Medical Journal of the
Islamic Republic of Iran

Volume 7
Number 1
Spring 1372
May 1993

STUDIES ON THE BINDING OF THE ALKYLATING AGENT SULFUR MUSTARD TO CALF THYMUS CHROMATIN

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ABSTRACT

In this study the effect of the alkylating agent, sulfur mustard, on calf thymus chromatin was investigated using UV/Vis spectroscopy, gel electrophoresis and thermal denaturation techniques. The results show that treatment of isolated chromatin with sulfur mustard releases histones from the core particles but does not affect histone H1 and nonhistone chromosomal proteins. The content of released proteins for the amounts lower than 50μg/mL are both dose and time dependent. It is suggested that they have cross linked reaction to other chromosomal proteins or to DNA. Thermal melting analysis also indicate that the interaction of sulfur mustard with DNA increases thermal melt(Tm) of DNA revealing condensation of DNA molecule by sulfur mustard. The results in relation to the functional states of chromatin are discussed.
MJIRI, Vol. 7, No.1, 43-46, 1993.

INTRODUCTION

Bifunctional alkylating agents are the most important and heterogeneous class of antineoplastic drugs in clinical use.[1,2] DNA is generally thought to be the principal target for these drugs. They alkylate DNA molecule preferentially at guanine-N7 position.[2-4] It has been reported that alkylating agents produce DNA inter-and intrastrand cross-links in a number of cell lines.[5-7] In addition, several workers have suggested that these drugs, especially nitrogen mustard, cause DNA-protein complexes in treated cells which are resistant to deproteination procedures.[8,9]

At the molecular level, in the cell nucleus, DNA is complexed with special sets of proteins called histones and nonhistone proteins. To date five main fractions of histones, namely H1, H2A, H2B, H3 and H4 have been isolated and characterized.[10] Interaction of the proteins with DNA molecule in the chromatin produces a bead on a string structure defined as nucleosomes.[10,12]

In this study we have attempted to examine the selective release and or cross-linking of chromatin components by treatment of calf thymus nuclei with the alkylating agent sulfur mustard.

MATERIALS AND METHODS

Calf thymus was obtained from Ziaran slaughter-house and transferred to the lab in ice. After removing the membranes, tissues were immediately frozen in liquid nitrogen before use. DNA was prepared from thymus according to Kay et al. method.[13] It was dissolved in 50 mM tris-HCl buffer by stirring overnight at 4°C. The amount of DNA was determined optically using A260=0.6. Sulfur mustard was obtained from Dr. Riasi in Defense Research Establishment. Stock solutions of 100μg/mL were made in 25 mM triethanolamine-HCl buffer pH 7.5 containing 1 mM EDTA and diluted to desired concentrations with the same buffer.

Isolation of nuclei: Nuclei from frozen calf thymus (10 g) were prepared essentially according to the procedure described by Hewish and Burgoyne.[14]

Nuclei pellet was washed three times with TMKC buffer and the sediment suspended in 10 mL of the same buffer. 100μL of the suspension was diluted into 3 mL of N NaOH and the absorbance at 260 nm was measured.

Binding assay: Nucleus suspension in TMKC buffer pH 7.4 diluted 1:1 with triethanolamine-EDTA buffer and

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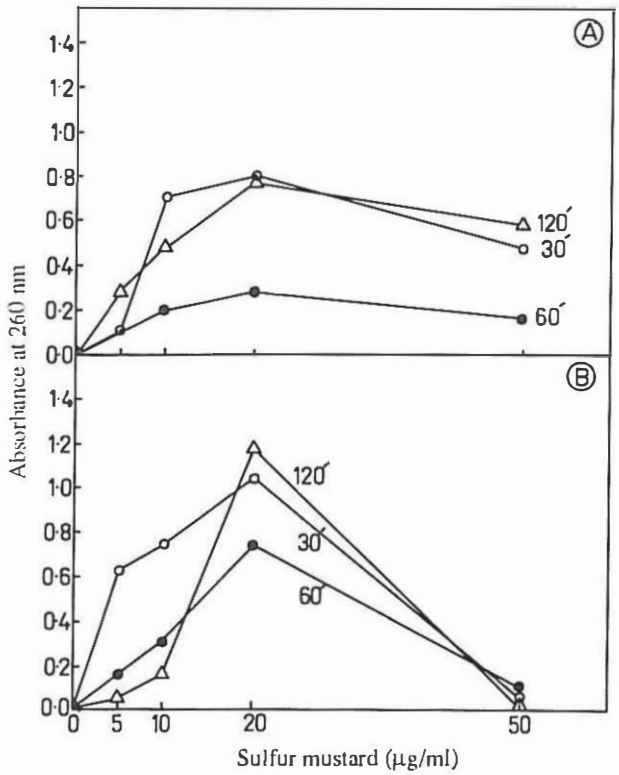

Fig. 1. Changes in the absorbance (260 nm) of the supernatants obtained from the treatment of calf thymus chromatin with different concentrations of sulfurmustard. Incubation times were 30, 60, and 120 min. at A, 25°C; and B, 37°C. Results are expressed as difference to controls and each point is the average of five determinations.

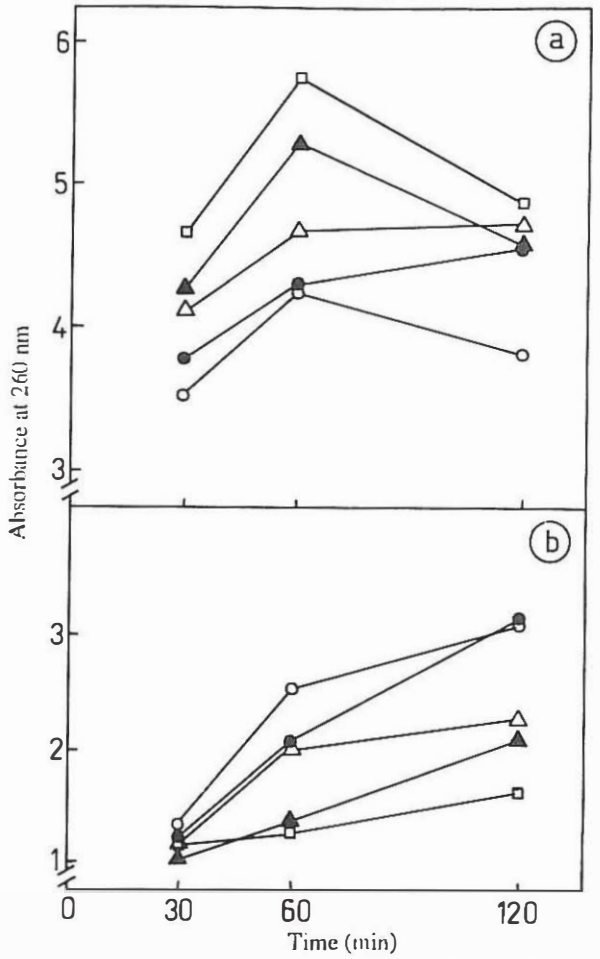

Fig. 2. Time course of sulfur mustard uptake by thymus chromatin. Measurements were performed at 260 nm. a: 25°C, b: 37°C.

the nuclei were lysed by a brief homogenization. Suspension was divided into several equal portions each containing 1 mg DNA per mL and sulfur mustard at 0.5, 1 0,2 0 and 50 μg/mL were added. The samples were incubated 30,60, and 120 min. at either 25°C or 37°C while occasionally mixing. Treated samples and the controls at time intervals were taken and centrifuged for 5 min at 6000g, 4°C. The supernatants and pellets were studied as follows:

The absorbance of the supernatants were monitored at 260 and 230 nm. The supernatants were made 10% trichloroacetic acid (TCA) by respect to 70% TCA. The precipitates were then collected by centrifugation at 6000 g for 5 min, dissolved in SDS sample solvent and loaded onto the gels. SDS-polyacrylamide gel electrophoresis was essentially carried out as described by Lammeli.[15] Destained gels were scanned by densitometric gel scanner Beckman Model (R-112) and the amount of each protein was determined by calculating the area under the peaks.

Thermal analysis: DNA was dissolved in 50 mM tris-HCl buffer pH 7.5 at a final concentration of 1 mg/mL. It was diluted into desired concentration and then mixed with different amounts of sulfur mustard. The mixtures were incubated for 30 min at 25°C. Treated and controls were-subjected to an increase in temperature at a rate of 1°C/min. The absorbance was read at 260 nm on a single beam Gilford 240 spectrophotometer using tris bufferas a blank. The derivative of hyperchromicity at temperature T was calculated by the equation $\frac{dhT}{dT} = \frac{h(T+1) - h(T-1)}{2}$

RESULTS AND DISCUSSION

Fig. 1 shows the effect of various concentrations of the alkylating agent sulfur mustard on the possible release of DNA from calf thymus chromatin into the supernatants as determined by measuring the absorbances at 260 nm. The results are expressed as difference to controls. Incubation was carried out at two different temperatures, 25°C and 37°C, and three time intervals 30, 60, and 120 minutes

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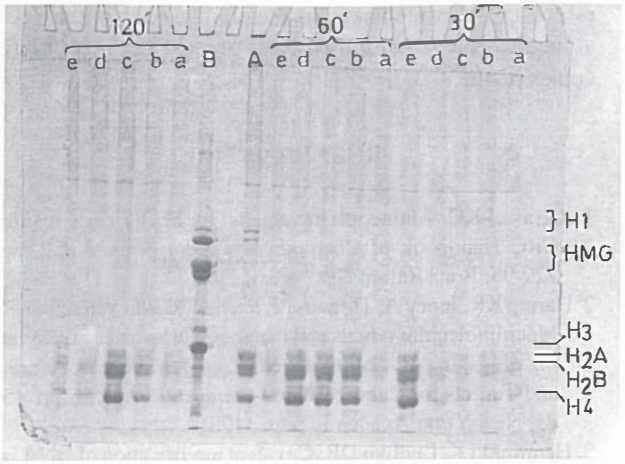

Fig. 3. SDS-polyacrylamide gel electrophoresis pattern of mustard treated and controls at 25°C. a-e of each experiment are zero to 50 ug/mL concentrations of sulfur mustard, respectively. A; standard calf thymus histones. B; standard nonhistone proteins (HMG).

were used. The results indicate that the release of DNA is proceeded in a dose dependent manner. In both conditions, the maximum release is obtained at 10-20 µg/mL of mustard level in absorbances. As is also seen in Fig. 2 there is a considerable difference between two incubation conditions as a function of time. At 30 min and 120 min of incubations at 25°C, the amount of released DNA is increased remarkably in comparsion to 60 min incubation. But when the experiment is performed at 37°C and 30 min incubation, the lowest absorbances are observed.

The proteins from treated and control samples were isolated and analyzed for identification of the materials remained in the supernatants. The proteins were precipitated with 10% TCA and the contents were electrophoresed on 15% SDS-polyacrylamide slab gels. The gel pattern is given in Fig. 3. It is shown that the highest release is at 20µg/mL of sulfur mustard, however, below this value the patterns are similar to controls. The results suggest an insufficient amount of mustard for acting on chromatin. On the other hand, at higher concentrations of mustard (50 µg/mL) although the gel pattern is identical to the controls, the interpretation is completely different from what has been given above, since in the latter presumably cross linking of proteins to proteins or to DNA has occurred by sulfur mustard action. Therefore after centrifugation the cross linked products remain attached to the insoluble part of the reaction mixtrue. According to the gel pattern, it is also apparent that the released proteins are only core histones H2A, H2B, H3 and H4. No histone H1 or nonhistone proteins especially high mobility group (HMG) is observed on the gel. To confirm the above results, data obtained from the 230 nm absorbance measurements is also given in Fig. 4. The results are in aggreement with the gel pattern.

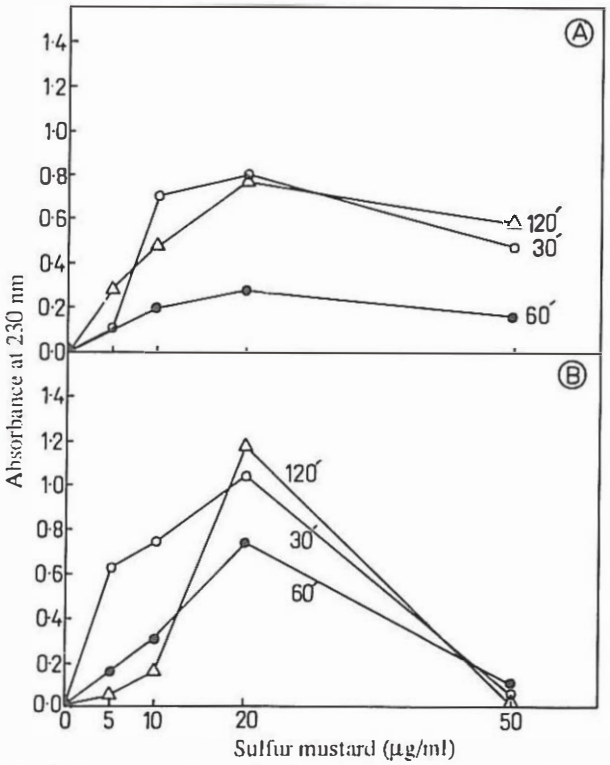

Fig. 4. Absorbance determination of the supernatants at 230 nm. Details are as indicated in Fig. 1.

Sediments from treated and controls were subjected to DNA and protein isolation procedures too. In the presence of alkylating agent DNA resists against extraction by phenol-chloroform procedure. Perchloric acid (5%) extraction and fractional acetone precipitation method was used to isolate H1 and HMG proteins. Analysis of the proteins on the SDS polyacrylamide gel electrophoresis revealed a complex pattern-indicating that both HMG and H1 are present in the sediment (data not shown).

In order to determine whether the binding of sulfur mustard alters isolated DNA structure, we examined the effect of different concentrations of mustard on free DNA in solution. Thermal denaturation analysis is a technique to demonstrate any structural changes occurred in DNA. Therefore DNA and sulfur mustard were mixed under experimental conditions mentioned in the methods section and incubated. Treated and controls were then thermally analyzed. Derivative melting profiles of the samples with 10 and 50 µg/mL sulfur mustard is shown in Fig. 5 in comparison to untreated DNA. It is seen that the binding of sulfur mustard protects DNA against thermal denaturation and shifts its Tm (thermal melts) to higher temperatures. This condensation pattern of DNA induced by mustard action can be explained by inter-or intrastrand cross linking of DNA as a function of alkylating agent.

Data mentioned so far indicate that when calf thymus chromatin is treated with sulfur mustard, DNA and pro-

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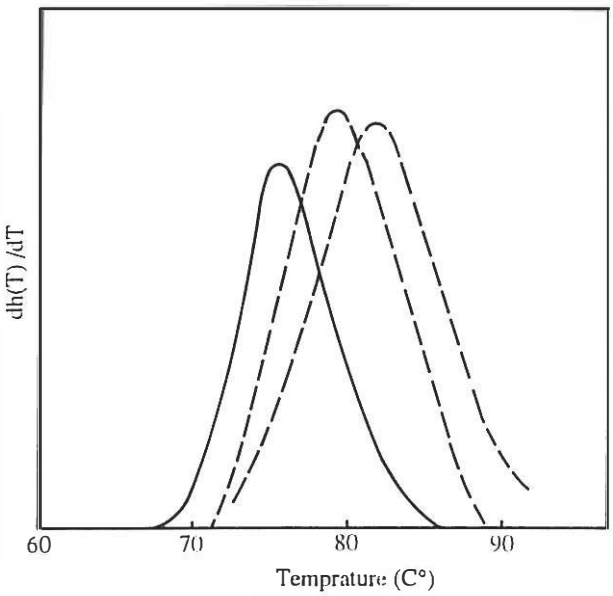

Fig. 5. Thermal denaturation profile of the interaction of DNA with sulfur mustard at 10 and 50μg/mL concentrations. Derivative of the points obtained from four separate experiments is shown.

___, DNA; - - - -, 20, μg/mL; ....., 50μg/mL mustards.

teins in the core particles are more susceptible to alkylation than linker DNA. Attachment of mustard to DNA in these regions render core histones to be released from the chromatin. On the other hand, when high concentrations of mustard is used, condensation of chromatin components occurs producing insoluble DNA-protein or protein-protein cross links. In vivo treatment of Yoshida sarcoma chromatin components with dibromodulcitol (DBD, 16) suggest that certain chromosomal proteins are degraded and subsequently newly synthesized resulting in an exchange of chromatin components. The formation of a nucleohistone complex between Hl and DNA is inhibited by pretreatment of Hl with DBD. The effect of other antitumor alkylating agents such as nitrogen mustard and trenimon on the nuclear proteins of chromatin has been reported by Riches and Harrap[17] and Wolf et al.[18] However, the type of alterations observed were quite different from the effects reported here. In spite of these findings our results do not show any reduced ability of histone Hl to interact with DNA but in contrast the interaction is quite stronger which tends for Hl to remain attached to DNA and cosediment with it.

From the results it is concluded that alkylating agents react chemically with chromosomal proteins some being more susceptible to alkylation that others. In view of the considerable importance of chromatin proteins in the cell functions such as replication and transcription they together with DNA are considered as important targets for alkylating agents. In studies on the mechanism of alkylating agents action it would seem desirable to measure the effects of these agents on the purified proteins and to look at the possible structural changes of individual chromatin components.

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