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Association of high molecular weight DNA fragmentation with apoptotic or non-apoptotic cell death induced by calcium ionophore

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Abstract Calcium ionophore (A23187)-induced high molecular weight (HMW) and internucleosomal DNA fragmentation were investigated in human leukemia cell lines. An apoptosis-sensitive cell line, HL-60, showed HMW, internucleosomal DNA fragmentation and morphological changes of apoptosis by A23187. MOLT-4, which is resistant to apoptosis, exhibited only HMW DNA fragmentation and died of necrosis under the same conditions. Autodigestion experiments suggested the endonucleolytic activity to cause HMW fragmentation in the cytoplasm of both cell lines. The activity was more dependent on Mg^{2+} than Ca^{2+} in HL-60, whereas it was Ca^{2+}-dependent in MOLT-4. These results suggest that HMW DNA fragmentation is not specific to apoptosis.

Key words: Apoptosis; Necrosis; High molecular weight DNA fragmentation; Internucleosomal DNA fragmentation; Endonuclease

1. Introduction

Two distinct patterns of cell death, termed necrosis and apoptosis, have been recognized on the basis of morphological and biochemical studies [1,2]. Morphologically, apoptosis is characterized by cell shrinkage, chromatin condensation and blebbing of the plasma membrane. The degradation of DNA into integer multiples of nucleosome-sized fragments has been considered another hallmark of apoptosis [3]. Recent investigators have postulated that internucleosomal DNA fragmentation is not necessarily a prerequisite for morphological changes of apoptosis. Instead, the formation of high molecular weight (HMW) DNA fragmentation, 200–250 or 30–50 kbp, without or prior to internucleosomal DNA fragmentation was reported [4–6]. However, the exact role of these two types of DNA fragmentation in the apoptotic process is still a matter of investigation.

Recently we have found that the sensitivity to apoptosis induced by calcium ionophore was different among human leukemia cell lines [7]. Namely, myeloid cell lines including HL-60 were highly sensitive to apoptosis, whereas T-lymphoblastic cell lines including MOLT-4 were resistant to apoptosis and died of necrosis. In order to clarify the association of HMW and nucleosomal-sized DNA fragmentation with apoptotic morphology, we tried to examine the occurrence of both types of DNA fragmentation during treatment with calcium ionophore (A23187) and an inhibitor of Ca^{2+}-dependent ATPase (Thapsigargin) in HL-60 and MOLT-4. The expression of the constitutive endonucleases presumably responsible for HMW or internucleosomal DNA fragmentation in these cells were also investigated by autodigestion experiments.

2. Materials and methods

2.1. Cell culture

Human myeloid leukemia cell line, HL-60 and T-lymphoblastic leukemia cell line, MOLT-4, were obtained from the Japanese Cancer Resources Bank (Tokyo, Japan). Cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2/95% air.

2.2. Determination of apoptotic cells

Cells at the density of 2×10^5/ml in their logarithmic growth phase were treated with A23187 or thapsigargin for the indicated intervals. Then, 100 μl of the cells were placed in a cytospin centrifuge and were centrifuged on a glass microscope slide at 800 rpm for 1 min. Apoptotic cells were evaluated on May-Grünwald-Giemsa stained preparations by scoring cells that were with fragmented nuclei and condensed chromatin.

2.3. Preparation of DNA for gel electrophoresis

Cell pellets (3×10^6 cells) with or without treatment of A23187 or thapsigargin were resuspended in 100 μl of a solution containing: 10 mM Tris, 20 mM NaCl, 50 mM EDTA, plus 100 μl of prewarmed 2% low-melting point agarose. Plugs were polymerized at 4°C for 10 min, transferred into a solution containing 100 mM EDTA, pH 8.0, 1% sodium lauryl sarcosine, and 1 mg/ml proteinase K, followed by overnight incubation at 50°C without agitation. Then plugs and supernatants were separated. The DNA in the supernatants was prepared and analyzed by conventional agarose gel electrophoresis with the method described before [8]. On the other hand, the plugs were washed four times in the washing buffer containing 20 mM Tris and 50 mM EDTA. The plugs were stored at 4°C in the washing buffer until use for pulsed-field gel electrophoresis.

2.4. Pulsed-field gel electrophoresis

Clamped homogeneous electric fields (CHEF) gel electrophoresis was used to resolve HMW DNA fragments. CHEF electrophoresis was carried out using a CHEF-DR II purchased from BioRad Laboratories, Japan. Horizontal gels of 1.5% agarose were run at 6 V/cm with a ramped switch time from 0.5 to 10 s for 9.5 h followed by a ramped switch time from 10 to 60 s for 9.5 h in 0.25 × TAE (10 mM Tris, 10 mM acetate, 0.5 mM EDTA) at 14°C. Two sets of pulse markers were used as standards: (i) 0.5–23 kbp fragments consisted of λDNA *Hin*dIII fragments; (ii) 225–2200 kbp consisted of chromosomes isolated from *Saccharomyces cerevisiae*. DNA gels were stained with ethidium bromide, and photographed in the UV light with a Polaroid camera.

2.5. Preparation of nuclei and whole cell lysates

Nuclei and whole cell lysates were prepared by the methods of Filipski et al. with slight modifications [9]. Briefly, 3×10^6 cells were incubated in ice-cold isolation buffer containing: 15 mM Tris, 0.25 M sucrose, 60 mM NaCl, 15 mM KCl, 0.5 mM spermine, 0.15 mM

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spermidine and 0.5% Nonidet-P40 for 10 min. After centrifugation at 2000 × g for 5 min, the supernatants were served as the cytosol fraction. The nuclei were prepared by washing the pellets twice with the isolation buffer without Nonidet-P40. The whole cell lysates were reconstituted by mixing the cytosol fraction with the nuclei.

2.6. Assay for the constitutive endonuclease activity

In order to determine the constitutive endonuclease activity in these cell lines, the whole cell lysates were incubated in the presence of 5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), 1 mM ATP and various combinations of excessive cations at 37°C for 12 h. The reaction was stopped by addition of EGTA to the final concentration of 10 mM and chilled on ice. The DNA was prepared by the procedures described above after an addition of equal volume of prewarmed 2% low-melting point agarose. On the other hand, the nuclei were incubated in the digestion buffer containing: 15 mM Tris, 0.25 M sucrose, 60 mM NaCl, 15 mM KCl, 1 mM ATP, 5 mM EGTA, and divalent cation, i.e. 10 mM $CaCl_2$ and/or 10 mM $MgCl_2$ at 37°C for 12 h. The digestion was stopped by addition of EGTA as described above.

2.7. Assay for the endonuclease activity in the nuclei treated by A23187

After treatment with 1 μM A23187 for 6 h, the cells were washed twice and the nuclei were separated from the cytosol fraction as described above. The extract of the nuclei was prepared and the chromatin was removed from the nuclei by the method of Gaido et al. [10]. The endonuclease activity in the nuclear fraction of A23187-treated cells was investigated by mixing the chromatin-depleted nuclear extract from A23187-treated cells with the nuclei prepared from the same cell line without A23187 exposure. They were incubated in the presence of 5 mM EGTA, 1 mM ATP and excessive cations, i.e. 10 mM $CaCl_2$ for MOLT-4 or 10 mM $MgCl_2$ for HL-60 at 37°C for 12 h. The reaction was stopped by addition of EGTA as described above.

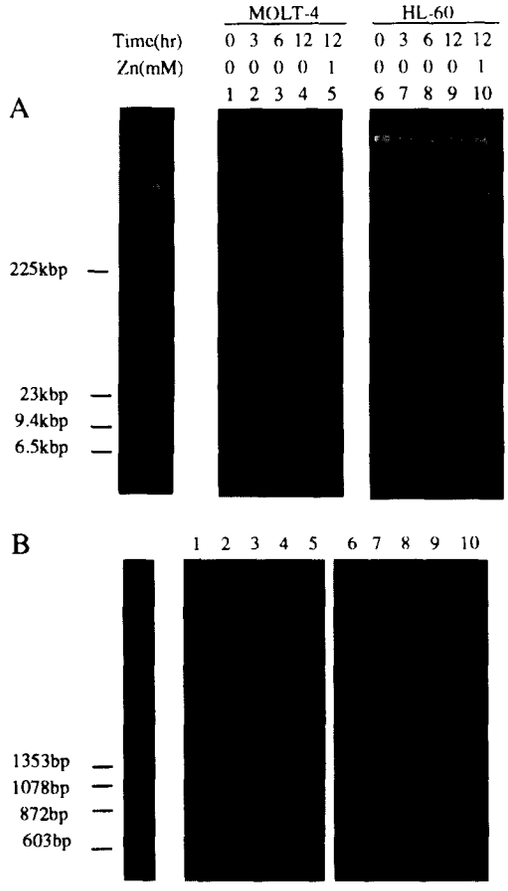

Fig. 1. A23187 induced (A) HMW and (B) internucleosomal DNA fragmentation. MOLT-4 (lanes 1–5) and HL-60 (lanes 6–10) were incubated in the presence of 1 μ A23187 for 0 h, 3 h, 6 h, or 12 h (lanes 1–4 and 6–9). Lanes 5 and 10 indicate the cells co-incubated with 1 μM A23187 and 1 mM zinc ion for 12 h. Then the cells were processed for either pulsed-field gel electrophoresis or conventional gel electrophoresis as described in section 2.

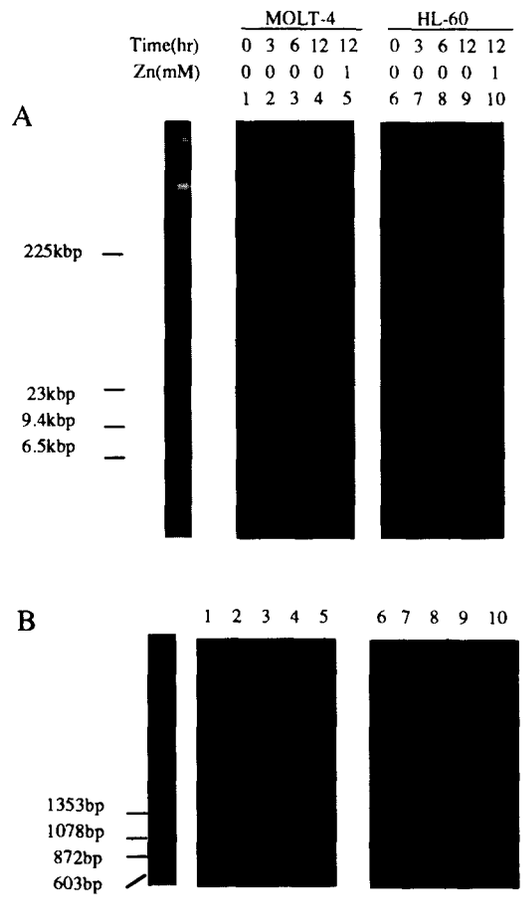

Fig. 2. Thapsigargin induced (A) HMW and (B) internucleosomal DNA fragmentation. MOLT-4 (lanes 1–5) and HL-60 (lanes 6–10) were incubated in the presence of 0.2 μM thapsigargin for 0 h, 3 h, 6 h, or 12 h (lanes 1–4 and 6–9). Other conditions are the same as described in Fig. 1.

3. Results

3.1. Analysis of HMW and internucleosomal DNA fragmentation induced by A23187 in HL-60 and MOLT-4 cells

When HL-60 cells were treated with 1 mM A23187, they started to show morphological changes characteristic of apoptosis at 2 h. After 6 h of incubation, approximately one-third of the cells had apoptotic morphology (data not shown). In the T-lymphoblastic leukemia cell line, MOLT-4, on the other hand, the percentage of apoptotic cells remained below 2% during 48 h of incubation with A23187 (1 mM). The cells were enlarged and had swollen mitochondria and vacuoles in the cytoplasm without chromatin condensation by electroscopic examination, which are features indicative of necrosis (data not shown). These results are consistent with our previous observation [7]. As shown in Fig. 1A, HMW DNA fragments of about 30 kbp were detected in both cell lines at 6 h, which were more prominent in MOLT-4 cells. Such HMW DNA fragmentation

was almost completely inhibited by 1 mM zinc ion. However, internucleosomal DNA fragmentation was seen only in HL-60 cells (Fig. 1B). Internucleosomal DNA fragmentation was again inhibited in the presence of zinc ion.

3.2. Analysis of HMW and internucleosomal DNA fragmentation induced by thapsigargin in HL-60 and MOLT-4 cells

Thapsigargin is the drug which can elevate intracellular calcium levels through inhibition of Ca^{2+}-dependent ATPase [11]. Recent investigators have reported that this drug induces apoptosis in androgen-independent prostatic cancer cells [12]. Incubation of HL-60 cells with thapsigargin (0.2 μM) caused apoptosis in the similar kinetics to A23187. The same concentration of thapsigargin failed to induce apoptosis in MOLT-4 (data not shown). Fig. 2 clearly demonstrates that thapsigargin could induce HMW DNA fragmentation in both cell lines, which was again inhibited by 1 mM zinc ion. On the other hand, HL-60 cells alone exhibited internucleosomal DNA fragmentation.

3.3. Endonucleolytic activity of isolated nuclei and whole cell lysates

In order to determine whether the endonuclease responsible for the DNA fragmentation described above is constitutive or newly synthesized, we carried out autodigestion experiments. First, the whole cell lysates of unstimulated HL-60 cells were capable of cleaving their DNA into about 20–300 kbp fragments (lane 6 in Fig. 3). This activity was more dependent upon Mg^{2+} than Ca^{2+}, since an addition of Mg^{2+} in excess had clearer bands than that of Ca^{2+}. Although the whole cell lysates of MOLT-4 cells had the similar activity, the cation dependency was quite opposite, i.e. it was more Ca^{2+}-dependent (lane 3 in Fig. 3). On the other hand, the activity to cause internucleosomal DNA fragmentation was observed only in HL-60 cells, which was also Mg^{2+}-dependent as described previously (data not shown) [13]. The incubation of the isolated nuclei from these cell lines with Ca^{2+} and/or Mg^{2+} could not produce either HMW or internucleosomal DNA fragmentation, suggesting that the endonucleolytic activities localized in the cytosol fraction (Fig. 4).

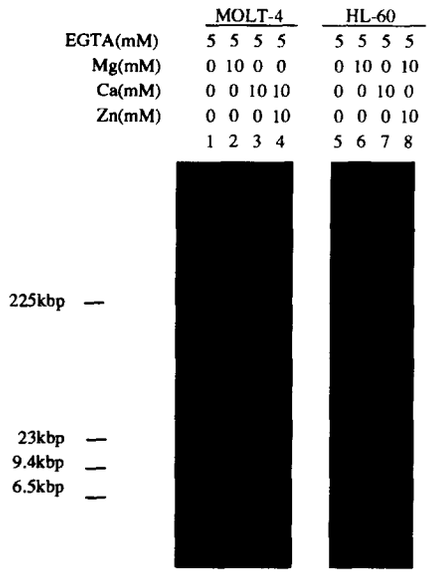

Fig. 3. Cation dependency of constitutive endonuclease responsible for HMW DNA fragmentation in the whole cell lysates. Whole cell lysates of either MOLT-4 or HL-60 cells were incubated for 12 h under the conditions described in the figure. Then the pulse-field gel analysis was performed.

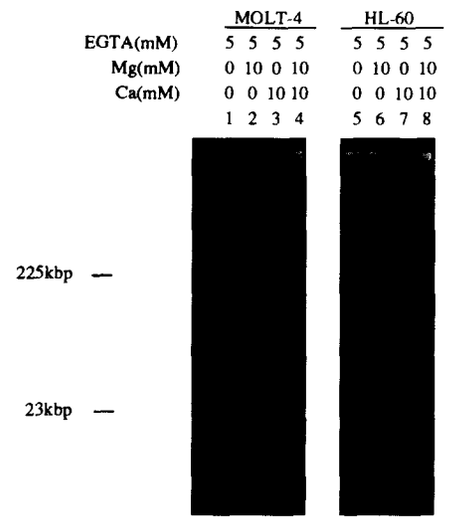

Fig. 4. Endonuclease activity to cause HMW DNA fragmentation in the isolated nuclei. Lanes 1–4 or 5–8 denote the pulse-field gel analysis of the DNA from the isolated nuclei of MOLT-4 or HL-60, respectively, after incubation for 12 h in the presence of 5 mM EGTA with 10 mM Ca^{2+} and/or 10 mM Mg^{2+}.

3.4. Endonucleolytic activity in the nuclei treated by A23187

Finally, we tried to examine the endonuclease activity to produce HMW fragmentation in the nuclear fraction of cells treated with A23187 (1 μM) for 6 h. In these experiments, the chromatin was first removed from the nuclei extract of A23187-treated cells by ultracentrifugation. Then, the assay was carried out by mixing the chromatin-depleted nuclear fraction of A23187-treated cells with the nuclei separately prepared from A23187-untreated cells. As shown in Fig. 5 (lanes 3 and 7), the activity to cleave DNA into HMW fragments was observed in the nuclear fraction obtained from cells incubated with A23187 in both cell lines.

4. Discussion

Previous reports indicated that the sensitivity to apoptosis differs among the different cell types. For example, topoisomerase inhibitors and calcium ionophore, both of which are potent inducers of apoptosis in HL-60 promyelocytic leukemia cells, can cause necrosis rather than apoptosis in a T-lymphoblastic leukemia cell line, MOLT-4 [7,14]. This may be partly attributed to the different expression of the constitutive endonuclease responsible for internucleosomal DNA fragmentation, which is only detected in the cytosol of HL-60 cells [7]. Accumulating evidences have suggested, however, that DNA fragmentation into oligonucleosomes and apoptotic chromatin condensation is separate phenomenon. In the isolated nuclei, Sun et al. have reported that micrococcal nuclease can cause internucleosomal DNA fragmentation without giving rise to chromatin condensation [15]. Furthermore, morphological changes indicative of apoptosis without typical DNA laddering

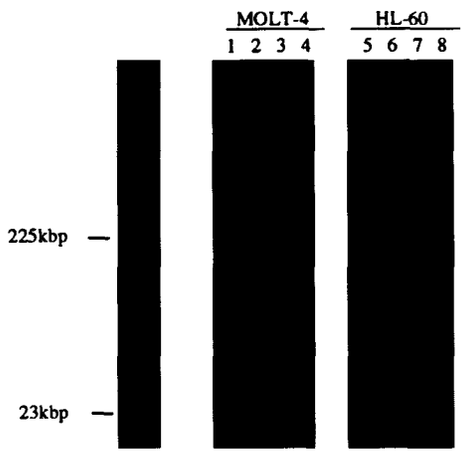

Fig. 5. Endonuclease activity to cause HMW DNA fragmentation in the nuclear fraction of cells treated with A23187. Lanes 1–4 = MOLT-4, lanes 5–8 = HL-60. The nuclei isolated from untreated MOLT-4 or HL-60 cells were incubated with either isolation buffer (15 mM Tris, 0.25 M sucrose, 60 mM NaCl, 15 mM KCl, 0.5 mM spermine, and 0.15 mM spermidine) (lanes 1 and 5) or the chromatin-depleted nuclear fraction of A23187-untreated cells (lanes 2 and 6) or the chromatin-depleted nuclear fraction of A23187-treated cells (lanes 3 and 7). In lanes 4 and 8, the chromatin-depleted nuclear fractions of A23187-treated cells were incubated without the addition of the nuclei of untreated cells. The incubation was carried out for 12 h in the presence of 5 mM EGTA with 10 mM Ca^{2+} for MOLT-4 or 10 mM Mg^{2+} for HL-60 before the pulse-field gel analysis.

are described in TGF-β1 treated hepatocytes [16] and cisplatin treated human ovarian carcinoma cell line [17]. On the other hand, the cleavage of DNA to large fragments, 200–250 and 30–50 kbp in length, has been reported, which occurs either prior to or in the absence of internucleosomal fragmentation [4,6]. Accordingly, some investigators propose that the formation of HMW DNA fragmentation represents a key committed step in apoptosis [18]. These reports led us to examine HMW and internucleosomal DNA fragmentation during calcium ionophore treatment in apoptosis-sensitive and -resistant human leukemia cell lines.

The important findings in the present study are two-fold. First, HMW DNA fragmentation can be induced by A23187 and thapsigargin without morphological features of apoptosis. The same phenomenon was also observed following treatment with etoposide, an inhibitor of topoisomerase II, in MOLT-4 cells. The DNA ladder formation occurred, on the other hand, in HL-60 cells which showed apoptotic morphology. Second, the endonuclease which presumably plays a role in the DNA cleavage into large fragments exits constitutively in the cytosol fraction of both cell lines. Such endonuclease may translocate into the nuclei during treatment with A23187. Whether the endonucleases identified in these cell lines are identical or not remains unelucidated. Interestingly, the cation dependency of the endonuclease is somewhat different between HL-60 and MOLT-4 cells. On the contrary, the endonucleolytic activity to cause internucleosomal DNA fragmentation was found only in HL-60 cells.

Several investigators have described the endonuclease which promotes internucleosomal DNA fragmentation [19]. For example, Tanuma et al. purified a neutral endonuclease that required both Ca^{2+} and Mg^{2+} for full activity in the nuclei of rat thymocytes [20]. However, as far as we know, the enzyme which is responsible for HMW DNA fragmentation has not been reported yet.

In summary, we demonstrate that: (i) HMW fragmentation is not specific to apoptosis and (ii) the endonuclease activity to cause HMW fragmentation constitutively exists in the cytoplasm of HL-60 and MOLT-4 cells, whose cation dependency is somewhat different among these two cell lines; and (iii) the endonuclease activity producing internucleosomal DNA fragmentation is only detected constitutively in HL-60 cells. These results suggest that the differential expression of the constitutive endonuclease may be related to the susceptibility of human leukemia cell lines to apoptosis.

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