RESEARCH COMMUNICATION

Chromatin condensation during apoptosis requires ATP

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The processes leading to morphological changes of the chromatin in cells that undergo apoptosis are presently unclear. We have recently shown that chromatin fragmentation and the nuclear morphological changes typically seen in apoptosis were reproduced in an *in vitro* system comprised of isolated rat thymocyte nuclei incubated in the presence of a lysate from Fas/APO-1-stimulated JURKAT cells [Chow, Weis, Kass, Holmström, Eriksson and Orrenius (1995) FEBS Lett. **364**, 134–138]. Using this *in vitro* system, we now report that the presence of ATP is necessary for chromatin condensation, its movement to the nuclear periphery and apoptotic body formation. In clear contrast, chromatin cleavage into high-molecular-mass and oligonucleosomal-length DNA fragments induced by lysates derived from Fas/APO-1-activated JURKAT cells did not require the presence of ATP. The induction of these morphological changes by ATP could not be substituted by the analogues, adenosine 5′-[β,γ-methylene]triphosphate and adenosine 5′-[α,β-methylene]-triphosphate, AMP, cAMP and UTP. However, adenosine 5′-[γ-thio]triphosphate, and to a lesser degree GTP and ADP, could partially replace ATP in inducing nuclear apoptotic morphological changes. It is concluded that ATP is essential for the morphological changes occurring in nuclei during apoptosis, but not for DNA fragmentation.

INTRODUCTION

Cell death through apoptosis or programmed cell death is used by multicellular organisms to eliminate superfluous, cancerous or virally infected cells [1,2]. As opposed to necrosis, apoptosis progresses through a series of well-defined morphological and biochemical stages which occur in the nucleus as well as in the cytoplasm of the dying cell. An early morphological event is the clumping and margination of the chromatin towards the inner nuclear membrane [1,3–5], followed by complete condensation of the chromatin and nuclear disruption into apoptotic bodies [1]. Biochemical analysis of nuclear DNA revealed that the early stages of apoptosis involve chromatin cleavage into distinct high-molecular-mass (HMM) fragments that correspond to > 700 kbp, followed by further degradation to 200–250 and 50 kbp fragments [6–9]. This initial chromatin degradation is attributed to an Mg^{2+}-requiring endonuclease [4,10–12]. It is currently thought that the 200–250 kbp fragments reflect hexameric loop structures of the DNA (known as rosettes), while the 50 kbp fragments would correspond to a single such loop [6]. These 50 kbp loops can then be further digested by a Ca^{2+}- and Mg^{2+}-activated endonuclease(s) [13], leading to the formation of oligonucleosomal-sized DNA fragments that produce the typical ladder appearance when resolved by agarose-gel electrophoresis [14]. The identity of the endonuclease(s) that participates in chromatin cleavage is still a matter of debate.

Several laboratories have shown that the early morphological changes of nuclear chromatin coincide with the appearance of HMM fragments, whilst the formation of the DNA ladder is a rather late event, occurring during or after apoptotic body formation has taken place [4,9,15]. Although the above studies have shed considerable light on the sequence of events that lead to the morphological changes of apoptosis, the relationship between DNA fragmentation and nuclear chromatin condensation is still unclear.

We have recently analysed the cellular events occurring during the induction of apoptosis following the stimulation of the Fas/APO-1 receptor in JURKAT T-lymphocytes [9,16,17]. This led us to report [18] that the nuclear morphological changes typically seen in apoptosis could be fully reproduced in an *in vitro* system composed of isolated thymocyte nuclei incubated in the presence of a lysate from Fas/APO-1-stimulated JURKAT cells. Similar results have recently been reported by several groups [19,20], indicating that the apoptotic nuclei promoting activity could also be obtained from UV-irradiated cells or in the presence of ceramide. In the present study, this *in vitro* system was used to investigate the relationship between the molecular aspects of the morphological changes occurring during nuclear chromatin condensation and DNA fragmentation. We show here that the presence of ATP is necessary for the movement of the chromatin to the nuclear periphery and for apoptotic body formation, whereas, in clear contrast, chromatin cleavage into HMM and oligonucleosomal-length DNA fragments still occurs in the absence of ATP.

MATERIALS AND METHODS

Chemicals

All media and serum were from Gibco (Paisley, Scotland, U.K.). Anti-Fas (clone CH-11) was obtained from Kamiya Biomedical

Abbreviations used: HMM, high molecular-mass; ATP[S], adenosine 5′-[γ-thio]triphosphate; AMP-CPP, adenosine 5′-[α,β-methylene]triphosphate; AMP-PCP, adenosine 5′-[β,γ-methylene]triphosphate; FCS, fetal calf serum.

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Company (Thousand Oaks, U.S.A.). ATP, ADP, AMP, UTP, GTP, creatine kinase and creatine phosphate were purchased from Boehringer Mannheim. Adenosine 5′-[γ-thio]triphosphate (ATP[S]), cAMP, adenosine 5′-[α,β-methylene]triphosphate (AMP-CPP) and adenosine 5′-[β,γ-methylene]triphosphate (AMP-PCP) were obtained from Sigma Chemicals. All other chemicals were of the highest purity commercially available.

Cell culture and preparation of 100 000 *g* supernatants

JURKAT T-cells, clone E-6 (American Type Culture Collection, Rockville, MD, U.S.A.) were maintained in suspension culture using RPMI 1640 medium supplemented with 10 % (v/v) heat-inactivated fetal calf serum (FCS). The cells (30×10^6 cells/ml) were stimulated with 20 ng/ml anti-human Fas antibody or left untreated for 60 min at 37 °C in RPMI 1640 [10 % (v/v) FCS]. The cells were washed twice with ice-cold RPMI 1640 (without FCS) and resuspended ($\approx 6 \times 10^6$ cells/10 μl) in buffer A (40 mM β-glycerophosphate/50 mM NaCl/2 mM $MgCl_2$/5 mM EGTA/10 mM Hepes, pH 7.0). After three cycles of freezing and thawing, the cell lysates were centrifuged for 30 min at 20 000 ***g*** at 4 °C and the pellet discarded. The cell lysate supernatants were centrifuged for a further 30 min at 100 000 ***g*** and the supernatants were then washed three times with a 5–10 vol. of buffer A in a Centricon ultrafiltration unit (10 000 Da molecular-mass cut-off) in order to remove low-molecular-mass components, in particular nucleotides. The washed supernatants were stored at −20 °C for up to 6 months without detectable loss of activity. The protein concentration was determined using the method of Lowry et al. [21] with BSA as standard.

Reconstituted *in vitro* system

The cell-free system was adapted from that of Lazebnik et al. [22] as described previously [18]. Rat thymocyte nuclei [23] were suspended in buffer B (5 mM $MgCl_2$/2.1 M sucrose/50 mM Tris/HCl, pH 7.5) at a final concentration of $(4–5) \times 10^8$ nuclei/ml before use. The reaction mixture (final volume of 30 μl) containing the washed cell lysates (10 μg of protein) and nuclei (5×10^6) was as described [18], and was supplemented with an ATP-regeneration system or ATP analogues (2 mM final concentration) as indicated in the text. The ATP-regenerating system consisted of (final concentration in the assay mixture) 2 mM ATP, 10 mM creatine phosphate and 75 μg of creatine kinase/ml.

Morphological and DNA analysis

Examination of the nuclear chromatin morphology was performed by confocal laser microscopy using propidium iodide as described previously [18]. Chromatin degradation into HMM and oligonucleosomal-length fragments was analysed as previously reported [8,24] using field-inversion gel electrophoresis and conventional agarose-gel electrophoresis respectively.

RESULTS AND DISCUSSION

Activation of the Fas antigen on the plasma membrane of JURKAT cells through cross-linking with anti-Fas antibody results in the rapid induction of apoptosis [9]. Using an *in vitro* system [18], we found that the 100 000 ***g*** supernatant from lysates derived from Fas-stimulated JURKAT cells, but not from control cells, induced the morphological and biochemical changes in isolated nuclei typically seen in apoptosis. Our results also suggested that the apoptotic nuclei-promoting activity involves protease(s), and that its formation upon Fas activation requires a cascade of proteases including an interleukin 1-β-converting enzyme-like protease [18].

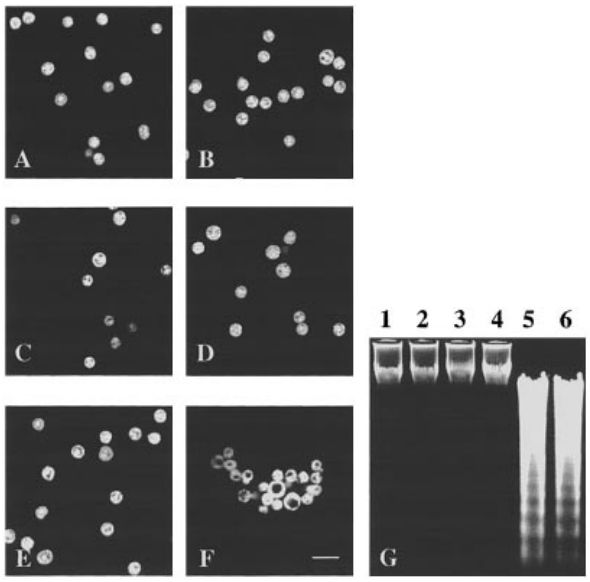

Figure 1 Morphological changes in isolated thymocyte nuclei incubated with lysate from Fas/APO-1-stimulated JURKAT cells require ATP

Isolated thymocyte nuclei were incubated at 37 °C with or without lysates derived from control and Fas/APO-1-stimulated JURKAT cells in the absence or presence of ATP. After 60 min the nuclei were processed for confocal laser microscopy and agarose-gel analysis as described in the Materials and methods section. (**A**) Nuclei alone without ATP; (**B**) nuclei alone with ATP; (**C**) nuclei + control cell lysates, without ATP; (**D**) nuclei + control cell lysates, with ATP; (**E**) nuclei + Fas-treated cell lysates, without ATP; (**F**) nuclei + Fas-treated cell lysates, with ATP. The bar represents 5 μm. (**G**) DNA fragmentation in isolated nuclei into oligonucleosomal-length fragments, visualized by ethidium bromide-stained agarose gel. Lanes 1–6 respectively represent chromatin integrity of isolated nuclei incubated under conditions that were identical with those described for (**A**) – (**F**).

Using this *in vitro* system to investigate the mechanisms causing the nuclear morphological changes, we now report that chromatin condensation and its migration towards the nuclear envelope occurred only when ATP was present in the incubation mixture. Thus, whilst the 100 000 ***g*** supernatant from lysates derived from Fas-stimulated JURKAT cells induced rat thymocyte nuclei to undergo the morphological changes typical of apoptosis in the presence of ATP (Figure 1F), rat thymocyte nuclei incubated under identical conditions, but in the absence of ATP, remained unchanged (Figure 1E) and were morphologically indistinguishable from control nuclei (Figures 1A and 1B) and nuclei incubated with control (untreated) cell lysates (Figures 1C and 1D). Incubating nuclei alone (Figure 1B) or with the 100 000 ***g*** supernatant derived from control JURKAT cell lysates in the presence of ATP (Figure 1D) did not result in chromatin condensation. However, examination of the integrity of the chromatin in isolated nuclei under similar treatments (Figure 1, A–F) revealed that nuclei incubated with Fas-stimulated JURKAT cell lysates in the presence or absence of ATP resulted in the formation of the characteristic laddering of the oligonucleosomal-length DNA fragments (Figure 1G, lanes 5 and 6). Both the nuclei alone, or incubated in the presence of control cell lysates, with or without ATP, did not show any DNA fragmentation (Fig. 1G, lanes 1–4).

Examination of the time course of the above morphological changes revealed that 50 % and 100 % of the thymocyte nuclei

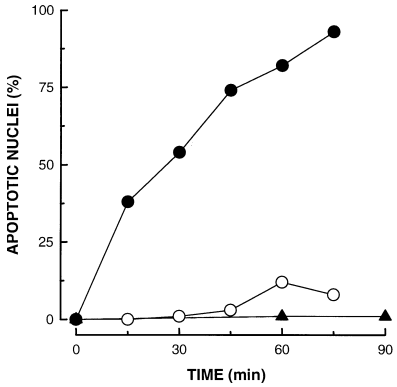

Figure 2 Time course for the induction of nuclear morphological changes

Isolated nuclei were incubated at 37 °C with lysates from Fas/APO-1-stimulated JURKAT cells in the absence or presence of ATP, and aliquots were removed at the indicated time points and processed for confocal laser microscopic analysis of chromatin using propidium iodide, as described in the Materials and methods section. Data are shown as the percentage of nuclei with chromatin condensed on the nuclear envelope or with fragmented DNA-containing vesicles. Symbols: ●, nuclei + lysates from Fas/APO-1-stimulated JURKAT cells, in the presence of ATP; ○, nuclei + lysates from Fas/APO-1-stimulated JURKAT cells, in the absence of ATP; ▲, nuclei with buffer (no lysate from Fas/APO-1-stimulated JURKAT cells), in the presence of ATP.

Table 1 Effect of ATP and nucleotide analogues on nuclear apoptotic morphology induced by Fas-treated cell lysate

Isolated thymocyte nuclei (5×10^6) were incubated with supernatant from untreated or anti-Fas-exposed JURKAT T-cells in the absence or presence of ATP or analogues (2 mM) for 60 min at 37 °C before preparing the samples for morphological examination using confocal laser microscopy as described in the Materials and methods section.

Treatment	Nucleotide	Apoptotic nuclei* (%)
Control cell lysate	None	0.4 ± 0.5 (7)
Control cell lysate	ATP	2.3 ± 2.9 (6)
Fas-treated cell lysate	None	8.5 ± 4.8 (7)
Fas-treated cell lysate	ATP	84.8 ± 10.3 (6)
Fas-treated cell lysate	ADP	18.4 ± 8.5 (5)
Fas-treated cell lysate	AMP	10.0 ± 7.2 (5)
Fas-treated cell lysate	ATP[S]	54.0 ± 6.1 (3)
Fas-treated cell lysate	AMP-CPP	9.3 ± 6.1 (4)
Fas-treated cell lysate	AMP-PCP	9.0 ± 5.3 (4)
Fas-treated cell lysate	cAMP	8.0 ± 2.6 (4)
Fas-treated cell lysate	UTP	9.2 ± 4.4 (5)
Fas-treated cell lysate	GTP	30.8 ± 11.7 (3)

* Percentage of nuclei with chromatin condensed on the nuclear envelope or with fragmented DNA-containing vesicles; numbers in parentheses represent the number of experiments performed.

were apoptotic after 30 and 75 min, respectively, of incubation with ATP and the 100000 ***g*** supernatant from lysates derived from Fas-stimulated JURKAT cells (Figure 2). In clear contrast, over the same period of time the thymocyte nuclei remained morphologically unaffected in the absence of ATP (but with the 100000 ***g*** supernatant from lysates derived from Fas-stimulated JURKAT cells) (Figure 2). Again, nuclei incubated with ATP and supernatant from control JURKAT cell lysates over the same time course remained morphologically unchanged (results not shown), which agrees with the results shown in Figure 1(D) and in our previous report [18]. Similarly, a time-dependent increase in the cleavage of chromatin into HMM DNA fragments (corresponding to > 700, 200–250 and finally < 50 kbp) was observed in nuclei incubated in the presence of Fas-stimulated cell lysates with or without ATP (Figures 3A and 3B). The subsequent degradation of the 50 kbp HMM DNA fragments to oligonucleosomal-sized fragments also did not require the presence of ATP (results not shown; Figure 1G). Similar to the results in Figure 1 (panels C and D) and our earlier work [18], nuclei incubated with control cell lysates did not show any DNA degradation into HMM or oligonucleosomal-length DNA fragments in the presence or absence of ATP over the same time course (results not shown). Thus, the formation of HMM fragments, and subsequently oligonucleosomal-length DNA fragments, in isolated thymocyte nuclei incubated in the presence of Fas-treated cell lysates, clearly does not require ATP.

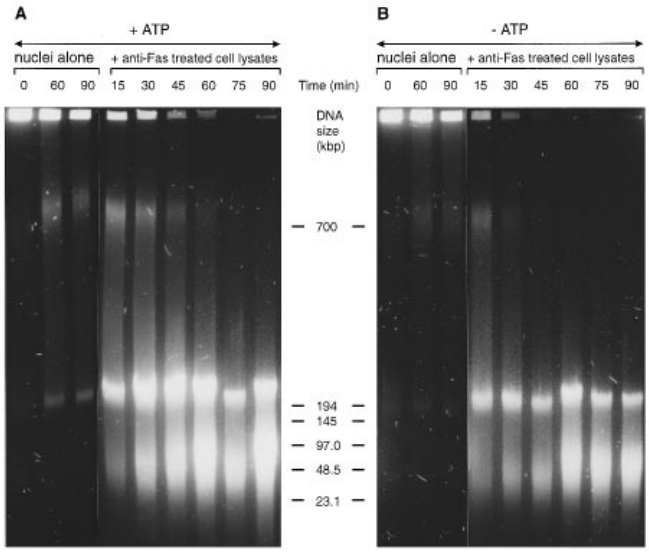

Figure 3 Time course for the formation of HMM DNA fragments in the presence or absence of ATP

Isolated nuclei were incubated at 37 °C with lysates from Fas/APO-1-stimulated JURKAT cells in the absence (**A**) or presence (**B**) of ATP, and aliquots were removed at the indicated time points and processed for analysis of HMM DNA fragments as described in the Materials and methods section.

We next investigated the degree of specificity for ATP versus other nucleotides, as well as the possible role of ATP in nuclear condensation. As summarized in Table 1, of all the nucleotides tested, ATP was the most potent at eliciting apoptotic nuclear morphology. The poorly hydrolysable analogues of ATP, AMP-CPP and AMP-PCP, did not produce any morphological changes in the presence of lysate from Fas/APO-1-stimulated JURKAT cells. Similarly, UTP, cAMP, AMP (Table 1) and the nucleoside adenosine (results not shown) had no affect on nuclear morphology. However, ATP[S] could partially substitute for ATP, with approximately half the nuclear population exhibiting chromatin condensation. GTP and ADP also had a chromatin condensing activity in the presence of lysate from Fas/APO-1-stimulated JURKAT cells (31% and 18% respectively), but they were much less potent than ATP. Substituting ATP for different analogues also did not alter DNA degradation into HMM DNA fragments (Figure 4) and the subsequent formation of oligonucleosomal-length DNA fragments (results not shown) by the Fas-treated cell lysates, which is in clear contrast with their effects on nuclear morphology in the presence of the active cell lysate shown in Table 1. Thus, chromatin degradation during

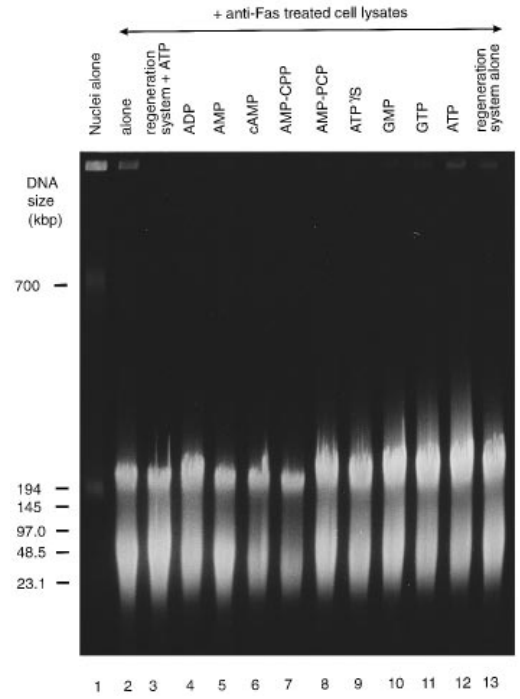

Figure 4 HMM DNA fragments formation in the presence of ATP analogues

Isolated nuclei were incubated at 37 °C with lysate from Fas/APO-1-stimulated JURKAT cells in the absence or presence of ATP or ATP analogues (ATPγS is ATP[S]) for 45 min before aliquots were removed and processed for analysis of HMM DNA fragments as described in the Materials and methods section.

apoptosis can occur without the formation of apoptotic nuclei morphology, but not vice versa. Taken together, our results show that the formation of nuclear apoptic morphology, but not chromatin degradation by lysate from Fas/APO-1-stimulated JURKAT cells, is highly dependent on the presence of ATP.

It should be noted that ATP was used with a regenerating system. This was to counteract its too rapid loss through metabolism by nuclear ATPases and a low level of mitochrondrial contamination (see [18]). ATP (2 mM), in the absence of a regenerating system, also induced the formation of apoptotic nuclei, often to the same extent as observed with the regenerating system, but with more variability between thymocyte nuclei preparations. Higher concentrations of ATP could not be used because they produced rapid nuclear swelling followed by lysis. However, the regenerating system did not affect nuclear morphology in the absence of added ATP.

How ATP could be affecting chromatin condensation and promoting its migration towards the inner membrane of the nuclear envelope is at present unclear. Several possibilities exist, including facilitation of the transport of molecules in and out of the nucleus through the nuclear pore complex [25] or nucleosome disruption [26,27]. The observation that ATP[S] could substitute to some extent for ATP, whereas the non-hydrolysable analogue AMP-PCP did not, would, however, suggest that a protein kinase, possibly $p34^{cdc2}$ or Nek-2, may be responsible for the effects of ATP on the nuclear morphology described here. The aforementioned protein kinases have recently been implicated in mitotic catastrophes that very much resemble apoptosis by inducing premature chromatin condensation [28–31].

In summary, this study reports that lysates from Fas/APO-1-stimulated JURKAT cells, the effector of Fas-induced apoptosis, require ATP to elicit the morphological nuclear changes typical of apoptosis but not chromatin degradation into HMM and oligonucleosomal-length DNA fragments. Further work is ongoing in our laboratories to identify the nuclear target(s) for ATP that elicit(s) chromatin condensation and abutting to the inner nuclear membrane.

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