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Replication-linked histone acetylation in rat liver tissue is sensitive to alkylating agents

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The effect of alkylating agents on histone acetyltransferase (EC 2.3.1.48) activity and thymidine incorporation was investigated in benign and malignant proliferating rat liver tissue and compared with the effect in normal non-proliferating rat liver tissue. In both, benign and malignant proliferating tissue, but not in quiescent tissue, the histone acetylation is depressed by alkylating agents and this depression correlates with the inhibition of the thymidine incorporation. This effect suggests that the depression of the replication associated histone acetylation may be an important factor for the antiproliferative activity of alkylating agents.

Histone; Acetylation; Replication; Hepatoma; Alkylating agent

1. INTRODUCTION

The posttranslational acetylation of histones is involved in a number of cell processes like cell differentiation [1], transcription [2] and replication [3,4]. Horiuchi et al. [5] first described differences between histone acetylation of normal and corresponding malignant cells and showed that histone acetylation and deacetylation occurs faster in nuclei from AH 66 hepatoma cells than in fetal, regenerating or adult normal liver. A replication associated histone acetylation was observed recently in rats after partial hepatectomy [6] as well as in various tumors [7].

It seemed interesting to investigate whether replication-linked histone acetylation is sensitive to alkylating agents. The capacity of alkylating agents like *N*-mustard or cyclophosphamide to depress the acetylation of core histones in tumor cells was shown in previous reports [8,9]. Keeping the above considerations in mind, we thought it reasonable to compare the depression of histone acetylation and proliferation in malignant tissue (hepatoma AS30D) with benign proliferating tissue (regenerating rat liver) and with normal non-proliferating untreated rat liver.

2. MATERIALS AND METHODS

2.1. *Preparation of cells*

Hepatoma AS30D was grown i.p. by inoculation in male Sprague–Dawley rats (250–300 g) and propagated by transplantation of the tumor every 12 days. Tumor cells were harvested at various days after inoculation. The alkylating antitumor agents were injected i.p. 4 h before harvesting the cells. Rats of the same sex, strain and weight as described above were partially hepatectomized [10]. The livers were isolated immediately and put into 0.14 M NaCl. The further procedure was as described previously [6]. The concentration of *N*-mustard was 5×10^{-5} mol/kg and of Mafosfamid (ASTA Z7557) 3×10^{-4} mol/kg. *N*-mustard (methyl-bis-(2-chloroethyl)amine) was purchased from Sigma Chemicals, Munich, FRG. 4-Sulfonatoethylthiocyclophosphamide (Mafosfamid, ASTA Z7557) was a gift from ASTA-Werke AG, Bielefeld, FRG.

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2.2. *Preparation of nuclei*

All the procedures were carried out at 0 to 4°C. Livers were homogenized in 3 vols STKM (0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.9) using a Potter homogenizer. Liver or hepatoma cells were suspended in STKM and sedimented by centrifugation at $1000 \times g$. Cells were resuspended in STKM containing 0.1% NP40, dounced 20–30 times and nuclei were pelleted at $1000 \times g$. Nuclei were washed two times using STC (0.34 M sucrose, 2 mM $CaCl_2$, 100 mM Tris, pH 7.9). All buffers contained 10 mM Na-butyrate, 0.1 mM phenylmethylsulfonylfluoride, 0.1% β-mercaptoethanol.

2.3. *Acetylation of histones in isolated nuclei*

Nuclei (1.5×10^7 approximately 1.2 mg protein) were preincubated in STC with 100 μM acetylcoenzyme A at 22°C for 5 min (total volume: 200 μl). The reaction was started by addition of [^{3}H]acetylcoenzyme A (1.6 Ci/mM, final concentration 3 μM) and terminated after 0, 1, 2, 4 and 8 min at 22°C by addition of 1 ml icecold STC buffer containing 160 μM unlabeled acetylcoenzyme A and subsequent centrifugation. Histones were extracted with 0.4 N H_2SO_4 at 4°C for 1 h, precipitated with TCA (final concentration 20%) and redissolved in 0.1 N NaOH. The radioactivity was counted in a liquid scintillation counter. Uptake of label was linear with time up to 4 min. Specific activities were indicated as pmol/min per mg histone. Histone protein concentration was determined according to the procedure by Lowry et al. [11].

2.4. *Gel electrophoresis of histones*

Isolated nuclei were lysed in sodium dodecyl sulfate sample buffer [12] and aliquots were subjected to electrophoretic analysis. An ali-

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quot of nuclei was used for determination of the protein content. Samples were analyzed by SDS-15% polyacrylamide slab gel electrophoresis (16 cm long) as described [12]. Equal amounts of protein were loaded on each lane. Gels were stained with Coomassie blue. Fluorography was performed according to a published procedure [13] using preflashed Kodak X-Omat RP film.

2.5. *Thymidine incorporation*

For in vivo labeling DNA with [^{3}H]thymidine, rats were i.p. injected with 1 μCi/g [^{3}H]thymidine (25 Ci/mmol) for 30 min. For in vitro labeling 1 ml of AS30D ascites was incubated with [^{3}H]thymidine at a final concentration of 20 μCi/ml at 37°C for 30 min. Nuclei were isolated as described above. Extraction of DNA and counting of radioactivity was done as described elsewhere [14]. DNA analysis was performed according to a published procedure [15].

3. RESULTS AND DISCUSSION

Table I shows histone acetyltransferase (HACT) activities of AS30D hepatomas of different age and of normal liver. The maximum of HACT activity occurred 6 days after i.p. inoculation and corresponds to an activity of 212% compared to the activity of untreated liver. After 12 days the activity decreased to values of about 144%. The results indicate a transient increase of HACT activity in AS30D hepatomas after i.p. inoculation of tumor cells. Whether this increased activity of histone acetyltransferase in hepatomas is due to elevated enzyme levels or whether it reflects alterations in the kinetic properties of the enzymes cannot yet be decided.

We used these results as a model to investigate the effects of alkylating agents on the HACT activity of AS30D hepatomas with different HACT activities. These tumors were compared with bening proliferating and non-proliferating tissues. It is well known that alkylating antitumor agents depress the acetylation of histones in tumor cells [8,16]. Our experiments showed that the extent of depression correlates with the height of the HACT activity. We found the greatest depression (62%) in 6-day-old tumors, 12-day-old tumors showed a depression of only about 46% (Table I). In normal liver we could not find an inhibition of HACT activity. These effects are not due to an increase in dead cells produced by the cytotoxic drugs, since the viability of cells treated with alkylating agents was 96.9 ± 2.3% when measured with the Trypan blue method.

Fig. 1A shows a representative histone acetyltransferase activity assay. Nuclei from AS30D hepatoma (6 days after i.p. inoculation) and liver in the presence and absence of *N*-mustard were compared. After treatment with alkylating agents the HACT activities of the tumors were lower than the activities of normal livers. These data were obtained by counting the labeled nuclei in a scintillation counter. Analyzing the same samples by SDS gel electrophoresis and fluorography produced the same results. Moreover, for all core histones the rate of [^{3}H]acetate incorporation was affected to about the same extent (Fig. 1B).

Table I

Histone acetyltransferase activity at various days after i.p. inoculation of AS30D tumor cells into rats

	n	Activity − *N*-mustard	*n*	Activity + *N*-mustard	*n*	Depression in %
AS30D						
5 days	3	197 ± 9.1	3	87 ± 7.0	3	56 ± 5.0
6 days	6	212 ± 8.6[a]	5	89 ± 4.1	5	62 ± 1.6[a]
8 days	3	167 ± 10.0	3	82 ± 7.0	3	50 ± 5.1
12 days	6	144 ± 5.7[a]	5	73 ± 2.7	5	46 ± 2.0[a]
Liver		100	5	102 ± 3.6		–

[a] The activity and the depression differed significantly between 6- and 12-day-old tumors ($P < 0.01$, *t*-test)

The activity was measured in hepatoma AS30D and normal rat liver with and without treatment of *N*-mustard. All activity values are given in % of untreated liver (100%). Each value is expressed as the mean ± SE of 3–6 experiments (*n* = number of experiments). 100% represents an activity of about 0.2 pmol/min/mg histone

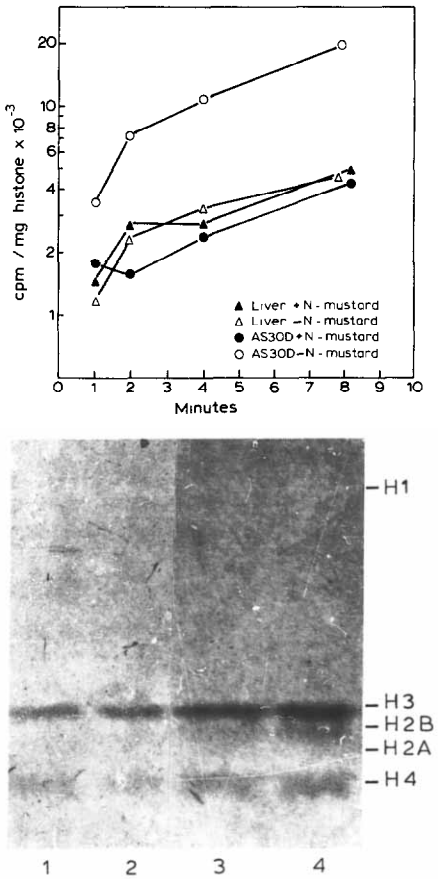

Fig. 1. (A) Effect of *N*-mustard on the histone acetyltransferase activity in hepatoma AS30D and host liver 6 days after i.p. inoculation. Histones were extracted and the incorporated label was measured as described in section 2. The values are normalized in terms of mg histone. (B) Fluorogram of acetylated histones. [^{3}H]Acetylcoenzyme A labeled nuclei from normal untreated liver (lane 1), 6-day-old hepatoma after treatment with *N*-mustard (lane 2), 12-day-old hepatoma (lane 3), 6-day-old hepatoma (lane 4).

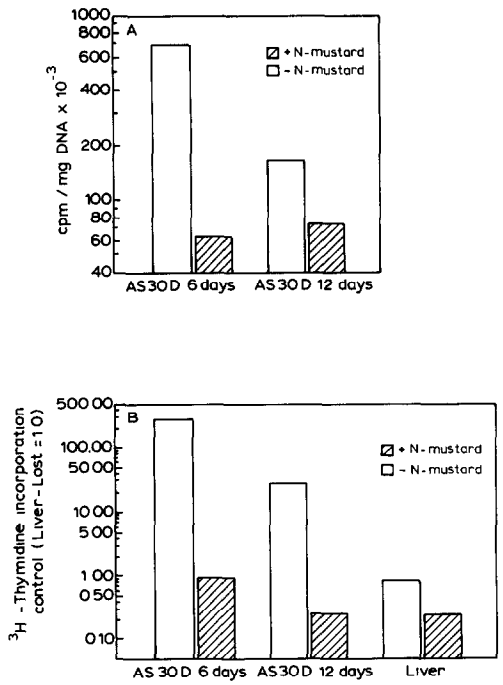

Fig. 2. Thymidine incorporation of *N*-mustard treated and untreated 6- and 12-day-old AS30D hepatomas in vitro (A) and compared with normal liver in vivo (B). Data are presented as the mean of two experiments.

In order to determine the antiproliferative effect of *N*-mustard at various days after i.p. inoculation of tumor cells we measured the thymidine incorporation in vivo and in vitro (Fig. 2). *N*-mustard has been shown to cause a direct block of DNA replication at the concentrations we used [17]. The in vivo thymidine incorporation correlates well with the data obtained after measuring the HACT activity. The 6-day-old tumor showed an increased thymidine incorporation and a stronger depression after treatment with *N*-mustard compared to 12-day-old tumor (Fig. 2B). The same data were obtained when thymidine incorporation was measured in vitro (Fig. 2A).

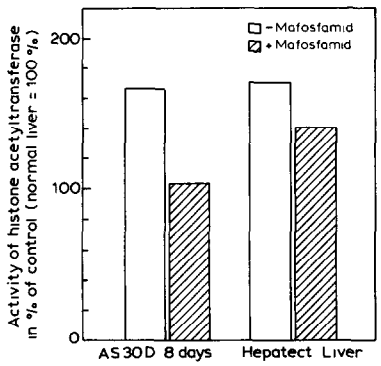

Fig. 3. The comparison of HCAT activity in benign (hepatectomized liver) and malignant (8-day-old AS30D hepatoma) proliferating tissue and the effect of Mafosfamid. The HACT activity was measured as described previously. Data are presented as the mean of two experiments.

The comparison of histone acetyltransferase activity in benign (hepatectomized liver) and malignant (AS30D-Hepatoma) proliferating tissue with non-proliferating tissue (normal liver) and the effect of cyclophosphamide (Mafosfamid) is shown in Fig. 3. Both proliferating tissues showed higher activities than liver and a depression of this activity after treatment with cyclophosphamide. The 6-day-old tumor showed a 300-fold decrease in thymidine incorporation and an inhibition of histone acetylation of about 62% after treatment with *N*-mustard. In normal liver the HACT activity was not influenced and only a 3-fold decrease in thymidine incorporation was seen (Fig. 2B and Table I). The thymidine incorporation in normal liver is probably not related to DNA replication – the small rate of thymidine incorporation may be due to DNA repair. The effect of *N*-mustard may be a combination of thymidine transport inhibition [18] and interaction with DNA repair.

These results suggest that the rate of inhibition of histone acetylation correlates with the rate of depression of thymidine incorporation in proliferating tissues. Therefore, the depression of histone acetylation may be an important factor for the antiproliferative activity of alkylating agents. This could be explained by a variety of possible mechanisms [19]. For example, proliferating tissue might take up more of the drug than non-proliferating tissue [20–23], or alternatively, a second more sensitive histone acetyltransferase might be present in proliferating cells [24]. A replication-linked destabilization of chromatin structure can make the core histones more vulnerable to alkylating agents, and, therefore, the acetylation of core histones will be depressed more strongly. This inhibition of acetylation would block the necessary displacement of core histones during DNA replication [1,25]. In any case, the data available so far demonstrate that replication-linked histone acetylation in proliferating malignant or benign tissues is particularly sensitive to alkylating agents.

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