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CLEAVAGE OF DNA TO LARGE KILOBASE PAIR FRAGMENTS OCCURS IN SOME FORMS OF NECROSIS AS WELL AS APOPTOSIS

Gareth R. Bicknell and Gerald M. Cohen*

MRC Toxicology Unit, Hodgkin Building, University of Leicester, PO Box 138, Lancaster Road, Leicester LE1 9HN, UK

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SUMMARY: In a number of cellular systems, DNA in apoptotic cells is initially degraded into large fragments of 30-50, 200-300 and ≥700 kilobase pairs, which may subsequently give rise to oligonucleosomal fragments often considered as the biochemical hallmark of apoptosis. In this study, necrosis was induced in U937 cells by incubation with water. Cells yielded large DNA fragments similar in size to those found in apoptosis, but the DNA was then degraded to a continuous spectrum of small fragments, confirming that death was necrotic. The results demonstrate that kilobase pair DNA fragments are formed in some instances of necrosis as well as in apoptosis. This indicates that apoptosis should not simply be assessed by the formation of kilobase pair DNA fragments, but that other criteria, such as cellular morphology, must also be used to verify apoptosis. © 1995 Academic Press, Inc.

Apoptosis, as opposed to necrosis, is an important mechanism for counteracting cell proliferation [1]. *In vivo*, apoptosis allows for clearing damaged or unwanted cells without causing inflammation: apoptotic cells play an active part in their own death and are rapidly engulfed by neighbouring cells [2-4]. Apoptosis may be induced by the activation of an endogenous program or by a wide variety of stimuli [5-7]. Necrosis, by comparison, is an uncontrolled event resulting from loss of homeostasis; cell contents are dispersed, which may then have adverse effects on neighbouring tissue [8-10]. Much interest has focused on the nuclear changes that occur during apoptosis, namely chromatin condensation and the enzymatic cleavage of DNA. Cleavage occurs in the internucleosomal regions, where histones provide little protection from endonucleolytic attack [5,11,12]. Fragmented DNA appears as a series of bands when viewed on agarose gels; this "DNA ladder" has often been

*To whom correspondence should be addressed.

Abbreviations: kbp, kilobase pairs; PBS, phosphate buffered saline; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(ß-amino-ethyl ether) N,N,N′,N′-tetraacetic acid.

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considered the biochemical hallmark of apoptosis [5,6,13]. In contrast, DNA fragmented during necrosis appears as a continuous spectrum of sizes resulting in a smear [9,14]. This is probably due to the effect of simultaneous, uncontrolled proteolysis removing histones and allowing endonucleases greater access to the DNA [5,11].

Results from our laboratory and others have suggested that, during apoptosis, DNA is initially degraded into large fragments of 30-50, 200-300 and $\geq$700 kbp which may subsequently give rise to the classical DNA ladder pattern [19-22]. The significance of the sizes of the large fragments is not known, but they may represent features of higher-order chromatin structure, such as loops or rosettes of DNA [23,24]. In the human leukaemic cell line, U937, DNA is similarly cleaved into 50 and $\geq$580 kilobase pair fragments during apoptosis [25]. The formation of these fragments is accompanied or followed by DNA laddering and is induced by a number of different apoptotic stimuli. However, in some cell systems, DNA laddering does not occur during apoptosis [15-18], and the temptation may arise to assess apoptosis simply by the formation of kilobase pair fragments. In the present study, we have found that degradation of DNA to large kilobase pair sized fragments also occurs under certain experimental conditions in necrotic cells. Necrosis has been confirmed both by the further degradation of the cellular DNA to a continuous spectrum of low molecular weight fragments (rather than to discrete oligonucleosomes) and also by microscopic appearance of the cells. The results show that it would be unwise to use fragmentation of DNA to large kilobase pair sizes alone to assess apoptosis.

MATERIALS AND METHODS

Materials

Reagents, unless otherwise stated, were from Sigma Chemical Company (Poole, UK). Materials for cell culture were obtained from Gibco BRL Life Technologies (Paisley, UK). Additionally, PBS (Dulbecco 'A') was obtained from Unipath Ltd. (Basingstoke, UK) and agarose from Pharmacia Biosystems (Milton Keynes, UK). DNA standards were obtained as follows: 123 base pairs DNA from Gibco; 245-2200 kbp *Saccharomyces cerevisiae* chromosomes from Clontech (Cambridge, UK); and 0.1-200 kbp DNA from Sigma. Solutions of EDTA/EGTA (1 mM/1 mM), $CaCl_2$ (10 mM stock) and $MgCl_2$ (10 mM stock) were made up in ultrapure water. Solution characteristics were as follows: PBS, pH 7.3 and 297 mOsm; water, pH 7.2 and 0 mOsm; EDTA + EGTA, pH 7.3 and 2 mOsm; $CaCl_2$ stock, pH 6.0 and 21 mOsm; $MgCl_2$ stock, pH 7.0 and 21 mOsm.

Cell Culture

U937 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK), and were deposited by Professor H. Harris/R. Sutherland of the Sir William Dunn School of Pathology, Oxford. Cells were maintained as a suspension culture at 37°C in a 95% humidified atmosphere containing 5% (v/v) CO_2. They were grown in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum, L-glutamine (2 mM), amphotericin B (2.5 μg/mL) and gentamicin (50 μg/mL). Cells were maintained in logarithmic growth by allowing each subculture to obtain a population density

of around 1.2 x 10^6 cells/mL before reseeding at 0.5-1 x 10^5 cells/mL. The population mean doubling time was between 20 and 25 hours.

Cell Treatments

Cells were counted with a model ZBI Coulter Counter and samples equivalent to 2 x 10^6 cells were prepared in 1% Agarose L essentially by standard methods [26] but with slight modifications: centrifuged cells were brought up to gel temperature (55°C) for 2 min in PBS (control) or water ± EDTA (1 mM) and EGTA (1 mM). Agarose, made up in appropriate solution, was mixed in and the plugs cooled at 4°C for 5 min. Plugs were then incubated as above in pre-warmed PBS or ultrapure water ± EDTA/EGTA (1 mM/1 mM) for the required time. After incubation, plugs were immersed into pre-chilled PBS at 4°C for 5 min. The purpose of incorporating cells into plugs before treatment was to avoid possible shearing of DNA during pipetting of fragile cells.

Gel Electrophoresis

Following treatment, plugs (1 x 10^6 cells) were immediately loaded onto a 1.8% Agarose 10 gel for conventional electrophoresis, which was performed as described [27]. Alternatively, plugs (0.5 x 10^6 cells) were digested and separated by field inversion gel electrophoresis [20]. Under the conditions used, the resolving power of this technique extends to 580 kbp. All gels shown are representative of at least 2 experiments.

RESULTS

In order to induce non-apoptotic death, U937 cells were subjected to hypotonic conditions. A time-dependent increase in kilobase pair sized fragments of DNA was observed in cells incubated in water at 37°C (Fig.1, lanes 2-7), which was not seen in cells incubated in PBS (Fig.1, lane 1). Little or no fragmentation was observed when cells were incubated with water for 60 min at 4°C or room temperature (20-24°C; Fig. 1, lanes 8 and 9, respectively). When examined by microscopy, all cells incubated as a suspension in water lost membrane integrity by 10 min, as assessed by uptake of propidium iodide (not shown). This contrasted with cells incubated in PBS, of which only a small proportion ($<10\%$) was non-viable. Similar results were observed in control and water-treated cells immobilised in the agarose plugs. Cells incubated in water swelled before losing viability, but were otherwise indistinguishable from control cells, in that there was no nuclear fragmentation or membrane blebbing characteristic of apoptosis. In comparison, cells induced to undergo apoptosis by teniposide (5 μM, 4 h; Fig.1, lane 10) showed degradation of DNA to fragments of 20-50 and $\geq$580 kbp in size that were similar, though not identical, to those found in cells incubated with water. Many of the cells treated with teniposide were blebbed in appearance and/or exhibited fragmented nuclei (results not shown).

It has been proposed that a Ca^{2+}/Mg^{2+}-dependent endonuclease cleaves DNA in many cases of apoptosis [28]. To investigate the possibility of a similar ionic dependence in necrosis, cells were incubated either with water alone or in the presence of EDTA/EGTA (1 mM/1 mM). EDTA/EGTA suppressed water-induced fragmentation to control levels (Fig.2,

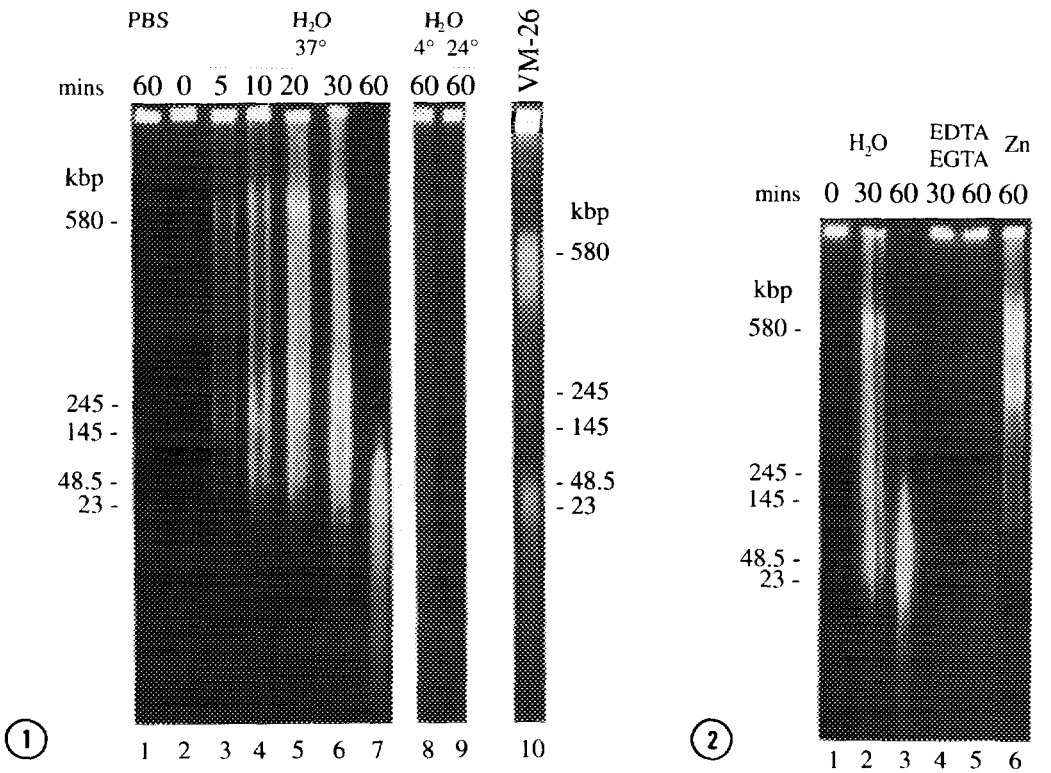

Fig. 1. Formation of kilobase pair DNA fragments during necrosis induced by water, as assessed by field inversion gel electrophoresis. Cell plugs were incubated in PBS (lane 1) or water at 37°C for 0-60 min (lanes 2-7) or at 4°C (lane 8) or 20-24°C (lane 9). For comparison, apoptosis was induced by teniposide (5 μM, 4 h; VM-26, lane 10).

Fig. 2. Inhibition of water-induced DNA fragmentation by co-treatment with EDTA/EGTA or Zn^{2+}, as assessed by field inversion gel electrophoresis. Cell plugs were incubated at 37°C in water either alone for 0-60 min (lanes 1-3) or in the presence of EDTA/EGTA for 30 and 60 min (1 mM/1 mM; lanes 4 and 5) or Zn^{2+} (1 mM; lane 6).

compare lanes 1-5). Microscopic examination revealed that ion-chelation did not improve cell viability at 10 min (not shown). Zn^{2+} (1 mM) significantly inhibited the formation of large fragments, though to a lesser extent than EDTA/EGTA (Fig.2, lane 6).

In order to investigate further the ionic dependence of DNA fragmentation, cells were incubated with water in the presence or absence of EDTA/EGTA and with or without counter-addition of Ca^{2+}/Mg^{2+} (2 mM/2 mM, final concentration; Fig. 3). The presence of EDTA/EGTA for 120 min caused significant, though not total, inhibition of DNA fragmentation (Fig. 3, compare lanes 3 and 4). In some experiments the inhibition was total. Restoration of Ca^{2+}/Mg^{2+} levels to cells treated with EDTA/EGTA greatly facilitated DNA fragmentation (Fig.3, lanes 5 and 6). By 60 min, nearly all the DNA had been degraded to sizes below the limit of resolution of field inversion gel electrophoresis. Neither the

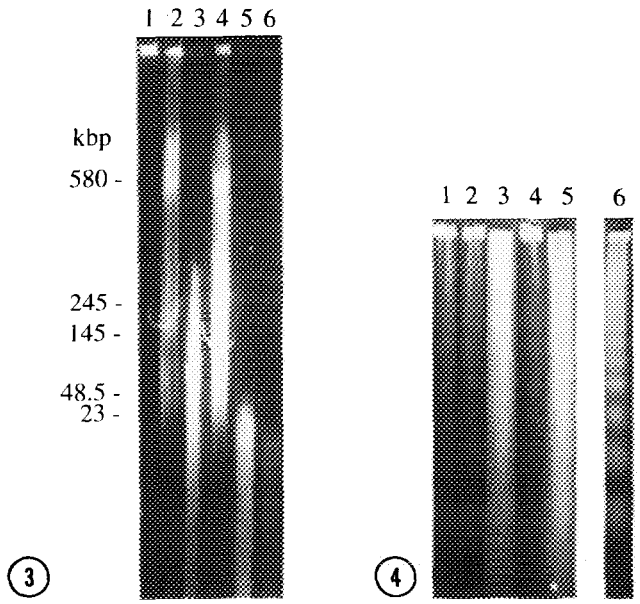

Fig. 3. Suppression by Ca^{2+}/Mg^{2+} of EDTA/EGTA-induced inhibition of DNA fragmentation, as assessed by field inversion gel electrophoresis. Cell plugs in lanes 1-3 were incubated at 37°C for 0, 30 and 60 min in water. Cell plugs in lanes 4-6 were incubated in water and EDTA/EGTA (1 mM/1 mM) for 60 min, then further incubated alone for 60 min (lane 4) or with addition of Ca^{2+}/Mg^{2+} (2 mM/2 mM) for 30 min (lane 5) or 60 min (lane 6).

Fig. 4. Fragmentation of DNA, as assessed by conventional gel electrophoresis. Cell plugs were incubated at 37°C for 120 min in PBS (lane 1) or for 0 and 60 min in water (lanes 2 and 3). Alternatively, cell plugs were incubated in water and EDTA/EGTA (1 mM/1 mM) alone for 120 min (lane 4), or for 60 min with subsequent counter-addition of Ca^{2+}/Mg^{2+} (2 mM/2 mM) for a further 60 min (lane 5). For comparison, cells were induced to undergo apoptosis by incubation with teniposide (5 μM, 4 h; lane 6).

inhibition of fragmentation by EDTA/EGTA nor its suppression by Ca^{2+}/Mg^{2+} were significantly affected by acid or alkaline conditions (pH 6.0 and 8.0; results not shown).

When plugs were run on conventional gels, fragmentation induced by water or by saturation of EDTA/EGTA with ions was in the form of a smear (Fig. 4, lanes 3 and 5 respectively). In contrast, cells induced to undergo apoptosis by teniposide produced characteristic internucleosomal cleavage (Fig 4., lane 6). Little fragmentation was observed in control cells or in cells incubated with water in the presence of EDTA/EGTA alone (Fig. 4, lanes 1 and 4).

DISCUSSION

Necrosis was induced in U937 cells by hypotonic incubation, as assessed by observations that viability was lost in all cells within 10 min of treatment with water and that

cell morphology was non-apoptotic when viewed by light microscopy (not shown). Water-treated cells also yielded a continuous spectrum of DNA fragments of low molecular mass (Fig. 4, lane 3), a phenomenon associated with necrosis [9,14]. This was in contrast to apoptotic cells, which yielded a characteristic DNA ladder (Fig. 4, lane 6) and were morphologically apoptotic (not shown). However, in both necrotic and apoptotic cells, we observed the formation of kilobase pair sized fragments of DNA (Fig. 1). The results are not peculiar to U937 cells, nor to death by hypotonic incubation, as we have found similar results with rat thymocytes incubated in water, and also with U937 cells incubated in 20% ethanol (not shown).

The formation of kilobase pair sized fragments in necrotic U937 cells was time- and temperature-dependent (Fig. 1) and was inhibitable both by ion chelators and Zn^{2+} (Fig. 2). Inhibition by the chelators was suppressed by counter-addition of Ca^{2+}/Mg^{2+} (Fig. 3). These results, whilst suggestive of an involvement of enzymic activity in the fragmentation, are by no means proof: large fragments may simply reflect the disintegration of DNA dictated by higher-order structure; Zn^{2+} may have acted via a number of indirect effects [29], rather than through direct inhibition of an enzyme [6,28].

We suggest that following the loss of membrane integrity, the release of ions from intracellular stores activated one or more endonucleases primarily responsible for cleavage of DNA into kilobase pair sized fragments. Ca^{2+}- and/or Mg^{2+}-dependent endonucleases are associated with degradation of DNA to kilobase pair sized fragments in rat thymocyte and rat liver nuclei [30,31], and with internucleosomal cleavage [28], though not necessarily in U937 nuclei [32]. The inhibition of fragmentation by EDTA/EGTA and the suppression of this inhibition by counter-addition of Ca^{2+}/Mg^{2+} did not prove that the crucial ions were Ca^{2+} and Mg^{2+}, but did demonstrate an ionic dependence of the process. The nature of the enzyme(s) is not clear, but degradation occurred similarly, whether in acid conditions (suitable for enzymes such as DNase II [33]), or in relatively neutral or alkaline conditions (suitable for the endonucleases involved in apoptosis [34,11]).

Fragmentation of DNA to large kilobase pair sizes may still be a valid indicator of apoptosis, providing it is observed in conjunction with appropriate morphology. It occurs in response to a wide variety of apoptotic stimuli in a number of cell systems at times prior to the loss of cell viability and when cell morphology indicates apoptosis or commitment to apoptosis [19-22,25]. The large fragments in necrotic cells are more smeared than their counterparts in apoptotic cells. This is probably due to release of non-specific proteases in necrosis, but not in apoptosis, which would negate any protective effect of histones and other proteins. DNA would therefore be more vulnerable to attack from nucleases. However, in cases where the mode of cell death is under investigation, the similarity of degradation of

DNA to large kilobase pair fragments in apoptosis and necrosis precludes using it as a sole indicator of either form of death.

In conclusion, we believe that the formation of large DNA fragments is a phenomenon occurring in some instances of necrosis as well as apoptosis. The cause of fragmentation may be similar in both modes of cell death and may reflect an enzymatic event or simply one dictated by higher-order chromatin structure. For this reason, if the large DNA fragments are to be used as evidence of apoptosis, we believe they should be presented in conjunction with other criteria, such as appropriate morphology.

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