Alteration in proportions of histone H1 variants during the differentiation of murine erythroleukaemic cells

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We have investigated the changes in the relative amounts of histone H1^o and all five H1 variants during the differentiation *in vitro* of Friend erythroleukaemic cells. Three different agents were used as inducers of differentiation: dimethyl sulphoxide, hexamethylenebisacetamide and sodium butyrate. By applying a combination of reverse-phase h.p.l.c. and one-dimensional gel electrophoresis we observed that, during differentiation *in vitro*, (1) the relative amount of each subtype changes upon induction and that (2) dimethyl sulphoxide and hexamethylenebisacetamide produce a similar histone H1 pattern with a strong increase in histones H1^o and H1c, a modest increase in histone H1e and a decrease in the relative amounts of histone H1a, H1b and H1d, whereas butyrate induces a different pattern, particularly with respect to both histones H1c and H1e: H1c increased slightly, and H1e strongly, during differentiation. These results are compared with changes in the histone H1 pattern during differentiation *in vivo* in the mouse [Lennox & Cohen (1983) *J. Biol. Chem.* **258**, 262–268] and in the rat [Pina, Martinez & Suau (1987) *Eur. J. Biochem.* **164**, 71–76], and similarities and deviations are discussed.

INTRODUCTION

The H1 histones are small basic proteins that play a fundamental role in organizing the structure of chromatin (Thoma & Koller, 1977; Boulikas *et al.*, 1980; Igo-Kemenes *et al.*, 1982). They are located in the region of the exit and entry points of DNA, stabilizing the nucleosome (Thoma & Koller, 1977; Boulikas *et al.*, 1980). They play a central role in forming and maintaining the higher-order structure of chromatin (Thoma & Koller, 1977). Chromatographic and electrophoretic studies demonstrated that the H1 histones can be resolved into several closely related subtypes that vary in amino acid sequence and molecular size. In addition, these H1 subtypes are non-uniformly distributed in chromatin and differ among themselves in their ability to condense DNA [for a review, see Cole (1987)]. It is generally assumed that different degrees of condensation accompany the chromatin changes during replication and differentiation. In fact, alterations of the H1 composition occur during postnatal development in liver, kidney and lung of the mouse (Lennox & Cohen, 1983) and in differentiating rat brain cortical neurons (Pina *et al.*, 1987). In order to obtain more information about the role of histone H1 subtypes we have started an investigation of erythroid differentiation using Friend murine erythroleukaemic (MEL) cells, a convenient model system for studying cellular differentiation *in vitro*. These cells are virus-transformed erythroid precursor cells blocked in a relatively early stage in the pathway of erythroid differentiation and can be induced to differentiate by a variety of agents, such as dimethyl sulphoxide (Me₂SO), hexamethylenebisacetamide (HMBA) or sodium butyrate (Marks & Rifkind, 1978). During MEL-cell differentiation, changes in the distribution of H1 histones have been described (Keppel *et al.*, 1977; Rousseau *et al.*, 1991). Using one-dimensional PAGE, they observed an increase of histone H1^o and a decrease of the subfraction named H1-1, whereas subfraction H1-2 remained constant. However, this analytical method is unable to resolve all of the H1 variants.

Recently, we have described rapid and simple methods for the separation of very-lysine-rich histones by reverse-phase h.p.l.c.

(Helliger *et al.*, 1988; Lindner *et al.*, 1988, 1990; Lindner & Helliger, 1990). By a combination of h.p.l.c. and one-dimensional gel electrophoresis it was possible to separate histone H1^o and all five H1 variants. By applying this method to our present study we were able to show that, during differentiation of MEL cells *in vitro*, the relative amount of each H1 subtype changes upon induction and that Me₂SO and HMBA produce about the same H1 pattern, whereas butyrate induces a different pattern which is similar to that seen in non-induced non-growing cells. In addition we have found that the changes in the proportions of the H1 variants during butyrate-induced differentiation of MEL cells *in vitro* resemble those observed *in vivo* in differentiating tissues of the young mouse (Lennox & Cohen, 1983).

MATERIALS AND METHODS

Chemicals

Me₂SO was obtained from Merck (Darmstadt, Germany), HMBA and trifluoroacetic acid were from Sigma Chemie (Munich, Germany), h.p.l.c.-grade acetonitrile (grade S) and water were from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland, U.K.) and ethylene glycol monomethyl ether from Aldrich-Chemie (Sternheim, Germany).

Cell culture

MEL cells (line F4N) were grown in suspension culture in Eagle's minimum essential medium with Earle's balanced salt solution (Biochrom, Berlin, Germany) supplemented with 10% (v/v) fetal-calf serum (Biochrom, Berlin, Germany) and amino acids as previously described (Krieg *et al.*, 1978). Penicillin (100 units/ml) and streptomycin (100 µg/ml) were added routinely. Cell cultures were incubated at 37 °C in air. Differentiation was induced by addition of 1.5% (v/v) Me₂SO, 4 mM-HMBA or 1.75 mM-sodium butyrate to cells growing exponentially at a density of 1 × 10⁵ cells/ml. At designated intervals, the number of cells in each culture was determined by counting in a haemocytometer. Induction of haemoglobin syn-

Abbreviations used: MEL cells, murine erythroleukaemic cells; Me₂SO, dimethyl sulphoxide; HMBA, hexamethylenebisacetamide; AU/PAGE, acid/urea/PAGE.

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thesis was monitored by benzidine (Serva, Heidelberg, Germany) staining as described (Orkin *et al.*, 1975).

Isolation of histones

All procedures were performed at 0–4 °C. To all buffers 10 mM-sodium butyrate, 10 mM-2-mercaptoethanol and 0.1 mM-phenylmethanesulphonyl fluoride were added. At indicated times the cells were collected by centrifugation (800 *g* for 10 min), washed twice with reticulocyte standard buffer [10 mM-Tris/HCl (pH 7.5)/10 mM-NaCl/5 mM-MgCl₂], pelleted and frozen at –70 °C. The frozen cell pellet was washed once in the same buffer. The crude nuclei were prepared as described by Kostraba *et al.* (1975). Cells were lysed in 10 vol. of distilled water containing sodium butyrate, 2-mercaptoethanol and phenylmethanesulphonyl fluoride with 30 strokes of the L-pestle of a Dounce homogenizer and then centrifuged at 10000 *g* for 10 min. Histones were extracted with 0.2 M-H₂SO₄, by incubating on ice for 60 min and centrifuging at 10000 *g* for 20 min. To the supernatant trichloroacetic acid was added to a final concentration of 20% (v/v). After incubating on ice for 2 h the precipitates were collected by centrifugation at 10000 *g* for 20 min. The sediments were washed once with acetone containing 1.2% (v/v) HCl. After centrifugation at 10000 *g* for 20 min the histones were dissolved in h.p.l.c.-grade water plus 1 mM-2-mercaptoethanol and freeze-dried.

H.p.l.c.

The equipment used for h.p.l.c. consisted of two Beckman Model 114M pumps, a 421A system controller and a model 165 variable-wavelength u.v./visible detector. The effluent was monitored at 210 nm, and the peaks were recorded using Beckman System Gold software. The protein separations were performed on a Nucleosil 300-5 C₄ column (4.0 mm internal diameter × 100 mm long; 5 µm particle size; 30 nm pore size; end-capped). The freeze-dried proteins were dissolved in water containing 0.1% (v/v) trifluoroacetic acid, and samples (100 µg) of histones were injected on to the column. At a constant flow rate of 0.5 ml/min the H1 histones were eluted within 30 min using a linear gradient starting at 40% (v/v) solvent A/60% B [solvent A was water containing 0.1% (v/v) trifluoroacetic acid and 10% (v/v) ethylene glycol monomethyl ether; solvent B was 10% (v/v) ethylene glycol monomethyl ether/70% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid].

Gel electrophoresis

The h.p.l.c. subfraction eluted with a retention time of 26–27 min and containing two H1 subtypes was collected, freeze-dried and analysed by acid/urea (AU)/PAGE [0.9 M-acetic acid/2.5 M-urea/15% polyacrylamide as described by Lennox *et al.* (1982)]. The gels were stained for 1 h with 0.1% (w/v) Serva Blue R in aq. 40% (v/v) ethanol/5% (v/v) acetic acid and destained overnight in 20% (v/v) ethanol/5% (v/v) acetic acid. Band intensities were quantified by gel scanning at 572 nm using a Beckman DU-8B spectrophotometer.

Statistics

The results shown in Fig. 3 (below) are presented as means ± S.D. for three to five independent experiments. Significance was determined by Student's *t* test.

RESULTS

Growth and haemoglobin accumulation of uninduced MEL cells and cells cultured in the presence of Me₂SO, HMBA and sodium butyrate

After exposure to specific chemical compounds such as Me₂SO,

HMBA or butyrate, MEL cells exhibit irreversible morphological and biochemical changes similar to those observed during differentiation *in vivo*. These include an accumulation of globin mRNA and haemoglobin (Ostertag *et al.*, 1972), a change in the cellular volume (Gazitt *et al.*, 1987), an altered protein kinase C activity (Melloni *et al.*, 1987) and a cessation of cell division (Friend *et al.*, 1971).

As shown in Fig. 1(a), the control cells grow exponentially for approx. 72 h, whereas the growth of cells treated with 1.5% (v/v) Me₂SO and 4 mM-HMBA decreases after 48 h and that of cells treated with 1.75 mM-butyrate after only 24 h. It should be noted that butyrate is metabolized by the cells, and after a certain time its concentration is reduced to a level below that required to stimulate differentiation (Friend *et al.*, 1987). Therefore, in the case of butyrate treatment, the studies were limited to the first 96 h. Fig. 1(b) shows that, in cultures with butyrate, 35% haemoglobin-containing (benzidine-positive) cells can be detected after 48 h and slightly less than 60% after 72 h. In cultures treated with Me₂SO or HMBA only 15% benzidine-positive cells are found after 48 h and approx. 60% after 96 h. However, throughout the entire period of investigation untreated cultures are found to contain less than 3% benzidine-positive cells.

H.p.l.c. analysis of histone H1^o and five H1 subtypes in untreated MEL cells after seeding and 144 h later and in MEL cells treated with HMBA for 144 h

As shown in Fig. 2, the peak eluted at about 26–27 min consists of a mixture of two subtypes. This subfraction was therefore collected and further analysed by AU/PAGE. The resulting electrophoretogram and the corresponding spectrophotometer scan is shown in the insets of Fig. 2. There are obvious changes in the proportions of the histone variants H1e and H1d between untreated MEL cells after seeding (Fig. 2a) and those 144 h later without any inducer (Fig. 2b) and in the presence of 4 mM-HMBA (Fig. 2c). Besides the increase of histone H1^o already described (Keppel *et al.*, 1977), there are less pronounced changes in the proportions of histones H1a and H1b; however, the strong increase of H1c in cells treated with HMBA is particularly evident.

Alteration in the proportions of each individual H1 variant of control cells as well as those after induction with Me₂SO, HMBA and butyrate as a function of time

Fig. 3(a) shows that the relative amount of histone H1a decreases continuously at a rate of approx. 30% within 144 h both in the uninduced and in the induced cells, independent of the inducer. However, the decrease in the case of the uninduced cells does not indeed begin until 48 h have elapsed.

The relative amount of histone H1b (Fig. 3b) shows a minimum of reduction in the control cells, whereas in the case of all induced cells a reduction to about 50% of the initial value occurs within the first 24 h, and this value remains essentially constant during the following 120 h.

Concerning H1c (Fig. 3c), there are obvious differences between control cells and those treated with butyrate on the one hand and Me₂SO- and HMBA-induced cells on the other hand. The strong increase of the relative amount of histone H1c after 144 h to the 2.5-fold level as a result of the addition of HMBA also takes place after induction with Me₂SO. In the untreated cells, however, there is only a slight increase of histone H1c of about 30–40%. Cells treated with butyrate behave similarly, containing the same relative amount of histone H1c after 96 h as the control cells at the same time. It is noteworthy in this context that the observed increase of histone H1c after treatment with

Fig. 1. Cell growth and haemoglobin production in untreated (control) and induced MEL cells

Cells were grown without inducer (●) or with 1.5% Me₂SO (△), 4 mM-HMBA (○) or 1.75 mM-sodium butyrate (□). (a) growth was assayed at the indicated times; (b) percentage of benzidine-positive (haemoglobin-producing) cells at 24 h intervals after induction.

butyrate is similar to the increase of this subtype in mouse neuroblastoma cells treated with millimolar concentrations of butyrate (Hall & Cole, 1985).

Fig. 3(d) shows the decline of histone H1d: in the case of butyrate and Me₂SO, a 96 h treatment leads to a 50% reduction of the original amount of H1d; in the case of HMBA this 50% reduction is achieved at 144 h. Also in untreated cells H1d is drastically reduced, but this decrease starts at 48 h only.

The changes of the fifth subtype, histone H1e, during differentiation are more strongly dependent on the inducer used (Fig. 3e). Butyrate leads to a 100% increase at 72 h, whereas Me₂SO leads to a 60% increase at only 120 h. In untreated cells histone H1e remains relatively constant for 48 h, then increases, and at 144 h the same amount of H1e is reached as in butyrate-treated cells at 72 h. If these results are compared with the growth curve shown in Fig. 1(a) it appears that histone H1e is related to the arrest of cell proliferation as assumed (Lennox & Cohen, 1983; Pina *et al.*, 1987). In HMBA-treated cells, however, which show a similar growth pattern as Me₂SO-treated cells, H1e scarcely increases during differentiation. After 144 h of HMBA treatment the cells contain the same small amount of histone H1e as at the time of seeding.

Histone H1° has been reported to be synthesized in a higher quantity in quiescent cells (Panyim & Chalkley, 1969; Pehrson & Cole, 1980). Furthermore, H1° accumulates in cells committed to differentiate (Keppel *et al.*, 1977; Pieler *et al.*, 1981; Hall & Cole, 1986). Our data concerning histone H1° agree with these observations. In untreated MEL cells (shown in Fig. 3f), we find an increase of histone H1° at 72 h. Upon induction a quick increase of H1° to the 4–5-fold level occurs during the first 24 h. Whereas after 72 h the amount of H1° is about the same upon induction by Me₂SO or butyrate (4-fold), it increases more strongly in the case of HMBA (6-fold at 72 h and 7-fold at

Fig. 2. H.p.l.c. analysis of histone H1° and five H1 subtypes in untreated MEL cells after seeding and 144 h later and in MEL cells treated with HMBA for 144 h

Histone H1 variants in untreated MEL cells immediately (a) and (b) 144 h after resuspending in fresh medium; (c) H1 variants in cells harvested 144 h after HMBA induction. H1 histones were isolated from MEL-cell nuclei and separated by reverse-phase h.p.l.c. as described in the Materials and methods section. The peak eluted at approx. 26–27 min contained two H1 subtypes and was further analysed by AU/15%-PAGE as described by Lennox *et al.* (1982). The inset shows the corresponding electrophoretogram and the scan of the gel.

120 h). These results resemble the results described by Pieler *et al.* (1981), who examined the effects of different inducers of differentiation on histone H1° accumulation in murine neuroblastoma cells. They found that these cells accumulated histone H1° when cultured in the presence of Me₂SO, HMBA or butyrate, and that they produced more H1° with HMBA than with the other two compounds.

Fig. 3. Changes in the proportion of histone H1° and the five H1 subtypes in control and induced MEL cells as a function of time

H1 histones were extracted from MEL cells grown without inducer (●) or in the presence of 1.5% Me₂SO (△) or 4 mM-HMBA (○) for 0, 24, 48, 72, 96, 120 and 144 h, and in the presence of 1.75 mM-butyrate (□) for 0, 24, 48, 72 and 96 h. The proteins were separated by reverse-phase h.p.l.c. in combination with gel electrophoresis, as shown in Fig. 2. The amount of each H1 variant was quantified using a Beckman System Gold software. The quantification of the subtypes H1d and H1e was performed by densitometric measurement of the stained gels using a Beckman DU-8B spectrophotometer. Results at each time point represent means $\pm$ s.d. for three to five independent experiments. The s.d. was within the limits of the symbols where no s.d. bars are shown. (a) Histone H1a. Significant differences at 48 h: ● versus □, $P < 0.05$. Significant differences at 144 h: ● versus ● (0 h), $P < 0.01$; ○ versus ○ (0 h), $P < 0.05$; △ versus △ (0 h), $P < 0.001$. (b) Histone H1b. Significant differences at 24 h: ● versus ○, △ or □, $P < 0.05$; ○ versus ○ (0 h), $P < 0.05$; △ versus △ (0 h), $P < 0.05$; □ versus □ (0 h), $P < 0.05$. Significant differences at 96 h: ○ versus □ or ●, $P < 0.01$; △ versus □ or ●, $P < 0.05$. Significant differences at 144 h: ● versus ● (0 h), $P < 0.01$; ○ versus ○ (0 h), $P < 0.01$; △ versus △ (0 h), $P < 0.001$. (d) Histone H1d. Significant differences at 48 h: ● versus ○, △ or □, $P < 0.01$. Significant differences at 96 h: ● versus ● (0 h), $P < 0.01$; ○ versus ○ (0 h), $P < 0.01$; △ versus △ (0 h), $P < 0.01$; □ versus □ (0 h), $P < 0.001$. Significant differences at 144 h: ● versus ● (0 h), $P < 0.001$; ○ versus ○ (0 h), $P < 0.01$; △ versus △ (0 h), $P < 0.001$; □ versus □ (0 h), $P < 0.001$. (e) Histone H1e. Significant differences at 24 h: ● versus ○, △ or □, $P < 0.01$; ○ versus ○ (0 h), $P < 0.001$; △ versus △ (0 h), $P < 0.001$; □ versus □ (0 h), $P < 0.01$. Significant differences at 72 h: ○ versus △ or □, $P < 0.05$. Significant differences at 144 h: ● versus ● (0 h), $P < 0.05$; ○ versus ○ (0 h), $P < 0.01$; △ versus △ (0 h), $P < 0.001$. (f) Histone H1°. Significant differences at 24 h: ● versus ○, △ or □, $P < 0.01$; ○ versus ○ (0 h), $P < 0.001$; △ versus △ (0 h), $P < 0.001$; □ versus □ (0 h), $P < 0.01$. Significant differences at 72 h: ○ versus △ or □, $P < 0.05$. Significant differences at 144 h: ● versus ● (0 h), $P < 0.05$; ○ versus ○ (0 h), $P < 0.01$; △ versus △ (0 h), $P < 0.001$.

DISCUSSION

It has been reported that there are changes in the relative amounts of the variants of the core histones H2A, H2B and H3 during Me₂SO- or HMBA-induced differentiation of MEL cells (Grove & Zweidler, 1981, 1984). These changes are shown to be similar to those occurring *in vivo* in terminally differentiating cells of the young mouse (Zweidler, 1980). By investigating modifications of the distribution of linker histones during the course of differentiation of MEL cells *in vitro*, Keppel *et al.* (1977) have demonstrated that the only change was an increase in 'protein IP₂₅' (which now termed 'histone H1°'). Rousseau *et al.* (1991) have reported a variation in the content of various H1 subfractions during 36 h of HMBA-induced differentiation of MEL cells: H1° increased by a factor of 3, subfraction H1-1 decreased and H1-2 remained constant. It should be noted, however, that the investigations by both Keppel *et al.* (1977) and Rousseau *et al.* (1991) were carried out using only one-dimensional SDS/PAGE.

In the present study we have examined the alterations in the

relative amounts of the very-lysine-rich histones during the time course of differentiation *in vitro* and during the same period of control (untreated) MEL cells by the combined use of a reverse-phase h.p.l.c. method previously described (Lindner & Helliger, 1990) and gel electrophoresis. For the induction of differentiation we have employed Me₂SO, HMBA and sodium butyrate, because we were interested to examine whether different inducers cause different changes in the content of H1 variants as well. In this context it should be noted that Gazitt & Friend (1980) divided the inducers into two classes based on their ability to stimulate ornithine decarboxylase activity. Me₂SO and HMBA are members of class 1, stimulating ornithine decarboxylase activity. Butyrate, an inducer belonging to class 2, has little or no stimulating effect on this enzyme. Gazitt & Friend (1980) assumed that different classes of inducers act through different mechanisms. Indeed, Me₂SO and HMBA produced a very similar pattern, whereas butyrate induced a different pattern bearing, aside from H1°, some resemblance to that of non-induced, non-growing cells (Fig. 3). In detail, we observed a remarkable accumulation of histone H1c upon induction by Me₂SO and

HMBA that we did not find using butyrate (Fig. 3c). Lennox (1984) suggested that H1c does not promote a higher-order chromatin structure. Since for differentiation and the maintenance of the differentiated state a low condensed chromatin structure very likely is necessary, we think that this state in the MEL cells might either be realized by the increasing presence of the variant H1c in chromatin (as happens upon induction by Me₂SO or HMBA) or by the hyperacetylation of core histones being induced by butyrate (Candido *et al.*, 1978; Ridsdale *et al.*, 1990; Bradbury, 1992). By using our new methods of analysis we were able also to demonstrate that, during the differentiation of MEL cells *in vitro*, the relative amounts of all H1 subtypes change, which contrasts with the results of investigations by Keppel *et al.* (1977) and Rousseau *et al.* (1991). Our observed changes of the H1 subtypes are, especially in the case of butyrate, very similar to the changes observed by Lennox & Cohen (1983) during murine postnatal maturation *in vivo* of liver, kidney and lung; these are a decline in subtypes H1a, H1b and H1d, a marked increase in H1e and H1^o, and an increase in H1c. According to Lennox & Cohen (1983) and Pina *et al.* (1987) an arrest of cell proliferation leads concomitantly to an increase in histone H1e. In contrast with this finding we found in our study that H1e was substantially increased only in case of MEL cells induced with Me₂SO or butyrate or in untreated cells, but not when cells were induced with HMBA. With the inducer HMBA, H1e modestly increases during the first 120 h, then declines during the next 24 h, leading after 144 h to the same amount of H1e as at the time of seeding.

Finally we could demonstrate untreated control cells also show a change in their H1 subtype composition when they pass from the growing to the non-growing state, although these changes are less pronounced than those observed during the chemically induced differentiation.

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