CONCISE REPORT

A Transient Increase in Histone H2A Ubiquitination Is Coincident With the Onset of Erythroleukemic Cell Differentiation

By Jack O. Hensold, Paul S. Swerdlow, and David E. Housman

Murine erythroleukemia cells are useful for studying the regulation of erythroid differentiation since these malignant pronormoblasts differentiate to orthochromatid normoblasts when treated with a variety of inducing agents. Changes in chromatin proteins have been described following inducing exposure. The significance of these changes, which are greatest in terminally differentiated cells remains unknown. Ubiquitin is a highly conserved 8.5 kilodalton peptide that is covalently linked to up to 10% of histone H2A. We demonstrate that following exposure of MEL cells to inducers of differentiation, a transient increase in ubiquitination of H2A occurs. This change is coincident with the onset of differentiation. This result suggests that ubiquitination of H2A may have a role in the nuclear changes necessary for erythroleukemic cell differentiation.

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MURINE ERYTHROLEUKEMIA (MEL) cells are a useful system for studying erythroid differentiation. These cells have features of pronormoblasts but undergo morphological and biochemical changes when treated with a variety of inducing agents, ultimately producing a cell with characteristics of a more differentiated orthochromatid normoblast. The mechanisms responsible for triggering this differentiation are not well understood. Previous studies have demonstrated that changes in intracellular calcium levels and changes in expression of the c-myc oncogene affect MEL differentiation. These observations suggest that signalling pathways common to a variety of cells are involved in initiating the differentiation process. However, the relationship of these and other changes to the altered expression of genes that occurs with MEL cell differentiation is unknown.

During MEL cell differentiation, changes in accumulation and modification of chromatin proteins have previously been observed. Changes in acetylation of histone H4 have been demonstrated in cells induced with sodium butyrate and increases in phosphorylation of H2A occur with exposure to inducer. Changes in the ratio of histone subtypes occur between 24 to 96 hours of inducer exposure and an increase in a 25-Kd nuclear protein identified as H1 also occurs with induction of differentiation. The significance of these changes and their relationship to alterations in gene expression remains undefined.

A protein modification common to all eukaryotes is ubiquitination. Ubiquitin is a small (8.5 Kd) protein that is conjugated to other proteins via an isopeptide bond involving the carboxy-terminal glycine of ubiquitin and free epsilon-amino groups of lysine or free amino-termini in target proteins. Ubiquitin has been implicated in mediating selective protein degradation, as well as in modifying chromatin structure, since up to 10% of H2A histone is ubiquitinated. Yeast deficient in ubiquitin have impaired viability following sporulation suggesting that ubiquitin may play a role in regulating this particular developmental event. Ubiquitination of proteins could play a role in regulating developmental events in higher organisms as well, either through its effects on chromatin structure or by selective degradation of specific regulatory molecules. We demonstrate here that a transient increase in ubiquitination of H2A is an early event in MEL cell differentiation. The time course of this change suggests that ubiquitination of H2A may play a role in the modification of chromatin structure that is necessary for the expression of genes characteristic of the differentiated erythroid phenotype.

MATERIALS AND METHODS

MEL cells (clone 745-PC4-B1-2) were grown in Dulbecco's modified eagle's medium (DMEM), supplemented with 15% fetal bovine serum (FBS) and L-glutamine (2mmol/L) at densities that ensured logarithmic growth (0.5-10 x 10^5/mL). Differentiation was induced by the addition of dimethyl sulfoxide (DMSO) 1.5% v/v or hypoxantine (500 mmol/L). Commitment to differentiation was assessed by clonal commitment assay.

For determination of ubiquitin-protein complexes, total cellular proteins were extracted from cells by boiling in 1% sodium dodecyl sulfate (SDS). Protein concentrations of extracts were determined using a LKB Ultrascan XL Laser Densitometer (LKB Instruments Inc, Gaithersburg, MD). The results were standardized to other ubiquitinated proteins in the same lane that were detected with longer exposures.

RESULTS

To determine if changes in ubiquitination of proteins occurred during exposure of MEL cells to inducers of differentiation, changes and their relationship to alterations in gene expression remain undefined.

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differentiation, we analyzed patterns of protein ubiquitination in control cells following four hours of DMSO exposure by immunoblotting. The early time of the inducer exposure was chosen to identify changes that might be responsible for differentiation and also to assure that observed changes in pattern of ubiquitination would not be the result of the appearance or disappearance of proteins associated with terminal differentiation. In preliminary studies, multiple proteins were found to be ubiquitinated in both control and DMSO-treated cells, but a prominent protein of approximately 22 Kd, representing the most heavily ubiquitinated species on the gel, was noted to increase following four hours of DMSO exposure (data not shown). Both the approximate molecular weight and its abundance suggested that this ubiquitinated species with H2A histone (µH2A).

To determine if this increase was in µH2A, immunoblots were repeated using calf thymus histone as marker and a gel system to enhance separation of histones. As demonstrated in Fig 1, an increase in a protein that comigrates with the ubiquitinated H2A in a preparation of purified calf thymus histone is seen in immunoblots of MEL cell proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A similar increase can be demonstrated in cells induced with hypoxanthine (Fig 1B), demonstrating that this is not a non-specific effect of DMSO. For both inducers, the magnitude of this increase following six hours of exposure to inducer was nearly two-fold, as determined by densitometry (Table 1). To further confirm that this protein was µH2A, immunoblots of acid extracted MEL nuclei separated on acid triton urea gels were performed. A similar increase in µH2A is seen on these gels following DMSO exposure (data not shown). These data provide evidence that this protein is µH2A since it comigrates with µH2A by charge as well as by molecular weight on SDS gels.

Since this increase in ubiquitination appeared to be transient in the preceding experiments, we examined more closely the kinetics of this change as well as their relation to the kinetics of differentiation. Cells were grown in DMSO for up to 72 hours. Ubiquitin-protein conjugates and commitment to differentiation was determined concurrently. An initial latent period of 11 hours occurred before any cells committed to differentiation (Fig 2A). Following this, a linear increase in committed cells occurred with ≈95% differentiation after 72 hours of DMSO exposure. These results are compared with the changes in µH2A (Fig 2B), it is seen that ubiquitination of H2A peaks following 11 hours of DMSO exposure and then slowly decreases toward control levels. A further decrease is not seen with increasing differentiation. Therefore, µH2A increases before any differentiation is apparent and returns toward control levels as the percentage of differentiated cells begins to increase.

**DISCUSSION**

These experiments demonstrate that increased ubiquitination of a chromatin protein, H2A histone, occurs shortly after exposure of MEL cells to an inducer of differentiation and then returns toward control levels as cells begin to differentiate. This increase in ubiquitination of H2A differs from previously described chromatin changes in its early onset and its transient nature. The time course of this change suggests that the increase in µH2A is not a characteristic of the terminally differentiated phenotype but rather is coincident with the changes that result in differentiation.

In inducer-exposed MEL cells a transient increase in G1 cells occurs near the time differentiation starts and a G1 arrest occurs in terminally differentiated cells. Ubiquitination of histone has been shown to occur throughout the cell cycle with a transient loss of µH2A occurring at the end of the G2 phase. It is unlikely though that the changes in

**Table 1. Densitometric Quantitation of µH2A**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Duration of Exposure</th>
<th>0 hr</th>
<th>2 hr</th>
<th>6 hr</th>
<th>13 hr</th>
<th>14 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td></td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.89</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td></td>
<td>1.00</td>
<td>1.51</td>
<td>2.35</td>
<td>1.82</td>
<td>1.12</td>
</tr>
</tbody>
</table>

The gels in Fig 1 were quantitated by densitometry and standardized as described in Materials and Methods. The numbers expressed are relative to the value in control cells (0 hr). The values for DMSO represent an average of the values obtained for both loading conditions (constant protein and constant cell number) indicated in Fig 1A.
Fig 2. The relationship of changes in histone H2A ubiquitination to the kinetics of DMSO-induced MEL cell differentiation. MEL cells were exposed to DMSO for the hours indicated in Figures 1 and 2. Commitment to differentiation and ubiquitin-protein conjugates were determined on cell aliquots as indicated in the text. A) Kinetics of DMSO-induced commitment to differentiation. The percentage of cells forming colonies that contained hemoglobin (as indicated by reactivity with benzidine) as a function of time of DMSO exposure. B) Immunoblot of MEL cell proteins following exposure to DMSO. The lanes are labelled above with the hours of inducer exposure. The lane labelled "H" contains calf-thymus histone.

μH2A shown here are due to any alterations in cell cycle. Induction of differentiation by hypoxanthine has been shown to be unaccompanied by a G1 arrest. Further, if cell cycle changes were solely responsible for the changes in extent of ubiquitination of H2A, then a large increase might be expected to occur in terminally differentiated cells that have arrested in G1. As shown here, μH2A does not further increase with terminal differentiation.

The function of ubiquitination of H2A is unknown, but the preferential association of μH2A with active chromatin suggests that it might have a role in the modification of chromatin structure associated with gene expression. It has been previously shown that following exposure of MEL cells to an inducer of differentiation, the turnover rate of H2A is increased. Since the turnover rate of H2A, the most heavily ubiquitinated histone, is greater than that of other histone proteins, and since ubiquitin has a demonstrated role in protein degradation, it may be speculated that ubiquitination of H2A is involved in the turnover of this protein. If this is the case, ubiquitination of chromatin-bound H2A could aid in altering chromatin structure by contributing to the disruption of histone-DNA binding. Such an effect might contribute to the creation of nucleosome-free hypersensitive sites that exist near active genes. Alternatively, removal of histones might facilitate replacement with histones of variant subtypes, thus producing chromatin alterations that might be necessary for changes in gene expression. The data presented here not only suggests that histone ubiquitination might have such functions, but also provides a system where changes in histone ubiquitination can be studied.

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