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Preclinical report

Anticancer drug-induced apoptosis in human monocytic leukemic cell line U937 requires activation of endonuclease(s)

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Anticancer agents effect tumor cell killing both *in vivo* and *in vitro* through the induction of apoptosis. Endonuclease-mediated internucleosomal DNA fragmentation, the most widely used biochemical marker of apoptosis, has been shown to play a central role in apoptosis in many experimental systems. In the present investigation, we report that activation of endonuclease(s) leading to oligonucleosomal DNA fragmentation is common and an essential event in apoptosis, induced by different anticancer drugs, adriamycin, etoposide and cisplatin. The endonuclease inhibitors aurintricarboxylic acid and zinc ion prevented apoptotic cell death in human monocytic leukemic cell line U937, as documented by DNA fragmentation, morphological and nuclear alterations, and cell viability assay. Additional studies suggest endonuclease(s)-mediated DNA fragmentation may not play a central role in apoptosis in the same cell line in response to other inducers such as heat shock and cells may undergo cell death showing all morphological features of apoptosis even in the absence of DNA fragmentation. [© 2000 Lippincott Williams & Wilkins.]

Key words: Anticancer drugs, apoptosis, DNA fragmentation, endonuclease inhibitors, U937.

Introduction

Although the primary targets for anticancer drugs seem to vary between tumor cell types and type of anticancer drugs, accumulating evidence suggests that most of the anticancer drugs effect tumor cell killing both *in vitro* and *in vivo* by triggering a conserved, gene-activated programme for cell death, referred to as apoptosis.[1–3] Since induction of apoptosis actively contributes to the cytotoxic effects of chemotherapeutic drugs, it could be expected that modulation in the molecular pathway of apoptosis can increase the sensitivity of cancer cells to anticancer drugs. Cleavage of DNA into oligonucleosomal fragments,[4–7] probably through the activation of endonuclease(s), has been shown to be an important event in the apoptotic mechanism.[7–9] Earlier, we have shown that the endonuclease inhibitor aurintricarboxylic acid (ATA) can block activation-induced apoptosis in murine peritoneal macrophages.[10] ATA and zinc ion have been reported to inhibit DNA fragmentation and cell death in H_2O_2-treated renal tubular epithelial cells.[11] ATA also inhibits DNA fragmentation and apoptosis in glucocorticoid-treated thymocytes *in vitro* and in activated T cell hybridomas.[12,13] However, not all forms of apoptosis necessarily depend upon DNA fragmentation and the phenomenon may be peripheral to the fundamental mechanism of apoptosis as recently it has been shown that cells can undergo apoptotic cell death with all the characteristics morphological features even in the absence of oligonucleosomal DNA fragmentation.[14–16] Moreover, apoptotic morphology can be induced in enucleated cells as well.[17]

In view of the above, the present investigation has been undertaken to study the role of endonuclease(s)-mediated DNA fragmentation in apoptosis induced by different anticancer drugs in human monocytic leukemic cell line U937.

Materials and methods

Chemicals

Adriamycin (ADM), etoposide (VP-16), cisplatin, medium RPMI 1640, propidium iodide (PI), RNase A and proteinase K were purchased from Sigma (St Louis, MO). FCS was purchased from Biological Industries (Haemek, Israel). [methyl-^{3}H]Thymidine was purchased from Bhabha Atomic Research Centre (Mum-

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bai, India). ATA and $ZnSO_4$ were purchased from Merck (Mumbai, India). All the reagents used were free of endotoxin contamination, as determined by the Limulus amoebocyte lysate assay (sensitivity limit 0.1 ng/ml).

Cell culture and drugs and heat shock treatment

The human monocytic leukemic cell line U937 was grown in suspension in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and gentamycin (20 μg/ml). U937 cells were seeded (10^6 cells/well) in polystyrene 24-well plates (Nunc, Roskilde Denmark) and treated with ADM (10 μM), VP-16 (10 μM) or cisplatin (10 μg/ml) in the presence or absence of ATA or Zn^{2+} for the indicated time period. For heat shock treatment, U937 cells were pelleted and resuspended in media prewarmed at 43°C and then maintained at that temperature for 1 h in a circulatory waterbath. Temperature was frequently monitored and cells were further cultured at 37°C for 12 h.

Percent DNA fragmentation assay

Quantitative measurement of DNA fragmentation was carried out following the method as described previously.[18] Briefly, U937 cells in the logarithmic phase of growth were radiolabeled in culture medium with 0.5 μCi/ml [^{3}H]thymidine for 18 h, washed thoroughly in RPMI 1640, and treated for various time periods and culture conditions as described. Cell-free culture supernatants (M) were collected at indicated timepoints and preserved at 4°C. The cells were lysed with 50 μl of ice-cold lysis buffer (25 mM sodium acetate, pH 6.6) for 1 h and DNA from cells was separated by centrifugation at 13 000 *g* at 4°C for 20 min into fragmented low molecular weight (supernatant; S) and intact high molecular weight (pellet; P) fractions. Radioactivity was determined by liquid scintillation counter (LKB, Wallac, Finland).

Percent DNA fragmentation was calculated as: $\{[\text{c.p.m. (M+S)}]/[\text{c.p.m (M+S+P)}]\} \times 100$.

Agarose gel electrophoresis of DNA

Extraction of U937 cell DNA was performed following the method as described previously.[18] U937 cells treated with anticancer drugs were washed 3 times in PBS and lysed in 0.5 ml of lysis buffer containing 10 mM Tris–HCl (pH 8.0), 75 mM NaCl, 10 mM EDTA, 0.5% SDS and 0.15 mg/ml proteinase K, and incubated for 3 h at 50°C. Lysate was spun down at 10 000 *g* for 20 min at 4°C. The supernatant was collected carefully and a solution of 0.5 M NaCl and 50% absolute ethanol was added to precipitate the DNA. The precipitated DNA was resolublized in 30 μl of TE buffer (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA, pH 8.0) for 1 h at 65°C and then incubated with 200 μg/ml RNase A for 2 h at 37°C. Thereafter, 10 μl of loading dye (0.025% bromophenol blue, 0.25% xylene cyanol and 30% glycerol in water) was added, and 40 μl of sample was loaded in the wells of 1.5% agarose gel and electrophoresed for 2 or 3 h at 75 V in TBE buffer in the presence of 0.5 μg/ml ethidium bromide. DNA was visualized and photographed on a UV transilluminator.

Morphological studies by phase contrast and fluorescence microscopy

Changes in the cellular and nuclear morphology associated with apoptosis were studied by phase contrast and fluorescence microscopy, using the DNA fluorescent dye PI. Cells were fixed in 80% cold ethanol, washed twice with cold PBS and stained with PI (50 μg/ml in PBS). The cells were washed twice with PBS and mounted with 50% glycerol. The same field of cells was visualized by phase contrast and fluorescence microscopy.

Determination of viable cell population and percent apoptotic cell population

Cell viability was determined by the MTT [3(4,5)-dimethylthiazol-2,5-diphenyl tetrazolium bromide] assay by the method as described previously.[19] Briefly, 10^4 cells/100 μl were placed in each well of a 96-well tissue culture plate (Falcon 3072; Becton Dickinson, Mountain View, CA) and incubated for 18 h in the presence or absence of different anticancer drugs and ATA or Zn^{2+}. After 18 h, 10 μl of 5 mg/ml MTT was added and the cells were incubated for 4 h. A purple formazan product was solubilized by the addition of 100 μl of acidic isopropanol (0.04 N HCl in isopropanol). The absorbance of each well was measured with a microplate ELISA reader using a wavelength of 570 nm. The relative cell viability was calculated according to the formula: (absorbance experimental/absorbance control) $\times$ 100.

The apoptotic cell population was determined by identifying and counting apoptotic cells on the basis of their characteristic cellular and nuclear morphology. The percentage of apoptotic cells was determined by counting more than 200 cells for at least three separate determinations.

Statistical analysis

Results are expressed as means ± SD of at least three independent experiments. The statistical significance of difference between test groups was analyzed by two-tailed Student's *t*-test. The level of significance was considered $p < 0.05$.

Results

Anticancer drugs induced endonuclease(s)-mediated DNA fragmentation in U937 cells

Treatment of U937 cells with anticancer drugs ADM (10 μM), VP-16 (10 μM) or cisplatin (10 μg/ml) induced DNA fragmentation in U937 cells. The above concentrations of the anticancer drugs were selected according to the dose–kinetic studies (data not shown) at which they caused 50% loss in the viable cell population after 24 h of treatment. As shown in Figure 1, results of the percent DNA fragmentation studies indicate that DNA fragmentation was detectable after 6 h of ADM, VP-16 and cisplatin treatment, which followed a time-dependent increase and reached a maximum of 55% by 18 h. All the anticancer drugs used followed almost similar kinetics of percent DNA fragmentation. Untreated control did not show significant DNA fragmentation.

To investigate the nature of DNA fragmentation induced by ADM, VP-16 or cisplatin in U937 cells, agarose gel electrophoresis was performed. DNA isolated from U937 cells treated with anticancer drugs showed oligonucleosomal cleavage giving a ladder pattern when run on agarose gel. DNA ladders were not observed upto 6 h of treatment (Figure 2, lanes 2–4). They appeared only after 12 h of post-anticancer drug treatment (Figure 2, lanes 6–8). DNA from untreated U937 cells did not show any ladder (Figure 2, lanes 1 and 5) even up to 24 h of culture.

To investigate the role of endonuclease(s) in anticancer drug-induced DNA fragmentation in U937 cells, the effect of endonuclease inhibitors ATA or Zn^{2+} was examined. ATA or Zn^{2+} have been shown to inhibit endonuclease(s) activity and have been utilized to delineate the role of endonuclease(s) in DNA fragmentation in apoptosis.[10–13] A dose of 100 μM of ATA and Zn^{2+} was found to inhibit DNA fragmentation completely in U937 cells induced by ADM, VP-16 or cisplatin, which was not detectable even after 18 h of culture (Table 1). The inhibitory effect of ATA and Zn^{2+} on DNA fragmentation was further confirmed by agarose gel electrophoresis. DNA extracted from U937 cells treated with ADM, VP-16 or cisplatin showed no

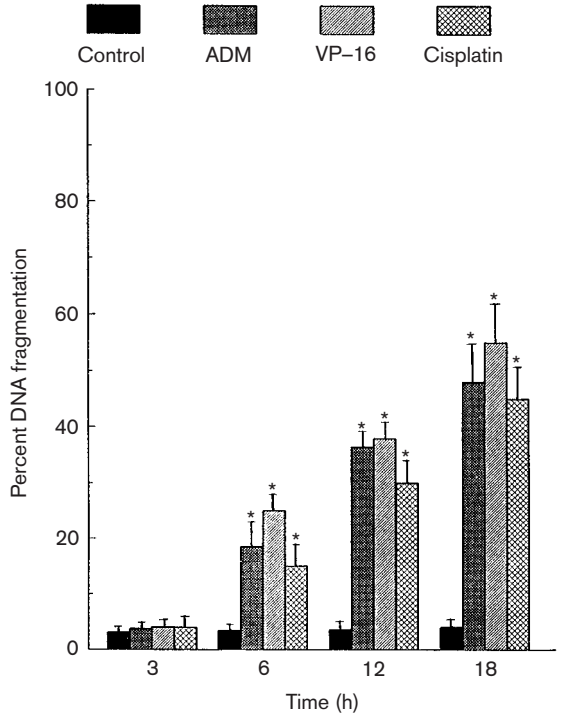

Figure 1. Kinetics of percent DNA fragmentation in U937 cells. Unlabeled or [^{3}H]thymidine-labeled U937 cells treated with ADM (10 μM), VP-16 (10 μM) or cisplatin (10 μg/ml) were cultured for the indicated time intervals. Percent DNA fragmentation was measured by the method as described in Materials and methods. Data shown are means ± SD and are representative of three independent experiments, done in triplicate. *$p < 0.05$ versus values for untreated control cultures.

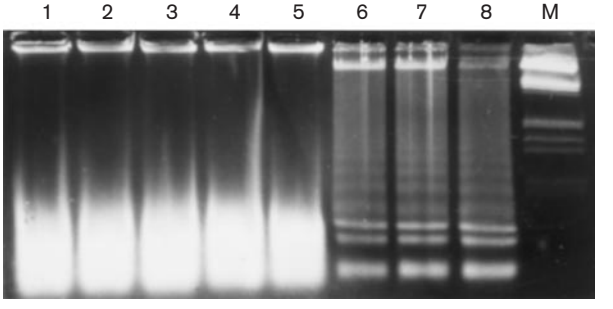

Figure 2. Agarose gel analysis of DNA of U937 cells. Untreated or U937 cells treated with ADM (10 μM), VP-16 (10 μM) or cisplatin (10 μg/ml) were cultured for the indicated time intervals. DNA extracted from cells was analyzed by agarose gel electrophoresis by the method as described in Materials and methods. Lanes 1 and 5, untreated U937 cells; lanes 2–4, U937 cells treated with ADM, VP-16 and cisplatin for 6 h; lanes 6–8, U937 cells treated with ADM, VP-16 and cisplatin for 12 h; M, marker.

sign of a DNA ladder when run on agarose gel even upto 18 h when cultured in the presence of 100 μM of ATA (Figure 3, lanes 6–8) or Zn^{2+} (Figure 3, lanes 10–12) .

Morphological and nuclear alterations in U937 cells induced by anticancer drugs were inhibited by endonuclease inhibitors

Figure 4(a) shows the typical cellular and nuclear morphology of U937 cells treated with ADM, VP-16 or cisplatin, such as membrane blebbing, cell shrinkage, and chromatin condensation and disintegration. These changes were apparent after 12 h of treatment and were further enhanced with time. Addition of 100 μM of ATA or Zn^{2+} prevented U937 cells from undergoing such morphological changes pertaining to apoptosis (Figure 4b). Untreated control did not show such alterations even upto a 18 h of culture.

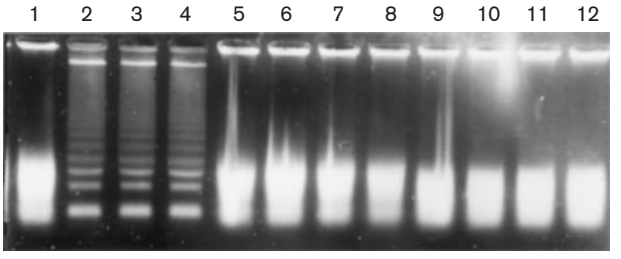

Figure 3. Effect of endonuclease inhibitors on oligonucleosomal DNA fragmentation in U937 cells. Untreated or U937 cells treated with ADM (10 μM), VP-16 (10 μM) or cisplatin (10 μg/ml) were cultured for the indicated time intervals in the presence or absence of 100 μM of ATA or Zn^{2+}. DNA extracted from cells was analyzed by agarose gel electrophoresis by the method as described in Materials and methods. Lane 1, untreated U937 cells; lanes 2–4, U937 cells treated with ADM, VP-16 and cisplatin; lanes 5–8, untreated U937 cells and U937 cells treated with ADM, VP-16 and cisplatin in the presence of ATA; lanes 9–12: untreated U937 cells and U937 cells treated with ADM, VP-16 and cisplatin in the presence of Zn^{2+}.

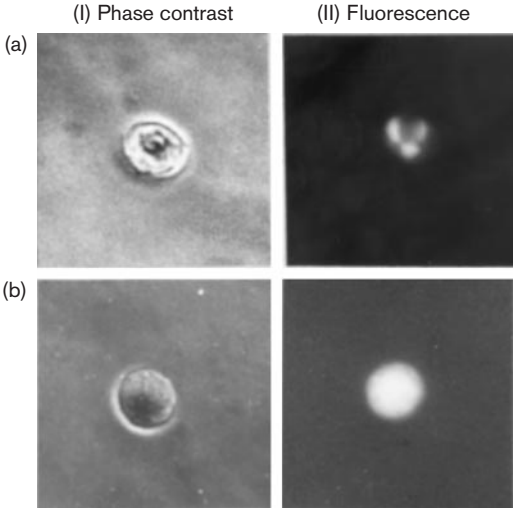

Figure 4. Morphology of U937 cells stained with PI. Untreated or U937 cells treated with ADM (10 μM), VP-16 (10 μM) or cisplatin (10 μg/ml) were cultured for 12 h in the absence or presence of 100 μM of ATA or Zn^{2+}. Cells were stained with PI (5 μg/ml) by the method as described in Materials and methods, and observed by fluorescence and phase contrast microscopy. The same field of cells was visualized by fluorescence and phase contrast microscopy. (a) Typical cellular and nuclear changes of apoptosis, such as membrane blebbing, cell shrinkage and nuclear breakdown, in the U937 cells treated with ADM, VP-16 or cisplatin. (b) Such changes were not observed in U937 cells treated with anticancer drugs in the presence of ATA or Zn^{2+} or in untreated U937 cells.

Table 1. Effect of endonuclease inhibitors on percent DNA fragmentation, percent apoptotic cell population and relative cell viability in U937 cells in response to anticancer drugs

Treatment	Percent DNA fragmentation			Percent apoptotic cell population			Relative cell viability		
	−Inhibitor	ATA	Zn^{2+}	−Inhibitor	ATA	Zn^{2+}	−Inhibitor	ATA	Zn^{2+}
Control	4±0.4[a]	5.2±1.4	4.8±1.1	5±1.1	4.8±2.0	5.2±1.5	95±4.5	95±4.3	95±3.2
ADM	48±8.3*	6.3±2.7**	5.8±2.3**	63±5.2*	7.3±1.3**	6.6±1.5**	70±6.6*	93±3.2**	93±3.7**
VP-16	55±9.1*	7.2±1.2**	8.6±3.4**	64±9.4*	6.3±1.6**	7.8±1.0**	72±8.9*	94±3.8**	95±4.0**
Cisplatin	45±6.5*	4.8±1.2**	5.3±2.3**	65±4.1*	7.2±2.1**	8.8±2.1**	78±9.0*	92±5.3**	94±0.4**

[a]The numbers represent mean±SD of triplicate cultures.
*$p<0.05$ versus values for untreated control cultures.
**$p<0.05$ versus values for treated cultures in the presence of ATA or Zn^{2+}.
Unlabeled or [^{3}H]thymidine-labeled U937 cells were treated with ADM (10 μM), VP-16 (10 μM) or cisplatin (10 μg/ml) for 18 h in the presence or absence of ATA (100 μM) or Zn^{2+} (100 μM). Percent DNA fragmentation, percent apoptotic cell population and percent viability of the cells were measured by the methods as described in Materials and methods.

Effect of endonuclease inhibitors on the apoptotic cell population and cell viability in U937 cells treated with anticancer drugs

Treatment of U937 cells with ADM, VP-16 or cisplatin resulted in an increase in the apoptotic cell population with a subsequent loss in the viable cell population. The apoptotic cell population was significant after 12 h (data not shown) and reached upto 63–65% after 18 h with a 22–30% loss in the viable cell population (Table 1). Untreated control showed less than 6% of the apoptotic cell population even after 18 h of culture with a viable cell population greater than 94%. Addition of 100 μM of ATA or Zn^{2+} prevented U937 cells treated with ADM, VP-16 and cisplatin from undergoing apoptotic cell death (Table 1).

Endonuclease-mediated DNA fragmentation acts as an irreversible step in anticancer drug-induced apoptosis in U937 cells

To investigate the time-point at which ATA or Zn^{2+} interferes with the anticancer drug-induced apoptotic process, detailed time-course studies were done. ATA (100 μM) or Zn^{2+} (100 μM) were added at different time-points post-anticancer drug treatment, then assessed for percent DNA fragmentation, agarose gel analysis, morphological and nuclear changes, apoptotic cell population, and relative cell viability. Addition of ATA or Zn^{2+} upto 4 h of after ADM, VP-16 and cisplatin treatment completely prevented percent DNA fragmentation, DNA laddering, cellular and nuclear changes, increase in the apoptotic cell population, and loss in cell viability (data not shown). However, delaying the addition of these inhibitors beyond 4 h post-anticancer drug treatment could not prevent percent DNA fragmentation (Figure 5a), DNA laddering (data not shown), nuclear and cellular alterations (Figure 4a), increase in the apoptotic cell populations (Figure 5b) and cell death (Figure 5c), and they followed a pattern similar to that of U937 cells treated with anticancer drugs in the absence of endonuclease inhibitors.

Suboptimal doses of endonuclease inhibitors caused partial inhibition of DNA fragmentation and delay in anticancer drug-induced apoptotic cell death in U937 cells

The effect of suboptimal doses of ATA or Zn^{2+} on anticancer drug-induced apoptosis in U937 cells was also studied. Results of the percent DNA fragmentation

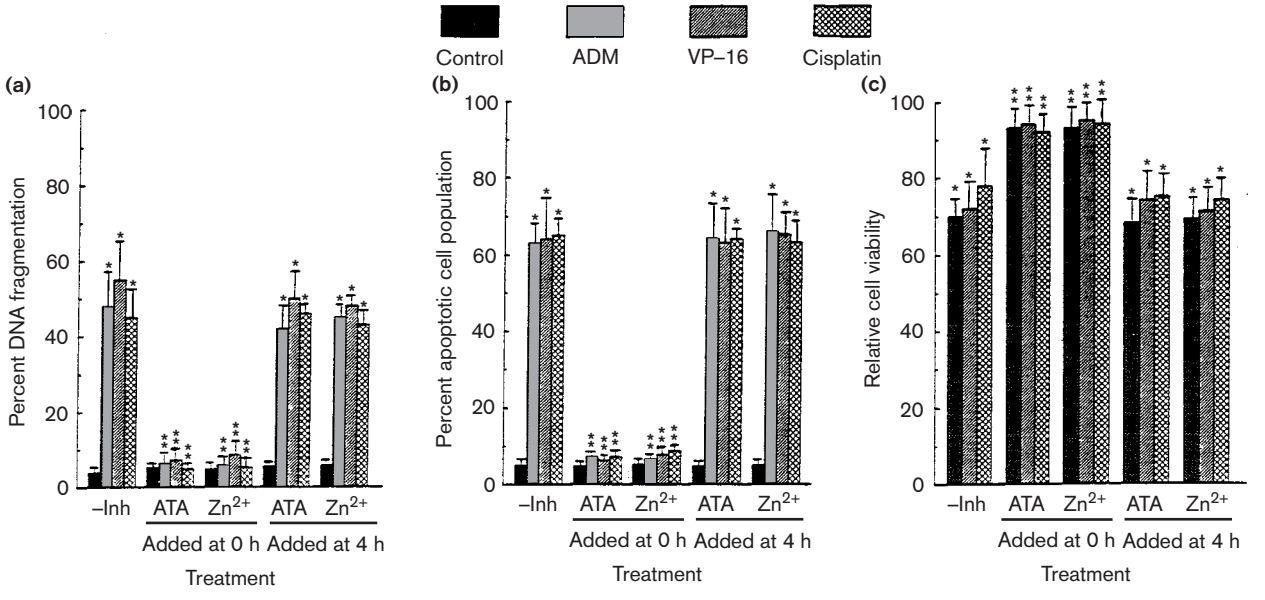

Figure 5. Effect of endonuclease inhibitors on (a) DNA fragmentation, (b) percent apoptotic cell population and (c) relative cell viability in U937 cells. Unlabeled or [^{3}H]thymidine-labeled U937 cells were treated with ADM (10 μM), VP-16 (10 μM) or cisplatin (10 μg/ml) and 100 μM of ATA or Zn^{2+} was added at the indicated time period. After 18 h of culture, percent DNA fragmentation, percent apoptotic cell population and relative cell viability were assayed by the methods as described in Materials and methods. Data shown are means $\pm$ SD and are representative of three independent experiments, done in triplicate. *$p < 0.05$ versus values for untreated control cultures. **$p < 0.05$ versus values for treated cultures in the presence of ATA or Zn^{2+}.

assay as shown in Figure 6, suggest that lower doses of endonuclease inhibitors were able to inhibit percent DNA fragmentation only partially. With 50 μM of ATA or Zn^{2+}, DNA fragmentation was found to be approximately 14–23% after 18 h of drug treatment. A further decrease in dose of ATA or Zn^{2+}, i.e. 25 μM, resulted in 24–33% DNA fragmentation. A dose of 100 μM of ATA or Zn^{2+} resulted in complete inhibition of DNA fragmentation induced by all anticancer drugs (Table 1).

We further investigated the effect of suboptimal doses of ATA on the viable cell population after different time intervals. In the presence of 100 μM of ATA, relative cell viability was found to be approximately 93, 87 and 83% after 18, 24 and 36 h of drug treatment, whereas in the presence of 50 μM of ATA, viable cell population was 83, 63 and 36% after 18, 24 and 36 h, respectively (Figure 7). Similar results were obtained with Zn^{2+} (data not shown).

Heat shock-induced cell death in U937 may not involve endonuclease-mediated DNA fragmentation as an essential step

To investigate whether DNA fragmentation plays a similar role in apoptosis in U937 cells in response to stimuli other than anticancer drugs, the effect of ATA or Zn^{2+} in heat shock-induced apoptosis in U937 cells was studied. Results of the percent DNA fragmentation and agarose gel electrophoresis suggested heat shock treatment of U937 cells induced oligonucleosomal DNA fragmentation which was inhibited by 100 μM of ATA or Zn^{2+} (Table 2 and Figure 8, lanes 2–4). However, results from the apoptotic cell population

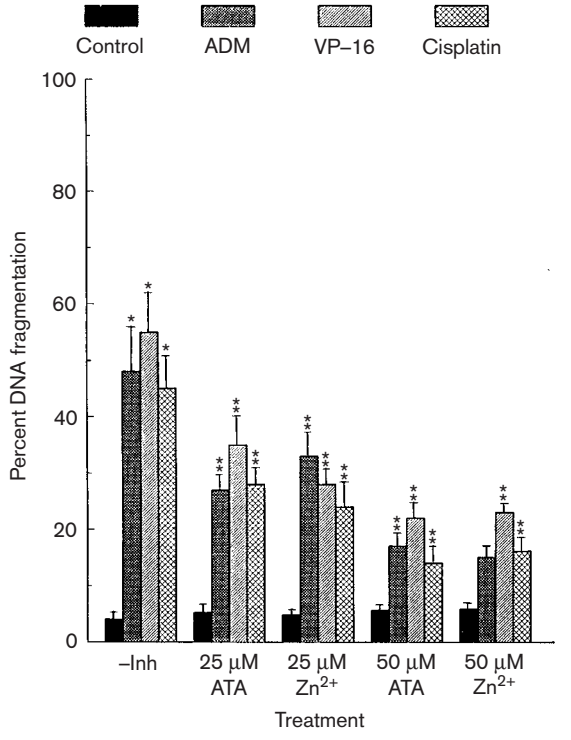

Figure 6. Effect of different doses of endonuclease inhibitors on percent DNA fragmentation in U937 cells. Unlabeled or [^{3}H]thymidine-labeled U937 cells were treated with ADM (10 μM), VP-16 (10 μM) or cisplatin (10 μg/ml) in the absence or presence of the indicated doses of ATA or Zn^{2+}. After 18 h of culture, percent DNA fragmentation was measured by the methods as described in Materials and methods. Data shown are means $\pm$ SD and are representative of three independent experiments, done in triplicate. *$p < 0.05$ versus values for untreated control cultures. **$p < 0.05$ versus values for treated cultures in the presence of ATA or Zn^{2+}.

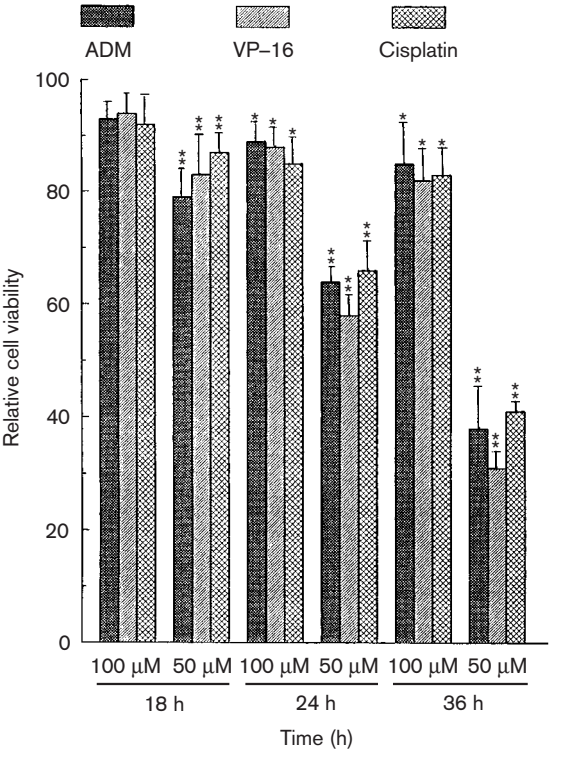

Figure 7. Kinetics of relative cell viability in U937 cells in response to different doses of endonuclease inhibitors. Untreated or U937 cells treated with ADM (10 μM), VP-16 (10 μM) or cisplatin (10 μg/ml) were cultured for the indicated time periods in the absence or presence of different doses of ATA. Relative cell viability was determined by the method as described in Materials and methods. Data shown are means $\pm$ SD and are representative of three independent experiments, done in triplicate. *$p < 0.05$ versus values for untreated control cultures. **$p < 0.05$ versus values for treated cultures in the presence of ATA.

Table 2. Effect of endonuclease inhibitors on percent DNA fragmentation, percent apoptotic cell population and relative cell viability in U937 cells in response to heat shock

Treatment	Percent DNA fragmentation			Percent apoptotic cell population			Relative cell viability		
	−Inhibitor	ATA	Zn^{2+}	−Inhibitor	ATA	Zn^{2+}	−Inhibitor	ATA	Zn^{2+}
Control	4±0.4[a]	5.2±1.4	4.8±1.1	5±1.1	4.8±2.0	5.2±1.5	95+4.5	95±4.3	95±3.2
Heat shock	58±5.9*	8.7±1.9**	8.6±2.1**	78±7.9*	83±7.9*	79±9.2*	16±2.9*	18±4.1*	21±2.9*

[a]The numbers represent mean±SD of triplicate cultures.
*$p<0.05$ versus values for untreated control cultures.
**$p<0.05$ versus values for treated cultures in the presence of ATA or Zn^{2+}.
Unlabeled or [^{3}H]thymidine-labeled U937 cells were given heat shock treatment at 43°C for 1 h in the presence or absence of ATA (100 M) or Zn^{2+} (100 μM) and cultured for 12 h. Percent DNA fragmentation, percent apoptotic cell population and relative cell viability were measured by the methods as described in Materials and methods.

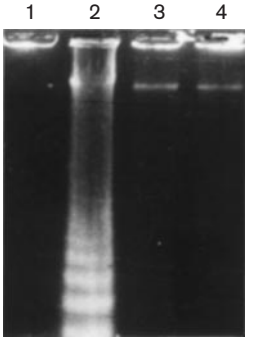

Figure 8. Effect of endonuclease inhibitors on heat shock-induced oligonucleosomal DNA fragmentation in U937 cells. U937 cells were given heat shock treatment at 43°C for 1 h in the absence or presence of 100 μM of ATA or Zn^{2+} and further cultured for 12 h. DNA extracted from the cells was analyzed by agarose gel electrophoresis by the method as described in Materials and methods. Lane 1, untreated U937; lane 2, U937 treated with heat shock; lane 3, U937 treated with heat shock in the presence of ATA; lane 4, U937 treated with heat shock in the presence of Zn^{2+}.

and viable cell population suggest that although DNA fragmentation in U937 cells induced by heat shock treatment was blocked by endonuclease inhibitors, they could not prevent cells from undergoing apoptotic morphology and finally cell death. As shown in Table 2, heat shock induced approximately 58% DNA fragmentation in U937 cells by 12 h. In the presence of 100 μM of ATA or Zn^{2+} DNA fragmentation was only 8.7%. Results of percent apoptotic cell population indicates ATA or Zn^{2+} failed to prevent U937 cells from undergoing apoptotic morphology and the apoptotic cell population was found to be approximately 81% after 12 h of heat shock treatment, which remained unchanged even after addition of endonuclease inhibitors. ATA or Zn^{2+} could not prevent loss of the viable cell population, which was decreased to 16–21% regardless of the presence or absence of endonuclease inhibitors.

Discussion

Many cancer chemotherapeutic drugs act primarily by inducing an apoptotic mode of cancer cell death both *in vitro* and *in vivo*.[1–3,20] However, the molecular pathway of apoptosis induced by these anticancer drugs is not well understood. Activation of an endonuclease(s) leading to oligonucleosomal DNA fragmentation has been implicated in the apoptotic pathways.[7–9] Evidence for involvement of endonuclease activation in apoptosis specifically includes effects mediated by certain inhibitors. Primary amongst these are the triphenylmethane dye ATA and Zn^{2+}.[8–11,21–24] The present investigation suggests the involvement of endonuclease(s) in apoptosis of U937 cells in response to different anticancer drugs ADM, VP-16 and cisplatin using endonuclease inhibitors ATA and Zn^{2+}.

Kinetics of DNA fragmentation indicate treatment of U937 cells with anticancer drugs induced DNA fragmentation which progressed with time in a linear pattern. Agarose gel analysis suggested this fragmentation to be of an oligonucleosomal nature. A comparison of the kinetics of percent specific DNA fragmentation with that of the appearance of a DNA ladder as detected by agarose gel electrophoresis shows that although DNA fragmentation was detectable at 6 h, it did not give any ladder pattern on agarose gel. The ladder pattern of DNA appeared only after 12 h of treatment, when percent specific DNA fragmentation was greater than 30%. The reason for this could be that low molecular weight DNA fragments may not be generated during early stages of apoptosis, despite the presence of extensive high

molecular weight DNA fragments, which are generated prior to oligonucleosomal fragments.[25,26]

Endonuclease inhibitors prevented percent DNA fragmentation and formation of a ladder pattern on agarose gel electrophoresis in U937 cells, suggesting activation of endonuclease(s) is required for the oligonucleosomal fragmentation of DNA induced by the anticancer drugs used. However, there are reports which suggest that cells can undergo cell death with all the morphogical features of apoptosis even in the absence of double-stranded DNA fragmentation and apoptotic morphology can be induced in enucleated cells as well.[14–17] Human erythroleukemic cell line K562 has been shown to undergo apoptotic cell death without internucleosomal DNA cleavage in response to a variety of apoptotic stimuli.[27,28] Recently, Sakahira *et al*. have also reported apoptotic nuclear morphological changes in human T cell lymphoma Jurkat cells despite blocked DNA fragmentation.[29] Hence, we also studied the effect of endonuclease inhibitors on morphological and nuclear changes in U937 cells in response to anticancer drugs by fluorescence and phase contrast microscopy. U937 cells treated with ADM, VP-16 or cisplatin showed cellular and nuclear morphological changes typical of apoptosis such as membrane blebbing, cell shrinkage and chromatin condensation. Addition of endonuclease inhibitors prevented these morphological changes also in U937 cells in response to anticancer drugs. Anticancer drug-induced DNA fragmentation and morphological studies were performed simultaneously to study the relationship between DNA fragmentation and morphological alterations. The observations that anticancer drug-induced morphological changes are blocked by endonuclease inhibitors suggest that these morphological changes are dependent on endonuclease(s)-mediated DNA fragmentation. A similar relationship between DNA fragmentation and morphological changes has also been proposed by others.[7,16]

Addition of endonuclease inhibitors upto 4 h post-anticancer drug treatment blocked DNA fragmentation, increased the apoptotic cell population and decreased the viable cell population (data not shown). Delaying the addition of inhibitors beyond 4 h could not block DNA fragmentation, which progressed with time, and the cells showed all the morphological features of apoptosis and decline in viable cell population. Once the DNA fragmentation has occurred, endonuclease inhibitors are unable to prevent death, suggesting the phenomenon to be irreversible.

We further studied the effect of suboptimal doses of endonuclease inhibitors on percent DNA fragmentation and relative cell viability in U937 cells in response to different anticancer drugs. The data suggests that lower doses of endonuclease inhibitors partially inhibited DNA fragmentation. Results of the kinetics of relative cell viability with suboptimal doses of endonuclease inhibitors and its comparison with that of percent DNA fragmentation suggest that partial inhibition of DNA fragmentation only delayed the process of apoptotic cell death, whereas the optimal dose (100 μM) of endonuclease inhibitors not only inhibited DNA fragmentation completely, but also extended cell survival. This suggests that apoptotic cell death is also dependent on the extent of DNA fragmentation, as reported by others also.[18]

Since the role of DNA fragmentation in apoptosis may differ depending upon the type of apoptotic stimuli, the role of endonuclease-mediated DNA fragmentation in response to heat shock-induced apoptosis in U937 cells was also studied. In contrast to the findings of anticancer drug-induced apoptosis, although endonuclease inhibitors prevented heat shock-induced DNA fragmentation, they could not rescue cells from undergoing cell death. Cellular and nuclear changes in U937 cells as studied by phase contrast and fluorescence microscopy in response to heat shock suggested the occurrence of apoptosis. Plasma membrane failure as studied by the PI permeability test[30] was observed at a later stage of apoptosis, followed by membrane disruption, a process called secondary necrosis[18] (data not shown). Moreover, most of the cells that underwent morphological changes were found to be viable at the early stages of heat shock treatment. The mechanism of heat shock-induced apoptosis is poorly understood. It is possible that heat shock may act primarily at different cellular targets which in turn may send signals for apoptosis to occur. A complete understanding of mechanisms of heat shock-induced apoptosis requires further investigation.

Results of the endonuclease inhibitors study suggested that apoptosis in U937 cells induced by heat shock may not involve endonuclease(s)-mediated DNA fragmentation as a prerequisite step and apoptosis occurred even in the abscence of DNA fragmentation. Our observation is consistent with that of Mogil *et al*. who reported that heat shock-induced apoptotic cell death in T cell hybridoma occurred regardless of the blockade to DNA fragmentation.[13] This raises the possibilities of involvement of pathways other than endonucleolytic cleavage of DNA fragmentation. The role of intracellular proteolysis involving different proteases has been well documented in apoptotic pathways.[31] They may act upstream to endonuclease activation or the two pathways may be completely independent.[30–32] Recently, Enari *et al*. have reported a caspase-activated DNase (CAD) and its inhibitor

ICAD.[33] Further, Sakahira *et al*. also shown that caspase-3 cleaves ICAD and inactivates its CAD inhibitory effrect, suggesting activation of CAD downstream of the caspase cascade is responsible for internucleosomal DNA cleavage during apoptosis.[34] The present study does not rule out the possibility of the involvement of other pathways or dependence of endonuclease(s) activation on other possible modulators of apoptosis in anticancer drug-induced apoptosis.

The anticancer drugs ADM, VP-16 and cisplatin are structurally and functionally unrelated, and have been reported to exert their cytotoxic actions against tumor cells by interaction with different cellular targets.[35–37] The present study indicates that these different anticancer drugs, despite their different cellular targets for their cytotoxic action, followed a common pathway, i.e. via activation of an endonuclease(s) to induce apoptosis. Endonuclease(s) involved in apoptotic pathways are poorly understood. ATA or Zn^{2+} used in this study have been reported to be general inhibitors of endonuclease(s) and and do not provide any idea about specific endonuclease(s) involved in anticancer drug-induced apoptosis. Identification of such endonuclease(s) will not only help to understand the molecular pathways of anticancer drug-induced apoptosis, but may also provide important insights for designing more effective anticancer drugs.

In summary, results of the present investigation suggest that anticancer drug (ADM, VP-16 and cisplatin)-induced apoptosis in U937 cells requires endonuclease-mediated DNA cleavage as a common prerequisite step.

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