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

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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 example, 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, H2 O2, 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
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 μM 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 × 10⁶), suspended them in 50 μL of phosphate-buffered saline, and lysed them in 50 μL 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 μm 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 × 10⁵ 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 μg/mL) (Dojindo, Kumamoto, Japan) 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-
Packing chromatin by histone H1

by performing electrophoresis on a 1.5% agarose gel in the presence of ethidium bromide (0.5 μg/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/DFF40 monoclonal antibody (R&D Systems, Minneapolis, MN, USA) and horseradish peroxidase-conjugated rabbit anti-mouse IgG (Fig. 2D). Glycer aldehyde 3-phosphate dehydrogenase (GAPDH) was the loading control, which was probed with mouse anti-
in a final volume of 60 μL contained $2.0 \times 10^5$ 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 μL 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 μL) to urine samples where indicated (Fig. 3B). After incubation, we lysed the reaction samples by adding 300 μL 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 μg/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).

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 ($1 \times 10^7$) and suspended them in 1 mL of cold isolation buffer (10 mM KCl, 250 mM sucrose, 4 mM MgCl₂, 1 mM DTT, and 20 mM HEPES) with 10 μL of Protease Inhibitor Cocktail (100×) (Thermo Fisher Scientific). We subsequently lysed the cells with the addition of 50 μL 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 $2.0 \times 10^7$ nuclei/μL with an equal volume of cold isolation buffer and 100% glycerol. The reaction mixture for the DNase sensitivity assay comprised 30 μL of nucleus suspension, 20 μL of biochemical buffer, and 100 μL of 4 M guanidine. After 2 h of incubation at 37°C, DNA was purified from the treated samples and separated on an agarose gel.

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, 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.

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