DNA fragmentation during apoptosis is caused by frequent single-strand cuts

Manuel C. Peitsch, Christian Müller and Jürg Tschopp*
Institute of Biochemistry, University of Lausanne, Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland

Received June 16, 1993; Revised and Accepted August 4, 1993

ABSTRACT

One of the hallmarks of apoptosis is the digestion of genomic DNA by an endonuclease, generating a ladder of small fragments of double-stranded DNA. We have examined the nature of the DNA breaks produced in mouse thymocytes triggered to undergo apoptosis by steroids or by stimulation of the T cell receptor. Whereas the typical ladder pattern of oligonucleosomal fragments was observed after agarose gel electrophoresis, numerous single-strand cuts were detected after electrophoresis under denaturing conditions. Single-strand nicks were found to be very frequent in the internucleosomal regions, but also to occur in the core particle-associated DNA. An identical pattern of single-strand nicks was obtained when chromatin DNA was exposed to the single-strand cleaving deoxyribonuclease I. The nicked DNA fragments, extracted from apoptotic thymocytes, were sensitive to the action of S1-nuclease. We propose that DNA fragmentation induced during apoptosis is not due to a double-strand cutting enzyme as previously postulated, but rather is the result of single-strand breaks. This ensures the dissociation of the DNA molecule at sites where cuts are found within close proximity.

INTRODUCTION

Programmed cell death or apoptosis is a mechanism of cell clearance in many physiological processes such as embryogenesis, metamorphosis and tumor regression (1-3). Apoptosis is also involved in the elimination of self-reactive T lymphocytes in the thymus (4,5), and in target cells attacked by cytolytic T cells (6,7).

Apoptosis is selectively triggered in cells by a variety of stimuli; however the mechanisms leading to the onset of apoptosis are as yet largely unknown. During apoptosis, a series of well defined morphological and biochemical changes occur within the cell (1,8). The process includes nuclear membrane breakdown, cytoskeletal reorganization, plasma membrane blebbing, and loss of adhesion. The most noticeable changes take place in the nucleus: shrinkage of the nucleus is observed, the chromatin condenses, and the nuclear material collapses into patches and dissociates into many lobes (8).

Associated with the loss of the integrity of the nucleus is digestion of the genomic DNA by an endonuclease (9,10). In cells of hematopoietic origin, cells undergo extensive DNA degradation, typically generating a ladder of small fragments of double-stranded DNA, as detected in nondenaturing agarose gel electrophoresis. The fragments are multiples of approximately 180 bp, reflecting the preferential accessibility of internucleosomal linker DNA to endonucleases. In epithelial and endothelial cells undergoing apoptotic cell death, the nucleosomal fragments are often absent; analysis of the DNA on denaturing sucrose density gradients, however, reveals that infrequent single-strand nicks are generated (11,12).

It is widely assumed that the DNA cleavage is a result of an endogenous Ca\(^{2+}\) and Mg\(^{2+}\)-dependent endonuclease activity capable of inducing double-strand breaks at internucleosomal sites. Several candidate nucleases of different cellular origins have been reported: DNase I (13,14), DNase II (15) or Nuc 18 (16).

To date, there is little information on the exact nature of the cuts produced by the apoptosis-associated endonuclease. Although the oligonucleosomal fragments appear to be the result of a nuclease cleaving in the linker region with double-strand cuts (9), a single-strand cleaving enzyme cannot formally be excluded, since the close proximity of two single-strand breaks on opposite strands will also result in the dissociation of double-stranded DNA.

This paper describes a more detailed analysis of the nature of the DNA cleavages occurring during apoptosis. We have analyzed DNA fragments produced in mouse thymocytes triggered to undergo apoptosis by the addition of dexamethasone or by stimulation of the T cell receptor-dependent signaling pathway. The results from this analysis provide strong evidence for a nuclease activated during apoptosis which produces numerous single-strand nicks on chromatin DNA, finally leading to its dissociation into oligonucleosome-sized fragments.

EXPERIMENTAL PROCEDURES

Cells and nuclei

Thymi of 4–5 week old OF1 mice (Ifa-Credo) were freshly collected. RG-17 cells (17) were harvested from exponentially growing cultures in DMEM medium containing 5% fetal calf

* To whom correspondence should be addressed
serum. Nuclei were isolated from single cell suspensions of thymocytes with 0.05% NP-40 according to Hewish and Burgoyne (18). Nuclei of RG-17 cells were prepared according to the same procedure except that 0.3% NP-40 was used.

Induction of apoptosis of $2 \times 10^7$ thymocytes was performed in 10 ml DMEM medium containing 5% fetal calf serum at 37°C for 5 hr, either by the addition of $10^{-6}$ M dexamethasone (Sigma, Buchs), or in 100-mm petri dishes precoated for 4 hr with the rat anti-mouse CD3 mAb 17A2 (19) at 20 μg/ml in 50 mM Tris–HCl, pH 9.5.

At the end of the incubation, the cells were harvested and resuspended in 500 μl DMEM medium and DNA was extracted twice with 500 μl phenol/chloroform/isoamyl alcohol (50/49/1). A RNase A digestion (10 μg/ml, 20 min at 37°C) was performed before the second DNA extraction. An aliquot of the samples was loaded onto a 1.8% agarose gel equilibrated in 40 mM Tris-acetate pH 8.0 containing 1 mM EDTA. After 1–2 hr migration at 3 Volts/cm, gels were stained with ethidium bromide and photographs were taken on a UV transilluminator.

DNA fragments corresponding to the mono- to tetranucleosomes were purified by excision from agarose gels after separation and using the GeneClean II Kit (Bio 101, La Jolla, CA).

DNA degradation in isolated nuclei
To activate the endogenous endonuclease, isolated mouse thymocyte nuclei ($1 \times 10^7$) were incubated at 37°C for 2 hr in 100 μl of 15 mM HEPES buffer (pH 7.5) containing 60 mM KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β-mercaptoethanol and 10% sucrose in the presence of 2 mM of both Ca$^{2+}$ and Mg$^{2+}$. RG-17 nuclei were incubated at 37°C with five-fold diluted normal rat serum as source of DNase I for 2 or 5 hr in the same buffer as thymocyte nuclei. Reactions were stopped by the addition 10 μl ice cold 500 mM EDTA, and DNA was extracted twice with 200 μl phenol/chloroform/isoamyl alcohol (50/49/1). In order to see only fragmented DNA on the agarose gels, we omitted the proteinase K digestion step used by other investigators (20). Samples were centrifuged at 12 000 x g for 10 min and the aqueous phase analyzed by agarose gel electrophoresis as described above.

DNA labeling and analysis
Total extracted DNA fragments and the individual fragments purified as described above were labeled at the S' end with the T4 polynucleotide kinase according to standard methods (21). Typical labeling reactions were carried out with 20 to 200 ng of DNA in 50 μl incubated with 10 μCi [γ-32P] ATP (Amersham) and 15 units of T4 polynucleotide kinase (Boehringer, Mannheim) in the appropriate kinase buffer at 37°C for 1 hr. Non-incorporated nucleotides were removed using adsorption on glass beads of the GeneClean II Kit.

End-filling reactions were performed with 20 to 200 ng (20 μl) of DNA, in the presence of 10 μCi [α-32P] dCTP, 25 μM of each of the three other deoxynucleotides, 2 units of the Klenow polymerase in the appropriate polymerase buffer (21). Non-incorporated nucleotides were removed using the GeneClean II Kit.

Separation of the DNA fragments was performed under denaturing conditions (7 M urea) on a 0.25 mm-4 mm sequencing gel (5% polyacrylamide).

S1-nuclease digestions of DNA were done with 50 ng of di-nucleosomal-sized fragments in 50 mM Na-acetate pH 4.5, 14 mM β-mercaptoethanol and 10% sucrose in the presence of 2 M/4 M KCl, 15 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 14 mM β-mercaptoethanol and 10% sucrose in the presence of 2 mM of both Ca$^{2+}$ and Mg$^{2+}$. RG-17 nuclei were incubated at 37°C with five-fold diluted normal rat serum as source of DNase I for 2 or 5 hr in the same buffer as thymocyte nuclei. Reactions were stopped by the addition 10 μl ice cold 500 mM EDTA, and DNA was extracted twice with 200 μl phenol/chloroform/isoamyl alcohol (50/49/1). In order to see only fragmented DNA on the agarose gels, we omitted the proteinase K digestion step used by other investigators (20). Samples were centrifuged at 12 000 x g for 10 min and the aqueous phase analyzed by agarose gel electrophoresis as described above.

**Figure 1.** Purified apoptotic DNA fragments. DNA extracted from thymocytes induced to apoptosis by (1) dexamethasone and (6) anti-CD3 antibodies was compared with DNA extracted from (7) Ca$^{2+}$/Mg$^{2+}$-treated thymocyte nuclei and (8) RG-17 nuclei treated with serum DNase I and both Ca$^{2+}$ and Mg$^{2+}$. DNA fragments corresponding to (2) tetra-, (3) tri-, (4) di- and (5) mono-nucleosomes were isolated from dexamethasone treated thymocytes as described in Materials and Methods.

**Figure 2.** End-labeling and end-filling experiments. (A) DNA extracted from: (1) dexamethasone-treated thymocytes; their corresponding purified (2) tetra-, (3) tri-, (4) di- and (5) mono-nucleosomal fragments; (6) anti-CD3-treated thymocytes; (7) Ca$^{2+}$/Mg$^{2+}$-treated thymocytes nuclei; (8) RG-17 nuclei treated for 2 hr with serum DNase I and (9) RG-17 nuclei treated for 5 hr with serum DNase I were end-labeled as described in Materials and Methods, before electrophoretic analysis under denaturing conditions. Seq represents a DNA sequencing reaction run on the same sequencing gel. DNA extracted from (lane a) dexamethasone-treated and (lane b) anti-CD3-treated thymocytes were radioactively end-filled and run in parallel on the same gel. The center of each population corresponding to mono- to tetra-nucleosomes, as well as the position of bases 140 and 195, are indicated in the left margin. (B) Enlargement of the lower portion of the gel, spanning the data collected from the isolated thymocyte and RG-17 nuclei. (1) corresponds to lane 7; (2) corresponds to lane 8 and (3) corresponds to lane 9 in A. The relative positions of the first 30 bases are shown in the right margin.
containing 1 mM ZnSO$_4$ and 200 mM NaCl for 30 min. at 37°C in a final volume of 15 $\mu$l. In the assays, 0.4 to 1.6 units of S$_1$-nuclease (Boehringer, Mannheim) were used. The products of the reaction were separated by 1.8% agarose gel electrophoresis as described above.

Computer modeling

The program APOPTOSIS was written in FORTRAN 77 on a Silicon Graphics personal Iris. A DNA molecule of 100 kbp was defined as a double array of integer numbers, each array corresponding to one strand. These values were set to one in positions corresponding to the nucleosomal segments (145 bp), and zero in the linker regions (50 bp). The DNase I single-strand cleavage sites were simulated by integer random numbers generated with the Silicon Graphics random generator. Each randomly chosen value was mapped as a coordinate on the DNA arrays, and the strand to be nicked was chosen based on the parity of another randomly generated number. Cuts were only allowed in linker regions. If two single-strand cuts were found within 14 bases, the double-stranded DNA molecule was considered cleaved (intervals of 4 to 14 bases were tested and found to influence the kinetics but not the final pattern of ladder formation). The lengths of the resulting fragments were computed and plotted logarithmically and displayed as to reflect the usual agarose gel aspect. The intensity of the bands is proportional to the number of fragments of a given size.

RESULTS AND DISCUSSION

In order to study the nature of apoptotic DNA degradation, we analyzed the DNA fragments produced by dexamethasone- and anti-CD3-mediated apoptosis in thymocytes (Fig. 1, lane 1 and 6) by agarose gel electrophoresis. The typical ladder pattern of oligonucleosomal fragments was observed with both treatments. The fragments were identical in size to those obtained when isolated thymocyte nuclei were treated with Ca$^{2+}$ and Mg$^{2+}$ to activate the endogenous endonuclease (Fig. 1, lane 7).

Since analysis in nondenaturing agarose gels does not allow the detection of single-strand breaks, separation on denaturing polyacrylamide gels was performed. The 5' end of the DNA fragments were radioactively labeled with T4 polynucleotide kinase. Electrophoretic separation then revealed not only fragments corresponding to multiples of 140 to 195 base pairs (major band populations), but also a number of additional fragments (minor band populations) with a regular spacing of approximately 10 base pairs (73 to 74 bases separate the 7 first minor band populations, which corresponds to a spacing of 10.4 bases) were observed (Fig. 2A and 2B). Compared to the major bands reflecting cuts in internucleosomal linker region, their intensities were at least 100-fold lower, indicating that single-strand cuts in the DNA wrapped around the histones were much less frequent (Fig. 2A).

This pattern is reminiscent of the pattern reported for nucleosomal core particles digested by the single-strand cutting deoxyribonuclease I (DNase I) (22,23). We therefore treated isolated RG-17 (17) nuclei (which harbor only limited amounts of endogenous endonuclease) with the DNase I contained in normal rat serum (13,14). Serum was taken as a source of DNase I, since we observed that treating isolated nuclei with purified DNase I does not produce a clean ladder pattern, whereas DNase I preincubated with normal serum gains the ladder forming activity (13,14). Furthermore, it has been convincingly demonstrated that the neutral DNase activity of serum is solely due to DNase I (13,24). The patterns of degradation of RG-17 nuclear DNA was identical to that obtained from apoptotic thymocytes on both agarose (Fig. 1) and denaturing polyacrylamide gels (Fig. 2A, lanes 8 and 9).

In a further attempt to characterize these fragments, mononucleosomal fragments were isolated. They migrated as single homogeneous bands on agarose (Fig 1) and on nondenaturing polyacrylamide gels (data not shown). However, under denaturing conditions, single-stranded fragments corresponding to all smaller band populations observed with total apoptotic DNA were revealed (Fig. 2A, lane 2—5). For example, the apparently homogenous population of isolated tetra-nucleosomal fragments also contain single-stranded segments corresponding to mono-, di- and tri-nucleosomes, as well as the minor band populations with an approximate spacing of 10 base pairs (Fig. 2A, lane 2).

End-filling of oligonucleosomal fragments using the Klenow fragment of the E.coli DNA polymerase in presence of [$\alpha$-$^{32}$P] dCTP yielded radioactive DNA fragments for all major bands in the apoptosis ladder (Fig 2A), lanes a and b), confirming the observations of ROsl (25). These results demonstrate that at least a portion of the DNA fragments produced during apoptosis have 5'-protruding ends.

Further support for the generation of single-strand nicks during apoptosis came from digestions of the fragments with the apparent homogenous population of isolated mono-nucleosomal fragments was observed with both treatments. The intensity of the bands is proportional to the number of fragments of a given size.

Figure 3. S$_1$-nuclease digestion of di-nucleosomal fragments. Fifty nanograms of di-nucleosomal fragments were digested with (2) 0 units, (3) 0.4 units, (4) 0.8 units and (5) 1.6 units of S$_1$-nuclease for 30 min at 37°C. Lane one shows the relative migration of the mono-nucleosomal fragment population.

Figure 4. Computer simulation of chromatin DNA cleavage. The cleavage of a 100 Kbp double-stranded DNA molecule was simulated with (1) 0, (2) 6000, (3) 18 000, (4) 30 000, (5) 42 000, (6) 56 000 and (7) 66 000 DNase I molecules (see Materials and Methods) generating single-strand nicks in the inter nucleosomal regions. The generated fragments were plotted on a logarithmic scale to simulate the appearance of bands generated by agarose gel electrophoresis. The similarity between the theoretical banding pattern and the experimental apoptosis ladder is striking (See Figure 1).
S₁-nuclease, which is known to cleave the second strand opposite of nicked DNA (21,26). From the above results we know that single-stranded nicks are more frequent in the inter-nucleosomal linker regions than on the nucleosomal core particles. Thus, digestion of the purified di-nucleosomal fragments with S₁-nuclease should yield fragments corresponding to mono-nucleosomes. This was indeed the case, when 50 ng of di-nucleosomal fragments were treated with 400 mU of S₁-nuclease (Fig. 3). Increased concentrations of S₁-nuclease resulted in the gradual disappearance of nucleosomal sized fragments due to the cleavage occurring in the nucleosomal DNA. S₁-nuclease recognizes a string of three to four exposed phosphates (27). Whereas in a double helix the two sugar-phosphate strand protect one another from attack by the nuclease, single-strand access becomes possible in loops, close to single-strand nicks and at helix termini (27). S₁-nuclease thus also cleaves the duplex DNA from and both ends (27); this may explain the gradual size reduction of the dinucleosomal fragment observed in figure 3 (lane 2 to 5).

Our data demonstrate that predominantly single-stranded nicks are generated during apoptotic DNA degradation. The apparent double-stranded breaks are thus due to the close proximity of single-strand nicks on opposite strands in the linker region of chromatin DNA, rather than to double-strand cleavages as previously proposed (9). Indeed, computer simulations of the DNA ladder formation (see Materials and Methods) based on the above observations confirmed this hypothesis. Two single-strand nicks on opposite strands within 14 bases (in the linker regions) were considered sufficient for the dissociation of the DNA molecule. The theoretical pattern of fragments obtained reproduced the experimentally observed ladder pattern (Fig. 4,1).

It is conceivable that the observed single-strand nicks are the result of a secondary set of events, following a double strand cleavage. However, the pattern of single strand nicks obtained with DNase I-treated RG-17 nuclei is identical to those obtained by autodigestion of isolated thymocyte nuclei and during thymocyte apoptosis, suggesting that no exclusively double-strand cutting enzyme is involved in this process.

In conclusion, we propose that the DNA fragment ladder observed during apoptosis is the result of an endonuclease with a single-strand cleaving activity. We show that single-strand nicks are generated at a high frequency in the linker regions of the chromatin DNA, leading to the dissociation of the DNA molecule. At least a portion of these fragments have 5' protruding ends. Furthermore, additional single-strand nicks were found even in the core particle-associated DNA. Since the complementary strand was apparently not nicked in the close vicinity of these nicks, no dissociation of the DNA molecule occurred under nondenaturing conditions.

This is in agreement with our previous finding that the single-strand cleaving enzyme DNase I is responsible for the DNA ladder observed during apoptosis (14). Indeed, the results presented here show that an identical single-strand cleavage pattern was generated not only by the action of the endogenous endonuclease in situ in thymocytes and isolated thymocyte nuclei, but also in isolated RG-17 nuclei by added DNase I. The 10.4 base pair spacing of the subnucleosomal fragments previously described during DNase I digestion of nucleosomal core particles at neutral pH (23) is also observed in all our samples of DNA from apoptotic cells, lending further support to the involvement of DNase I in apoptotic DNA degradation (14).

In light of our results, which demonstrate that the nucleosomal fragments found in cells of hematopoietic origin are most likely due to a single-strand nicking enzyme, we suggest that a similar if not identical enzyme is responsible for the single-strand nicks observed during apoptosis in epithelial cells (11,12). The lack of apparent double-strand cleavage in these cells as compared to thymocytes may be due to a lower levels of the single-strand nicking endonuclease; we are currently testing this hypothesis.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the technical assistance of M.A.Bettex and Mme E.Burnier. This work was supported by grants of the Swiss National Science Foundation and the Swiss Cancer League.

REFERENCES