Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 9003–9006, December 1988
Biochemistry

Relationship between transient DNA hypomethylation and erythroid differentiation of murine erythroleukemia cells

(3-deazaadenosylhomocysteine/cycloheximide/dexamethasone)

AHARON RAZIN*, ALEX LEVINE*, TAL KAFRI*, SERGIO AGOSTINI†‡, TOMAHARU GOMI†§, AND GIULIO L. CANTONI†¶

*Department of Cellular Biochemistry, The Hebrew University-Hadassah Medical School, Jerusalem, 91010, Israel; and †Laboratory of General and Comparative Biochemistry, National Institute of Mental Health, Bethesda, MD 20892

Contributed by Giulio L. Cantoni, September 14, 1988

ABSTRACT **The state of DNA methylation in mouse erythroleukemia (MEL) cells has been analyzed in relation to commitment to differentiation in response to treatment with hexamethylenebisacetamide (HMBA). Previous experiments have shown that induction by HMBA involves transient genome-wide hypomethylation of DNA that is achieved by replacement of 5-methylcytosine with cytosine residues. The experiments described in the present communication revealed that hypomethylation is a very early event in the process of differentiation. Exposure of the cells to 3-deazaadenosine, an adenosine analog, in combination with homocysteine, resulted in the intracellular accumulation of 3-deazaadenosylhomocysteine, which caused an inhibition of HMBA-induced hypomethylation that was correlated with a comparable inhibition of differentiation. While these experiments suggest that hypomethylation is a necessary step in the process of differentiation, other experiments reported here indicate that hypomethylation of DNA may be necessary but not sufficient to trigger the whole program of differentiation in MEL cells. We found, for example, that exposure of the cells to cycloheximide during the first 24 hr of induction by HMBA resulted in complete inhibition of differentiation without significant effect on the HMBA-induced hypomethylation. This result also indicates that the enzymatic machinery required for the hypomethylation of DNA is present in uninduced cells.**

Murine erythroleukemia (MEL) cells can be induced to differentiate by a variety of agents. It has become increasingly clear that the decision to "switch" upon induction from a state of continuous proliferation to terminal differentiation involves a complex multistep mechanism, and that the response of MEL cells to induction proceeds through various stages. Terminal differentiation is a late response, usually measured 72–96 hr after induction and first detectable at 40 hr. However, as clearly demonstrated by the elegant studies of Marks and his collaborators (1), terminal differentiation is preceded by a state of "commitment" that can be detected as early as 18 hr after induction.

Recently, two cytoplasmic factors that act synergistically in the process of MEL differentiation have been isolated and characterized (2, 3). A variety of events, such as changes in membrane permeability, chromatin structure, and expression of oncogenes take place before cells become committed (1). Clearly, these changes reflect a major alteration in the repertoire of genes expressed in these cells before and after induction. Activation and silencing of genes have been shown in many instances to be associated with a change in the DNA methylation pattern (4). It is evident that a change in the methylation pattern of DNA may involve both the loss of methylated cytosines at some sites of the genome and methylation of previously unmethylated cytosines at other sites.

We have previously shown (5) that when MEL cells are treated with a variety of inducers of terminal differentiation, their DNA undergoes a transient and quickly reversed genome-wide decrease in the content of 5-methylcytosine. Further experiments revealed that hypomethylation is accomplished by an active mechanism whereby, in the absence of DNA replication, a large fraction (as much as 60%) of the 5-methylcytosine residues in the DNA are replaced by cytosine residues (6), which subsequently become methylated at position 5. In biochemical terms, this experiment indicates that the modulation of the pattern of DNA methylation involves at least two enzymatic steps catalyzing, respectively, the replacement of a number of methylcytosine residues by cytosine and methylation of the newly incorporated cytosines by a *de novo* methylase.

We report here the results of experiments designed to explore further the possibility that these changes in DNA methylation play a role, together with a number of other processes, in setting the stage for cell commitment and to define more precisely the relationship between the observed transient hypomethylation and the process of terminal differentiation of the erythroleukemia cells.

METHODS AND MATERIALS

Cell Culture and Materials. MEL cell line clones 745, DR10, and R1 were maintained in Dulbecco's modified Eagle's medium supplemented with 15% fetal calf serum (Beit Haemek, Israel). Cells were diluted 48 hr before use to a freshly split culture (1.5×10^5 cells per ml) in the medium described above. MEL clone 745 cells were used in all experiments unless specified in the text. Deazaadenosine (deaza-Ado) was a gift from Burroughs Wellcome. L-Homocysteine thiolactone (Hcy) was purchased from Calbiochem, and hexamethylenebisacetamide (HMBA) and cycloheximide were purchased from Sigma.

Assays. The content of 5-methylcytidine in DNA was assayed by HPLC (7); commitment of MEL cells (8) and benzidine-reactive cells were determined as described (9). Intracellular levels of deaza-AdoHcy were analyzed as described (10).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: deaza-Ado, 3-deazaadenosine; HMBA, hexamethylenebisacetamide; Hcy, L-homocysteine thiolactone; deaza-AdoHcy, 3-deazaadenosylhomocysteine; MEL, murine erythroleukemia.

‡Present address: Department of Human Biopathology, University "La Sapienza", Rome, Italy.
§Present address: Department of Biochemistry, Toyama Medical and Pharmaceutical University, Faculty of Medicine, Sugitani, Toyama 930-01, Japan.
¶To whom reprint requests should be addressed.

RESULTS

The replacement of a fraction of the methylcytosine residues by cytosine in the DNA of MEL cells induced with HMBA is a very early event. Fig. 1 shows that DNA hypomethylation reached its maximum ≈12 hr after induction and was followed by a methylation process that was essentially complete by 18 hr. These events precede both differentiation, which can first be detected at 40 hr, and commitment, which begins 18–20 hr after induction.

To examine whether both hypomethylation and methylation of hypomethylated DNA are involved in the process of differentiation, we used deaza-Ado, expecting that this compound might specifically inhibit the methylation process. Deaza-Ado is an adenosine analog that in the presence of homocysteine can be utilized as a substrate by *S*-adenosylhomocysteine hydrolase and converted to deaza-AdoHcy (10). Deaza-AdoHcy like AdoHcy is a potent inhibitor of transmethylation reactions, but it differs from the natural congener in its affinity for various methyltransferases, and, hence, in the spectrum of its biological activity (12, 13).

As a first step, we have confirmed earlier observations (14), which demonstrated that HMBA-induced differentiation can be inhibited completely by deaza-Ado in a dose-dependent manner. We have also confirmed the fact that the inhibition produced by deaza-Ado is potentiated by Hcy (14). This observation provides very strong evidence that the inhibitory effect of deaza-Ado is due to its conversion to deaza-AdoHcy. In further support of this conclusion, we have established (data not shown) that in MEL cells, as in other eukaryotic cells, deaza-Ado is rapidly and effectively converted to deaza-AdoHcy, which accumulates intracellularly.

An experiment designed to establish the relationship between the deaza-Ado-dependent inhibition of differentiation and changes in the pattern of DNA methylation yielded an interesting and unexpected result. We observed that administration of deaza-Ado and Hcy to HMBA-induced MEL cells inhibited both the differentiation and the DNA hypomethylation induced by HMBA. Fig. 2 and Table 1 show that these inhibitory effects depend strictly on the duration and timing of the exposure of the cells to deaza-Ado and Hcy. When deaza-Ado and Hcy were added at the same time as the inducer and removed 4, 6, or 8 hr later, there was no effect on differentiation; if the cells were exposed to deaza-Ado and Hcy for 12 or 18 hr after the beginning of induction, differentiation was inhibited by 20% and 90%, respectively; exposure of the cells to deaza-Ado and Hcy for the first 24 hr after induction resulted in complete inhibition of differentiation (Fig. 2). Table 1 shows that if exposure to deaza-Ado and Hcy was terminated 8 hr after induction, the extent of HMBA-induced hypomethylation was the same as in control (HMBA-induced) cells, whereas exposure to deaza-Ado and Hcy for 24 hr resulted in complete inhibition of hypomethylation. The inhibitory effects of deaza-Ado and Hcy on DNA hypomethylation were greatly decreased if Hcy was omitted (data not shown).

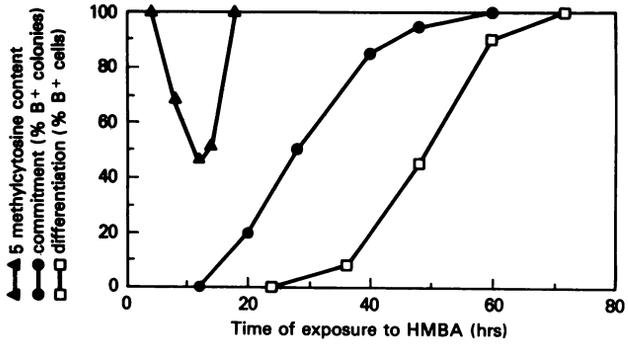

FIG. 1. Time course of changes in DNA methylation with respect to commitment and differentiation. MEL cell cultures at 2×10^5 cells per ml in Dulbecco's modified Eagle's medium and 15% fetal calf serum were treated with 5 mM HMBA. At 6-hr intervals cell culture aliquots were withdrawn for DNA preparation, plating on semisolid medium, and benzidine staining. The extent of DNA methylation was determined by HPLC (7) or nearest-neighbor analysis (11). Cell commitment and differentiation were analyzed, respectively, by benzidine staining of colonies formed on semisolid medium (9) or staining the cells grown in suspension in Dulbecco's modified Eagle's medium/15% fetal calf serum (8). ▲, Hypomethylation; ●, commitment; □, differentiation. 5-Methylcytosine content: 100% methylation corresponds to 3.1 ± 0.2 mol % of total cytosine.

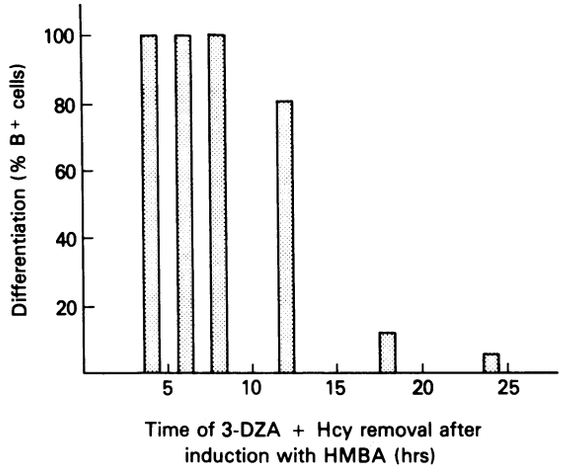

FIG. 2. Relationship between differentiation and length of exposure of HMBA-induced cells to deaza-Ado and Hcy. MEL cells were cultured and treated with HMBA as described in Fig. 1 except 20 μM deaza-Ado (3-DZA) and 50 μM Hcy were added together with HMBA. Cell culture aliquots were withdrawn at the times indicated, and cells were washed with Dulbecco's modified Eagle's medium and recultured in fresh medium/serum with 5 mM HMBA alone. Differentiation was estimated by benzidine staining of the cells in suspension 96 hr after induction.

In another set of experiments, we observed that when the addition of deaza-Ado and Hcy was delayed with respect to induction, the effect on differentiation was progressively diminished (Fig. 3). If the administration of deaza-Ado and Hcy was delayed by 12, 18, or 24 hr after the beginning of induction, differentiation was inhibited by 80%, 68%, or 48%, respectively. Addition of deaza-Ado and Hcy 48 hr after induction had no inhibitory effect on differentiation.

The experiments described above show that exposure to deaza-Ado and Hcy during the period from 8 to 18 hr after induction results in the inhibition of DNA hypomethylation

Table 1. Effect of 3-deaza-Ado and Hcy on the hypomethylation of DNA from HMBA-induced MEL cells

Addition(s)	Time of exposure to HMBA, hr		
	6	12	18
None	3.2	1.8	3.3
Deaza-Ado + Hcy	3.35	3.15	3.0
Deaza-Ado + Hcy (removed at 8 hr)	—	1.65	3.1

MEL cells were induced by HMBA in the presence of deaza-Ado and Hcy as described in the legend to Fig. 3. Aliquots for DNA preparation were taken 6, 12, and 18 hr after induction. DNA was analyzed for the extent of methylation as described in the legend to Fig. 2 and it is expressed as methylcytosine (mol % of total cytosine). Deaza-Ado and Hcy were removed as described in the legend to Fig. 3.

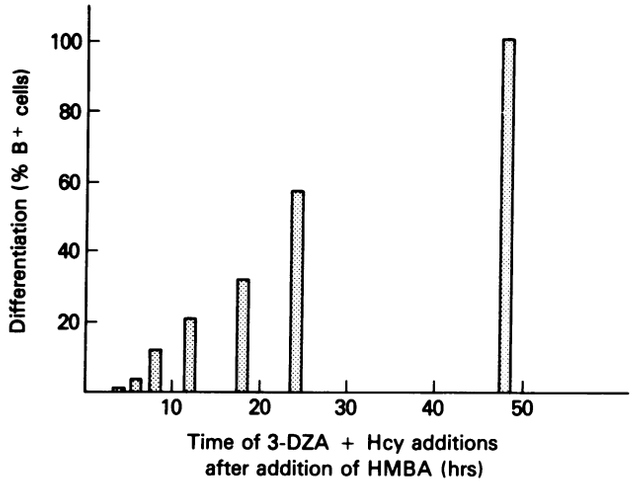

FIG. 3. Relationship between differentiation and the time of addition of 3-deaza-Ado and Hcy after induction. MEL cells were treated with 5 mM HMBA as described in the legend to Fig. 1 and 20 μM 3-deaza-Ado (3-DZA) and 50 μM Hcy were added at the times indicated. Differentiation was determined by benzidine staining of the cell suspensions 96 hr postinduction.

and commitment to terminal differentiation. Thus, the experiments described in Figs. 2 and 3 and in Table 1, taken together, support the idea that genome-wide hypomethylation may be a necessary step in the process of commitment of MEL cells to differentiate.

The inhibition of differentiation resulting from administration of deaza-Ado and Hcy is highly specific. This conclusion stems from the observation that intracellular accumulation of AdoHcy in response to the administration of adenosine (80 μM) and Hcy in the presence of erythro-9-(2-hydroxy-3-nonyl)adenine, a potent inhibitor of adenosine deaminase, had no effect either on HMBA-induced differentiation or on the concomitant decrease in the methylcytosine content of DNA.

The next series of experiments was designed to determine whether the transient decrease of the methylcytosine content of DNA is necessary and sufficient to trigger the process of differentiation, or whether it is only one factor in a series of reactions, acting in concert or in a way-station manner. We have approached this question by examining whether hypomethylation takes place under the following conditions in which treatment with HMBA does not result in cell differentiation. (*i*) When cells were exposed to cycloheximide for an 18-hr period, from 6 to 24 hr after induction with HMBA, DNA hypomethylation proceeded normally (Table 2), whereas cell differentiation was completely inhibited (Fig. 4). As a control, HMBA-induced MEL cells were treated with cycloheximide for the same length of time between 0 and 18 hr (data not shown) or between 18 and 36 hr after induction; under these conditions, differentiation was delayed but not

Table 2. Extent of DNA methylation of HMBA-induced MEL cells in the presence of cycloheximide

Addition	Time of exposure to HMBA, hr		
	6	12	18
None	3.2	2.05	3.15
Cycloheximide	3.24	2.32	3.12

MEL cells were induced by HMBA as described in Fig. 1 in the presence of cycloheximide (0.5 μg/ml) added at the beginning of the induction. Aliquots for DNA preparation of 5-methylcytosine in the DNA samples was carried out as described in the legend to Fig. 1 and is expressed as methylcytosine (mol % of total cytosine).

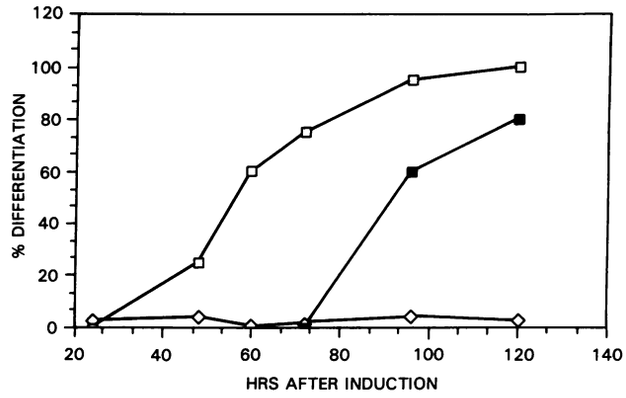

FIG. 4. Effect of cycloheximide on differentiation. MEL cells were cultured and treated with HMBA as described in Fig. 1. Cycloheximide (0.5 μg/ml) was added between 6 and 24 hr (◇) or 18 and 36 hr (■) after HMBA addition. □, Control cells (HMBA alone). Extent of differentiation was estimated by benzidine staining of the cells at the times indicated.

inhibited (Fig. 4). It is clear from this experiment that the expression of differentiation requires the synthesis of several factors that are synthesized in succession and at specific times after induction. At least one of these has to be produced during the period between 6 and 24 hr after induction since exposure to cycloheximide during this time inhibits differentiation. (*ii*) MEL cell clones R1 and DR10, which have lost the capacity to differentiate in response to HMBA, underwent normal DNA hypomethylation 12 hr after exposure to HMBA (data not shown). (*iii*) It has been previously suggested that dexamethasone inhibits events critical for the initiation of commitment. We have observed that while HMBA-induced commitment was prevented in the presence of dexamethasone, the HMBA-induced DNA hypomethylation was not prevented, albeit somewhat delayed (data not shown).

The results of these experiments indicate that in MEL cells it is possible to uncouple DNA hypomethylation from terminal differentiation. The results also support the idea that in these cells genome-wide transient DNA hypomethylation may be a necessary, but not sufficient, event in the cascade required for the expression of the terminally differentiated phenotype.

DISCUSSION

The association of gene activity with specific methylation patterns in mammalian DNA is now well established. Housekeeping genes are, in general, hypomethylated in all tissues, including sperm; by contrast, many but not all tissue-specific genes are heavily methylated in sperm and in somatic tissues but undermethylated in the tissues in which they are expressed (5). The hypomethylation of genes is usually correlated with the presence of DNase-sensitive and hypersensitive sites, reflecting an open chromatin structure characteristic of active genes (15). It is, therefore, probable that the differentiation process is associated with changes in DNA methylation and chromatin structure.

We have previously observed a genome-wide transient reduction in the methylcytidine content of DNA in MEL cells induced by agents such as HMBA. We show here that this hypomethylation is an early event that precedes commitment. It should be noted that in MEL cells hypomethylation coincides with the first appearance of DNase I-sensitive sites in the α- and β^{maj}-globin genes (16, 17). This suggests that changes in the pattern of DNA methylation may be one of the preparatory events that are essential for commitment of the cells to terminal differentiation. In fact, a complete inhibition

of the demethylation process by a combination of deaza-Ado and Hcy is accompanied by a complete inhibition of cell differentiation. There is every reason to believe that the effects of the administration of deaza-Ado and Hcy are due to the intracellular accumulation of deaza-AdoHcy, a congener of AdoHcy, which is a product and an inhibitor of methyl transfer reactions from *S*-adenosylmethionine. The spectrum of action of deaza-AdoHcy differs from that of AdoHcy, as has been shown (12, 13).

The fact that hypomethylation is not sufficient for terminal differentiation is demonstrated by the fact that in a variety of conditions induction of MEL cells with HMBA results in hypomethylation but not in differentiation. The conclusion that the hypomethylation of DNA, while necessary, may not be sufficient is in accord with the observation that certain specific genes are undermethylated but not expressed. There is compelling evidence to suggest that the hypomethylated state sets the stage for gene expression, but it is also known that for expression to occur tissue-specific trans-acting factors have to be present in the cell to bind to the cis-acting sequences and thereby activate the gene.

A second conclusion that emerges from the data is that, although hypomethylation takes place only when MEL cells are induced, the enzymatic machinery responsible for this process is already present in the uninduced cells, as shown by the fact that hypomethylation is observed in the presence of cycloheximide.

The biological effects of deaza-AdoHcy have been generally ascribed to the ability of this compound to interfere with AdoMet-dependent methyl transfer reactions (18). In MEL cells, however, deaza-AdoHcy was shown to inhibit the HMBA-induced hypomethylation of DNA, a reaction whose mechanism is not known but presumably does not involve the participation of *S*-adenosylmethionine. Further work will be required to elucidate the molecular basis of the inhibitory effect of deaza-AdoHcy on DNA hypomethylation.

We are indebted to Burroughs Wellcome for a gift of 3-deazaadenosine and to Drs. H. Nash, S. H. Mudd, and R. R. Aksamit for critical and constructive reading of the manuscript. This research was supported in part by U.S.–Israel Binational Foundation Grant 00015/85 to A.R.

1. Marks, P. A., Sheffrey, M. & Rifkind, R. A. (1987) *Cancer Res.* **47,** 659–666.
2. Nomura, S., Yamagoe, S., Kamiya, T. & Oishi, M. (1986) *Cell* **44,** 663–669.
3. Watanabe, T. & Oishi, M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 6481–6485.
4. Razin, A. & Szyf, M. (1984) *Biochim. Biophys. Acta* **782,** 331–342.
5. Razin, A., Feldmesser, E., Kafri, T. & Szyf, M. (1985) in *Biochemistry and Biology of DNA Methylation*, eds. Cantoni, G. & Razin, A. (Liss, New York), pp. 239–253.
6. Razin, A., Szyf, M., Kafri, T., Roll, M., Giloh, H., Scarpa, S., Carotti, D. & Cantoni, G. L. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2827–2831.
7. Singer, J., Stellwagen, R. H., Roberts-Ems, J. & Riggs, A. D. (1977) *J. Biol. Chem.* **252,** 5509–5513.
8. Fibach, E., Reuben, R. C., Rifkind, R. A. & Marks, P. A. (1977) *Cancer Res.* **37,** 440–444.
9. Reuben, R. C., Wife, R. L., Breslow, R., Rifkind, R. A. & Marks, P. A. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 862–866.
10. Chiang, P. K., Richards, H. & Cantoni, G. L. (1977) *Mol. Pharmacol.* **13,** 939–977.
11. Gruenbaum, Y., Stein, R., Cedar, H. & Razin, A. (1981) *FEBS Lett.* **124,** 67–71.
12. Aksamit, R. R., Falk, W. & Cantoni, G. L. (1982) *J. Biol. Chem.* **257,** 621–625.
13. Scarpa, S., Strom, R., Bozzi, A., Aksamit, R. R., Backlund, P. S., Jr., Chen, J. & Cantoni, G. L. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 3064–3068.
14. Sherman, M. L., Shafman, T. D., Spriggs, D. R. & Kufe, D. W. (1985) *Cancer Res.* **45,** 5830–5834.
15. Conklin, K. & Groudine, M. (1984) in *DNA Methylation: Biochemistry and Biological Significance*, eds. Razin, A., Cedar, H. & Riggs, A. D. (Springer, New York), pp. 293–351.
16. Sheffrey, M., Marks, R. A. & Rifkind, R. A. (1984) *J. Mol. Biol.* **172,** 417–436.
17. Salditt-Georgieff, M., Sheffrey, M., Kranter, K., Darnell, J. E., Jr., Rifkind, R. & Marks, P. A. (1984) *J. Mol. Biol.* **172,** 437–450.
18. Cantoni, G. L. (1986) in *Biological Methylation and Drug Design*, eds. Borchardt, R. T., Creveling, C. R. & Ueland, P. M. (Humana Press, Clifton, NJ), pp. 227–238.