Spermine induces haemoglobin synthesis in murine erythroleukaemia cells

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The naturally occurring polyamine spermine induces haemoglobin synthesis in murine erythroleukaemia (MEL) cells. Haemoglobin production was accompanied by accumulation of cytoplasmic β -globin mRNA and growth inhibition, but not by cell-cycle block or changes in cell volume. Hexamethylene-

bisacetamide (HMBA), a well known differentiating agent, also induces haemoglobin production, but causes a G_1 block and decreases cell volume. These findings indicate that HMBA and spermine affect MEL cells differently, even though both induce haemoglobin production.

INTRODUCTION

Murine erythroleukaemia (MEL) cells are virus-transformed erythroid precursors that differentiate in response to many stimuli [1]. In this process, the cells undergo an irreversible commitment to terminal differentiation, which is followed by loss of proliferative capacity, decrease in cell volume, chromatin condensation, synthesis and accumulation of globin mRNA, production of globin protein, increase in iron uptake, increase in haem synthesis and appearance of erythroid membrane antigens [2–7]. These processes are stimulated by various chemicals and proteases [2,8–17].

Polyamines are ubiquitous polycations whose intracellular concentrations regulate cellular growth and differentiation and are regulated by a system of uptake, efflux, synthesis and catabolism [18,19]. There is contradictory evidence concerning their role in MEL cell differentiation. One important point is that compounds structurally related to polyamines, such as canavanine [20] and the acetylated diamines hexamethylene-bisacetamide (HMBA) [1,17] and diacetylputrescine [21], stimulate MEL cell differentiation. Other observations that support an essential function for polyamines in MEL cell differentiation include a transitory stimulation of ornithine decarboxylase activity (an important polyamine biosynthetic enzyme) early in the differentiation process, obligatory changes in intracellular polyamine concentration that accompany differentiation, and a requirement for spermidine after induction of differentiation by a variety of agents [22–27]. However, other investigators have found that the stimulation of ornithine decarboxylase activity depends on experimental conditions, and is not required for differentiation [28,29]. Furthermore, pharmacological inhibition of polyamine biosynthesis can either constrain or stimulate MEL cell differentiation [22,25,28,30,31]. Therefore it is unclear whether specific alterations in polyamine metabolism and/or intracellular polyamine concentrations are necessary for terminal differentiation in MEL cells. As a first step towards understanding the functions of polyamines in MEL cell differentiation, we examined the effect of spermine, a physiological product of polyamine biosynthesis, on the expression of the differentiated phenotype by MEL cells. We observed that spermine induces

haemoglobin synthesis in MEL cells. However, the effects of spermine and the differentiating agent HMBA on cell growth, cell-cycle progression and cell volume differed.

EXPERIMENTAL

Chemicals

HMBA, aminoguanidine, propidium iodide, and RNase A were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Spermine was obtained from Calbiochem (La Jolla, CA, U.S.A.). All other chemicals were of reagent grade.

Cell culture and treatment

MEL cells (clone DS-19) were kindly provided by Dr. S. Sassa (Rockefeller University, New York, NY, U.S.A.). The original clone was treated by hyperthermia for mycoplasma infection as described by Ho and Deen [32]. Briefly, MEL cells were grown for 5 days at 41 °C in an atmosphere of 5% CO_2 . Cells were assayed for the presence of mycoplasma by staining with Hoechst 33258 [33], measurement of uridine/uracil uptake [34] and fluorescent antibody [35]. All experiments were performed on mycoplasma-free cells, although mycoplasmas infection did not affect the results (results not shown).

MEL cells were maintained in continuous exponential growth in Eagle's modified minimum essential medium supplemented with 10% fetal calf serum at 37 °C in an atmosphere of 5% CO_2 . Cultures were seeded at 5×10^4 cells/ml in a medium containing test compounds (5–100 μ M spermine or 5 mM HMBA). Aminoguanidine (1 mM) was always added before test compounds to inhibit serum diamine oxidase; this by itself did not affect growth or haemoglobin production (results not shown). In some experiments, 48 h after seeding, cells were diluted into fresh medium to which spermine was or was not added ('spermine split'). In other experiments, cells were grown in the presence of spermine for different times, pelleted and resuspended in fresh medium without spermine.

Cell density was determined by electronic particle counting. Growth curves were obtained by plotting cell density versus days

Abbreviations used: MEL, murine erythroleukaemia; HMBA, hexamethylenebisacetamide.

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in culture. Doubling times were determined graphically on the growth curves between days 0 and 2 for continuous culture and days 2 and 4 for split cultures. To determine the size distribution of cells after spermine or HMBA treatment, cell volume was measured using a Coulter S-Plus VI particle counter (Coulter Electronics, Hialeah, FL, U.S.A.) after 96 h in culture.

Polyamine measurement

Between 5×10^5 and 2×10^6 cells were harvested, washed twice with cold PBS, sonicated in 250 μ l of 8% sulphosalicylic acid, danylated and analysed for fluorescence intensity using HPLC [36].

Haemoglobin synthesis

MEL cells were stained for haemoglobin content by the tetramethylbenzidine reaction in acetic acid [37]. We report the percentage of blue-stained cells out of at least 100 counted under a microscope.

Cell-cycle analysis

Cell-cycle distribution was determined using specimens that were fixed in ethanol, treated with 100 μ g/ml RNase and stained with 50 μ g/ml propidium iodide. We used a Becton Dickinson (Mountain View, CA, U.S.A.) Facscan flow cytometer equipped with a doublet discriminator as previously described [38]. We used the polynomial fit method [39] to determine the percentage of cells in each phase of the cell cycle.

Dot-blot analysis for β -globin mRNA

Cytoplasmic RNA from MEL cells purified using RNazol (Tel-Test, Friendswood, TX, U.S.A.) was dotted on to nitrocellulose filters and hybridized sequentially with 32 P-labelled mouse β -globin cDNA and actin cDNA (kindly provided by Dr. P. Curtin, Department of Molecular Genetics and Hematology and Dr Y. De Keyser, Hormone Research Center, University of California, San Francisco, CA, U.S.A.), according to the method described by Sambrook et al. [40]. Analysis of mRNA by Northern blots using these probes showed the presence of a single transcript (results not shown).

RESULTS

Haemoglobin synthesis induced by spermine

Table 1 shows that spermine induced haemoglobin production in a dose-dependent fashion in MEL cells, with the maximum

Table 1 Effect of spermine on haemoglobin production in MEL cells

The cells were incubated with various concentrations of spermine. The fraction of benzidine-positive cells was determined in cells harvested after 96 h in culture. Results are the means $\pm$ S.D. of three determinations.

Spermine (μ M)	Benzidine-positive cells (%)
0	2 $\pm$ 2
5	21 $\pm$ 4
10	34 $\pm$ 5
20	35 $\pm$ 5
50	48 $\pm$ 5
100	45 $\pm$ 3

reached between 20 and 50 μ M. Figure 1 shows that approximately 40% of cells were benzidine-positive after 2 days in 50 μ M spermine and that 45% were positive at 4 days. Haemoglobin production in MEL cells was dependent on the total time of spermine treatment: 2 h of 50 μ M spermine induced 10% of cells to produce haemoglobin at day 4; 24 h of treatment induced 30% of cells, and both 2 days and 4 days of treatment induced approximately 50% of cells to produce haemoglobin at day 4 (results not shown).

Figure 1 also shows that 80% of cells exposed to 50 μ M spermine split culture were benzidine-positive at 4 days, regardless of whether spermine was present after the split. Only 30% of cells were benzidine-positive at 4 days of exposure to 5 μ M spermine when spermine was present after the split. A 48 h exposure to 5 mM HMBA resulted in greater than 90% benzidine-positivity.

β -globin for mRNA

The results of the dot-blot analyses using cloned β -globin and actin cDNAs are shown in Figure 2. Continuous treatment with 50 μ M spermine or 5 μ M HMBA increased the level of cytoplasmic β -globin RNA as early as 24 h after drug addition, and levels continued to increase with time. β -Globin RNA increased faster and to higher levels in cells treated with HMBA. Actin RNA levels changed much less than those of β -globin after treatment with either HMBA or spermine.

MEL cell growth inhibition by spermine

Figure 3 shows the growth curves of MEL cells treated with 5 μ M or 50 μ M spermine. Untreated controls grew exponentially and reached a saturation density of approx. 3×10^6 cells/ml at day 3. Spermine at 5 μ M did not appreciably affect the growth rate (doubling time approx. 10 h). However, 50 μ M spermine decreased the growth rate (doubling time 14.4 h), but growth continued between days 3 and 4, a time when it plateaued in control cells. When cells were diluted into fresh medium at 48 h, controls continued to grow at the same rate (Figure 3). The doubling times of 5 μ M spermine split cultures (14.4 h) were similar to those of cultures treated with 50 μ M spermine prior to dilution. Spermine (50 μ M) split cultures grew slightly slower than they did before dilution (doubling times between 17 and 20 h). Cultures of MEL cells continuously exposed to 5 mM HMBA did not grow after 48 h of treatment.

Cell cycle

Table 2 shows that 93% of HMBA-treated cells and 90% of control confluent cells were in G₁ phase after 4 days in continuous culture. Treatment with 5 μ M and 50 μ M spermine resulted in fewer cells in G₁ (80% and 56% respectively) and more in S phase (13% and 36% respectively). In spermine split cultures, even fewer cells remained in G₁, and more were in S.

Cell volume

Control and spermine-treated cells had very similar size distributions, with a modal value of approx. 200 fl, but cells treated with HMBA were much smaller, with a modal value of approx. 100 fl.

Polyamine levels

Treatment with 50 μ M spermine for 1 day depleted putrescine and spermidine; these two polyamines remained nearly un-

Figure 1 Time course of haemoglobin production in MEL cells treated with spermine or HMBA

The cells were harvested at the times indicated and the proportion of benzidine-positive cells was determined. (a) Unsplit cells: control cells (Δ), and cells cultured in the presence of 5 μ M spermine ($\diamond$), 50 μ M spermine ($\square$) or 5 mM HMBA ($\circ$). (b) Cells after splitting at day 2: cells grown in the presence of 5 μ M spermine were split into fresh medium with ($\diamond$) or without ($\blacklozenge$) spermine; cells grown in the presence of 50 μ M spermine were split into fresh medium with ($\square$) or without ($\blacksquare$) spermine. The points are the averages of two determinations from a single experiment and are representative of four experiments with similar results.

Figure 2 Effects of spermine and HMBA on actin (a) and β -globin (b) expression

MEL cells were incubated in the presence of 50 μ M spermine or 5 mM HMBA. Cells were harvested at various times for total RNA. RNA was dotted on to nitrocellulose filters and hybridized with 32 P-labelled probes for β -globin and actin, as described in the Experimental section.

detectable during the next 2 days (Figures 4a and 4b). Intracellular spermine increased by 25% on day 1 and decreased to initial levels thereafter (Figure 4c). Cells treated continuously with spermine contained more spermine than controls throughout the experiment. The transfer of control cells to fresh medium 2 days after seeding boosted their polyamine levels. Levels of spermine were similar in spermine-treated cells transferred to fresh medium in the presence or absence of spermine and in spermine-treated cells that were not transferred.

HMBA treatment quickly depleted putrescine in MEL cells (Figure 4a). Spermidine levels also dropped, but remained significantly higher than levels reached in the presence of spermine

(Figure 4b). After 2 days of exposure to HMBA, spermine levels were approx. 50% of control (Figure 4c).

DISCUSSION

Our study of MEL cells shows that spermine induces benzidine-positivity in a dose-dependent fashion and increases the cellular content of RNA coding for β -globin. Haemoglobin production began during the first day of spermine treatment, and was taking place in nearly 40% of cells by day 2, when it began to level off. In contrast, after 2 days of HMBA treatment, 90% of cells produced haemoglobin.

Other variables that mark terminal differentiation of MEL cells, and that differed in cells treated with spermine and cells treated with HMBA, include cell volume, measures of cell cycle progression and growth rate. We noticed that the haemoglobin production occurring with 50 μ M spermine treatment was not associated with the immediate growth arrest and smaller cell volume observed after HMBA treatment. Instead, for the first 3 days of treatment, the growth rate was slightly lower than the initial growth rate in controls and the cell size did not decrease. Because haemoglobin production was associated with growth arrest and a decrease in cell volume in HMBA-treated cells, and because growth was inhibited only slightly over the first 96 h of spermine treatment, we reseeded cells at day 2 to see whether more uniform haemoglobin production and more profound growth inhibition would occur at a later time.

We found that haemoglobin synthesis was greater in the 50 μ M spermine split cultures than in continuous cultures, regardless of whether the diluted cells were cultured in the presence or absence of spermine. Haemoglobin synthesis occurred in 80% of cells at day 4, a percentage that approached the 90–95% caused by HMBA treatment at a 100 times higher concentration; only 40% of cells synthesized haemoglobin in continuous culture. This implies that an additional 40% of cells were committed to haemoglobin production after 2 days of treatment, and simply required dilution and time to proceed. However, haemoglobin production was only slightly greater in

Figure 3 Effects of spermine or HMBA on MEL cell growth

(a) Unsplit cells: control cells (Δ), and cells cultured in the presence of 5 μ M spermine ($\diamond$), 50 μ M spermine ($\square$) or 5 mM HMBA ($\circ$). (b) Cells after splitting at day 2: control cells ($\blacktriangle$); cells cultured in the presence of 5 μ M spermine were split into fresh medium with ($\diamond$) or without ($\blacklozenge$) spermine; cells grown in the presence of 50 μ M spermine were split into fresh medium with ($\square$) or without ($\blacksquare$) spermine. The points shown are the averages of two determinations in a single experiment and are representative of four experiments with similar results.

Table 2 Effects of spermine and HMBA on MEL cell-cycle distribution

Cells were harvested after 96 h in culture, prepared and analysed for cell-cycle distribution as described in the Experimental section.

Treatment	Cells in each phase (%)		
	G ₁	S	G ₂ M
Control	90	8	2
Control split	30	60	10
Spermine (5 μ M)	80	13	7
Spermine (5 μ M) split plus spermine	35	59	6
Spermine (5 μ M) split minus spermine	34	60	6
Spermine (50 μ M)	56	36	7
Spermine (50 μ M) split plus spermine	36	52	13
Spermine (50 μ M) split minus spermine	35	54	11
HMBA (5 mM)	93	3	4

cultures treated with 5 μ M spermine when spermine was included in the culture medium after the split. This result was similar to those found with such compounds as haemin or tiazofurin [41,42]. Only an additional 10% of cells treated with 5 μ M spermine were induced after being diluted in the presence of spermine, and no further cells differentiated when spermine was absent. This implies that cells treated for 2 days with 5 μ M spermine were not committed to haemoglobin production.

When the concentration of spermine was increased, MEL cell doubling clearly slowed. Control cells doubled fastest and cells treated with 5 and 50 μ M spermine showed progressively longer doubling times. As these doubling times increased, the percentage of benzidine-positive cells also increased; 5 and 50 μ M spermine induced 15% and 40% benzidine-positivity at 48 h and 30% and 80% positivity at 48 h after dilution. Doubling time therefore seems to be correlated with haemoglobin induction.

The observation that massive haemoglobin production after treatment with HMBA was associated with quick growth arrest corroborates this correlation; this is further supported by the fact that 5 μ M spermine split and undiluted 50 μ M spermine-

treated cultures had similar doubling times (14.4 h) and percentages of benzidine-positive cells (30–40%).

Because the cell densities of control and spermine-treated cultures assayed at 4 days without dilution were high (i.e. at the plateau phase), the cell-cycle distributions were skewed towards G₁ phase (Table 2). Cultures treated with HMBA exhibited G₁ arrest that could be directly associated with haemoglobin production because they never reached a high cell density. Spermine split cultures (5 and 50 μ M) assayed during exponential growth had G₁ fractions similar to those of control split cultures, even though their growth rates and haemoglobin production differed from controls and from each other. In addition, even though more than 80% of 50 μ M spermine split cells produced haemoglobin, their G₁ fractions were 2.5-fold less than in HMBA-treated cells. Thus, in contrast to the results with HMBA, growth inhibition associated with spermine treatment was not tightly associated with a G₁ block. If it had been, we would have expected most of the 80% benzidine-positive cells in 50 μ M spermine split cultures to be blocked in G₁, not the 35% we observed, which was similar to that in control split cultures. Instead, these results support the idea that the growth inhibition observed after spermine treatment slows all phases of the cell cycle without specifically blocking a particular one. Since we know that MEL cells are capable of being blocked in G₁ (as shown in the HMBA results), 50 μ M spermine split treatment may not provide a signal to stop in G₁ by 96 h. Alternatively, spermine may block the signals to stop in G₁ that are associated with haemoglobin synthesis in terminal differentiation.

The addition of spermine to MEL cells had a dramatic effect on intracellular polyamine levels (Figure 4). The spermine level peaked at day 1 and remained high during the following days, even in cells split into fresh medium without spermine. The appearance of early benzidine-positive cells accompanied the peak of intracellular spermine. However, no change in spermine accompanied the further production of haemoglobin in spermine split cells. This suggests that elevated spermine levels are involved in the induction of haemoglobin synthesis.

Earlier studies have suggested that the cellular level of free spermidine is a key factor in MEL cell differentiation: its presence is required at the time of induction [25–27], but the spermidine concentration must drop for the process to continue [20,30].

Figure 4 Putrescine (a), spermidine (b) and spermine (c) levels in MEL cells treated with spermine (50 μ M) or HMBA (5 mM)

△, Control cells; ▲, control cells split into fresh medium. Cells cultured in the presence of 50 μ M spermine were either unsplit (◇) or were split into fresh medium with (■) or without (□) spermine, ●, HMBA-treated cells. Polyamine levels of split cells are shown in inserts. Values are means $\pm$ S.D. of three determinations from a single experiment and are representative of two experiments with similar results.

HMBA-induced differentiation is stimulated by α -difluoromethylornithine (an inhibitor of ornithine decarboxylase). This has been attributed to its ability to deplete spermidine [30]. Our

results suggest that spermidine depletion may also be involved in the induction of haemoglobin synthesis in MEL cells by spermine. Spermidine levels decreased in our HMBA-treated cells, and decreased even more abruptly in spermine-treated cells. Spermine increased the rapidity and the amplitude of the changes in intracellular spermidine that were associated with the induced differentiation. However, this resulted in slower and less profound haemoglobin induction than in HMBA-treated cells, and suggests that other mechanisms are involved.

The pathways involved in MEL cell differentiation have been divided into two stages: events that lead to a commitment to cell differentiation before (early events) and after (late events) the cells undergo cycle arrest [1]. Events that occur before G_1 arrest have been called 'early erythroid functions' [43]. These events include expression of β -globin mRNA and the appearance of haem-synthesis enzymes. 'Late erythroid functions' take place after the cells are arrested in G_1 phase, and include the final synthesis of haemoglobin. Our observations document early events (β -globin mRNA increase) and later functions as well (final synthesis of haemoglobin). However, cells treated with spermine did not exhibit the G_1 arrest and the decrease in cell volume also associated with later functions. We speculate that spermine affects only haemoglobin synthesis, whereas terminal differentiation also requires regulation of many other processes. Spermine causes alterations of DNA conformation *in vitro* and of chromatin structure *in vivo* [38,44,45,46]. Since gene expression is related to changes in chromatin conformation [47,48], a spermine-dependent change in chromatin structure could affect the transcriptional events that regulate the expression of genes controlling haemoglobin synthesis.

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