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Utilization of an *in vitro* assay to evaluate chromatin degradation by candidate apoptotic nucleases

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Abstract

Apoptosis is commonly associated with the catabolism of the genome in the dying cell. The chromatin degradation occurs in essentially two forms: (1) internucleosomal DNA cleavage to generate oligonucleosomal-length fragments (180–200 bp and multiples thereof), and (2) cleavage of higher order chromatin structures to generate ≈30–50 Kb fragments. To investigate this component of apoptosis and identify the nuclease(s) responsible, we have developed and utilized an *in vitro* assay that recapitulates the genomic destruction seen during apoptosis *in vivo* and allows the simultaneous analysis of both forms of DNA degradation from the same sample. Using this assay we evaluated the digestion patterns of several candidate apoptotic nucleases: DNase I, DNase II, and cyclophilin (NUC18) as well as the bacterial enzyme micrococcal nuclease (not thought to be involved in apoptosis). Chromatin degraded by DNase I formed a smear of DNA on conventional static-field agarose gels and ≈30–50 Kb DNA fragments on pulsed field gels. In contrast, DNase II, at a physiologically relevant pH, had no effect on the integrity of HeLa chromatin in either analysis. Similar to DNase I, cyclophilin C produced only ≈30–50 Kb DNA fragments but did not generate internucleosomal fragments. In contrast, micrococcal nuclease generated both oligonucleosomal and ≈30–50 Kb DNA fragments. Nuclear extracts from glucocorticoid-treated apoptotic thymocytes generated oligonucleosomal DNA fragments and the larger ≈30–50 Kb DNA fragments, fully recapitulating both types of apoptotic DNA degradation. Previously, differential sensitivity of nucleases to inhibition by Zn^{2+} was used to argue that two distinct enzymes mediate ≈30–50 Kb DNA cleavage and internucleosomal DNA degradation. While, the nuclease activity present in thymocyte nuclear extracts was differentially sensitive to inhibition by Zn^{2+} during short term incubations it was not during prolonged digestions, suggesting that differences in DNA detection are likely to account for previous results. Together our studies show that none of the nucleases commonly associated with apoptosis could fully recapitulate the DNA degradation seen *in vivo.*

Keywords: apoptosis, cell death, endonuclease, nuclease assay

Abbreviations: MARS, matrix attachment regions; MN, micrococcal nuclease; TE buffer, Tris, EDTA buffer; TPE buffer, Tris, Phosphate, EDTA buffer

Introduction

Recently much attention has been focused on the process of apoptosis or programmed cell death. Correct implementation of this death process is critical to many physiological phenomena such as development, immune function and tissue homeostasis. In addition to its role in normal physiology, deregulation of apoptosis appears to play a central role in many pathological phenomena including AIDS, cancer and neurodegenerative diseases such as Alzheimer's (Häcker and Vaux, 1995). Thus an understanding of this process may lead to novel and innovative avenues for therapeutic intervention in disease.

Apoptosis is characterized by several well-defined morphological and biochemical hallmarks. Morphologically the cells shrink, the chromatin condenses around the periphery of the nucleus and the entire cell eventually buds into small spherical strutures known as apoptotic bodies which contain organelles, fragments of the nucleus and other cytoplasmic components. Membrane changes facilitate the rapid endocytosis of these dying cells and apoptotic bodies by neighboring cells or resident macrophages wherein they are completely degraded and their components recycled. Many biochemical changes are associated with these morphological alterations and much controversy exists over which of these changes are part of the common pathway of apoptosis. By far the most widely recognized biochemical change associated with the vast majority of apoptotic systems is the degradation of genomic DNA in the internucleosomal or linker DNA regions (Bortner *et al*, 1995; Hughes and Cidlowski, 1994; Schwartzman and Cidlowski, 1993a; Wyllie, 1980). Cleavage in these regions releases DNA of nucleosomal or oligonucleosomal lengths which, when analyzed by conventional static-field agarose electrophoresis, forms a very definitive banding pattern known as the 'apoptotic ladder'. This pattern of DNA fragmentation occurs prior to morphological changes and has been suggested to be the first irreversible event in the death process (Arends *et al*, 1990; Compton and Cidlowski, 1986; McConkey *et al*, 1989a).

Although initially observed in conventional gels (Kokileva, 1979), the recent application of pulsed field electrophoretic techniques to the study of apoptosis has led to the identification of an additional form of genomic degradation (into ≈30–50 Kb DNA fragments) associated with dying cells (Cohen *et al*, 1994; Oberhammer *et al*, 1993; Walker

et al, 1993). These fragments have been hypothesized to arise from the cleavage of higher order chromatin configurations known as 'loops' (Filipski *et al*, 1990; Kokileva, 1994; Oberhammer *et al*, 1993; Walker *et al*, 1995) that are presumed to be attached to the nuclear matrix through A–T rich regions known as matrix attachment regions (MARs) (Laemmli *et al*, 1992; Mirkovich *et al*, 1984; Saitoh and Laemmli, 1994). These $\approx$30–50 Kb DNA fragments have been proposed to be generated by cleavage at or near the MARs and have been proposed to provide the substrate for subsequent internucleosomal cleavage (Kokileva, 1994; Walker *et al*, 1993), however, no definitive cleavage site or substrate-product relationship has been shown. The enzyme(s) responsible for this type of chromatin cleavage are unknown and a current topic of debate is whether the two forms of DNA degradation are mediated by separate or identical enzymes. Several studies have attempted to differentiate the activities of these nucleases, primarily on the basis of their ionic requirements and sensitivities to inhibitors. For example, recent studies in both thymocytes (Brown *et al*, 1993; Kokileva, 1995) as well as isolated liver nuclei (Kokileva, 1995; Walker *et al*, 1994) have reported that the generation of oligonucleosomal fragments was more sensitive to inhibition by Zn^{2+} than was the cleavage of chromatin into $\approx$30–50 Kb DNA fragments. These data were used to infer the existence of separate nucleases mediating the different forms of DNA degradation, and further, that the large fragments were substrate for internucleosomal cleavage enzymes. Similarly, others have suggested that the generation of $\approx$30–50 Kb DNA fragments is dependent only on Mg^{2+}, whereas further degradation to oligonucleosomal DNA fragments required both Ca^{2+} and Mg^{2+} (Cain *et al*, 1994; Sun and Cohen, 1994; Walker *et al*, 1994, 1995).

Additional forms of DNA cleavage into even larger fragments (such as $\approx$300 Kb fragments) have also been detected in some models of apoptosis. From these studies, a stepwise degradation of chromatin was proposed in which successively smaller fragments of DNA (each representing a progressively smaller structure of chromatin) are generated from their larger predecessors (Cain *et al*, 1995; Kokileva, 1994; Walker *et al*, 1995). However, unlike the $\approx$30–50 Kb DNA fragments, such very large fragments are not consistently observed in all apoptotic cells, are dependent on the cell type as well as inducing agent, and may even be reversible (Beere *et al*, 1995; Bortner *et al*, 1995; Cohen *et al*, 1994; Oberhammer *et al*, 1993; Ormerod *et al*, 1994; Walker *et al*, 1994).

The study of DNA degradation during apoptosis has benefited from several *in vitro* models (Cohen and Duke, 1984; Hewish and Burgoyne, 1973; Lazebnik *et al*, 1993; McConkey *et al*, 1989b; Nieto and Lopez-Rivas, 1989; Sun *et al*, 1994; Walker *et al*, 1994) which have provided a wealth of information on internucleosomal cleavage and, recently, on the generation of large fragments. Similarly, our laboratory previously developed an *in vitro* assay (the HeLa nuclei assay) designed to measure the internucleosomal cleavage activity associated with these dying cells (Schwartzman and Cidlowski, 1991, 1993b). In the current study we have modified and extended the use of this assay to simultaneously analyze chromatin degradation into fragments ranging from 10–500 Kb. Specifically, we evaluated the degradation patterns evoked by several nucleases proposed to mediate DNA degradation during apoptosis. These include DNase I (Peitsch *et al*, 1993, 1994), DNase II (Barry and Eastman, 1993; Eastman, 1994) and cyclophilin (related to NUC18) (Montague *et al*, 1994, 1996). We have also studied micrococcal nuclease (MN), which, though not implicated in apoptosis, specifically cleaves chromatin at internucleosomal regions and thus generates an oligonucleosomal ladder indistinguishable from that produced during apoptosis *in vivo*. Finally, we examined the ability of nuclear extracts from apoptotic thymocytes to produce $\approx$30–50 Kb DNA and oligonucleosomal DNA fragments and the sensitivity of their production to inhibition by Zn^{2+}.

Results

Previous studies from our laboratory have shown that HeLa nuclei make an excellent source of substrate for the analysis of internucleosomal DNA cleavage activity because the cells are highly resistant to apoptosis and the isolated nuclei do not spontaneously degrade (i.e. autodigest) their DNA internucleosomally even in the presence of exogenous Ca^{2+} or Mg^{2+} (Schwartzman *et al*, 1991, 1993b). However, it has been suggested that other cells can autodigest their DNA into large ($\approx$30–50 Kb) fragments without generating internucleosomal fragments (Walker *et al*, 1994). Therefore, to determine if HeLa nuclei are a suitable substrate to use in an assay for the generation of large DNA fragments, we examined the integrity of their DNA over time in the absence of added nuclease. As shown in Figure 1, HeLa chromatin was not degraded into either low (Figure 1A) or high (Figure 1B) molecular weight fragments, even up to 5 h of incubation, although some signal was lost from the pulsed field plugs over time. The intensity of the compression band on the pulsed field gel (unresolved

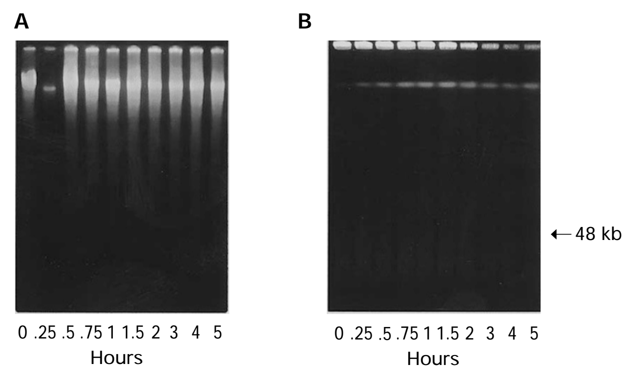

Figure 1 Autodigestion of HeLa nuclei. HeLa nuclei were incubated in the presence of 2 mM $MgCl_2$ and 1 mM $CaCl_2$ for increasing periods of time. At the indicated time, samples were divided and aliquots processed for conventional or pulsed field analysis as described in Materials and Methods. (A) Ethidium bromide-stained agarose gel of HeLa DNA subjected to conventional static-field electrophoresis. (B) Ethidium bromide-stained agarose gel of HeLa DNA, from the same samples as in A, subjected to pulsed field electrophoresis.

DNA $>$500 Kb) is slightly increased over time suggesting that there is a small amount of endogenous nuclease activity. This slight degradation can be prevented by the inclusion of EDTA (data not shown), implicating the involvement of specific, divalent cation-dependent enzymes in the generation of this background degradation. The limited amount of degradation is significantly below the level of total genomic catabolism this assay is designed to detect and therefore should not interfere with interpretation of the results.

We have utilized this assay to explore the patterns of DNA degradation evoked by nucleases proposed to mediate DNA degradation during apoptosis. DNase I is one such candidate nuclease (Boone *et al*, 1995; Peitsch *et al*, 1993, 1994) and Figure 2(A and B) depicts the pattern of both low and high molecular weight DNA fragments generated by 100 ng DNase I in this asssay. DNase I degraded HeLa chromatin in a time-dependent fashion, generating a smear of low molecular weight DNA fragments on a conventional agarose gel (Figure 2A). These results are consistent with previously published data (Peitsch *et al*, 1993; Vanderbilt *et al*, 1982). Pulse field analysis of these same samples (Figure 2B) allowed the detection of larger forms of fragmentation in which chromatin is degraded from intact chromosomes (which remain in the preparatory agarose plugs) through a continuum of sizes that eventually center on $\approx$30–50 Kb and become nearly undetectable thereafter. This pattern is similar to that seen in thymocyte nuclei induced to undergo autodigestion by addition of Ca^{2+} and Mg^{2+} and thymocyte autodigestion is a well characterized model thought to accurately reflect the DNA degradation seen during apoptosis (Cohen *et al*, 1984; Hewish *et al*, 1973; McConkey *et al*, 1989b; Nieto *et al*, 1989; Sun *et al*, 1994; Walker *et al*, 1994). Loss of signal on these gels indicates degradation of DNA into $<$2 Kb fragments which diffuse out of the preparatory agarose plug during processing. These results demonstrate that DNase I can produce high molecular weight apoptotic-like $\approx$30–50 Kb DNA fragments although the low molecular weight analysis reveals a smear of DNA not generally associated with the apoptotic process.

DNase II has also been proposed as a candidate apoptotic nuclease (Barry *et al*, 1993; Eastman, 1994). Although the pH optimum of DNase II is 5.5, the most extreme acidic conditions an apoptotic cell could be expected to achieve is 6.3 (Barry *et al*, 1993). Thus pH 6.3 was used in this experiment to more accurately reflect the possible *in vivo* situation. In addition, an increased amount of DNase II was used to provide similar units of activity (at an optimal pH) as compared to DNase I. As seen in Figure 2, very little or no chromatin degradation was detected during the course of this experiment on either conventional (Figure 2C) or pulsed field gels (Figure 2D). In the pulsed field analysis a slight increase in the intensity of the compression band was detected which is probably attributable to endogenous HeLa activities observed in the absence of divalent cation chelation (Figure 1). The results

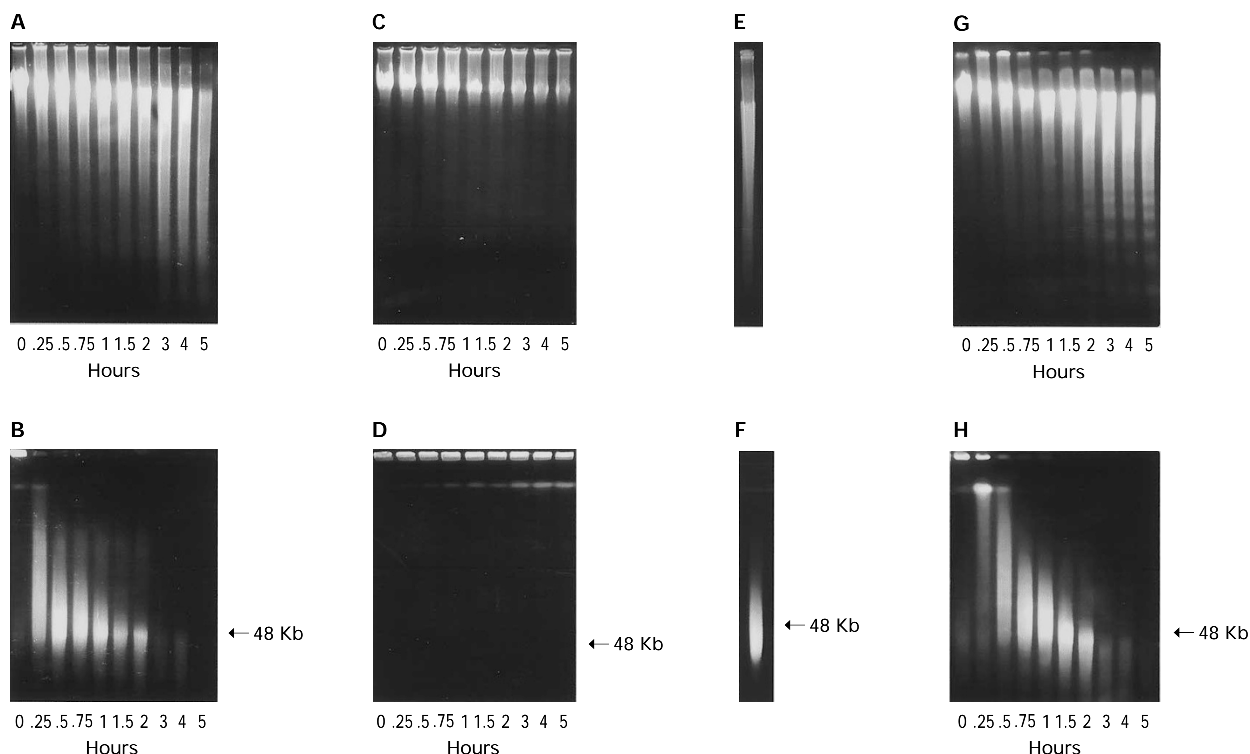

Figure 2 HeLa nuclei assay performed as in Figure 1 in the presence of 50 ng DNase I (A and B), 100 ng DNase II (C and D), 3.5 μg cyclophilin C (E and F) or 1 ng micrococcal nuclease (G and H). DNase I: (A) Conventional agarose gel of HeLa DNA isolated at the indicated time periods. (B) Pulsed field agarose gel of HeLa DNA from the same samples as in A. DNase II: (C) Conventional agarose gel. (D) Pulsed field agarose gel. Cyclophilin C: (E) Conventional agarose gel. (F) Pulsed field agarose gel. Micrococcal nuclease: (G) Conventional agarose gel. (H) Pulsed field agarose gel.

of this assay suggest that DNase II, at a physiologically relevant pH, does not possess sufficient activity to evoke the type of catastrophic genomic degradation seen during apoptosis.

Our laboratory has previously isolated an 18 Kd nuclease (NUC18) active in apoptotic thymocytes but only present in control cells in an inactive high molecular weight complex (Gaido and Cidlowski, 1991). Sequencing of fragments of this enzyme revealed a high homology with the cyclophilin family of immunophilins. Subsequent analysis revealed that the three members of this family (Cyclophilins A, B and C) have significant nuclease activity with cyclophilin C being the most potent (Montague *et al*, 1994). In the present study we evaluated the effects of the most active homologue of cyclophilin (cyclophilin C) on HeLa nuclear chromatin integrity. A single time point (5 h) was chosen because of the limited supply of pure enzyme. As shown in Figure 2E, cyclophilin C did not produce any specific low molecular weight DNA fragments, although there was a slight increase in DNA smearing compared to the time 0 control. Similarly, cyclophilin A does not produce internucleosomal fragments (data not shown). Cyclophilin C did, however, generate a very strong $\approx$30–50 Kb DNA signal during the incubation (Figure 2F). The results indicate that cyclophilins are unlikely to be involved in the production of internucleosomal DNA fragments during apoptosis but may mediate the generation of $\approx$30–50 Kb DNA fragments. Similar results are being reported in an additional manuscript on the nuclease activities of cyclophilin (Montague *et al*, 1996). Together the results indicate that none of the enzymes proposed to mediate DNA fragmentation during apoptosis can completely recapitulate all the DNA degradation seen in apoptotic cells *in vivo*.

Next we investigated the effects of micrococcal nuclease (MN). Although this bacterial enzyme has not been implicated in apoptosis, it is well known to cleave chromatin in the internucleosmal regions and generate a DNA ladder equal to that seen during apoptosis. As shown in Figure 2G, 1 ng MN degraded the HeLa chromatin in a time dependent fashion, generating an apoptotic-like oligonucleosomal DNA ladder within 2 h of incubation. In the pulsed field analysis (Figure 2H) MN generates a pattern similar to DNase I in that it rapidly degrades all the intact chromatin into a continuum of sizes and finally to $\approx$30–50 Kb DNA fragments before becoming absent from

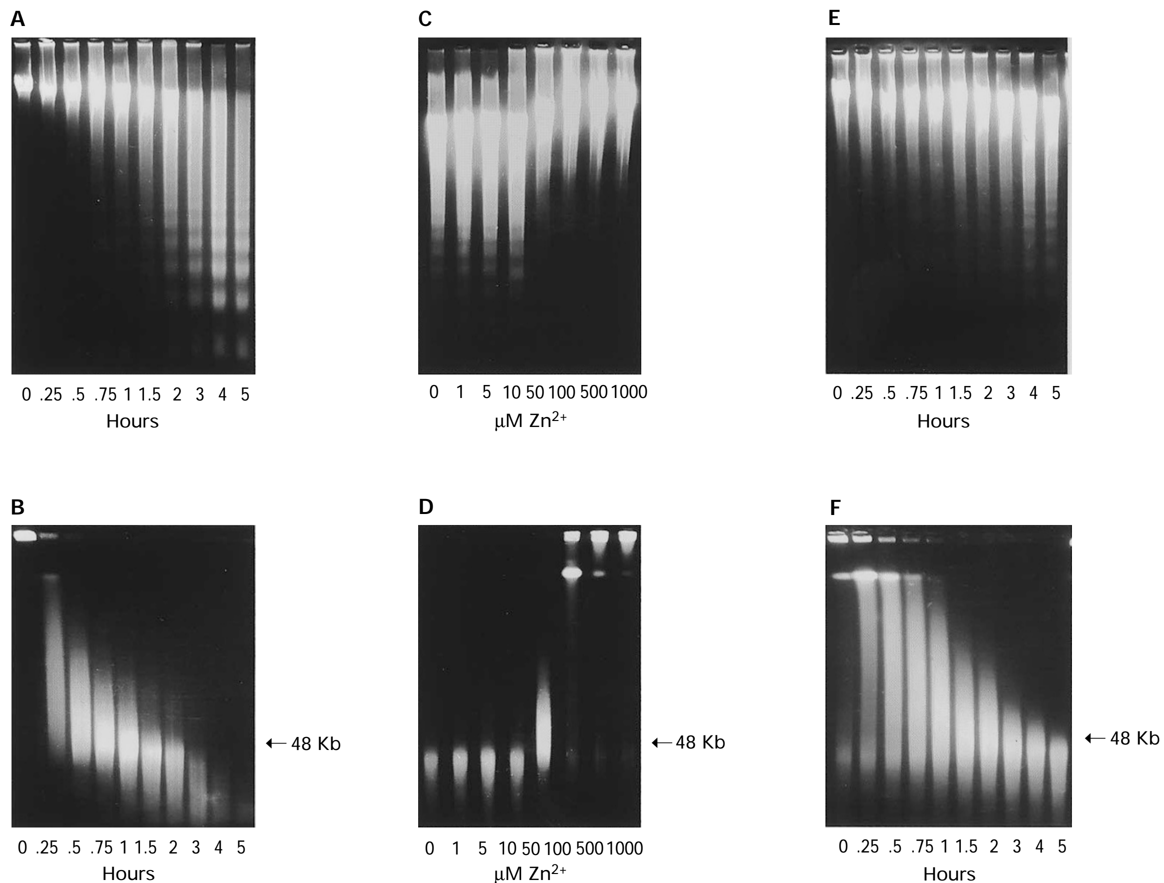

Figure 3 HeLa nuclei assay performed as in Figure 1 in the presence of 100 μg thymocyte nuclear extract either alone (A and B), with increasing concentrations of Zn^{2} (C and D) or 50 μM Zn^{2+} for increasing periods of time (E and F). (A) Conventional agarose gel. (B) Pulsed field agarose gel. (C) Conventional agarose gel. (D) Pulsed field agarose gel. (E) Conventional agarose gel. (F) Pulsed field agarose gel.

the gel completely. Thus, the single enzyme MN, generates both oligonucleosomal and ≈30–50 Kb DNA fragments, suggesting that a single enzyme can mediate both types of DNA degradation. These results do not, however, rule out the potential involvement of different nucleases mediating each type of DNA degradation during apoptosis *in vivo*, but only suggest that one enzyme can generate both types of DNA degradation.

We have previously shown that nuclear extract from apoptotic thymocytes can also generate internucleosomal DNA fragments in this assay. We next sought to determine if such extracts could also produce the large ≈30–50 Kb DNA fragments. As shown in Figure 3A, treatment of HeLa nuclei with 100 μg nuclear extract resulted in a significant time-dependent degradation of the HeLa genome into oligonucleosomal DNA fragments. Pulsed field analysis (Figure 3B) revealed a fragment pattern similar to DNase I, cyclophilin C and MN in which there was rapid degradation of HeLa chromatin to ≈30–50 Kb fragments which eventually become almost completely absent from the gel. Thus, activity(s) in nuclear extract from apoptotic thymocytes generates both types of apoptotic-like DNA fragments (oligonucleosomal and ≈30–50 Kb), essentially recapitulating the *in vivo* patterns of genomic degradation.

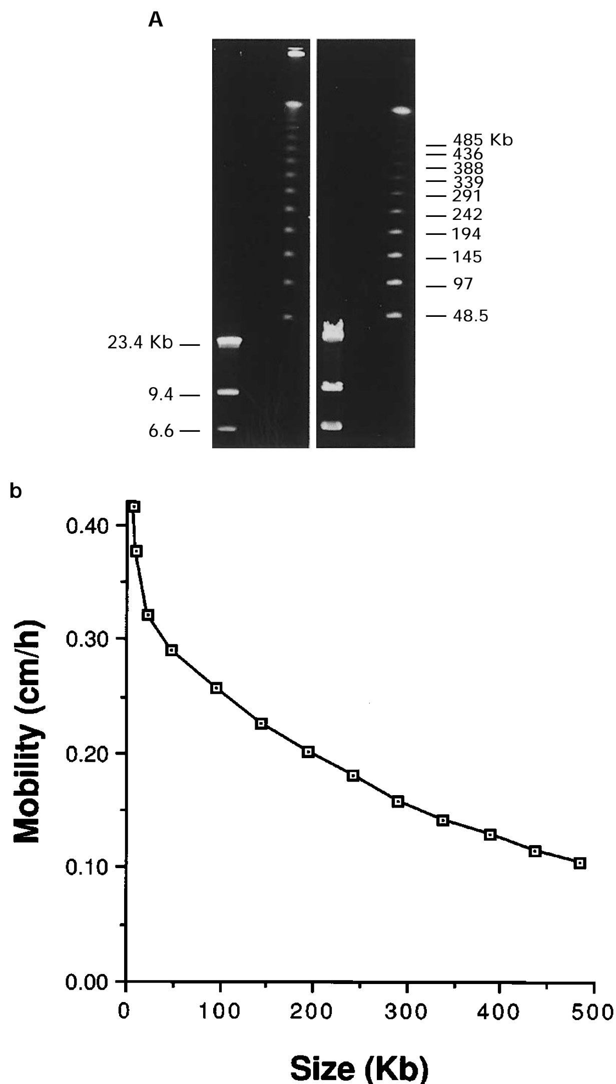

Figure 4 Molecular weight standards for pulsed field electrophoresis. DNA fragments obtained from a HinD III digest of λ DNA (lanes 1 and 3) or concatamers of the intact λ genome were used as size standards. Prior to electrophoresis, one set of standards (lanes 1 and 2) were encapsulated in agarose and processed in an identical manner as sample plugs as a control for the diffusion of fragments out of the sample plugs and electrophoresis off the end of the gel. (A) Pulsed field agarose gel showing the migration of standards. Lane 1 (HinD III digest of λ DNA) and 2 (λ concatamers) were first encapsulated in agarose and treated as described above. Lanes 3 and 4 are non-processed standards. (B) The standards shown in A were graphed according to their mobility (cm/h) as a function of their size. From the resulting graph, the linear range of size separation can be directly read off (50–500 Kb).

Recently, other investigators have reported that, in intact thymocytes and nuclear autodigestion experiments, the generation of ≈30–50 Kb DNA fragments and oligonucleosomal DNA fragments is differentially sensitive to Zn^{2+} during short term incubations (Brown *et al*, 1993; Kokileva, 1995; Walker *et al*, 1994). They interpreted their data to mean that different enzymes mediate each form of DNA degradation. Figure 3C and D depicts the effects of increasing concentrations of Zn^{2+} on the degradation of HeLa chromatin by thymocyte nuclear extracts during short term digestions (3 h). Following this incubation, internucleosomal fragmentation remains clearly evident in the presence of 10 μM Zn^{2+} but is inhibited by 50 μM or greater of this ion. The generation of ≈30–50 Kb DNA fragments (Figure 3D) is not efficiently inhibited by 50 μM Zn^{2+}, although the modal DNA fragment size is slightly shifted up in size (quantitative data not shown). However, the generation of ≈30–50 Kb DNA fragments is clearly inhibited at higher concentrations of Zn^{2+} (>100 μM). These results are consistent with the previously published literature (Brown *et al*, 1993; Kokileva, 1995; Walker *et al*, 1994) which suggests these two forms of DNA degradation are mediated by separate enzymes, differing in their sensitivity to Zn^{2+}. However, when comparing pulsed field and conventional agarose gels, it should be noted that detection of large DNA fragments is considerably more sensitive than small fragments because of the increased number of ethidium bromide molecules each DNA fragment binds. Accordingly, the pulsed field and conventional gels cannot be directly compared because of the 150–250 fold difference in DNA fragment size. This, in conjunction with the substantially fewer number of cuts in a chromosome needed to detect a decrease in size on the pulsed field gel, makes the pulsed field analysis a significantly more sensitive measurement of nuclease activity than the conventional agarose gel. Because of this difference in sensitivity of detection of DNA fragments, the interpretation of the data derived from Zn^{2+} studies may be in question and the data of Figure 3C and D may have resulted from a partial inhibition of a single nuclease. If this hypothesis is correct, then extending the time of incubation in the presence of 50 μM Zn^{2+} should allow the attenuated digestion reaction to go further toward completion, resulting in the delayed appearance of oligonucleosomal DNA fragments. Figure 3E and F shows the result of an extended digestion time course with nuclear extract in the

presence of 50 μM Zn^{2+}. During this incubation, internucleosomal DNA fragments became apparent (albeit weakly) by 4 h of incubation, suggesting that the internucleosomal enzyme was not completely inhibited by this treatment but only partially reduced in activity. Incubation with this ion also slowed the degradation seen on pulsed field gels (Figure 3F), further suggesting that the high molecular weight degradation was not mediated by a separate enzyme whose activity was not affected by 50 μM Zn^{2+}. Taken together the results are consistent with a single, Zn^{2+} sensitive, nuclease mediating both types of DNA degradation by the nuclear extract.

Discussion

Apoptosis has recently been associated with the degradation of chromatin into large ($\approx$30–50 Kb) DNA fragments in addition to the classical oligonucleosomal fragments often used as a hallmark of this process. In the present study we utilized an *in vitro* assay to examine the ability of several candidate nucleases to recapitulate both types of DNA degradation. DNase I reproduced the $\approx$30–50 Kb apoptotic-like DNA fragments but did not produce internucleosomal fragments. Likewise, cyclophilin C (related to NUC18) produced large $\approx$30–50 Kb DNA fragments but not internucleosomal fragments. In contrast, DNase II did not catalyze significant DNA fragmentation of any size. Interestingly, micrococcal nuclease was able to completely mimic both types of chromatin degradation, demonstrating that separate enzymes may not be necessary to generate both types of DNA degradation. These results do not, however, rule out the possibility that separate nucleases exist *in vivo*. Nuclear extracts from apoptotic thymocytes also produced both types of *in vivo* apoptotic DNA fragmentation, suggesting that these extracts possess all the enzymatic requirements for this process. Finally, although previous reports suggested that large fragment and small fragment generation were differentially sensitive to inhibition by Zn^{2+} (Brown, *et al*, 1993; Kokileva, 1995; Walker *et al*, 1994), extended digestions suggest that these results may reflect an inherent difference in the sensitivity of ethidium bromide staining of the large and small DNA fragments. Together the results are consistent with a single nuclease mediating the DNA degradation evoked by the thymocyte nuclear extracts.

Our results demonstrate that DNase I, cyclophilin C (related to NUC18), MN and thymocyte nuclear extract can all generate $\approx$30–50 Kb apoptotic-like DNA fragments, despite the radically different patterns produced on the conventional gels. The $\approx$30–50 Kb DNA fragments are postulated to result from cleavage of chromatin at or near the region attached to the nuclear matrix (the MARs) (Filipski *et al*, 1990; Kokileva, 1994; Oberhammer *et al*, 1993; Walker *et al*, 1995), although this selectivity has not been conclusively demonstrated. Such areas are known to be centers of active transcription and actively transcribed areas are preferentially susceptible to digestion by nucleases such as DNase 1 (Gromova *et al*, 1995). Thus it is possible that MARs, and the areas that surround them form natural hypersensitive sites. In this regard it is interesting that the generation of these large DNA fragments has also been detected in two separate studies of necrosis (Bicknell and Cohen, 1995; Kataoka *et al*, 1995) during which many nonspecific enzymes are activated.

DNA degradation during apoptosis has been proposed to occur stepwise, proceeding downward in size through a $\approx$300 Kb DNA species to the $\approx$30–50 Kb DNA fragments. However, many studies have not detected the $\approx$300 Kb fragments. Likewise, our analysis did not reveal any specific fragment bands larger than $\approx$30–50 Kb, even though we significantly slowed the kinetics of the reaction in order to detect any such fragments that may transiently appear. Instead, we detect an initial smear of very large fragments which are rapidly degraded to $\approx$30–50 Kb. We suspect that the early smear represents chromosomes that are in early stages of degradation. For example, if we assume that cleavage occurs at intervals of $\approx$30–50 Kb, then a single cut in one chromosome would generate two large DNA fragments that average one-half of a chromosome in length. If the cut is chosen randomly the actual fragments generated would form a continuum of sizes (which constitute a smear) ranging from a single $\approx$30–50 Kb DNA fragment to an intact chromosome minus $\approx$30–50 Kb. As degradation continues the modal fragment size decreases rapidly to $\approx$30–50 Kb. Interestingly, *in vivo* we and others detect only $\approx$30–50 Kb DNA fragments without an early smear (Bortner *et al*, 1995; Cohen *et al*, 1994; Oberhammer *et al*, 1993, 1994; Walker *et al*, 1994). We speculate that the lack of a detectable smear reflects the fact that, *in vivo*, a cell population undergoes apoptosis asynchronously with only a small number of cells showing characteristic apoptotic changes at any given time. Accordingly, at the onset of the experiment, most cells would have intact DNA, but a small number may be rapidly degrading their DNA through the continuum of sizes down to $\approx$30–50 Kb DNA fragments where they persist for a relatively longer period of time. During the period that these fragments persist, other cells may begin rapidly degrading their DNA down to $\approx$30–50 Kb. Thus, the number of cells containing $\approx$30–50 Kb DNA fragments will increase over time while the number of cells possessing a smear of DNA sizes would remain exceedingly low and beyond the threshold of detection. This would give the appearance that $\approx$30–50 Kb DNA fragments are generated without any intermediate sizes. In contrast, in the HeLa nuclei assay all nuclei are acted upon simultaneously by the exogenous nuclease activity, causing a synchronized degradation of HeLa chromatin and making an early smear clearly apparent. Further evidence of the asynchronous nature of *in vivo* apoptosis versus the synchronous nature of the HeLa assay can be seen by examining DNA remaining in the preparatory agarose plugs. When $\approx$30–50 Kb DNA fragments are seen *in vivo*, significant amounts of DNA remains in the plug (Bortner *et al*, 1995) indicating the presence of intact DNA from nondying cells. However, in the HeLa assay as the $\approx$30–50 Kb DNA fragments are formed, all signal has been lost from the plugs (Figures 2 and 3), demonstrating that the chromatin in all nuclei had been degraded. Thus the DNA smear we detect early in the synchronized HeLa assay may actually reflect the degradation pattern occurring asynchronously in a cell population.

One interesting result of this study is that a single nuclease, such as MN, can generate both the $\approx$30–50 Kb apoptotic-like DNA fragments and oligonucleosomal fragments. Thus a single nuclease with an internucleosomal specificity could potentially mediate both types of DNA degradation during apoptosis. These results do not, however, rule out a separate $\approx$30–50 Kb DNA fragment-generating enzyme in apoptotic cells *in vivo* or in our thymocyte nuclear extracts, nor do they discount the possibility of more than one nuclease mediating either type of DNA fragmentation, rather they simply show that there is no physical need for multiple enzymes since one nuclease can generate both types of fragments. Analysis of types of nucleases involved in apoptosis and their specificities for chromatin structures is currently being investigated in our laboratory.

Materials and Methods

Materials

Micrococcal nuclease (16,322 Units/mg) was obtained from Worthington Biochemical Corp. (Freehold, NJ) while DNase I (1900 Units/mg), DNase II (900 Units/mg) and RNase A were purchased from Sigma Chemical Corp. (St. Louis, MO). Cyclophilin C was a generous gift from Sandoz Pharmaceuticals (Bazel, Switzerland). Dexamethasone was received from Steraloids (Wilton, NH). Tris was obtained from Fisher Scientific (Norcross, GA) whereas EDTA was received from EM Science (Curtin Matheson Scientific, Houston, TX), chloroform from Mallinckrodt Chemicals (Paris, KY) and phenol from United States Biochemical (Cleveland, OH). Proteinase K was purchased from Boehringer Mannheim (Indianapolis, IN) while Joklik's Minimum Essential Medium was obtained from Flow Laboratories (McLean, VA). Fetal calf serum was purchased from Irvine Scientific (Santa Ana, CA) and calf serum received from Intergen (Purchase, NY). Insert agarose from FMC Bioproducts (Rockland, ME). The HinD III digest of λ DNA was obtained from Gibco/BRL (Gaithersburg, MD) while the λ concatamers were purchased from New England BioLabs (Beverly, MA).

HeLa nuclei assay

The HeLa nuclei assay was modified from previously published accounts (Schwartzman *et al*, 1991, 1993b) to include analysis of large DNA fragments by pulsed field electrophoresis. Briefly, HeLa cells were maintained ($10^5 - 7 \times 10^5$ cells/ml) in suspension culture at 37°C in Joklik's minimum essential medium supplemented with 2% fetal calf serum, 2% calf serum, 2 mM glutamine, 75 U/ml penicillin and 50 U/ml streptomycin sulfate. Prior to assay, cells were counted, pelleted and resuspended in 10 mM $MgCl_2$, 0.25% Nonidet P-40 to isolate nuclei. Nuclei were then pelleted, resuspended in 50 mM Tris (pH 7.4) and 2×10^6 nuclei added to 1.5 ml microcentrifuge tubes containing final concentrations of 50 mM Tris (pH 7.4 or 6.3 for DNase II), 2 mM $MgCl_2$, 1 mM $CaCl_2$ and experimental treatments in a total volume of 400 μl. The parameters of this assay have been previously optimized for the detection of internucleosomal cleavage activity (Schwartzman *et al*, 1991, 1993b). The effects of all nucleases on chromatin integrity were dose-dependent and the concentrations of active enzymes were chosen to provide similar amounts of DNA degradation during the course of the experiment.

DNA fragmentation during apoptosis *in vivo* is very rapid, requiring only 15–60 min. Because these changes occur in such a short period, the sequence of events has been difficult to evaluate. Thus, in the present studies the concentrations of enzyme under investigation were chosen to allow the reaction to occur over an extended period of time in order to facilitate data analysis. Preliminary experiments revealed that high concentrations of enzyme would lead to kinetics of DNA degradation comparable to that seen *in vivo*. In addition, the relatively low ionic strength conditions of the assay has been shown to allow degradation of both relaxed, transcriptionally active chromatin and bulk inactive chromatin (Walker and Sikorska, 1986).

For pulsed field analysis, 200 μl was removed at the end of the incubation and mixed with 200 μl of 1% Incert agarose at 37°C until nuclei were evenly dispersed. The mixture was transferred to a 0.5 cm^2 mold and placed at 4°C for 5 min to solidify. Agarose plugs were then extruded into 10 ml of 100 mM EDTA, 1% (w/v) N-lauroylsarcosine and incubated at 37°C overnight. Plugs were subsequently transferred to 1 ml of the same buffer containing 100 μg proteinase K and incubated a minimum of 16 h at 50°C. No effects of longer incubations were noted. Plugs were stored at 4°C in this buffer until used.

Following removal of the aliquot for pulsed field analysis, conventional electrophoresis samples were prepared from the remaining nuclei by first adding EDTA, NaCl and sodium dodecyl sulfate to a final concentration of 25 mM, 540 mM, and 0.5%, respectively. The EDTA serves a dual purpose; to chelate divalent cations, effectively inhibiting cation-dependent nucleases, and to maximize the solubility of the released chromatin fragments (Walker *et al*, 1986). TE buffer (10 mM Tris (pH 7.4), 1 mM EDTA) was added to bring the final volume to 400 μl and Proteinase K added to 0.5 mg/ml. Samples were then incubated for 1 h at 50°C, extracted twice with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and once with chloroform/isoamyl alcohol (24 : 1) alone. DNA was subsequently precipitated by adding 10 μl of 5 M NaCl plus 1 ml of ice-cold 100% ethanol and incubating at -70°C for $>$20 min. DNA was collected by centrifugation, dried on a Savant Speed Vac (Savant Instruments, Inc., Holbrook, NY) for 20 min with mild heating and resuspended by adding 30 μl TE buffer and 1 μl 10 mg/ml DNase-free RNase A to the tube and incubating overnight at 37°C. DNA concentrations were determined spectrophotometrically using the absorbance at 260 nm. Ratios of absorbance at 260 nm/280 nm consistently gave values of $\approx$1.8 reflective of high purity of DNA.

Preparation of thymocyte nuclear extract

Thymocyte nuclear extract was prepared as previously described (Cidlowski, 1982; Schwartzman *et al*, 1991). Briefly, male Sprague-Dawley rats (100–250 g body weight) were bilaterally adrenalectomized at least 5 days prior to use and maintained on 0.85% NaCl and rat chow *ad libitum*. Four hours prior to sacrifice, rats were injected i.p. with 5 mg/Kg body weight of the synthetic glucocorticoid dexamethasone suspended by sonication in phosphate buffered saline. Previous studies from our laboratory have shown that this paradigm results in $\approx$50% of the genome degraded while maintaining a high cellular viability ($\approx$98%) (Compton and Cidlowski, 1986). Following a 4 h *in vivo* exposure period, rats were killed by decapitation and the thymus removed and placed in ice-cold PBS. Thymocytes were released from the tissue by mincing with scissors followed by gentle homogenization in a loose fitting Kontes no. 22 glass/glass homogenizer (Kontes Co., Vineland, NJ) on ice. Suspensions were filtered through 202 μm Nitex mesh (Tetko, New York, NY) and pelleted at 4°C. Cells were then washed once in ice-cold PBS and the plasma membranes lysed (10 mM $MgCl_2$, 0.25% Nonidet P-40). Nuclei were pelleted and resuspended in extraction buffer (300 mM NaCl, 1 mM EDTA, 20 mM Tris (pH 7.4); 0.5 ml/

thymus) and rotated at 4°C for 1 h. Extracts were then cleared of debris and endogenous chromatin by ultracentrifugation at 165 000 × g for 1 h at 4°C. Following this centrifugation, the supernates were removed and protein concentrations determined by the method of Bradford (Bradford, 1976). Extracts were aliquoted to avoid repeated freeze/thaw cycles and stored at −70°C until used.

Electrophoresis

For conventional static-field electrophoretic analysis 1.8% agarose gels were submerged in 0.5 × TPE (40 mM Tris-phosphate, 4 mM EDTA) and 15 μg of HeLa DNA added per lane. An electric field was applied for 3.25 h with constant voltage (80 V). For pulsed field analysis a 2.0 mm thick slice was cut from the preparatory agarose plug and equilibrated in 50 ml of 0.5 × TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA) for >3 h. To insure that these conditions did not result in significant loss of smaller DNA fragments, size standards (Hind III digest of λ DNA or λ concatamers) were encapsulated in agarose and processed in an identical manner as experimental samples (Figure 4A-Lane 1 and 2). These standards were then electrophoresed and compared to non-processed standards (Figure 4A-lane 3 and 4). As shown in Figure 4A, no significant losses of DNA fragments ⩾6.6 Kb were seen during processing. To prepare the gel, slices were placed against a gel comb and 1% molten agarose added and solidified around the plugs. The comb was removed and the gel placed in a Clamped Homogenous Electric Field (CHEF) pulsed field system (Bio-Rad Laboratories, Hercules, CA) filled with 0.5 × TBE and allowed to equilibrate to 14°C. The gel was then electrophoresed for 19 h at 6.0 V/cm with a linear switch interval ramp from 0.5 s to 45.0 s. The parameters were chosen to optimally separate DNA fragments ranging from 10 – 500 Kb ((Birren and Lai, 1993), E. Lai, personal communication, information also available from Bio-Rad). As shown in Figure 4A, these parameters retained fragments in the gel as small as 6.6 Kb. To insure that these parameters provide a linear separation of fragments in the target size, the mobility of the size standards shown in Figure 4A were plotted as a function of size (Figure 4B). From this graph the linear range of separation can be directly read off (≈50 – 500 Kb) (Birren and Lai, 1993). Size standards also included chromosomes from *S cerevisiae* and 8 – 48 Kb standards obtained from Bio-Rad. Following electrophoresis, both pulsed field and conventional gels were stained with 1 mg/ml ethidium bromide and photographed (Polaroid type 55 film) using UV transillumination.

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