

# Involvement of histone hyperacetylation in triggering DNA fragmentation of rat thymocytes undergoing apoptosis

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**Abstract** The treatment of rat thymocytes with trichostatin A and sodium butyrate, which are inhibitors of histone deacetylase, resulted in an increase in DNA fragmentation in a concentration-dependent manner. A significant increase in DNA fragmentation induced by these compounds was observed after a lag time of 2 h. Analysis of the fragmented DNA revealed the production of approximately 50 kb DNA fragments and DNA ladders, the biochemical hallmarks of apoptotic cell death. Judging from a laser scanning microscopic analysis, the inhibitors of histone deacetylase induced nuclear condensation, the morphological feature of apoptosis. Biochemical and morphological analyses demonstrated that trichostatin A and sodium butyrate induced thymocyte apoptosis. Furthermore, hyperacetylation of nuclear histones was observed in thymocytes treated with the inhibitors of histone deacetylase. These effects of sodium butyrate and trichostatin A were seen 0.5 and 1 h, respectively, after incubation of the cells. These results thus indicate that hyperacetylation of nucleosomal histones precedes DNA fragmentation in thymocytes undergoing apoptosis induced by trichostatin A and sodium butyrate.

**Key words:** Histone hyperacetylation; Apoptosis; DNA fragmentation; Thymocyte; Chromatin structure

## 1. Introduction

The influence of chromosomal structure on gene expression is the current focus of molecular biology and genetics. Chromosome units named nucleosomes consist of DNA and histones, in which two turns of DNA are wrapped around the core histones [1]. Since the nucleosomal arrays are folded and packed into 30 nm chromatin fibers, the structure of chromatin is seemed to prevent access of a variety of enzymes [2]. Recently, it has been shown that acetylation, one of the post-translational modifications of core histones [3], induces unfolding of the chromatin fiber [4,5].

Apoptosis is a characteristic type of cell death and plays an important role in cell growth, differentiation and tissue development [6,7]. Although many biochemical changes, including the characteristic internucleosomal DNA fragmentation, correlate with apoptosis [8], little is known about the molecular mechanism of the onset of the apoptotic process and of DNA fragmentation. Since nuclear DNA is tightly packaged with histones into chromatin [9], relaxation of the chromatin structure is supposed to be necessary for endonucleases to catalyze

the reaction of DNA fragmentation. However, there have so far been no reports describing apoptosis from this point of view. With regard to loosening of chromatin, it has been shown that treatment of cells with trichostatin A [10] and sodium butyrate [11], well known inhibitors of histone deacetylase, leads to changes in the chromatin structure due to hyperacetylation of histones. In this study, these inhibitors were therefore used to study whether the apoptotic process is accompanied by such a modification of the chromatin structure.

The results obtained here have demonstrated for the first time that the acetylation of nuclear histones can induce thymocyte apoptosis with biochemical and morphological characteristics. A possible mechanism of DNA fragmentation in thymocytes undergoing apoptosis will be discussed.

## 2. Materials and methods

### 2.1. Materials

Trichostatin A was obtained from Wako Pure Chemicals Co. (Osaka, Japan). Butyrate was from Nacalai Tesque, Inc. (Kyoto, Japan) and used as sodium salt. [ $1-^{14}\text{C}$ ]Acetic acid sodium salt (59 mCi/mmol) was from Amersham International plc. All other reagents were of analytical grade.

### 2.2. Cell culture of thymocytes

Sprague-Dawley rats (4 weeks) were obtained from Japan S.L.C. (Hamamatsu, Japan). Isolated thymocytes from rats were incubated with RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) at a density of  $10 \times 10^6$  cells/ml under 5%  $\text{CO}_2$  in air as reported previously [12].

### 2.3. DNA fragmentation

After the incubation of thymocytes in the absence or presence of the compounds, the cells were collected and washed twice with phosphate buffered saline (PBS). The intact and fragmented DNAs in the cells were assayed as described previously [12]. DNA fragmentation was expressed as a percentage of total DNA (intact plus fragmented DNA).

### 2.4. Analysis of fragmented DNA

To separate large DNA fragments, the cells were resuspended in 50  $\mu\text{l}$  of PBS ( $5 \times 10^6$  cells) after the and 50  $\mu\text{l}$  of prewarmed low melting point agarose (1% in PBS) was added. Plugs were formed at  $4^\circ\text{C}$  for 30 min and then transferred into 1 ml of 0.5 M EDTA, 1% sarcosyl and 0.5 mg/ml proteinase K and incubated for 24 h at  $50^\circ\text{C}$ . The plugs were used for biased sinusoidal gel electrophoresis [13] using the Genofield apparatus (Atto Co. Ltd., Japan). To examine DNA laddering, fragmented DNA was analyzed by 1.8% agarose gel electrophoresis as described previously [12].

### 2.5. Laser scanning microscopy

After incubation of the thymocytes, the cells were fixed with 10% formaldehyde in PBS for more than 15 min at  $4^\circ\text{C}$ . The cells were collected by centrifugation at  $300 \times g$  for 10 min and the resulting pellet was suspended with PBS. The cell suspensions ( $10 \times 10^6$  cells/ml) were stained with 6.15  $\mu\text{g/ml}$  Hoechst 33342 for 10 min at room temperature. A laser scanning microscope (Olympus, LSM GB 200)

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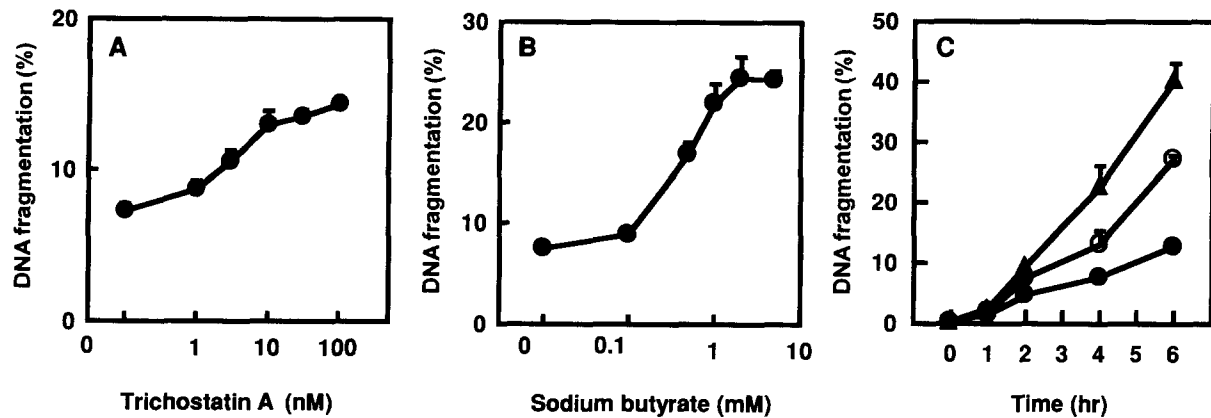


Fig. 1. Effect of various concentrations of trichostatin A (A), sodium butyrate (B) and incubation time (C) on DNA fragmentation in rat thymocytes. The thymocytes were incubated with different concentrations of trichostatin A and sodium butyrate for 4 h. In C, the thymocytes were incubated in the absence (●) and presence of 10 nM trichostatin A (○) or 5 mM sodium butyrate (▲) for different times. Then, fragmented and intact DNAs were determined as described in Section 2. The data are expressed as means  $\pm$  S.E.M. from 4–6 separate experiments.

equipped with a UV argon laser and a 40 $\times$  water-immersion objective was used to visualize individual nuclei as described elsewhere [14].

#### 2.6. Acetylation of histones

To extract histones, the nuclear fraction was prepared as follows. Thymocytes ( $100 \times 10^6$  cells) were suspended with 10 mM Tris-HCl

(pH 7.6) containing 150 mM NaCl, 1.5 mM  $MgCl_2$ , 3% glycerol and 10 mM EDTA (buffer A). The cell suspension was lysed with 0.1% Triton X-100 in buffer A. The lysate was put on the solution containing 10 mM Tris-HCl (pH 7.6), 1.5 mM  $MgCl_2$  and 25% glycerol and then the nuclei were collected by centrifugation at  $350 \times g$  for 10 min. Histones were extracted with 0.4 M sulfuric acid from the resulting

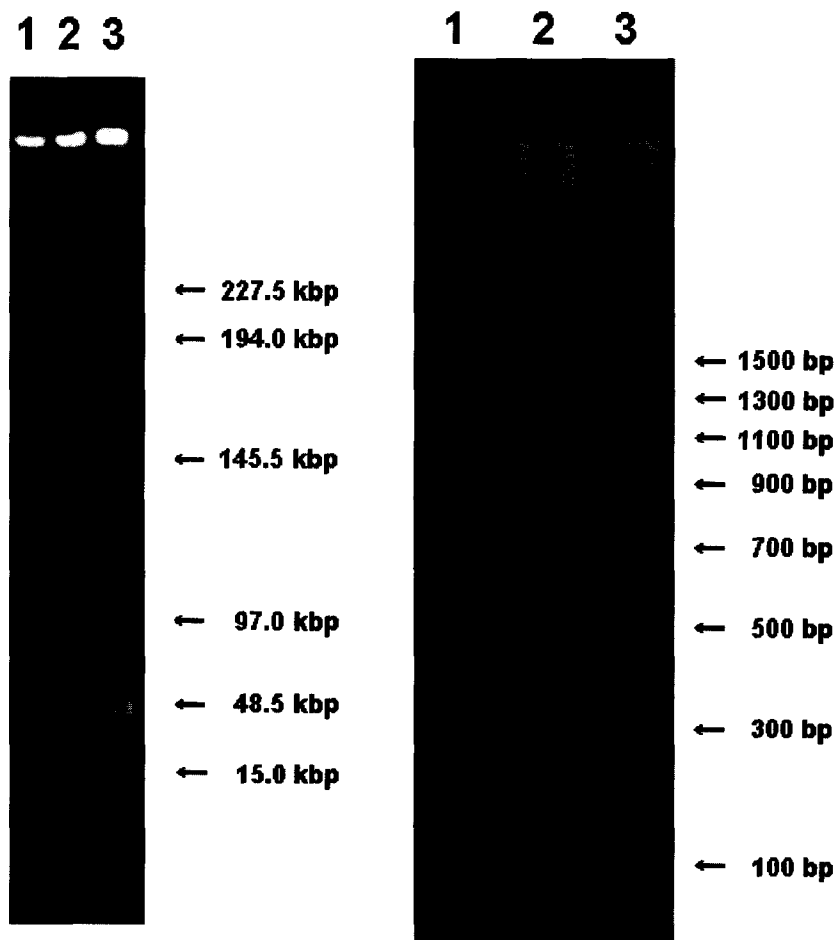


Fig. 2. Biased sinusoidal field gel electrophoresis (left) and conventional gel electrophoresis (right) of DNA from thymocytes treated with inhibitors of histone deacetylase. The thymocytes were treated with 10 nM trichostatin A or 5 mM sodium butyrate for 6 h. Lane 1, control; lane 2, trichostatin A-treated; lane 3, sodium butyrate-treated thymocytes.

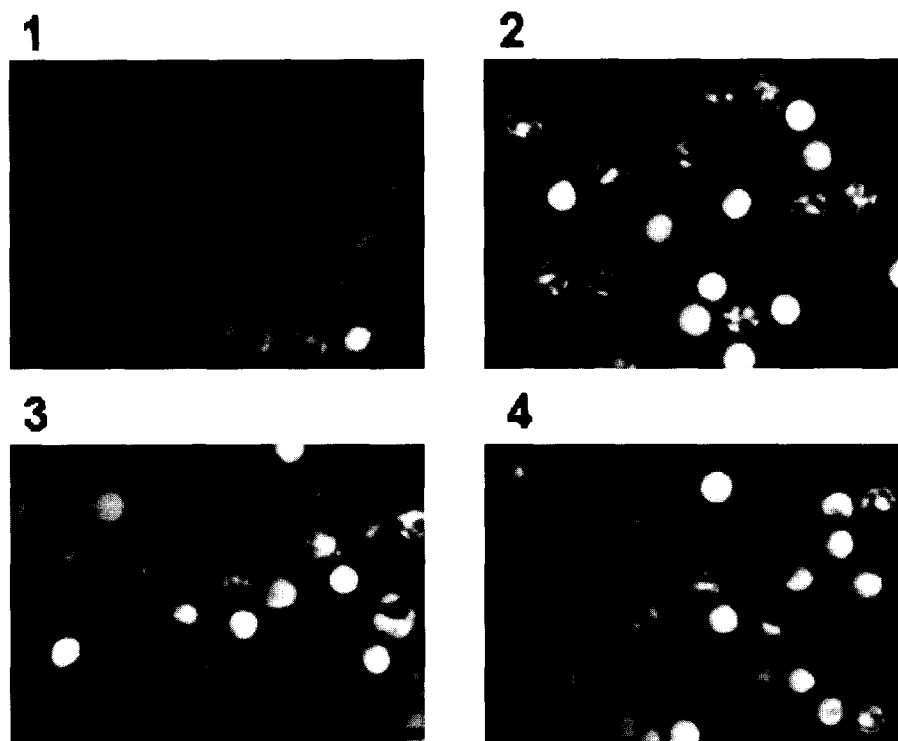


Fig. 3. Laser scanning microscopic images of thymocytes treated with inhibitors of histone deacetylase. The thymocytes were treated with 1  $\mu$ M dexamethasone, 10 nM trichostatin A or 5 mM sodium butyrate for 6 h. The cells were fixed with formaldehyde after the treatment and were stained with Hoechst 33342. Lane 1, control; 2, dexamethasone-treated; 3, trichostatin A-treated; 4, sodium butyrate-treated thymocytes.

nuclear fraction. Acetylation of histones was examined by gel electrophoresis using acid urea Triton gels as described by Hoshikawa et al. [15]. The gel was stained with Coomassie brilliant blue.

### 3. Results and discussion

#### 3.1. DNA fragmentation of thymocytes induced by inhibitors of histone deacetylase

In apoptosis as well as gene expression, lack of compactness of nascent chromatin is assumed to be a prerequisite for triggering the process. To determine whether modification of the chromatin structure with acetylation of nuclear histones elicits genome degradation, immature rat thymocytes were incubated with trichostatin A, a potent and specific inhibitor of histone deacetylase [10]. As shown in Fig. 1A, treatment of thymocytes with trichostatin A resulted in an increase in DNA fragmentation. In addition to trichostatin A, sodium butyrate, another inhibitor of histone deacetylase [11], was also effective in causing DNA fragmentation (Fig. 1B). The DNA fragmentation induced by these compounds was dependent on their concentrations (Fig. 1A,B) and on incubation time (Fig. 1C). Although DNA fragmentation induced by trichostatin A and sodium butyrate required a lag time of at least 2 h, these compounds significantly increased DNA fragmentation after incubation for 4 h (Fig. 1C).

To analyze the size of DNA fragmentation, the fragments of nuclear DNA were examined by the use of agarose gel electrophoresis. As shown in Fig. 2 (right), agarose gel electrophoresis of the DNA from thymocytes treated with trichostatin A and sodium butyrate revealed the typical ladder pattern of DNA fragments, showing that these compounds caused internucleosomal DNA fragmentation in the cell. In

addition, it has recently been reported that cleavage of nuclear DNA to large fragments preceding the oligonucleosomal fragmentation is an initial step of apoptosis [16,17]. The production of approximately 50 kb fragments of DNA induced by trichostatin A and sodium butyrate was clearly observed in this experiment (Fig. 2, left). Data from electrophoretic analyses of nuclear DNA support that the apoptotic process is promoted by these compounds.

#### 3.2. Morphological characterization of thymocyte apoptosis induced by inhibitors of histone deacetylase

The laser scanning microscopic image of control thymocytes showed a core-like structure and unequal contours of fluorescent intensity in most cells, being responsible for the nuclear chromosome (Fig. 3). As a positive control of apoptotic cell death, the thymocytes were treated with glucocorticoid, well known to induce typical apoptosis [8]. Treatment of the cells with dexamethasone revealed a peculiar feature of nuclei, having an equal distribution of much more intensive fluorescent and smaller diameter than the control-like nuclei. The formation of this type of nuclei was dependent on the incubation time and paralleled the increase in DNA fragmentation, thus accounting for the apoptotic nuclei. However, no significant difference in the shape and size of cells between control and apoptotic thymocytes was observed using a phase-contrast microscope (data not shown). In trichostatin A- or butyrate-treated cells, the distinct features of apoptotic nuclei were also observed (Fig. 3). When these nuclei were counted in the field of vision using confocal microscopy, the percentages of this type of nuclei were 10, 50, 35 and 37 in control, dexamethasone-, trichostatin A- and butyrate-treated cells, respectively. The evidence presented here fulfils all the

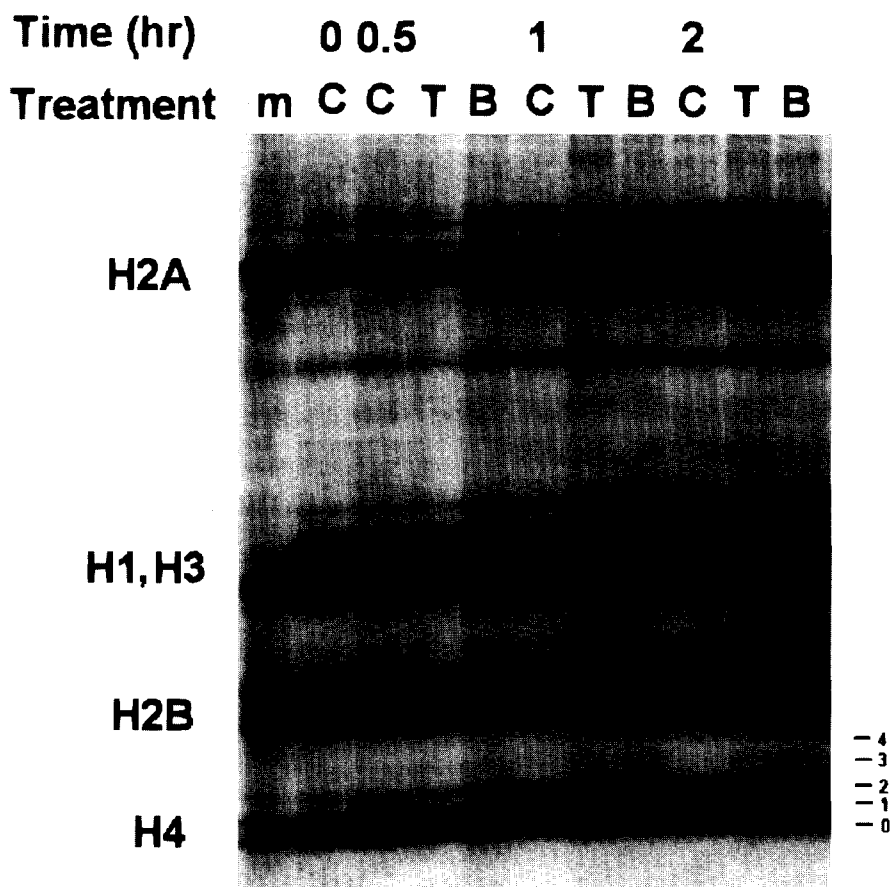


Fig. 4. Effect of trichostatin A and sodium butyrate on histone acetylation. The thymocytes were treated with or without the inhibitors of histone deacetylase for the time indicated. Histones were extracted with 0.4 M sulfonic acid from the cells and analyzed on AUT gel electrophoresis as described in Section 2. C, control; T, 10 nM trichostatin A; B, 5 mM sodium butyrate; m, standard histone mixture. The small numbers on the right side point to the different extents of acetylation of histone H4.

criteria for apoptosis so far proposed, demonstrating that inhibitors of acetylation of histones induce thymocyte apoptosis.

### 3.3. Hyperacetylation of histones in thymocytes treated with inhibitors of histone deacetylase

It has been reported that sodium butyrate induces apoptosis in the human promyelocytic leukemia cell line HL-60 [18,19]. Chang and Yung showed that sodium butyrate-induced apoptosis was accompanied by an increase of tyrosine phosphorylation of cellular proteins [19]. However, little is known about the mechanism of apoptotic cell death induced by this compound. In order to clarify the inhibition of histone deacetylation with trichostatin A and sodium butyrate in thymocytes, the effect of these compounds on the acetylation of histones was determined using thymocytes preloaded with [ $^{14}$ C]acetate as a donor of histone acetylation. The activity of histone deacetylation was inhibited 43% and 65% by treatment with 10 nM trichostatin A and 5 mM sodium butyrate, respectively, consistent with previous reports [10,11].

Fig. 4 shows an AUT gel electrophoresis on which core histones such as H2A, H3, H2B and H4 and H1 were observed in the extracts from control cells. Each histone was separated into additional bands, showing different extents of acetylation. As reported previously [15], band H4 was separated into mono-, di-, tri-, and tetra-acetylated forms. Among

the core histones, H4 was hyperacetylated only 1 h after the incubation of thymocytes with 10 nM trichostatin A. 5 mM of sodium butyrate caused hyperacetylation as well after 30 min incubation. These results demonstrate that trichostatin A and sodium butyrate inhibit histone deacetylation and thus accelerate hyperacetylation of histones, especially H4, in thymocytes. It should be noted that these compounds (Fig. 1C) induced DNA fragmentation a few hours later than the hyperacetylation of histone.

The primary proteins whose properties mediate the folding of DNA into chromatin are the histones [1]. The acetylation of these basic proteins is regulated by histone acetyltransferase and histone deacetylase. Since exposure of HeLa cells to butyrate leads to an increase in acetylated histones with a concomitant increase in DNase I sensitivity [20], histone acetylation is involved in the modulation of the nucleosome structure [21]. In our preliminary experiments, we also observed that the sensitivity of DNase I toward the nuclei of cells treated with the inhibitors was significantly higher than that toward control nuclei. This evidence shows that hyperacetylation of nucleosomal histone with butyrate or trichostatin A increases the accessibility to DNase I of the chromatin DNA. Therefore, it is likely that histone hyperacetylation triggers DNA fragmentation in thymocytes due to an increase in the accessibility of DNA to constitutive endonuclease.

Although the physiological roles of histone acetylation are

not fully understood, there is some evidence that actively transcribed genes contain enriched acetylated histones [22–24]. In addition, modification of cellular events, including inhibition of the cell cycle in fibroblasts [25] and induction of erythroid differentiation in leukemia cells [26], was observed after treatment with trichostatin A; this may help to elucidate the role of histone acetylation. The compound also induces morphological changes and gelsolin expression in carcinoma cell lines [27]. The present study clearly shows that histone acetylation by trichostatin A induces thymocyte apoptosis. Therefore, we propose that a similar modification of chromatin structure with histone hyperacetylation initially happens during apoptosis and thus the endogenous endonuclease can easily attack linker DNAs, resulting in internucleosomal DNA fragmentation. The present results suggest that histone hyperacetylation in the chromatin structure is one of the molecular mechanisms of the onset of the apoptotic process.

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