

Formation of high molecular mass DNA fragments is a marker of apoptosis in the human leukaemic cell line, U937

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SUMMARY

Inhibitors of macromolecular synthesis and topoisomerases induce apoptosis in the human leukaemic cell line, U937. In this study, U937 cells were treated with the RNA synthesis inhibitor, actinomycin D (1 μ M), the protein synthesis inhibitors, emetine (1 μ M) and cycloheximide (100 μ M), the topoisomerase II inhibitor, teniposide (5 μ M), or the topoisomerase I inhibitor, camptothecin (1 μ M). Apoptotic cell death was assessed both by flow cytometry and agarose gel electrophoresis, and was correlated to the appearance of large (20 to \geq 580 kilobase pairs) DNA fragments, as assessed by field inversion gel electrophoresis. In all cases, the appearance of DNA fragments of 20-50 kilobase pairs accompanied the appearance of an apoptotic population and of internucleosomal cleavage.

However, teniposide additionally induced a marked increase in fragmentation to \geq 580 kilobase pairs. The co-treatment of cells with zinc (1 mM) inhibited the formation of all large DNA fragments, internucleosomal cleavage and the appearance of an apoptotic population.

We conclude that the generation of large DNA fragments is characteristic of apoptosis induced by various stimuli in U937, as has been found previously in rat thymocytes. However, unlike what occurs in rat thymocytes, zinc treatment does not dissociate the formation of large fragments from conventional markers of apoptosis.

Key words: apoptosis, U937, DNA fragmentation, zinc

INTRODUCTION

Apoptosis is a form of cell death likened to a suicide program (Kerr et al., 1972) that may be triggered by widely diverging stimuli (Lennon et al., 1991) or hormonal action (Wyllie, 1980). It was originally defined by purely morphological features, such as chromatin condensation, but is often characterised by endonucleolytic cleavage of nuclear DNA into multiples of 180-200 base pairs, the length of DNA contained in a nucleosome (Wyllie, 1980; Schwartzman and Cidlowski, 1993). This oligonucleosomal DNA appears as a series of bands when viewed on agarose gels - a 'DNA ladder' - and is inhibitable by zinc (Cohen and Duke, 1984; Martin et al., 1990; Cohen et al., 1992). Formation of DNA ladders is used by many as the only marker of apoptosis, but there is a growing number of cases where morphological apoptosis occurs without internucleosomal cleavage (Cohen et al., 1992; Oberhammer et al., 1993a; Tomei et al., 1993; Sun et al., 1994). It is inadvisable to define apoptosis as simply the appearance of a DNA ladder, and to date the most reliable diagnostic tool is morphological appearance (Kerr et al., 1972; Wyllie et al., 1980). Recently, however, it has been suggested that an early stage in apoptosis may be characterised by cleavage of DNA into large fragments (Walker et al., 1991; Brown et al., 1993). It is probable that these large fragments, ranging from 50 to \geq 700 kilobase pairs (kbp) in size, are precursors of the oligonucleosome ladders (Brown et al., 1993; Oberhammer et al.,

1993b). Since they have been shown to occur in cases of apoptosis where there is no internucleosomal cleavage (Brown et al., 1993; Oberhammer et al., 1993b), large fragments may represent a more reliable biochemical marker than DNA ladders.

In two of the cell systems studied most extensively, rodent thymocytes and T cell hybridomas, RNA and protein syntheses are required for the induction of apoptosis and the subsequent formation of large DNA fragments and DNA ladders (Cohen and Duke, 1984; Wyllie et al., 1984; Cohen et al., 1994). Proposed roles of the newly synthesized molecules include the $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease (Compton and Cidlowski, 1987) and a protein required to transport Ca^{2+} into the nucleus (Cohen and Duke, 1984). Alternatively, inhibition of macromolecular synthesis may block apoptosis by depleting nuclei of an endonuclease that is undergoing rapid turnover (McConkey et al., 1990). However, in other cell systems, including the human tumour cell lines, U937 and HL-60, treatment with cycloheximide or actinomycin D alone induces apoptosis (Martin et al., 1990; Owens and Cohen, 1992). These results suggest that some cells already have the apoptotic machinery in place and require continuous synthesis of a signal to suppress spontaneous apoptosis (Martin, 1993). Candidate signals have been reported, including the *ced-9* product from *Caenorhabditis elegans* (Hengartner et al., 1992), the mammalian Bcl-2 (Bissonnette et al., 1992) and the Abelson virus tyrosine kinase, BCR-ABL (Evans et al., 1993).

U937 cells also undergo apoptosis in response to treatment with topoisomerase inhibitors, such as teniposide and camptothecin (Lorico et al., 1990; Bertrand et al., 1993). These inhibitors stabilise the 'cleavable complex', i.e. a reaction intermediate in which the topoisomerase is bound to cleaved strands of DNA (D'Arpa and Liu, 1989; Cummings and Smyth, 1993). Topoisomerase II inhibitors yield double-stranded breaks, while topoisomerase I inhibitors yield single-stranded breaks, though it is unlikely that the strand breaks alone are sufficient to induce apoptosis (Holm et al., 1989; Bertrand et al., 1991).

In order to investigate more fully the relationship between the formation of large DNA fragments and apoptosis, U937 cells were treated with actinomycin D, emetine, cycloheximide, teniposide or camptothecin. The effect of zinc co-treatment was also investigated. The results demonstrated that the formation of 20-50 kbp DNA fragments is a characteristic of apoptosis in U937 cells, as is the case in rat thymocytes (Brown et al., 1993). However, in U937 zinc co-treatment did not separate the formation of these fragments from the appearance of DNA laddering and apoptotic morphology, in contrast to the effect in rat thymocytes.

MATERIALS AND METHODS

Materials

Most reagents were obtained from the Sigma Chemical Company (Poole, UK). Those for cell culture were obtained from Gibco BRL Life Technologies (Paisley, UK). Additionally, teniposide and camptothecin were obtained from Bristol Myers Squibb (Evansville, IN), phosphate buffered saline (Dulbecco 'A') from Unipath Ltd (Basingstoke, UK) and agarose from Pharmacia Biosystems (Milton Keynes, UK). DNA standards were obtained as follows: 123 base pair DNA from Gibco; 245-2200 kbp *Saccharomyces cerevisiae* chromosomes from Clontech (Cambridge, UK); and 0.1-200 kbp DNA from Sigma.

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) CO₂. 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 gentamycin (50 µg/ml). Cells were maintained in logarithmic growth by allowing each subculture to obtain a population density of around 1.2×10⁶ cells/ml before reseeding at 0.5×10⁵ to 1×10⁵ 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 resuspended to a population density of 1×10⁶ cells/ml. Samples of the suspension were pipetted into the wells of a culture plate and mixed with the desired concentration of agent(s). The cells were then incubated as above for the required time. Emetine was dissolved in absolute ethanol, the topoisomerase inhibitors in dimethylsulphoxide and the other agents in distilled water. The concentration of each vehicle was never allowed to exceed 1% (v/v) and was previously confirmed not to induce apoptosis by itself.

Measurement of apoptotic cells by flow cytometry

We have recently described a flow cytometric method for the separa-

tion and quantification of normal and apoptotic thymocytes (Sun et al., 1992). This method was modified for use with U937 as follows: cells (1×10⁶) were incubated for 5 minutes at 37°C with Hoechst 33342 (1 µM) and then centrifuged immediately at 90g for 6 minutes at 4°C to prevent further uptake of the dye. The pellets were resuspended in propidium iodide (8 µM) in phosphate buffered saline. Analysis was carried out at a flow rate of 200 cells/second using a Becton Dickinson FACS Vantage linked to a Hewlett Packard 9000/340. The Hoechst 33342 and propidium iodide were excited using the 352 nm ultraviolet line of a krypton laser and the resultant blue (400 to 500 nm) versus red (>630 nm) fluorescence recorded using linear amplification. Non-viable, red fluorescing cells were gated out. The remaining cells were displayed as a cytogram of blue fluorescence versus forward light scatter (indicative of cell size).

Cells treated with Hoechst 33342 as above were also examined by light microscopy under ultraviolet illumination (352 nm).

Gel electrophoresis

Cells (1×10⁶) were loaded onto a 1.8% agarose 10 gel for conventional electrophoresis, as described by Sorensen et al. (1990). Alternatively, plugs of cells (2×10⁶) were prepared in 1% agarose L by standard methods (Anand and Southern, 1990), and then digested and run on a field inversion gel, as described previously (Brown et al., 1993). Under the conditions used, the resolving power of this technique extends to 580 kbp.

RESULTS

Separation and quantification of apoptotic and non-apoptotic U937 cells

Actinomycin D and cycloheximide are known to induce apoptosis in U937 cells (Martin et al., 1990). When incubated with Hoechst 33342 and analysed as described in Materials and Methods, control cells yielded only one main population, exhibiting low blue fluorescence and high forward scatter (Fig. 1A, region 3). In contrast, cells exposed to actinomycin D (1 µM for 14 hours, Fig. 1B) or cycloheximide (100 µM for 14 hours, Fig. 1C) showed three populations. Cells in region 1 exhibited high blue fluorescence and high forward scatter; region 2 comprised low blue fluorescing, low forward scatter particles; cells in region 3 exhibited low blue fluorescence and high forward scatter. The appearance of cells in region 1 was coincidental with the emergence of a hypodiploid peak on DNA analysis (data not shown). Such a peak represents partially degraded DNA and is a measure of apoptosis (Nicoletti et al., 1991; Darzynkiewicz et al., 1992).

In our previous studies with rat thymocytes, we demonstrated that the cells exhibiting high blue fluorescence were apoptotic, based on a number of criteria including DNA laddering and ultrastructure (Sun et al., 1992). In the present study, we sorted cells from regions 1 and 3 using fluorescence activated cell sorting. The population from region 1 showed extensive DNA laddering and comprised primarily cells with fragmented nuclei (Fig. 2A). In contrast, the nuclei from cells in region 3 were indistinguishable from those of untreated cells and no evidence of internucleosomal cleavage was observed (Fig. 2B). The population in region 2 was significantly increased by treatment with actinomycin D (28%) or cycloheximide (6%), compared to control cells (1%). The increase coincided with the appearance of small cellular fragments that excluded propidium iodide (Fig. 2A, arrow). It is probable, therefore, that region 2 corresponded to apoptotic bodies.

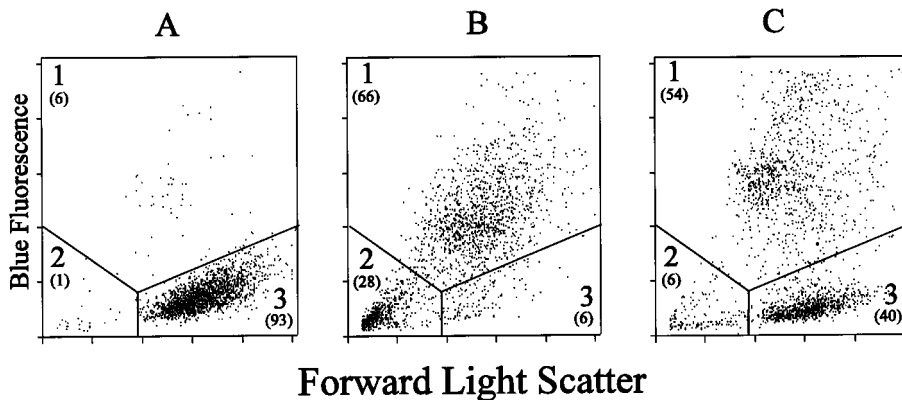


Fig. 1. Separation of apoptotic and non-apoptotic cells by Hoechst flow cytometry. U937 cells were incubated for 14 hours alone (A) or with actinomycin D (1 μ M; B) or cycloheximide (100 μ M; C). They were then analysed by flow cytometry, as already described. Control cells yielded only one major population. Cells treated with actinomycin D or cycloheximide showed three populations. The numbers in parentheses refer to the percentage of cells in each population. All populations excluded propidium iodide. The results are representative of at least five experiments.

These results clearly demonstrate that apoptotic and non-apoptotic U937 cells can be separated by flow cytometry using Hoechst 33342.

Little or no differences were observable in forward light scatter between apoptotic and non-apoptotic cells (Fig. 1, compare regions 1 and 3). Forward light scatter is an indication of cell size and so, in this respect, U937 cells differ from rat thymocytes (Thomas et al., 1983).

Induction of apoptosis by RNA and protein synthesis inhibitors

Actinomycin D (0.05–1 μ M, 14 hours) caused a concentration-dependent induction of apoptosis, as determined by flow cytometry with Hoechst 33342 (Fig. 3A). The critical concentration lay within a tight range: 0.1 μ M actinomycin D gave no significant apoptosis (5% of viable cells), while 0.25 μ M caused a marked induction of apoptosis (55% of viable cells).

When incubated for 0–14 hours, actinomycin D (1 μ M) caused a time-dependent induction of apoptosis (Fig. 3B). The number of apoptotic cells did not rise significantly above control levels (5% of viable cells) until 6 hours, but thereafter rose steadily (55% of viable cells at 14 hours). Similar results were found with cycloheximide (1–100 μ M) and emetine (0.05–10 μ M; data not shown).

Large DNA fragments are formed during apoptosis induced by actinomycin D

Recent studies from our laboratory and others have demonstrated that high molecular mass DNA fragments (30–700 kbp) are formed at an early stage in apoptosis (Walker et al., 1991; Brown et al., 1993; Oberhammer et al., 1993b). In this study, cells were incubated for increasing times with actinomycin D (1 μ M) and examined by gel electrophoresis. A time-dependent formation of large DNA fragments was observed, predominantly of 20–50 kbp with much smaller amounts of 145–245

and \geq 580 kbp (Fig. 4A). Large fragments were clearly detected at 6 hours and were accompanied by an increase in internucleosomal cleavage (Fig. 4B). In all experiments, formation of

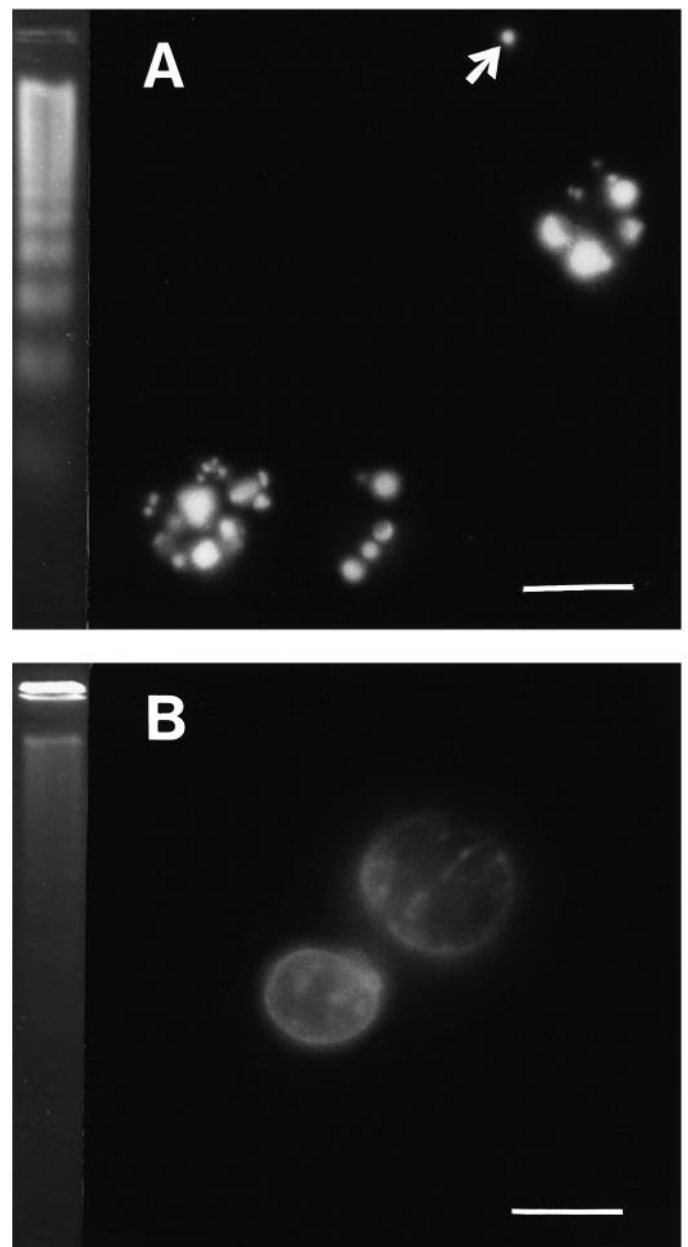


Fig. 2. Characterisation of sorted cell populations by gel electrophoresis and ultraviolet microscopy. Cells were incubated with actinomycin D (0.5 μ M) for 9 hours. Cells from regions 1 and 3 (Fig. 1B) were then sorted by flow cytometry, and analysed by gel electrophoresis and microscopic appearance. Cells in region 1 (A) showed extensive DNA laddering, and exhibited nuclear fragmentation. Cells in region 3 (B) yielded neither DNA laddering nor nuclear fragmentation. The arrow (in A) indicates an apoptotic body, which probably became separated from one of the cells during sorting. The results are representative of three experiments. Bars, 10 μ m.

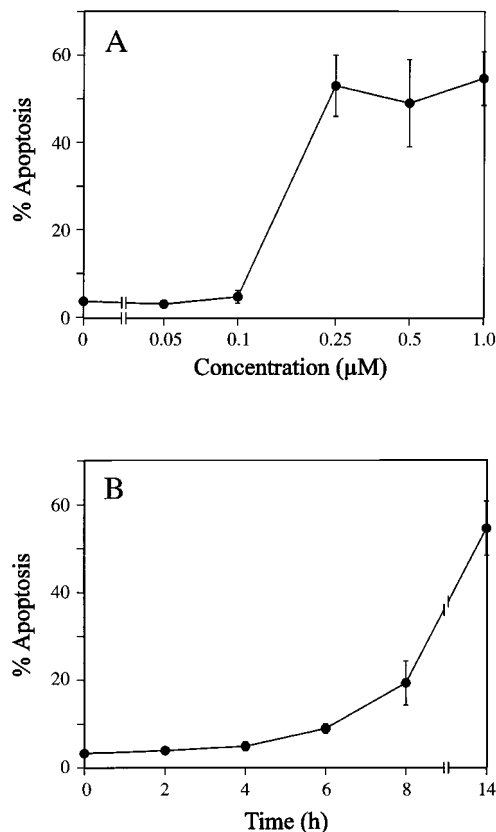


Fig. 3. Time- and concentration-dependent induction of apoptosis in U937 cells by actinomycin D. (A) Cells were treated for 14 hours with actinomycin D (0.05–1 µM). (B) Cells were treated for 0–14 hours with actinomycin D (1 µM). The percentage of apoptotic cells excluding propidium iodide was determined by flow cytometry. Results are the mean \pm s.e.m. of three experiments.

large fragments either preceded or was accompanied by internucleosomal cleavage.

Inhibitors of topoisomerases and protein synthesis also induce large fragments

Cells were incubated for 1–12 hours, either alone or in the presence of teniposide (5 µM) or camptothecin (1 µM), and analysed by gel electrophoresis (Fig. 5). Control cells exhibited little or no DNA cleavage, either to large fragments or to oligonucleosomes. In contrast, teniposide and camptothecin induced the formation of 20–50 kbp fragments (Fig. 5A). Only teniposide produced large amounts of ≥ 580 kbp fragments. These were clearly visible at 1 hour and preceded the formation of 20–50 kbp fragments. The appearance of large fragments preceded or accompanied both DNA laddering (compare Fig. 5A,B) and an increase in the apoptotic population when measured by flow cytometry (data not shown). When cells were incubated with emetine (1 µM) or cycloheximide (100 µM), the formation of large DNA fragments was observed, similar to that following treatment with camptothecin (results not shown).

Zinc co-treatment inhibits the formation of large DNA fragments

Recently, we have shown that in rat thymocytes, zinc inhibits DNA laddering, but not the cleavage of DNA into large

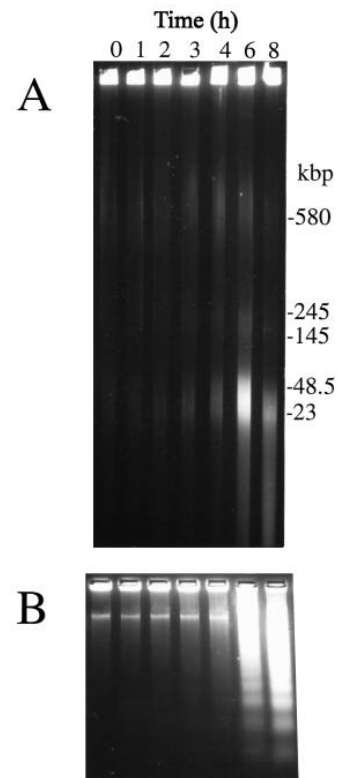


Fig. 4. Formation of large DNA fragments and oligonucleosomal ladders during apoptosis of U937 cells induced by actinomycin D. The time course of the formation of large fragments (A) and oligonucleosomal fragments (B) was assessed by field inversion and conventional gel electrophoresis, respectively. Cells were incubated with actinomycin D (1 µM) for 0–8 hours, as indicated. The appearance of 20–50 kbp fragments was closely associated with that of oligonucleosomal fragments. The 0 hour treatment actually refers to an 8 hour control. The results are representative of four experiments.

fragments (Brown et al., 1993). In order to investigate the effect of zinc in the U937 line, cells were incubated either alone or for 7 hours with actinomycin D (1 µM) in the presence or absence of zinc chloride (1 mM). A clear increase in 20–50 kbp fragments (Fig. 6A) and DNA ladders (Fig. 6B) was observed in cells incubated with actinomycin D over control cells. Both the laddering and the larger fragments were inhibited by zinc co-treatment. There was also a marked reduction in the number of apoptotic cells (Fig. 6C), which was not due to increased necrosis or to simple suppression of fluorescence by zinc.

Similar results were found with emetine (1 µM, 11 hours), cycloheximide (100 µM, 10 hours), teniposide (5 µM, 6 hours) and camptothecin (1 µM, 6 hours; results not shown). If zinc co-treatment continued past 12 hours, a loss of cell viability was observed. This appeared to be necrotic, as defined by microscopic appearance, showing that zinc was initially cytoprotective but ultimately toxic.

DISCUSSION

We have shown that our previously described flow cytometric method for the separation and quantification of normal and

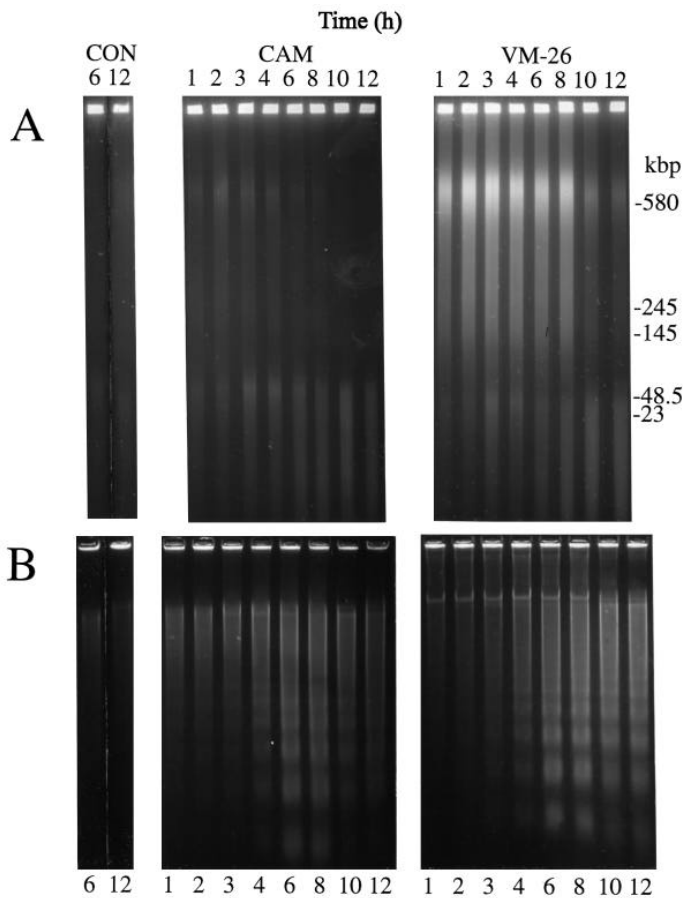


Fig. 5. Formation of large DNA fragments and oligonucleosomal ladders during apoptosis of U937 cells induced by teniposide and camptothecin. The time course of the formation of large fragments (A) and oligonucleosomal fragments (B) was assessed by field inversion and conventional gel electrophoresis, respectively. Cells were incubated for the indicated times alone (CON) or in the presence of camptothecin (1 μ M; CAM), or teniposide (5 μ M; VM-26). The appearance of 20-50 kbp fragments was closely associated with that of DNA laddering. Teniposide additionally produced large amounts of fragments of \geq 580 kbp in size. For clarity, the incubation time for each treatment is repeated at the bottom of the figure. The results are representative of three experiments.

apoptotic thymocytes can be modified successfully for use in U937 cells (Figs 1, 2). Apoptotic bodies were also separated by this method (Fig. 1B, region 2). Quantification of apoptosis without the need to damage cell integrity is therefore possible in U937, which is an advantage compared to several other flow cytometric methods.

Incubation of U937 cells with inhibitors of macromolecular synthesis or of topoisomerases resulted in a significant induction of apoptosis, as assessed by flow cytometry (Fig. 3) and/or conventional gel electrophoresis (Figs 4B, 5B). In all cases where apoptosis was induced, the formation of large fragments of DNA was closely associated with internucleosomal cleavage (compare Figs 4A,B and 5A,B). Our data support the hypothesis that the two are linked, possibly with the large fragments preceding the DNA ladders: in no experiments did internucleosomal cleavage precede higher-order fragmentation and it seems unlikely that the two were parallel, unconnected events.

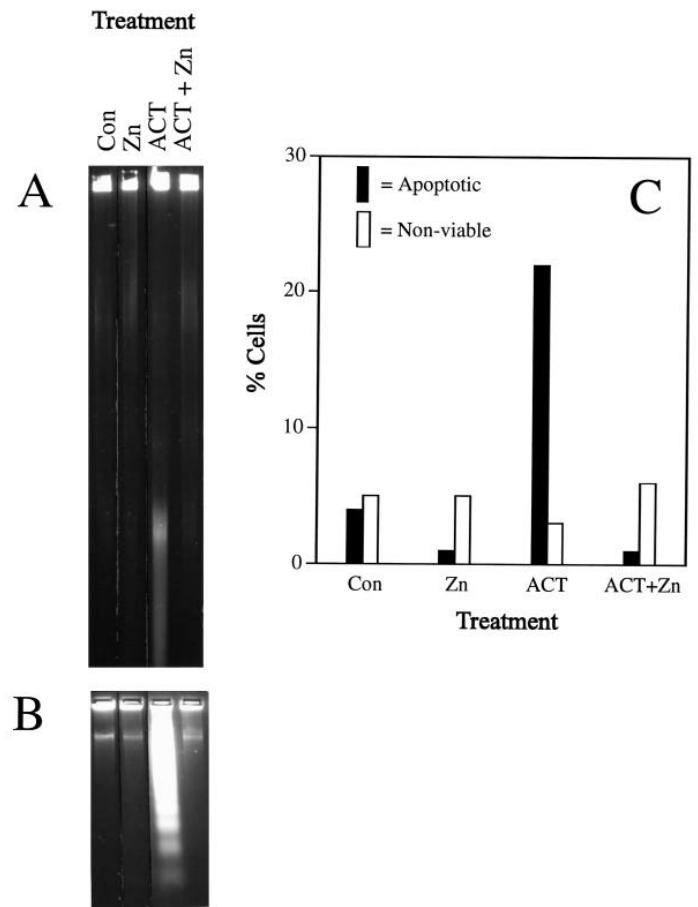


Fig. 6. Inhibition of apoptosis by zinc in U937 cells treated with actinomycin D. The appearance of large DNA fragments (A), internucleosomal cleavage (B) and apoptotic cells (C) was assessed by field inversion gel electrophoresis, conventional gel electrophoresis and flow cytometry, respectively. Cells were incubated for 7 hours alone (Con) or in the presence of actinomycin D (1 μ M; ACT) either with or without zinc (1 mM; Zn). Co-treatment with zinc inhibited the appearance of 20-50 kbp fragments, internucleosomal cleavage and the percentage of apoptotic cells (A, B and C, respectively). Inhibition was not due to an increase in non-viable cells (C). Results are representative of four experiments.

The appearance of 20-50 kbp fragments during apoptosis of U937 agrees with previous findings in other cells. Treatment of thymocytes with etoposide or dexamethasone results in the formation of fragments of 30-50, 200-300 and \geq 700 kbp in size (Walker et al., 1991; Brown et al., 1993). Similarly, thymocyte nuclei exposed to Mg^{2+} or Ca^{2+}/Mg^{2+} show DNA fragmentation to 200-250 and 30-50 kbp (Sun and Cohen, 1994). It is suggested that the appearance of each lower size-range of fragments represents cleavage of DNA into the next lower order of chromatin structure. DNA of 30-50 and 200-245 kbp in length may correspond to chromatin loops and rosettes, respectively (Filipski et al., 1990; Solov'yan et al., 1991). The \geq 700 kbp fragments represent an unidentified higher-order structure.

In the present study, U937 only yielded large amounts of DNA fragmentation above 20-50 kbp in size when incubated with teniposide (Figs 4A, 5A). This agrees with the observations of Elias and Berry (1991), who reported that tumour

necrosis factor induced apoptosis in U937 cells but that fragments of 200-400 kbp were not observed. In the present study, teniposide probably induced most of the ≥ 580 kbp fragments via direct topoisomerase II effects, i.e. by causing double-stranded breaks, in agreement with Filipinski et al. (1990). This is supported by the results with camptothecin, a topoisomerase I inhibitor causing single-stranded DNA breaks, which gave much smaller amounts of ≥ 580 kbp fragmentation (Fig. 5A). However, we cannot exclude the possibility that some higher-order fragments were also generated through apoptosis. Their dearth could be attributed to rapid cleavage to 20-50 kbp fragments.

Actinomycin D is generally regarded as inducing apoptosis in U937 by inhibiting RNA synthesis (Martin et al., 1990). Nevertheless, we cannot rule out the possibility that other mechanisms were also involved. Actinomycin D intercalates in DNA (Reich and Goldberg, 1964), induces hypersensitivity of DNA to DNase I (Bishop et al., 1991), and inhibits both topoisomerase I and II (Wasserman et al., 1990). Any one of these secondary effects may have induced or enhanced apoptosis and may in part explain why actinomycin D induced more apoptosis than cycloheximide (Fig. 1; and Martin et al., 1990). However, since fragments of ≥ 580 kbp were not generated in large amounts, we suggest that topoisomerase II inhibition was not a major factor in the induction of apoptosis by actinomycin D.

There were at least two major differences in the response of U937 and thymocytes to zinc, namely the inhibition of large fragments and the effect on the percentage of apoptotic cells. In thymocytes, zinc inhibits the internucleosomal cleavage of DNA but not the formation of large fragments (Cohen and Duke, 1984; Cohen et al., 1992; Brown et al., 1993). The net effect is a stabilisation of fragments of 30-50, 200-245 and ≥ 700 kbp in size. In U937, however, zinc co-treatment inhibited the formation of large DNA fragments as well as internucleosomal cleavage. This contrast may have been due to different enzyme(s) being involved in DNA degradation with differing susceptibilities to zinc. It is possible that zinc exerted its effect solely by indirect mechanisms (Waring et al., 1991), but there is evidence that it does act at the nuclear level (Lohmann and Beyersmann, 1993; Fernandes and Cotter, 1993), even if not on the endonuclease itself (Compton and Cidlowski, 1987; Peitsch et al., 1993).

In U937, zinc inhibited the appearance of the apoptotic cells in region 1 (Fig. 6C). This was in contrast to its effect in rat thymocytes, where the apoptotic population induced by dexamethasone or etoposide was not inhibited (Cohen et al., 1992; Sun et al., 1994). Given, in U937, the close temporal relationship between the appearance of cells in region 1 and DNA cleavage, together with the ability of zinc to inhibit both phenomena, it seems likely that they were linked. We do not believe this link to be direct, as previous studies suggest that increased Hoechst 33342 staining is a result of increased uptake (Ormerod et al., 1993), not of DNA cleavage (Darzynkiewicz et al., 1992; Telford et al., 1992). It is possible, therefore, that a common process signals for both the change in cell membrane permeability (observed as the increased Hoechst fluorescence) and the earliest DNA cleavage, and that it was this signal that was modified by zinc.

In summary, we have shown that the appearance of large DNA fragments is a significant feature of apoptosis in the

human leukaemia cell line, U937. Such large fragments were induced by a diverse range of stimuli, but in all cases they were inhibited by zinc. The latter effect contrasts with results found in rat thymocytes, where large fragments are stabilised by zinc. Nevertheless, the appearance of large DNA fragments may well prove to be a more general marker of apoptosis than DNA ladders.

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