Histone H1 quantity determines the efficiencies of apoptotic DNA fragmentation and chromatin condensation

Marie KIJIMA and Ryushin MIZUTA

Research Institute for Biomedical Sciences, Tokyo University of Science, Noda, Chiba 278-0022, Japan

(Received 11 September 2018; and accepted 13 November 2018)

ABSTRACT

Oligonucleosomal DNA fragmentation and chromatin condensation are two hallmarks of apoptosis. However, their generation mechanisms are not entirely understood. Histone H1, a positively charged nuclear protein located in the linker region of chromatin, is involved in higher-order chromatin structures and tight chromatin packing. On the basis of the physical and biochemical characteristics of histone H1, we hypothesized that histone H1 plays a role in determining the efficiencies of apoptotic DNA fragmentation and chromatin condensation. Therefore, we examined histone H1 quantity in five human leukemia cell lines and compared the efficiencies. The cell lines were categorized into two groups according to their origins: (i) Ramos and Molt-4 cells of lymphoid origin and (ii) U937, ML-1, and HL60 cells of myeloid origin. Compared to the lymphoid-origin group, the myeloid-origin group had lower levels of histone H1 but more open chromatin. Furthermore, the myeloid-origin group showed marked DNA fragmentation but less chromatin condensation during apoptosis. These results suggested that histone H1 determined chromatin structure and that its quantity affected the efficiencies of DNA fragmentation and chromatin condensation in apoptosis.

DNA fragmentation and chromatin condensation are well-known hallmarks of apoptosis (10). However, how they are generated and the factors involved in these phenomena are not completely understood. DNA fragmentation creates structural DNA fragments of multiples of the 180-bp nucleosomal unit, which appears as a DNA ladder in agarose gel electrophoresis (11). Although caspase-activated DNase (CAD), also known as DNA fragmentation factor 40 (DFF40), has been proposed as an apoptotic endonuclease (14), other endonucleases such as DNase γ /DNase1L3 and DNase I also cause oligonucleosomal DNA fragmentation (8). Moreover, DNA fragmentation does not always occur in apoptotic cells, and the efficiency varies from cell to cell. For ex-

ample, human T-cell leukemia cells (Molt-4), erythroleukemia cells (K562), and glioblastoma cells (T98G) do not undergo internucleosomal DNA cleavage upon treatment with various apoptotic stimuli (*e.g.* etoposide, H_2O_2 , TNF, and irradiation) (9). Particularly, Molt-4 is known as the cell line that does not show oligonucleosomal DNA fragmentation during apoptosis even in the activation of apoptosis signal cascade and the presence of CAD (5). In the case of chromatin condensation, neither the generation mechanism nor the factors involved are understood. We previously studied the roles of three endonucleases in cell death—CAD/DFF40, DNase γ /DNase1L3, and DNase I—and found the possibility that linker histone H1 may affect DNase accessibility to chromatin in dead cell nuclei (8).

Histone H1, an abundant positively-charged nuclear protein, is located in the linker region of chromatin. Several *in vitro* studies using reconstituted chromatin suggested that histone H1 plays a role in the formation of higher-order chromatin structures

Address correspondence to: Dr Ryushin Mizuta, Research Institute for Biomedical Sciences, Tokyo University of Science, Noda, Chiba 278-0022, Japan
Tel: +81-4-7121-4071
E-mail: mizuta@rs.noda.tus.ac.jp

Fig. 1 Detection of more abundant histone H1 protein in Ramos and Molt-4 cells than in U937, ML-1, and HL60 cells. **(A)** Western blot analysis of histone H1 and H3 proteins in Ramos, Molt-4, U937, ML-1, and HL60 cells. The anti-histone H1 antibody has cross-reactivity with histone H3. Thus, the level of histone H3 protein is the protein loading control. **(B)** Quantification of histone H1 protein levels shown in (A). Data are presented as mean $\pm$ standard deviation (SD) of four independent experiments. * $P < 0.05$ ($n = 4$, Tukey's multiple comparison test).

and tight chromatin packing (4, 6). On the basis of the physical and biochemical characteristics of histone H1, we hypothesized that histone H1 plays a role in determining the efficiencies of apoptotic DNA fragmentation and chromatin condensation. Thus, we examined the quantity of histone H1 in various cell lines and compared the efficiencies of chromatin condensation and DNA fragmentation in apoptosis.

We chose five human leukemia cell lines: Ramos and Molt-4 cells, which are of lymphoid origin; and U937, ML-1, and HL60 cells of myeloid origin (9, 13). We used western blot analysis to quantify histone H1 protein. We cultured cells in RPMI-1640 medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 50 μ M β -mercaptoethanol, 2 mM penicillin/streptomycin, and 10% fetal calf serum (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C under 5% CO₂ in a humidified incubator. We harvested cells (1.0×10^6), suspended them in 50 μ L of phosphate-buffered saline, and lysed them in 50 μ L of sample buffer: 4% sodium dodecyl sulfate (SDS), 100 mM Tris-HCl (pH 6.8), 2 mg/mL bromophenol blue, 20% glycerol, and 200 mM dithiothreitol (DTT). After 5 min of sonication, we separated the samples on an SDS-15% polyacrylamide gel and electrophoretically transferred them onto the nitrocellulose membrane Amersham protran 0.45 μ m NC (GE Healthcare Lifesciences, Chicago, IL, USA). We probed membrane-immobilized proteins with mouse anti-linker histone H1 monoclonal antibody clone AE-4 (Upstate Biotechnology, Lake Placid,

NY, USA) and visualized them with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Thermo Fisher Scientific). The AE-4 antibody cross-reacts with core histone H3 (8). Thus, we used the signal of histone H3 as the loading control. Images were collected with Fujifilm LAS-3000 Imager and analyzed with Image Gauge software (Fujifilm, Tokyo, Japan). We found that histone H1 was more abundant in cells of lymphoid origin (Ramos and Molt-4) than cells of myeloid origin (U937, ML-1, and HL60) (Fig. 1A and B). In particular, histone H1 levels were significantly higher in Ramos cells than U937, ML-1, or HL60 cells (Fig. 1B).

Next, we investigated the sensitivity of chromatin condensation and DNA fragmentation in apoptosis using these five cell lines. We induced apoptosis in each cell line (5.0×10^5 cells/mL) with ultraviolet (UV) irradiation (40 mJ/cm²) using a GS Gene Linker UV chamber (Bio-Rad Laboratories, Hercules, CA, USA). Condensed chromatin was stained with Hoechst 33342 (1 μ g/mL) (Dojindo, Kumamoto, Japan) at 24 h after UV-irradiation and examined with an Olympus IX71 fluorescence microscope (Olympus, Tokyo, Japan). We detected the difference of condensation signals (*i.e.*, strong signals in lymphoid-origin cells vs weak signals in myeloid-origin cells) and expected that the levels of histone H1 correlated with the difference. To quantify chromatin condensation, we counted cells exhibiting a single large clump of chromatin as positive cells with strong chromatin condensation. We counted more than 100 cells in the different fields and calcu-

Fig. 2 Correlation between chromatin condensation and DNA fragmentation in apoptosis. **(A, B)** Stronger chromatin condensation was detected in Ramos and Molt-4 cells than in U937, ML-1, and HL60 cells after apoptosis induction. Apoptosis was induced with UV-irradiation. Chromatin was stained with Hoechst 33342 and examined under a fluorescent microscope after 24 h of incubation. Magnified images are shown in insets (A). Ctrl: non-irradiated samples. Scale bar: 100 μ m. **(B)** The quantification of chromatin condensation was determined as the ratio of cells having a single large clump of chromatin. Data are presented as mean $\pm$ SD of three independent experiments. In each experiment, images were collected from three different fields and used for the analysis. $*P < 0.05$, $**P < 0.01$ ($n = 3$, Tukey's multiple comparison test). **(C)** Efficient DNA fragmentation was detected in U937, ML-1, and HL60 cells after apoptosis induction with UV-irradiation. DNA was purified from the irradiated samples after 3 or 24 h of incubation, lanes 6–10 or 11–15, respectively. Purified DNA was separated on an agarose gel and visualized with ethidium bromide. Ctrl: non-irradiated samples (lanes 1–5). 100 bp: 100-bp DNA ladder marker. 1 kb: 1-kb DNA ladder marker. **(D)** Comparable levels of CAD protein were detected in all five cell lines by western blot analysis of CAD and GAPDH proteins.

lated the number of positive cells as the percentage of total counted cells. Then, we concluded that the lymphoid-origin cells with abundant histone H1 showed stronger chromatin condensation than myeloid-origin cells with scarce histone H1 (Fig. 2A and B).

To examine apoptotic DNA fragmentation, we harvested UV-irradiated cells (5.0×10^5) after 3 or 24 h of incubation with centrifugation at $200 \times g$ for 5 min at 4°C and suspended in 60 μ L of phosphate-buffered saline. We lysed the harvested cells by adding 300 μ L of 8 M guanidine and isolated DNA from the lysate with the Wizard DNA purification resin (Promega, Madison, WI, USA) as described previously (13). We measured DNA fragmentation

by performing electrophoresis on a 1.5% agarose gel in the presence of ethidium bromide (0.5 μ g/mL).

The U937, ML-1, and HL60 cells (Fig. 2C, lanes 8–10 and 13–15) had DNA that was easier to degrade than Ramos or Molt-4 cells (Fig. 2C, lanes 6, 7, 11, and 12) and exhibited typical DNA fragmentation or DNA ladder formation after 3 h of incubation. Then, we examined the expression of CAD protein by western blot analysis using mouse anti-CAD/DF40 monoclonal antibody (R&D Systems, Minneapolis, MN, USA) and horseradish peroxidase-conjugated rabbit anti-mouse IgG (Fig. 2D). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the loading control, which was probed with mouse anti-

Fig. 3 Chromatin of U937, ML-1, and HL60 cells is more sensitive to DNases than that of Ramos and Molt-4 cells. Nuclei isolated from lymphoid-origin (Ramos and Molt-4) and myeloid-origin cells (U937, ML-1, and HL60) were treated with DNases for 2 h (DNase I) or 6 min (MNase) at 37°C. DNA was purified from the treated samples and separated on an agarose gel. (A) DNase I (lanes 8–12) or MNase treated samples (lanes 15–19) are exhibited. Ctrl: untreated samples (lanes 1–5). (B) Nuclei isolated from Ramos and U937 cells were treated with the urine of C57BL/6 wild-type mice at 37°C for 2 h in the presence (+, lanes 2 and 4) or absence of protease inhibitor (lanes 1 and 3). 100 bp: 100-bp DNA ladder marker. 1 kb: 1-kb DNA ladder marker.

GAPDH monoclonal antibody (MBL, Aichi, Japan) and horseradish peroxidase-conjugated rabbit anti-mouse IgG. We detected comparable levels of CAD-protein expression between lymphoid- and myeloid-origin cell lines. These results suggested that the efficiency of DNA fragmentation was not dependent on the quantity of CAD but on chromatin structure.

Finally, we examined the DNase sensitivity of chromatin in nuclei prepared from cultured cells, which has been used as an indicator of accessible chromatin or open chromatin (17). We used micrococcal nuclease (MNase) and DNase I (Takara Bio Inc., Osaka, Japan). We also used mouse urine from C57BL/6 wild-type mice, because mouse urine contains abundant DNase I (7). To prepare nuclei, we harvested cultured cells (1×10^7) and suspended them in 1 mL of cold isolation buffer (10 mM KCl, 250 mM sucrose, 4 mM $MgCl_2$, 1 mM DTT, and 20 mM HEPES) with 10 μ L of Protease Inhibitor Cocktail (100 $\times$) (Thermo Fisher Scientific). We subsequently lysed the cells with the addition of 50 μ L of 0.5% NP-40. After 10 min of incubation on ice, we harvested cell nuclei by centrifugation at $300 \times g$ for 10 min at 4°C, rinsed precipitated nuclei twice with 1 mL of cold isolation buffer, and adjusted the concentration to 2.0×10^4 nuclei/ μ L with an equal volume of cold isolation buffer and 100% glycerol. The reaction mixture for the DNase sensitivity assay

in a final volume of 60 μ L contained 2.0×10^5 nuclei, Dulbecco's modified Eagle medium (Nissui Pharmaceutical Co.) with 25 mM HEPES, and DNases—DNase I (0.02 U), MNase (0.2 U), or 1.5 μ L mouse urine (Fig. 3). The reactions were performed at 37°C for 2 h, 6 min, or 2 h, respectively. We added Protease Inhibitor Cocktail (0.6 μ L) to urine samples where indicated (Fig. 3B). After incubation, we lysed the reaction samples by adding 300 μ L of 8 M guanidine and isolated DNA from the lysate with the Wizard DNA purification resin as described previously (13). We analyzed DNA by electrophoresis on a 1.5% agarose gel in the presence of ethidium bromide (0.5 μ g/mL). No DNA degradation was detected in the control (without DNases) cell nuclei (Fig. 3A, lanes 1–5). However, with DNase I (lanes 8–12) and MNase (lanes 15–19), the chromosomal DNA of U937, ML-1, or HL60 nuclei was more efficiently degraded than that of Ramos or Molt-4 nuclei; moreover, higher-molecular-weight DNA was detected in Ramos and Molt-4 nuclei. The difference was particularly evident with DNase I treatment (lanes 8–12). Ramos and Molt-4 nuclei exhibited a smear pattern containing higher-molecular-weight DNA on the agarose gel (lanes 8 and 9); whereas U937, ML-1, and HL60 nuclei showed a ladder pattern (lanes 10–12) similar to that of apoptotic DNA fragmentation (Fig. 2C).

The urine-treated samples exhibited similar results

to DNase I treatment (Fig. 3B). Cleaved chromosomal DNA from the Ramos nuclei exhibited a smear pattern (lane 1), whereas that from U937 nuclei exhibited a ladder pattern (lane 3); the latter was cleaved more efficiently than the former. Furthermore, DNA degradation was inhibited in the presence of protease inhibitor (lanes 2 and 4)—suggesting that, in urine, DNase I cleaves chromatin in collaboration with proteases that degrade proteins associated with DNA, such as histones. DNase I is rather resistant to proteases and cleaves naked DNA more efficiently than protein-associated DNA (*e.g.*, nucleosome-associated DNA) (8). The preference of DNase I to naked DNA also suggests that the linker region of U937 chromatin was more open than that of Ramos and explains the generation of nucleosomal unit-DNA fragments. The results collectively suggest that the chromatin of myeloid-origin cells was more sensitive to DNases than that of lymphoid-origin cells, indicating that the myeloid-origin cells had more open chromatin.

Our results show that the amount of histone H1 was related to apoptotic chromatin condensation and DNA fragmentation, and also determined the structure of chromatin (*i.e.*, open or closed) in living cells. Specifically, cells with a low amount of histone H1 tended to have open chromatin and exhibited marked DNA fragmentation but less chromatin condensation during apoptosis. Experiments involving knockdown or overexpression of histone H1 protein might be necessary to confirm these findings, although such experiments are technically challenging. Because humans have 11 distinct variants of the *histone H1* gene (3), it is extremely difficult to generate null mutant cells. Furthermore, histone H1 is one of the most abundant proteins in eukaryotic cells (1), making it difficult to obtain histone H1-overexpressing clones.

In this study, we found that the myeloid-origin cells (U937, ML-1, and HL60) had less histone H1 than the lymphoid-origin cells (Ramos and Molt-4). The myeloid-origin group also exhibited marked DNA fragmentation and low chromatin condensation during apoptosis. Notably, the myeloid-origin cells (U937, ML-1, and HL60) are cell lines known to be blocked at an early stage of their development and fail to differentiate into functionally mature cells—several reports suggest that phorbol diesters and retinoids can induce them to terminal differentiation and apoptosis (2, 12, 15). Moreover, it was reported that condensed chromatin is resistant to the damage generated by γ -irradiation and cisplatin treatment, which are usually used in anti-tumor therapy (16).

Thus, histone H1 quantity may determine the sensitivity to anti-cancer treatments. Future studies should examine the relationships among histone H1 quantity, chromatin state, cell differentiation, and sensitivity to anti-cancer therapy.

Acknowledgements

This work was supported by Grants-in Aid for Scientific Research (C) 17K08891 from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan (to RM).

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