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Effect of α-difluoromethylornithine on DNA methylation in murine erythroleukaemic cells

Relationship to stimulation of induced differentiation

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Murine erythroleukaemic (MEL) cells cultured with α-difluoromethylornithine (DFMO) accumulated decarboxylated *S*-adenosylmethionine (decarboxylated AdoMet). In the absence of the inducer hexamethylenebisacetamide (HMBA), this accumulation of decarboxylated AdoMet was associated with a concomitant and proportional increase in DNA hypomethylation. In the presence of HMBA, DFMO, which stimulates the erythrodifferentiation of MEL cells, enhanced the differentiation-associated DNA hypomethylation. However, this differentiation-associated DNA hypomethylation was neither temporally nor quantitatively correlated with the accumulation of decarboxylated AdoMet in these cells. Therefore DFMO probably stimulates the HMBA-induced differentiation of MEL cells and the asociated DNA hypomethylation via the effect of this drug on polyamine biosynthesis.

INTRODUCTION

α-Difluoromethylornithine (DFMO), which is an enzyme-activated irreversible inhibitor of ornithine decarboxylase (EC 4.1.1.17) [1], the first enzyme in the polyamine-biosynthetic pathway, can stimulate the differentiation of murine erythroleukaemic (MEL) cells induced by hexamethylenebisacetamide (HMBA) [2]. DFMO can also stimulate or induce differentiation in a number of other cellular systems [3–6]. The differentiation process induced in MEL cells by HMBA is associated with changes in intracellular polyamines which are qualitatively similar to those caused by DFMO [2]. These and other observations [7] suggest that DFMO stimulates or induces the differentiation of MEL cells by exacerbating the normal differentiation-associated changes in intracellular polyamines.

However, in addition to inhibiting polyamine biosynthesis, DFMO has also been reported [8,9] to cause a massive increase in intracellular decarboxylated *S*-adenosylmethionine (decarboxylated AdoMet), the aminopropyl donor in the synthesis of spermidine and spermine from putrescine and spermidine respectively. In addition, this decarboxylated AdoMet can be acetylated *in vivo* and *in vitro* to form *N*-acetyl decarboxylated AdoMet [10,11]. Since *S*-adenosylmethionine (AdoMet) is the substrate for most intracellular methylase reactions, it was suggested [8,9] that this increase in decarboxylated AdoMet may indirectly affect the methylation of cellular constituents. The differentiation process induced in MEL cells is associated with a genome-wide hypomethylation [12,13], and in addition, hypomethylating agents can induce these cells to differentiate [12]. Therefore the experiments described here were undertaken in order to ascertain firstly whether an increase in intracellular decarboxylated AdoMet is associated with a change in DNA methylation, and secondly whether the intracellular accumulation of decarboxylated AdoMet and the eventual modification of DNA methylation could be responsible for the stimulation by DFMO of the MEL-cell differentiation.

MATERIALS AND METHODS

Cell culture

Maintenance of MEL cells (clone DS19) and measurements of cell growth, cell viability and the proportion of cells committed to differentiate were performed as previously described [14]. Experimental cultures were established as follows: cells, maintained in exponential growth for 3 days by daily dilution in Eagle's modified minimum essential medium supplemented with 5% (v/v) foetal-calf serum, were diluted to 2×10^5 cells/ml into medium of the same composition. HMBA (4 mM; stock solution 100 mM in culture medium without serum) and/or DFMO (5 mM; stock solution 300 mM in 15 mM-Hepes, pH 7.4) were added to the cultures 15 h after this dilution.

Analytical methods

Preparation of acid-soluble extracts for polyamine and AdoMet analysis were performed as previously described [2]. Intracellular polyamines, AdoMet and decarboxylated AdoMet were analysed simultaneously by h.p.l.c. using a LKB model 2150 GTi pump and a model-2152 gradient controller, coupled to a low-pressure gradient mixer, a manual injector with a 200 μl loop and an Ultropac Spherisorb ODS-2 column (particle size 3 μm, column dimensions 4.6 mm × 50 mm; LKB France). The column was developed at 37 °C with the chromatographic system described by Wagner *et al.* [15], except that

Abbreviations used: DFMO, α-difluoromethylornithine; MEL, murine erythroleukaemic; HMBA, hexamethylenebisacetamide; AdoMet, *S*-adenosylmethionine.

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octane-sulphonate was used at a concentration of 5 mM and the EDTA was omitted. The adenosyl-group-containing molecules were detected by u.v. absorption at 258 nm (LKB model-2151 variable-wavelength monitor) and the eluant was then mixed with *o*-phthalaldehyde reagent [16] for fluorescent detection of the polyamines as previously described [17]. The amounts of the individual molecules were determined by planimetry, as described elsewhere [17]. 1, 7-Diaminoheptane and Ado[*carboxy*-^{14}C]Met were added to the cell extracts as internal standards. Polyamine standards were from Fluka, AdoMet and *S*-adenosylhomocysteine were from Boehringer-Mannheim and decarboxylated AdoMet was kindly supplied by Dr. K. Samejima, Faculty of Pharmaceutical Sciences, Josai University, Saitama, Japan.

Determination of methylation *in vitro*

DNA was prepared from MEL cells [(2–3) × 10^6 cells] by standard procedures [18]. DNA methyltransferase was extracted from nuclei (10^8) of untreated exponentially growing MEL cells as described by Bestor & Ingram [19]. The crude extract (total volume 1.5 ml) was used immediately to determine the '*in vitro*' methylation capacity of DNA prepared from the various cell cultures. The assay was performed in duplicate and contained (for a 100 μl reaction volume) 1.5–5 μg of purified DNA, 2 μM-Ado[*methyl*-^{3}H]Met (20 Ci/mmol) and 40 μl of the methylase extract. In these conditions the incorporation of *methyl*-^{3}H into poly(dG-dC): poly(dG-dC) was proportional to the amount of DNA present (≤ 20 μg). The reaction mixture was incubated at 37 °C for 1 h and then stopped by cooling on ice. Proteinase K (0.4 mg/ml; Boehringer-Mannheim) and *N*-dodecylsarcosine (0.6%; Sigma) were added and the reaction mixtures reincubated for 1 h at 37 °C, followed by a further treatment with NaOH (0.3 M, 60 °C, 10 min). The DNA was precipitated with 7% (w/v) $HClO_4$ and collected on glass-fibre filters (GF/C; Whatman). The filters were washed three times with $HClO_4$ and twice with 95% (v/v) ethanol. The radioactivity collected on the filters was determined in liquid-scintillation counter using a Triton X-100-based scintillation fluid.

RESULTS

We verified initially that the changes in polyamine biosynthesis, which we have previously shown to be associated with the HMBA-induced differentiation of MEL cells [2,7], also occurred under the culture conditions used here (exponentially growing cells), namely that the intracellular putrescine and spermidine decreased in the cells cultured in the presence of inducer (results not shown). DFMO, which rapidly depleted the cells in these two polyamines, exacerbated these differentiation-associated changes in intracellular polyamines. In the absence of inducer and DFMO, no changes in the intracellular polyamines were detected during the 24 h period investigated in the experiments below.

Simultaneously with these polyamine analyses, the intracellular AdoMet and decarboxylated AdoMet were also measured. Decarboxylated AdoMet was only detected in extracts from MEL cells cultured in the presence of DFMO for more than 12 h (Figs. 1*a* and 1*b*). HMBA alone did not cause an intracellular accumulation

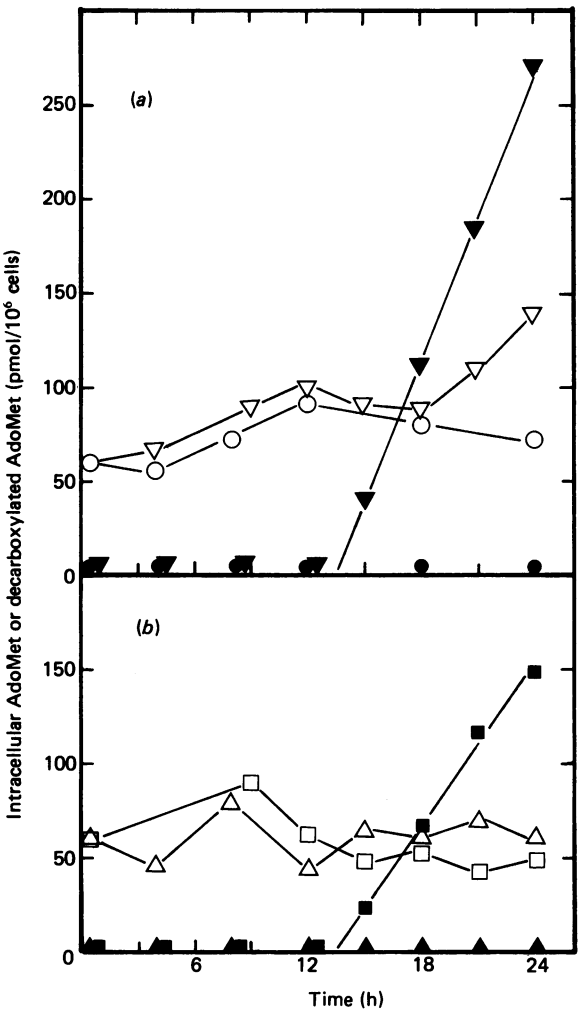

Fig. 1. Effect of DFMO and HMBA on intracellular AdoMet and decarboxylated AdoMet

Experimental cultures of exponentially growing cells were established as described in the Materials and methods section in the presence of (*a*) no drugs (○, ●) or 5 mM-DFMO (▽, ▼) and (*b*) 4 mM-HMBA (△, ▲) or 4 mM-HMBA with 5 mM-DFMO (□, ■). At the indicated times after the addition of the drugs, samples (1 × 10^6 cells) were taken and the intracellular acid-soluble AdoMet (open symbols) and decarboxylated AdoMet (closed symbols) were analysed by h.p.l.c. Quantification was performed by comparing the u.v. absorbance at 258 nm with that of a known amount of the corresponding standard molecule. The results shown are from two separate experiments.

of decarboxylated AdoMet, and the presence of HMBA in the culture medium with the DFMO (Fig. 1*b*) did not modify the time at which decarboxylated AdoMet was first detected in the cell extracts. However, the amount of this molecule that had accumulated intracellularly after 24 h of culture with HMBA and DFMO was about 33% less than that accumulated in the presence of DFMO alone. The basal level of decarboxylated AdoMet in MEL cells cultured without DFMO or HMBA, determined in extracts from 10^8 cells, was 4 pmol/10^6 cells. This is just below the detection limit for this molecule (6 pmol/10^6 cells) in the analyses described here. The amount of AdoMet in cells cultured in the

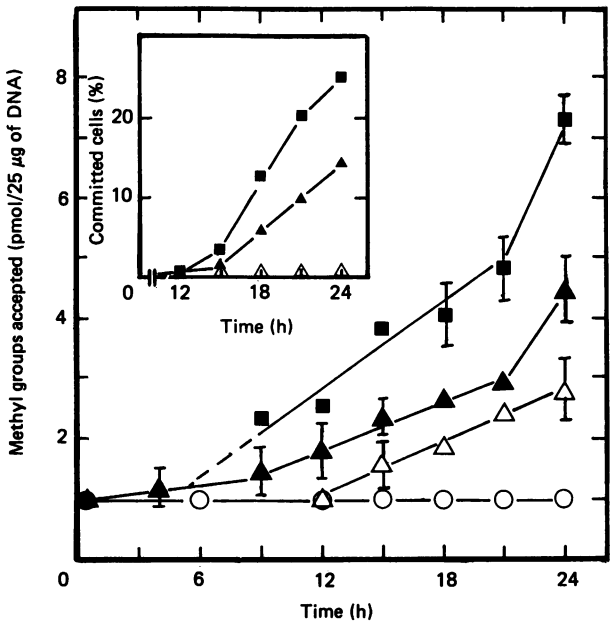

Fig. 2. Effect of DFMO and HMBA on DNA methylation

Experimental cultures were established as described in the legend to Fig. 1, in the presence of no drugs (○), 5 mM-DFMO (△), 4 mM-HMBA (▲) or 5 mM-DFMO with 4 mM-HMBA (■). At the indicated times, DNA was purified from samples [(2–3) × 10^6 cells]. The methyl-group-accepting capacity of this DNA was then measured (see the Materials and methods section) in the presence of Ado[*methyl*-^{3}H]Met and methyltransferase extracted from nuclei of MEL cells cultured without drugs. The results shown are the averages ± maximum range from six experiments (no drugs), four experiments (DFMO or HMBA alone) and two experiments (DFMO with HMBA). Where no error bars are shown, they were smaller than the symbols. The inset shows the time course of the appearance of committed cells for a typical experiment. Symbols are as for the main Figure.

presence of DFMO alone increased towards the end of the period investigated, whereas the intracellular AdoMet decreased slightly in the cells cultured for more than 12 h in the presence of HMBA with or without DFMO. *N*-Acetyl decarboxylated AdoMet was not detected in the cellular extracts from any of these cultures. Whether *N*-acetyl decarboxylated AdoMet accumulated in MEL cells cultured with DFMO for more than 24 h was not determined. In previous studies in which the acetylation of decarboxylated AdoMet was shown to occur, the animals [10] or cells [11] were treated with DFMO for 4 days.

The degree of DNA methylation in the various cell cultures was ascertained by measuring the capacity of purified DNA to be methylated in an '*in vitro*' reaction. The data in Fig. 2 shows that the methylation status of the DNA from the cells cultured in the absence of DFMO and HMBA was constant during the period investigated. For cells cultured in the presence of DFMO, however, a hypomethylation of the DNA was first detected after 15 h of culture with the drug, and it continued to increase during the entire 24 h period. DFMO did not induce these cells to differentiate (see the inset to Fig. 2) even after 3 days in culture with the drug.

When these MEL cells were induced to differentiate with HMBA, the differentiation-associated decrease in DNA methylation was initiated about 6 h before the appearance of committed cells in the culture (Fig. 2). In agreement with our previous results [2], when DFMO was present with the inducer, the accumulation of committed cells was stimulated once their presence in the culture with HMBA alone was detected (see the inset to Fig. 2). In these cells cultured with HMBA and DMFO an early decrease in DNA methylation was also observed, and this hypomethylation of the DNA was maintained throughout the 24 h period studied. It is noteworthy that the hypomethylation associated with the induced differentiation (HMBA alone) was greater than that observed in the presence of DFMO alone. This could explain why DFMO does not act as an inducer of MEL cells, whereas ethionine, which causes a much greater hypomethylation of the DNA, is an inducer of this differentiation process [12].

From the data in Fig. 2, the increase in the DNA hypomethylation for the cells culture with HMBA and DFMO, relative to that for the cells cultured with HMBA alone, was calculated for each time point. Throughout the period investigated the ratio of these pairs of values (HMBA + DFMO/HMBA) was found to have a constant value (2.0 ± 0.4). This is close to the 1.5–2.0-fold stimulation by DFMO of the induced differentiation (see the inset to Fig. 2).

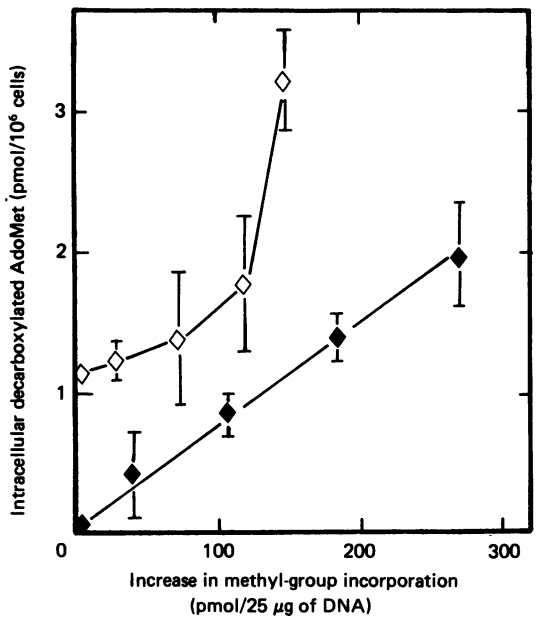

Fig. 3 Relationship between intracellular decarboxylated AdoMet and DNA methylation

Experimental cultures were established as described in the legend to Fig. 1. At 12 h after the addition of the drugs, and at 3 h intervals thereafter, aliquots were taken for the determination of both decarboxylated AdoMet and the methyl-group-accepting capacity of the DNA. The increase in methyl incorporation measured in the DNA purified from cells cultured in the presence of DFMO alone (◆) or DFMO with HMBA (◇), above the basal level measured respectively in extracts from cells cultured with no drugs or HMBA alone, was then calculated. The error bars show the maximum ranges of values from four experiments.

These decreases in DNA methylation were compared with the changes in intracellular polyamines and decarboxylated AdoMet observed in the presence of DFMO with or without HMBA. No temporal or quantitative correlation could be found in either culture between the decreased DNA methylation and the decrease in intracellular putrescine and spermidine. For the cells cultured in the presence of HMBA and DFMO the enhancement of DNA hypomethylation, which was correlated with the stimulation by DMFO of the differentiation (see above), occurred at least 6 h before the accumulation of intracellular decarboxylated AdoMet was detected. At later times, when decarboxylated AdoMet was detected in the extracts from these cells (after 15 h), the increase in DNA hypomethylation and the intracellular accumulation of this metabolite did not appear to be directly related (Fig. 3). However, for the cells cultured in the presence of DFMO alone, the accumulation of decarboxylated AdoMet in the cell extracts and the change in DNA methylation were both detected at the same time (compare Fig. 1*a* with Fig. 2). In addition, the increase in DNA hypomethylation was directly proportional to the amount of intracellular decarboxylated AdoMet (Fig. 3).

DISCUSSION

One important implication of the results presented here is that, in MEL cells cultured in the presence of DFMO alone, not only are the polyamines putrescine and spermidine depleted [2] by the direct inhibitory action of this drug on ornithine decarboxylase [1], but also a hypomethylation of the nuclear DNA can occur. In these cells cultured in the presence of DFMO alone the hypomethylation of the DNA was observed to be temporally and quantitatively correlated with the accumulation of decarboxylated AdoMet. It is important to note that, quantitatively, only very small increases in intracellular decarboxylated AdoMet above the basal level would not have been detected. These results imply that DFMO can indirectly cause a hypomethylation of the DNA in MEL cells, possibly via the modification that occurs in AdoMet metabolism. DFMO has often been used to assess the role that polyamines may play in cellular differentiation [20], a process frequently associated with changes in the methylation status of the DNA [21]. Hence the possible hypomethylation of nuclear DNA, caused by the presence of DFMO in the culture medium, must now be considered when evaluating the effect of this drug in differentiating cellular systems. This may be especially important in cases where the differentiation can be induced by DFMO alone [4–6].

Efforts to ascertain whether decarboxylated AdoMet acted as a competitive or non-competitive inhibitor of the methylase enzyme were inconclusive. The methylase reaction *in vitro* was not inhibited by decarboxylated-AdoMet/AdoMet molar ratios of 2–3, which were similar to that observed in cells cultured for 24 h with DFMO. However, for molar decarboxylated-AdoMet/AdoMet ratios of 20–40, this reaction appears to be inhibited by about 25% (P. Papazafiri and H. B. Osborne, unpublished results). It is important to underline several differences between this reaction *in vitro* and the real intracellular situation. Intracellularly, DNA is associated with structural and regulatory proteins, whereas purified DNA stripped of these proteins is used in the reaction *in vitro*. Secondly, only the total intracellular AdoMet and decarboxylated AdoMet can be measured. Since intracellular compartmentation of enzyme substrate could, and probably does, occur, the actual intracellular concentrations of AdoMet and decarboxylated AdoMet accessible to the enzyme are not known.

The genome-wide hypomethylation associated with the HMBA-induced differentiation of MEL cells is shown here to precede the irreversible commitment event by about 6 h (Fig. 2). The stimulation of this differentiation by DFMO was associated with a quantitatively similar enhancement of the DNA hypomethylation. However, the increase in decarboxylated AdoMet observed in the presence of DFMO and HMBA occurred after the appearance of hypomethylated DNA in these cells. Hence this differentiation-associated change in DNA methylation cannot initially be due to the intracellular accumulation of decarboxylated AdoMet, but rather it is the consequence of earlier differentiation-associated event(s) which were stimulated by DFMO (e.g. changes in intracellular polyamines). Like most DNA–protein interactions [22], the intracellular methylase reaction is sensitive to chromatin structure [23], which in turn could be affected by changes in intracellular polyamines. However, other factors must also be important in the intracellular regulation of this reaction, since in cells cultured with either HMBA and DFMO, or DFMO alone, the changes in intracellular polyamines are very similar, whereas the decreases in DNA methylation are not.

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REFERENCES

1. Metcalf, B. W., Bey, P., Danzin, C., Jung, M. J., Casara, P. & Vevert, J. P. (1978) J. Am. Chem. Soc. **100**, 2551–2553
2. Meilhoc, E., Moutin, M.-J., Hugues-Romani, B. & Osborne, H. B. (1986) Exp. Cell Res. **162**, 142–150
3. Käpyaho, K. & Jänne, J. (1983) Biochem. Biophys. Res. Commun. **113**, 18–23
4. Chen, K. Y., Nau, D. & Liu, A. Y. -C. (1983) Cancer Res. **43**, 2812–2818
5. Schindler, J., Kelly, M. & McCann, P. P. (1985) J. Cell. Physiol. **122**, 1–6
6. Oredsson, S. M., Billgren, M. & Heby, O. (1985) Eur. J. Cell Biol. **38**, 335–343
7. Meilhoc, E., Moutin, M.-J. & Osborne, H. B. (1986) J. Cell. Physiol., in the press
8. Mamont, P. S., Danzin, C., Wagner, J., Siat, M., Joder-Ohlenbusch, A. -M. & Claverie, N. (1982) Eur. J. Biochem. **123**, 499–504
9. Pegg, A. E., Pösö, H., Shuttleworth, K. & Bennett, R. A. (1982) Biochem. J. **202**, 519–526
10. Wagner, J., Hirth, Y., Piriou, F., Zakett, D., Claverie, N. & Danzin, C. (1985) Biochem. Biophys. Res. Commun. **133**, 546–553
11. Pegg, A. E., Wechter, R. S., Clark, R. S., Wiest, L. & Erwin, B. G. (1986) Biochemistry **25**, 379–384
12. Christman, J. K., Price, P. & Acs, G. (1977) Eur. J. Biochem. **81**, 53–61
13. Bestor, T. H., Hellewell, S. B. & Ingram, V. M. (1984) Mol. Cell. Biol. **4**, 1800–1806
14. Osborne, H. B., Bakke, A. C. & Yu, J. (1982) Cancer Res. **42**, 513–518
15. Wagner, J., Danzin, C. & Mamont, P. (1982) J. Chromatogr. **227**, 349–368

16. Seiler, N. & Knödgen, B. (1980) J. Chromatogr. **221**, 227–235
17. Meilhoc, E., Moutin, M.-J. & Osborne, H. B. (1986) Biochem. J. **238**, 701–707
18. Maniatis, T., Fritsch, E. & Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, New York
19. Bestor, T. H. & Ingram, V. M. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 5559–5563
20. Pegg, A. E. & McCann, P. P. (1982) Am. J. Physiol. **243**, C212–C221
21. Taylor, J. H. (1984) DNA Methylation and Cellular Differentiation: Cell Biology Monographs, vol. 11, Springer-Verlag, Vienna and New York
22. Jackson, D. A. (1986) Trends Biochem. Sci. **11**, 249–252
23. Davis, T., Rinaldi, A., Clark, L. & Adams, R. L. P. (1986) Biochim. Biophys. Acta **866**, 233–241

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