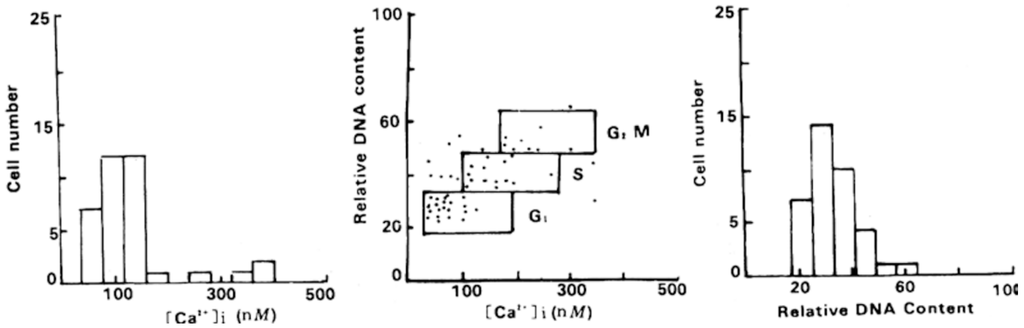

Fig 3. Two parameter dot-plot for DNA vs. [Ca^{2+}]i and histoigrams of DNA and [Ca^{2+}]i in MELC

Tab 1. The intracellular free calcium concentration in MELC and HMBA-treated MELC ($\overline{X} \pm s$)

	[Ca^{2+}]i (n*M*($\overline{X} \pm s$))		
	G_1	S	G_2M
Control	93.3 ±8.3	147.6± 15.8	252±24.6
5 m*M* HMBA 36 h	115.0±18.1	205.5±6.35	371.5±5.10
t test	$p<0.05$	$p<0.05$	$p<0.05$
5 m*M* HMBA 60 h	142.1 ±8.83	217.6±6.74	385.9±4.59
t test	$p<0.005$	$p<0.01$	$p<0.05$
5 m*M* HMBA 96 h	143.5±7.64	288.1±9.42	426.8±8.85
t test	$p<0.01$	$p<0.001$	$p<0.025$

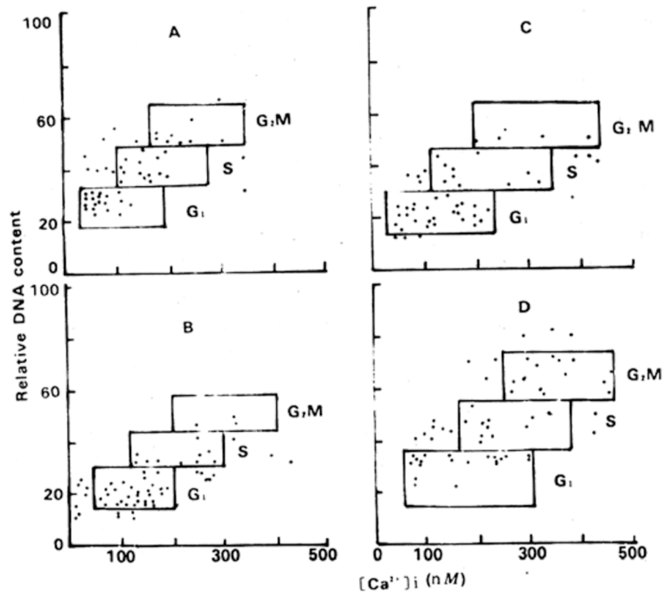

Fig 4. Two parameter dot-plot for DNA vs. $[Ca^{2+}]i$ in MELC (A) and the cells treated with 5 mM HMBA for 36 h (B), 60 h (C) and 96 h (D)

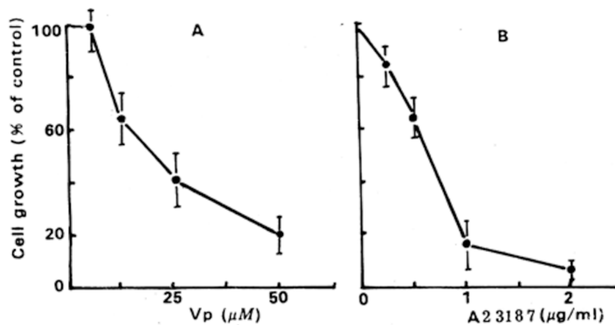

Fig 5. The cytotoxic response of exponentially growing MELC to A23187 (B) and Vp (A)

When MELC were treated with 5 m*M* HMBA for 36, 60 and 96 h, the percentage of differentiated cells was 25%, 72% and 90% respectively. The results of microphotometry (Fig 4, Tab 1) showed that $[Ca^{2+}]i$ increased in all cell cycle phases during this process: G1 phase 1.24, 1.53, 1.53 times vs control; S phase 1.39, 1.48, 1.96 times vs control; and G_2M phase 1.48, 1.53, 1.69 times vs control. The difference was statistically significant ($p<0.01$).

4. Effects of A23187 and Vp on MELC differentiation

A23187 or verapamil can inhibit the growth of MELC. The results were shown in Fig 5. According to these results, we chose the dose with low effects on the MELC growth.

As shown in Fig 6A, a culture treated with 5 m*M* HMBA only attained a level of 83% differentiated cells at 96 h. A second culture was treated with A23187 + 5 m*M* HMBA simultaneously, and this culture had reached a 90% differentiated cells by 96 h. A third culture was treated with A23187 alone, there was no obvious sign of differentiation during the entire period.

As shown in Fig 6B, a culture treated simultaneously with V p + 5 m*M* HMBA only attained 75% differentiated cells at 96 h.

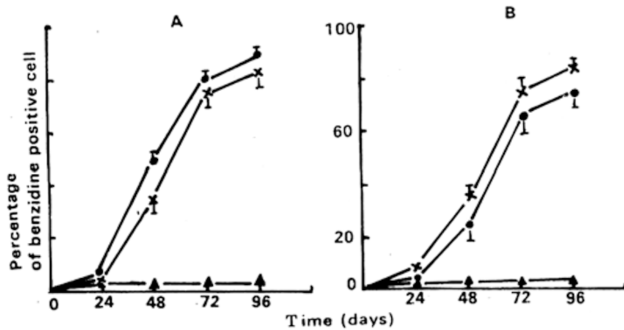

Fig 6. Effects of A23187 (A) and Vp (B) on differentiation MELC **X** HMBA alone ▲ drug alone ● HMBA + drug

DISCUSSION

The results reported in the present study suggested that the intracellular level of calcium ions may have critical functions during MELC differentiation.

By employing the sensitive calcium fluorescent probe fiuo-3 AM and DNA binding dye Hoechst 33342, the correlated measurements of $[Ca^{2+}]i$ and DNA contents during MELC differentiation were performed. We have also measured the change of relative volume of MELC during differentiation. The results showed that during MELC differentiation , $[Ca^{2+}]i$ increased at all cell cycle phase while the relative volume of cells decreased. Thus, the increase of $[Ca^{2+}]i$ during MELC differentiation was confirmed critically.

Our results (employing A23187 or Vp) also showed that alterations in the calcium transport may play an important role in initiating the MELC differentiation. The observation that A23187 alone was not sufficient to induce commitment as shown in Fig 6 implied that a change in the rate of calcium transport alone is not a significant

parameter for the initiation of MELC differentiation.

Early experiments suggested that an increase in cytosolic calcium concentration played a critical role in inducing the commitment of differentiation[3,4,6,9]. Recently, Shibata[12] have also demonstrated that MELC induced to differentiate by the β-subunit of the hormone inhibin increased their cytosolic Calcium. By using HMBA (5 m*M*) as inducing agent, a cell population with a high percentage of differentiated cells was obtained. Furthermore, we measured the $[Ca^{2+}]i$ with respect to DNA contents and compared the $[Ca^{2+}]i$ in all cell cycle phases. Thus we believed that the observed increase in $[Ca^{2+}]i$ was a true reflection of the change of $[Ca^{2+}]i$ during MELC differentiation. Inducing agents may give rise to $[Ca^{2+}]i$ increase through several pathways: 1, the inducer increased the Ca^{2+} influx by activating the Na^{+} /Ca^{2+} antiport[7]; 2, depolarization of cell mitochondria led to the release of mitochodrial calcium stores[10]; 3, the inducer caused the release of calcium from non-mitochondrial stores especially those sensitive to caffeine[11, 12]. These observations and the data presented in the present study suggested that calcium may play a key role in cell differentiation.

Calcium is known to activate a number of cellular enzyme systems involved in cell proliferation and differentiation, such as: protein kinases, phospholipases, phosphodiesterases and adenylate cyclases. Changes in inositol phosphate levels and protein kinase C activity[13] may constitute the regulatory mechanism. Although the mechanism by which cytosolic calcium might affect cell differentiation is yet uncertain, it seemed likely that an increase in cytosolic calcium concentration is one of the possible mechanisms that may be affected by inducing agents to trigger the differentiation.

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