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INVOLVEMENT OF Ca^{2+} IN THE FORMATION OF HIGH MOLECULAR WEIGHT DNA FRAGMENTS IN THYMOCYTE APOPTOSIS

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Internucleosomal DNA fragmentation (DNA laddering) and formation of apoptotic bodies have long been considered characteristic features of apoptosis. However, recent work has shown that formation of high molecular weight DNA fragments precedes internucleosomal cleavage and may involve mechanisms that differ from those responsible for DNA laddering. Here, we show that glucocorticoid treatment of human thymocytes stimulated the formation of high molecular weight DNA fragments by Ca^{2+}- and endonuclease-mediated mechanisms. Either the removal of Ca^{2+} from the medium or pretreatment of the cells with the intracellular Ca^{2+} chelator, BAPTA-AM, prevented the formation of large DNA fragments. Further, treatment of the thymocytes with the microsomal Ca^{2+}-ATPase inhibitor, thapsigargin, which caused a sustained increase in intracellular Ca^{2+} concentration, was in itself sufficient to activate high molecular weight DNA fragmentation. Our results show that Ca^{2+}-dependent mechanisms promote the multistep chromatin cleavage in human thymocyte apoptosis.

Apoptosis, a genetically controlled cell deletion process, is characterized by cell shrinkage, nuclear condensation and extensive chromatin degradation (1, 2). Cleavage of the internucleosomal linker regions by Ca^{2+}-Mg^{2+}-dependent endonuclease(s) (DNA laddering) has long been considered the most reliable biochemical marker of apoptosis (3).

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Abbreviations: ATA, aurintricarboxylic acid; EGTA, ethylene glycol-0.0[1]-bis(2-aminoethyl)-N,N,N[1],N[1]-tetraacetic acid; EDTA, (ethylenedinitro)tetraacetic acid; HMW DNA, high molecular weight DNA; BAPTA-AM, bis-(o-aminophenoxy)-ethane-N,N,N[1],N[1]-tetraacetic acid/tetra (acetoxymethyl)-ester.

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Ca^{2+} signals are sufficient to promote endonuclease activation and formation of apoptotic bodies in several experimental models of apoptosis (4). Buffering intracellular Ca^{2+}, or removal of extracellular Ca^{2+}, has been shown to protect from DNA laddering and apoptotic body formation in lymphoid and other cells (5, 6). Thus, the Ca^{2+} involvement in apoptosis has primarily been linked to the Ca^{2+} dependency of the endonuclease(s) (7). However, experiments using Zn^{2+} to inhibit endonuclease activity have recently suggested that apoptotic body formation can be dissociated from DNA laddering in rat thymocytes (8). Further, recent work has indicated that high molecular weight (HMW) DNA fragments are formed prior to internucleosomal fragmentation in several cell systems, and that the latter is not a prerequisite for apoptotic morphology (9 - 11).

To date, only a few studies have investigated the early phase of chromatin degradation in apoptosis. These studies have concluded that the formation of HMW DNA fragments can occur in the absence of endonuclease activation (9, 11). Additional observations that multiple enzymes, including DNase I (12) and DNase II (13), or Mg^{2+}-dependent nucleases (14), can be involved in apoptotic cell killing have provided further support for the assumption that internucleosomal DNA cleavage may be a late event in apoptosis and not necessarily mediated by a specific Ca^{2+}-dependent enzyme. On the other hand, we have recently reported that treatment of thymocytes with the microsomal Ca^{2+}-ATPase inhibitor, thapsigargin, was sufficient to promote all the characteristic features of apoptosis, i.e. cell shrinkage, apoptotic body formation and DNA laddering (6). All these events could be prevented by the removal of Ca^{2+} from the incubation medium.

In an attempt to further elucidate the role of Ca^{2+} and endonuclease-mediated DNA cleavage in apoptosis, we have now studied the multistep chromatin degradation following exposure of human thymocytes to methylprednisolone or thapsigargin. Our results suggest that Ca^{2+}-dependent mechanisms, including endonuclease activation, are involved in the early steps of chromatin fragmentation.

MATERIALS AND METHODS

Materials. Methylprednisolone, aurintricarboxylic acid and $ZnSO_4$ were obtained from Sigma. BAPTA-AM was from Calbiochem and proteinase K from Boehringer Mannheim. Thapsigargin was purchased from Gibco. All other reagents were of the highest possible grade of purity and came from local commercial sources. All agents tested as possible inhibitors of HMW DNA cleavage were added to the cell suspensions immediately after treatment with methylprednisolone or thapsigargin.

Thymocyte Incubation and Treatment. Thymocyte suspensions were prepared from the thymus gland of young patients (less than 1.5 year old) undergoing corrective surgery. The glands were minced in a RPMI 1640 medium (Gibco Ltd.) supplemented with 2% heat-inactivated fetal calf serum. Thymocytes were then passed through three layers of gauze and diluted to a final

concentration of $5x10^6$ cells/ml before incubation at 37°C in a humidified incubator under an atmosphere of 5% CO_2 in air. Cell viability was measured by trypan blue exclusion. A total of 96-100% of cells routinely excluded trypan blue after the isolation procedure. Thymocyte suspensions were treated with either 0.1 μM methylprednisolone or 0.1 μM thapsigargin. In the experiments designed as "Ca^{2+}-free", cells were transferred to a nominally Ca^{2+}-free RPMI 1640 medium (NORD CELL) after treatment in normal RPMI 1640 medium.

Preparation of Agarose Plugs for Pulsed-Gel Electrophoresis. Cell pellets ($6x10^5$ cells) were resuspended in 50 μl of a solution containing: 0.15 M NaCl, 2 mM KH_2PO_4/KOH, 1 mM EGTA, 5 mM $MgCl_2$, pH 6.8, plus 50 μl of prewarmed 1% low-melting point agarose added while gently mixing. Plugs were polymerized on ice for 10-15 min, transferred into a solution containing: 10 mM NaCl, 10 mM tris-HCl, pH 9.5, 25 mM EDTA, 1% N-lauroyl sarcosine buffer and 200 μg/ml proteinase K (Boehringer Mannheim) and incubated for 24 h at 50°C with continuous agitation. Thereby plugs were rinsed three times for periods of 2 h in 10 mM tris-HCl, pH 8.0 and 1 mM EDTA at 4°C. The plugs were then stored until use at 4°C in 50 mM EDTA, pH 8.0.

Pulsed-Field Gel Electrophoresis. Countour-clamped homogeneous electric field (CHEF) gel electrophoresis was used to resolve high molecular weight (HMW) DNA fragments. Electrophoresis was carried out using a horizontal gel chamber, a power supply and a 2015 Pulsaphor apparatus (LKB-Pharmacia, Sweden). Total run time was 22 h at 180 V. Pulse time changed from 90 sec to 15 sec. The running buffer was 0.5 x TBE (45 mM tris, 1.25 mM EDTA, 45 mM boric acid, pH 8.0). Two sets of pulse markers obtained from Sigma were used as standards: i) 0.1 - 200 kbp fragments consisted of a mixture of λ DNA-Hind III fragments, λ DNA and λ concatemers; ii) 225 - 2200 kbp consisted of chromosomes isolated from *Saccharomyces cerevisiae*. Each gel lane represents a typical run of 3-4 samples prepared independently. DNA gels were stained with ethidium bromide, visualized using a 305 nm UV light source and photographed using a Polaroid 655 positive-negative film.

Conventional Agarose Gel Electrophoresis. Low molecular weight DNA released from the plugs during the incubation with proteinase K was precipitated over a 48 h time period by 5M NaCl in absolute ethanol. The DNA was subsequently loaded on conventional 1.8% agarose gels. Electrophoresis was run with constant current set at 60 mA. Gel staining and photography were performed as described above.

RESULTS

Human thymocyte suspensions were exposed to 0.1 μM methylprednisolone and - at different time points - cell samples were removed, placed in agarose plugs, lysed and run on horizontal agarose gels using CHEF. Two large DNA fragments of about 300 kbp and 50 kbp were formed between 3h and 6h after exposure of the thymocytes to methylprednisolone (FIG.1A). Up to 6 h, internucleosomal DNA degradation was not detectable by DNA laddering on agarose gels (FIG. 1B). When the incubation time was extended to 12 h, the bulk of DNA fragments accumulated and internucleosomal fragmentation became evident (FIG.1B). A band corresponding to DNA sizes between 700 kbp and 1000 kbp was noticeable in all samples, including untreated cells, in accordance with previous observations by Walker et al. (10) and Filipski et al. (15). Migration of any DNA fragment > 750 kbp entering the gels may have been responsible for the formation of this band (16).

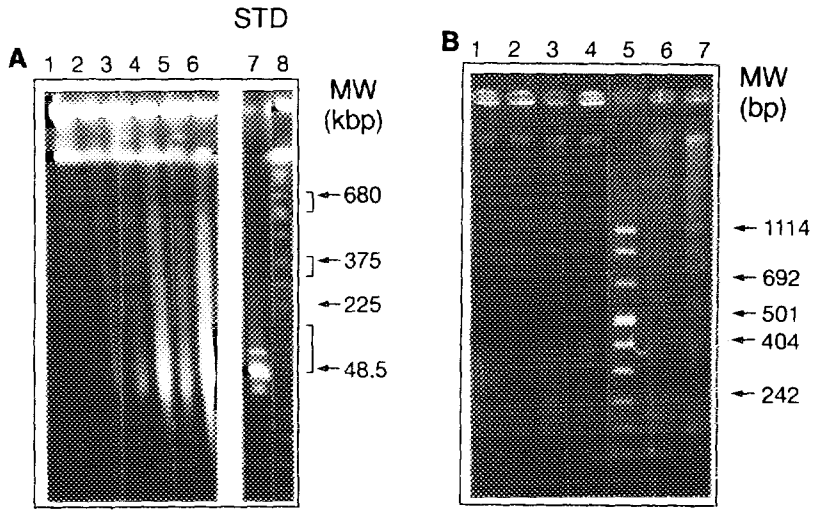

Figure 1. Size distribution of DNA fragments in methylprednisolone-treated human thymocytes. A. Time course of formation of HMW DNA fragments. Control (lanes 1, 3 and 5), and methylprednisolone-treated (lanes 2, 4 and 6) thymocytes were incubated for 3, 6 or 12 h and examined for HMW DNA fragments by pulsed-field gel electrophoresis. Lanes 7 and 8 contain the standards (STD) "i" and "ii", respectively (see Materials and Methods). B. Time course of formation of low molecular weight DNA fragments. DNA that had leached from the agarose plugs was analyzed by conventional gel electrophoresis. Control (lanes 1, 3 and 6) and methylprednisolone-treated (lanes 2, 4 and 7) thymocytes were incubated for 3, 6 or 12 h. Lane 5 is a mixture of pBR 328 DNA-Bgl1 and pBR 328 DNA-Hinf1.

Sensitivity to zinc ions and to aurintricarboxylic acid (ATA) is characteristic of several endonucleases (17). As shown in FIG. 2, addition of 100 μM Zn^{2+} to human thymocytes efficiently blocked HMW DNA fragmentation caused by methylprednisolone at 3h or 6h, suggesting the involvement of an endonuclease-sensitive step in the formation of HMW DNA fragments. To further study whether endonuclease activity was involved in the formation of HMW DNA fragments in this experimental system, we investigated the effect of aurintricarboxylic acid, another agent known to inhibit nuclease activity (17, 18). At 100μM, ATA fully prevented methylprednisolone-induced DNA fragmentation (FIG.2) without affecting chromatin structure and cell viability when used alone (data not shown).

Studies in our and other laboratories have shown that Ca^{2+} signals can activate apoptosis in a variety of cell systems. The role of Ca^{2+} in thymocyte apoptosis has largely been linked to endonuclease activation, although Ca^{2+}-dependent alterations of chromatin structure and gene expression may also play a role (4). To investigate the role of Ca^{2+} in the formation of HMW DNA fragments in methylprednisolone-induced apoptosis, we performed experiments with thymocytes pretreated with the intracellular Ca^{2+} chelator BAPTA-AM, or incubated in a nominally Ca^{2+}-free medium. As shown in FIG.2, both treatments prevented HMW DNA

fragmentation. The protective effect of BAPTA was reduced with time, presumably due to its leakage from cells, whereas under Ca^{2+}-free conditions (i.e. incubation in nominally Ca^{2+}-free RPMI 1640 medium) protection was maintained for up to 12 h (data not shown).

To investigate whether a sustained Ca^{2+} elevation *per se* was sufficient to trigger the formation of HMW DNA fragments, we used the microsomal Ca^{2+}-ATPase inhibitor thapsigargin. Thapsigargin is known to elicit a sustained intracellular Ca^{2+} increase that can cause thymocyte apoptosis (6). The results of these experiments are illustrated in FIG.3 and show that thapsigargin treatment caused the formation of 300 kbp and 50 kbp DNA fragments. Treatment with 100μM Zn^{2+} (FIG. 3), or incubation of thymocytes under Ca^{2+}-free conditions (data not shown), prevented the generation of HMW DNA fragments also in this case. As reported previously (6), thapsigargin treatment was also associated with DNA laddering, apoptotic body formation, and loss of thymocyte viability. All these effects of thapsigargin were sensitive to Ca^{2+}-free conditions. Notably, also in this system the treatments which prevented HMW DNA fragmentation (FIG. 3) blocked apoptotic body formation and subsequent cell lysis.

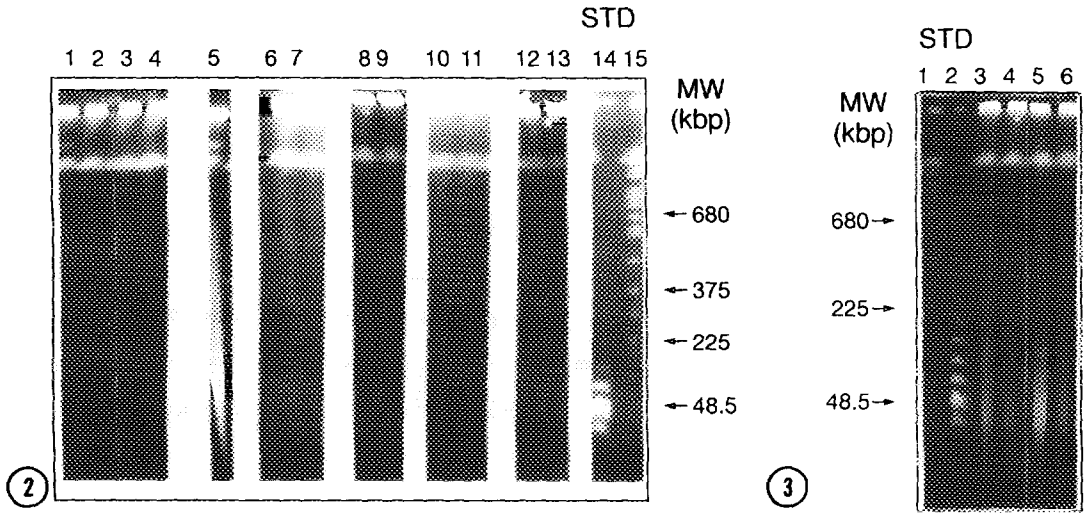

Figure 2. Effects of various agents on the formation of HMW DNA fragments. Human thymocytes were incubated in the absence (lanes 1 and 3) or in the presence of 100 μM $ZnSO_4$ (lanes 2 and 4) for 3 h (lanes 1 and 2) or 6 h (lanes 3 and 4). Methylprednisolone-treated cells were incubated in the absence (lane 5) or in the presence of 100 μM $ZnSO_4$ (lanes 6 and 7), 100 μM aurintricarboxylic acid (lanes 8 and 9), 8μM BAPTA-AM (lanes 10 and 11), or in nominally Ca^{2+}-free medium (lanes 12 and 13) for 3 h (lanes 6, 8, 10, and 12) or 6 h (lanes 7, 9, 11, and 13), respectively. Lanes 14 and 15 contain the same standards as in FIG. 1A.

Figure 3. Thapsigargin-induced formation of HMW DNA fragments. Human thymocytes were incubated with thapsigargin (0.1 μM) in the absence (lanes 3 and 5) or in the presence of 100 μM $ZnSO_4$ (lanes 4 and 6) for 3h (lanes 3 and 4) or 6h (lanes 5 and 6). Lanes 1 and 2 contain the same standards as in FIG. 1A.

DISCUSSION

The results of our experiments suggest that Ca^{2+}-dependent and endonuclease- mediated DNA cleavage is responsible for the generation of HWM DNA fragments in human thymocytes exposed to methylprednisolone or thapsigargin. The Ca^{2+} requirement for the formation of HMW DNA fragments is clearly demonstrated by the experiments with Ca^{2+} chelators. The involvement of endonuclease(s) is suggested by the inhibitory effects seen with Zn^{2+} and aurintricarboxylic acid. Both agents also prevented the appearance of HMW DNA fragments in isolated liver nuclei incubated with Ca^{2+} and Mg^{2+} (B. Zhivotovsky, D. Wade, S. Orrenius, and P. Nicotera, unpublished observations). Our finding of an inhibitory effect of zinc ions differs from the observation by Brown et al., who reported the formation of 50 kbp fragments in the presence of 1 mM Zn^{2+} in rat thymocytes treated with dexamethasone (9). This discrepancy may be related to the different Zn^{2+} concentrations used in our experiments (100 μM) and in those of Brown et al. (1 mM). Thus, while pretreatment of human thymocytes with 100 μM Zn^{2+} prevented methylprednisolone- and thapsigargin-induced large fragment formation (FIG. 2 and 3), exposure of untreated cells to 1mM Zn^{2+} caused in itself the formation of HMW DNA fragments (data not shown).

According to a current model of chromatin arrangement proposed by Filipsky et al. (15), the chromatin structures corresponding to the HMW DNA fragments observed during the early phase of apoptosis would represent single (50 kbp) or folded hexameric (300 kbp) loop structures released from the nuclear scaffold proteins. While the nature of these fragments is still unclear, the mechanism(s) responsible for their formation have become the subject of intense studies in several laboratories. A mechanism involving topoisomerase II activation has been invoked by Walker et al. (10) and Brown et al. (9), whereas the possibility that proteolytic cleavage would sever the connections between scaffold or matrix associated proteins and chromatin loop domains has also been proposed (10). In the latter case, chromatin loops detaching from nuclear scaffold elements would become more sensitive to endonuclease cleavage.

In addition to causing endonuclease activation, Ca^{2+} may also affect chromatin organization by other mechanisms. Recent findings in our laboratory indicate that nuclear Ca^{2+} sequestration is associated with chromatin unfolding (4). Chromatin unfolding initiated by ion changes in the nuclear interior may facilitate H1 redistribution (19), or topoisomerase II activation (20) leading to changes in supercoiling and increasing DNase sensitivity in particular chromatin domains (21). The importance of chromatin arrangement in determining the sensitivity of cells to apoptosis is also suggested by the observation that agents promoting chromatin compaction can also prevent thymocyte killing (22). Further, it has recently been demonstrated that Ca^{2+}-activated proteases are involved in the cleavage of nuclear lamins (23). Current studies in our laboratory have shown

that Ca^{2+}-dependent proteolysis in required for the formation of the 50 kbp DNA fragments in isolated rat liver nuclei and rat thymocytes (B. Zhivotovsky, D. Wade, S. Orrenius and P. Nicotera, unpublished observations). Thus, Ca^{2+} may play a key role in both the activation of the endonuclease-dependent cleavage and the dissociation of chromatin loops from scaffold constituents (i.e. lamins). At present, we favour a mechanism whereby changes in the supercoiling of a particular chromatin domain, reducing chromatin torsional stress, would promote endonuclease-mediated DNA cleavage. Proteolytic cleavage of scaffold-loop interaction would enhance chromatin unfolding and allow further chromatin fragmentation resulting in the typical DNA laddering observed in apoptotic cells.

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