Alteration of Methylation Patterns in Rat Liver Histones following Administration of Ethionine, a Liver Carcinogen

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ABSTRACT

The possible role of alterations of histone methylation by ethionine in the mechanism of ethionine carcinogenesis was studied. In regenerating rat liver, histone synthesis was inhibited by only 20 to 30% with large doses of ethionine (0.75 to 1.0 mg/g body weight). The effect of ethionine on the in vivo methylation of histones was studied by giving 0.5 mg ethionine and [methyl-3H]methionine per g body weight. In vivo methylation of lysine was inhibited by 50%, whereas the arginine methylation was inhibited by 89%. The cellular localization of the methyltransferases and S-adenosyl-L-methionine may be related to this differential effect. Utilizing an in vitro assay for protein-lysine and protein-arginine methyltransferases, we have demonstrated that the methyl-deficient histones are transported to the nucleus and with time lose their ability to accept methyl groups in vivo.

INTRODUCTION

Histone methylation via AdoMet has been studied and reviewed by Paik and Kim (13). It appears that protein-lysine and protein-carboxyl methyltransferases are found in the cytosol (12). Although the exact function of the methyl group in histones is not understood, Duerre and Quick (5) have suggested that the methyl groups are involved in establishing the higher order of chromatin structure by interacting with neighboring proteins. Paik et al. (11) have presented evidence that enzymatic methylation of cytochrome c results in an increased binding of cytochrome c with mitochondria; this may be detrimental to the cell. Ethionine, a liver carcinogen (6), via methylation of histones is involved in their binding to nuclear chromatin (14), it was of interest to determine if in vivo histone methylation is altered following ethionine treatment.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass.

The animals weighed about 125 to 150 g at the time of sacrifice and were given free access to water and food (Purina rat chow). Partial hepatectomies were performed according to the procedure of Higgins and Anderson (7).

Chemicals. dl-Ethionine, AdoEth, and AdoMet were purchased from Sigma Chemical Co., St. Louis, Mo. S-Adenosyl-[methyl-3H]methionine (9.7 Ci/mmol), [methyl-3H]methionine (83 Ci/mmol), and [3H]lysine (1.8 Ci/mmol) were purchased from New England Nuclear, Boston, Mass.

Protein-Lysine Methyltransferase Assay. Protein-lysine methyltransferase was assayed in whole nuclei as described by Baxter and Byvoet (2). The standard reaction mixture contained 23 μM Tris-HCl buffer (pH 7.4), 125 μM sucrose, 9.2 μM KCl, 4.6 μM MgCl₂, 25 μM dithiothreitol, 2.5 μM (1 μM) S-adenosyl-[methyl-3H]methionine, and nuclei from 0.5 g liver. Total volume of the assay was 0.5 ml. This mixture was incubated for 10 min at 37°C, and the reaction was stopped by the addition of 2.5 ml of ice-cold 0.08 M NaCl containing 0.02 mM EDTA (pH 7.2). The nuclei were sedimented at 2000 x g, and total histones were isolated as described by Ueda et al. (16). The washed nuclei were extracted twice with 20 volumes of 0.25 M HCl by shaking in the cold for 30 min. The 0.25 M HCl extraction of proteins from rat liver nuclei is referred to in this report as histones, although some nonhistone proteins are present. The histones were then precipitated from the combined HCl extracts with 6 volumes of cold acetone. After standing overnight at 0°C, histones were collected, washed with acetone:1% HCl and acetone, air dried, and dissolved in water for determination of radioactivity. Protein concentration was determined by the procedure of Lowry et al. (10) using bovine serum albumin as a standard.

Protein-Arginine Methyltransferase Assay. Protein-arginine methyltransferase was isolated by the procedure described by Lee et al. (8). Thirty g of liver were homogenized in 4 volumes of 0.25 M sucrose containing 1 mM EDTA (pH 7.4), and the homogenate was centrifuged at 105,000 x g for 60 min. The supernatant was made 31.3% with respect to ammonium sulfate and centrifuged at 30,000 x g for 10 min. The pellet was dissolved in water, and calcium phosphate gel (Sigma) was added (17 mg of solid/ml). The suspension was then stirred for 20 min. The enzyme was eluted from the gel by suspension in 0.25 M sodium phosphate buffer (pH 7.2) for 15 to 20 min. After centrifugation, the enzyme was again precipitated from the supernatant with 31.1% ammonium sulfate. The precipitate was collected by centrifugation and dissolved in 5 mM sodium phosphate buffer (pH 6.0) containing 3 mg of dithiothreitol per 100 ml and 10% glycerol. The solution was dialyzed against water for 4 hr and then against overnight against 5 mM sodium phosphate buffer (pH 6.0); dithiothreitol (3 mg/100 ml); 10% glycerol. This was used as a source of protein-arginine methyltransferase for these studies.
The methyltransferase was assayed as described by Casel-lass and Jeanteur (3). The reaction mixture contained 114 µg of partly purified protein-arginine methyltransferase (prepared as described above), 18 µg of rat liver histone (isolated 1 day after ethionine treatment; see Table 2, legend), 4 µmol of Tris-HCl (pH 7.4), 2.5 µCi (1 µmol) of S-adenosyl-[methyl-3H]methionine, and 0.5 µmol of dithiothreitol in a total volume of 100 µl. After incubation for 10 min at 37°C, the reaction was stopped by the addition of 2 ml of cold 10% trichloroacetic acid. The mixture was heated at 90°C for 10 min. The precipitate was collected by filtration and washed successively with 5% trichloroacetic acid and 95% ethanol. The filters were dissolved in methyl cellulose and counted for radioactivity.

Hydrolysis and Chromatography. Histones were hydrolyzed in a vacuum in 6 n HCl for 24 hr at 110°C. HCl was removed by evaporation, and the residue was washed 3 times with water. A Technicon amino acid analyzer was used for chromatography of the amino acids. Elution was with 0.3 M sodium citrate (pH 5.25) with a flow rate of 48 ml/hr, using a column of Aminex A-5 (50 x 0.9 cm) at 53°C.

Histones from both protein-lysine and protein-arginine methyltransferase assays were hydrolyzed and chromatographed as described above. At least 95% of radioactivity was found cochromatographing with the methylated lysine or arginine derivatives (see Chart 1).

RESULTS AND DISCUSSION

AdoEth inhibits both protein-lysine and protein-arginine methyltransferase in vitro (1, 2). Chart 2 also demonstrates the inhibition of these histone methylases by AdoEth. From the Kᵢ values reported by Baxter and Byvoet (1, 2) and the data in Chart 2, it appears that the protein-arginine methyltransferase is more sensitive to inhibition by AdoEth. The protein-arginine methyltransferase was inhibited 47% by AdoEth at a ratio of AdoMet:AdoEth (10:1) (see 0.2 mM AdoEth concentration in Chart 2A), whereas the protein-lysine methyltransferase was inhibited by 78% at this same ratio (see 1.0 mM AdoEth concentration in Chart 2B). On a molar ratio basis, the protein-lysine methyltransferase required 10 to 15 times more AdoEth to obtain the same level of inhibition. However, this may be due to the use of a whole nuclear assay for the protein-lysine methyltransferase, which would contain other methylase enzymes, requiring a higher level of AdoEth to achieve comparable inhibition.

To study the effects of AdoEth on histone methylation in vivo, it was necessary to obtain a situation in rat liver where AdoEth levels are high (0.5 to 1.0 µmol/g liver) and where newly synthesized histones (unmethylated) are available. Therefore, we first examined the synthesis of histones in regenerating liver following ethionine treatment. Chart 3 demonstrates the effects of varying doses of ethionine on histone synthesis 24 hr after partial hepatectomy. A greater inhibition is seen with increasing amounts of ethionine. However, at the maximum dose of ethionine (1 mg/g body weight), the synthesis of histones is inhibited by only 28%. Thus, in the presence of high levels of AdoEth, the synthesis of histones in the rat liver continues at a rate near normal.

Histone methylation in the regenerating rat liver does not occur at the time of synthesis but several hr after synthesis is completed (9, 15). This makes the situation better for our in vivo study, because ethionine can be administered after histone synthesis but before methylation has occurred. It was necessary to determine the peak of histone methylation following a two-thirds partial hepatectomy in order to find the optimum time in our laboratory setting for studying in vivo methylation. The methylation of histones was followed in regenerating rat liver by isolating nuclei at various times following partial hepatectomy and measuring lysine methylation in vitro as described in "Materials and Methods." Chart 4 gives the results of such an experiment, which demonstrates that histone methylation is returned to a normal level by 2 to 3 days following partial hepatectomy in the animals used in these studies.

The ability of ethionine to inhibit the methylation of rat liver histones in vivo is demonstrated in Chart 5. Ethionine was
administered 40 hr after partial hepatectomy, and 5 hr later, a 2-hr pulse of \([\text{methyl}^3\text{H}]\)methionine was given i.p. In control rat liver, both lysine and arginine were methylated as seen in Chart 5A. Chart 5B shows the chromatographic profile of the hydrolyzed histones from an ethionine-treated animal. The incorporation of radioactivity from methionine via AdoMet into the methylated derivatives was inhibited. Table 1 gives the actual values from the data in Chart 5 plus the values obtained when adenosine was given to maintain ATP levels. The incorporation of radioactivity into lysine was inhibited by 50%, whereas such incorporation into arginine was decreased by 89%. The adenosine administration did not alter this inhibition and suggests a role of AdoEth in the inhibition. At this time, the in vivo data are merely suggestive. Data concerning the uptake of ethionine, pool sizes (nuclear and cytoplasmic) of AdoMet, and rates of passage of these molecules across cellular membranes are needed. One explanation of the data is that ethionine alters the uptake of precursor (methionine or AdoMet) such that the nuclear and cytoplasmic pool sizes are different, resulting in an observable effect on the incorporation of radioactivity into histones. However, the greater sensitivity of the protein-arginine methyltransferase to inhibition by AdoEth as mentioned above in the in vitro assay coupled with the in vivo experiment suggests that the level of AdoEth may be greater in the cytoplasm than in the nucleus, thus affecting the cytoplasmic enzyme, protein-arginine methyltransferase, to a greater extent.

We also isolated histones from control and ethionine-treated animals. In the in vitro assay for lysine, the histones from ethionine-treated rats were methylated at 72,000 dpm/mg of histone, whereas control animals gave a value of 21,000 dpm/mg of histone. Table 2 gives a time study with the protein-arginine methyltransferase. This experiment demonstrates that the histones lose their ability to accept methyl groups. Thus, histones deficient in arginine methylation may be turning over, being ethylated by AdoEth or methylated by AdoMet. Further experiments are needed to clarify this question.

These experiments suggest that ethionine inhibits the meth-

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**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lysine</th>
<th>Arginine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18,000 ± 643 (100)</td>
<td>29,000 ± 3,512 (100)</td>
</tr>
<tr>
<td>Ethionine</td>
<td>9,000 ± 764 (50)</td>
<td>3,200 ± 173 (11)</td>
</tr>
<tr>
<td>Ethionine and adenosine</td>
<td>9,000 ± 608 (50)</td>
<td>3,000 ± 200 (14)</td>
</tr>
</tbody>
</table>

* Average of 3 rats ± S.E.
* Numbers in parentheses, percentage of control.

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**Table 2**

A time study of histone methylation after ethionine treatment

Ethionine was given 40 hr after partial hepatectomy (500 mg/kg body weight) and then 3 more times at successive 12-hr intervals. Three animals were then sacrificed at various times after the last dose of ethionine. Histones were isolated from each rat separately as described in "Materials and Methods" and then used as a substrate for the protein-arginine methyltransferase assay.

<table>
<thead>
<tr>
<th>Days after ethionine</th>
<th>Control</th>
<th>Ethionine</th>
<th>% of difference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>434,000 ± 26,000</td>
<td>1,314,000 ± 110,000</td>
<td>67</td>
</tr>
<tr>
<td>7</td>
<td>483,000 ± 25,000</td>
<td>676,500 ± 29,000</td>
<td>28</td>
</tr>
<tr>
<td>14</td>
<td>483,000 ± 24,000</td>
<td>509,000 ± 52,000</td>
<td>5</td>
</tr>
</tbody>
</table>

* Change in accepted methyl groups between histones of control and ethionine-treated animals.
* Average ± S.E. of 6 assay tubes (6 from each rat).
ylation of lysine and arginine residues in vivo and strongly suggest that this inhibition is due to the accumulation of AdoEth in the liver. It is known that ethionine via AdoEth inhibits methylation reactions in vivo as demonstrated previously with DNA methylase in rat liver (4) and now with histone methylase as shown in this report.

REFERENCES

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