RESEARCH COMMUNICATION

Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation

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Apoptosis, a major form of cell death, is characterized by chromatin condensation, a reduction in cell volume and endonuclease cleavage of DNA into oligonucleosomal length fragments. The detection of these fragments by gel electrophoresis, as a DNA ladder, is currently used as the major biochemical index of apoptosis. Here we report that key morphological changes of apoptosis can be dissociated experimentally from the DNA fragmentation produced by endonuclease activity. Internucleosomal cleavage of DNA is thus likely to be a later event in the apoptotic process.

INTRODUCTION

Apoptosis is a major form of cell death [1] found in the deletion of autoreactive T-cell clones in thymic maturation [2], in the senesence of neutrophils [3] and in target cells attacked by cytotoxic T-lymphocytes and natural killer cells [4–6]. It is characterized morphologically by chromatin condensation and by a reduction in cell volume [1,4,5,7]. These morphological changes have been associated with endonuclease cleavage of DNA into nucleosomal size fragments of 180–200 bp or multiples thereof [4,5,8] in many systems, including immature thymocytes exposed to glucocorticoids [8,9], γ-irradiation [10], the calcium ionophore A23187 [11] and anti-CD3 antibodies [12]. There has been a general tendency in the literature to equate this nucleosomal 'ladder pattern' with apoptosis [5]. In this study, we clearly demonstrate that key morphological changes of apoptosis precede internucleosomal DNA cleavage.

MATERIALS AND METHODS

Materials

Dexamethasone, actinomycin D, cycloheximide, aurintricarboxylic acid, the dye Hoechst 33342, propidium iodide and Percoll were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Foetal calf serum and RMPI-1640 were from Gibco, Paisley, Scotland, U.K.

Thymocyte incubations

Thymocytes were isolated from Male Fischer 344 rats (4–5 weeks old) as described previously [13]. The resulting cell suspension was diluted with RPMI-1640 containing 10% foetal calf serum to give a final cell suspension of $(15–20) \times 10^6$ cells/ml. Incubations were carried out for 4 h at 37 °C under an atmosphere of 95% air/5% CO_2, in the presence of dexamethasone (0.1 μM).

Percoll gradients

Purified populations of normal and apoptotic thymocytes were prepared by discontinuous Percoll gradients as described previously [14].

Flow cytometry

Following culture, thymocytes (2×10^6 cells/ml) were incubated with Hoechst 33342 (1 μg/ml) in RPMI-1640 containing 10% foetal calf serum at 37 °C for 10 min. The cells were cooled to 4 °C, centrifuged at 400 g for 5 min, resuspended in phosphate-buffered saline containing propidium iodide (5 μg/ml) and examined by flow cytometry [15].

Flow cytometric analyses were carried out at a flow rate of 200 cells·s^{-1} using an Ortho Cytofluorograph 50-H linked to a 2150 computer system. Hoechst 33342 and propidium iodide were excited using the 352 nm u.v. line of a krypton laser, and the resultant blue (400–500 nm) versus red (> 630 nm) fluorescence was recorded using linear amplification. The blue fluorescent cells were gated and displayed as a two-dimensional cytogram of fluorescence intensity versus forward light scatter. Sub-populations of cells were sorted at a flow rate of 800 cells·s^{-1} based on these parameters.

Gel electrophoresis and DNA fragmentation

Agarose gel electrophoresis was used to detect DNA laddering in whole cells (1×10^6) by the method of Sorenson et al. [16]. DNA fragmentation was measured by the percentage of diphenylamine-reactive material present in the 13000 g supernatant fractions of lysed cells [8,17].

Electron microscopy

Suspensions (2.5×10^5 cells) were fixed overnight in 2% (v/v) glutaraldehyde in 0.1 M-sodium cacodylate buffer (pH 7.2). They were subsequently spun down in microcentrifuge tubes and the pellets were post-fixed for 1 h in 1% (w/v) osmium tetroxide in buffer 0.1 M with respect to sodium cacodylate and 0.04 M with respect to potassium ferrocyanide. All pellets were stained en bloc for 1 h with 2% aqueous uranyl acetate before being dehydrated through a series of ethanol concentrations and embedded in Araldite.

Semi-thin (1 μm) sections were cut axially through all layers of each pellet to select areas for ultramicrotomy. Ultrathin sections were stained with lead citrate and examined in a Jeol 100-CX electron microscope.

RESULTS AND DISCUSSION

The introduction of apoptosis in immature thymocytes by glucocorticoids, such as dexamethasone and methylprednisolone, has been well characterized [8,9]. We have quantified the formation of apoptotic thymocytes, induced by dexamethasone (0.1 μM), by flow cytometric analysis of the differential blue
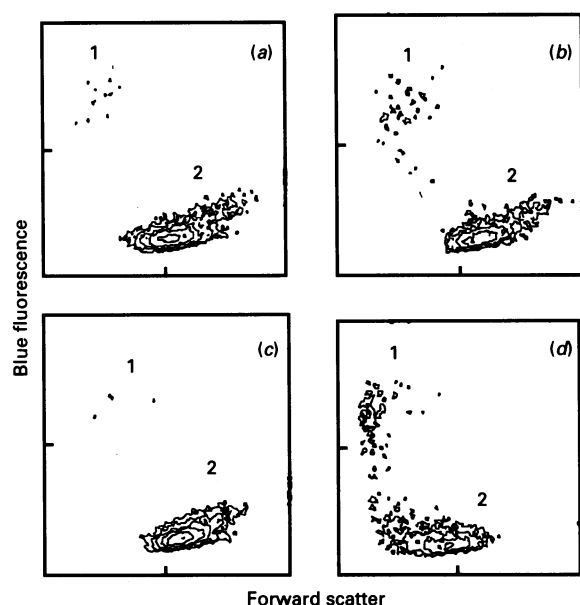
Fig. 1. Zn^{2+} does not prevent the formation of apoptotic thymocytes by dexamethasone

Freshly isolated thymocytes were incubated for 4 h either alone (a) or with dexamethasone (0.1 μM) in the absence (b) or in the presence of either cycloheximide (10 μM) (c) or zinc acetate dihydrate (1 mM) (d). Normal (region 2) and apoptotic (region 1) thymocytes were separated by flow cytometry following incubation with Hoechst 33342 (1 μg/ml) and propidium iodide [15]. Non-viable cells, which included propidium iodide, were gated out. Apoptotic cells, with high blue fluorescence and low forward light scatter, were distinguished from normal thymocytes, with low blue fluorescence and high forward light scatter. Forward scatter gives an indication of size, i.e. apoptotic cells were smaller than normal cells.

Table 1. Dexamethasone-induced thymocyte apoptosis is inhibited by cycloheximide, actinomycin D and aurin, but not by zinc

Thymocytes were incubated for 4 h as described in the Materials and methods section, either alone or with dexamethasone (Dex; 0.1 μM) in the absence or presence of various reported inhibitors of apoptosis. The percentage of apoptotic cells was determined by flow cytometry following incubation with Hoechst 33342. Values are means $\pm$ S.E.M. for at least three separate determinations.

Treatment	Apoptotic cells (%)	DNA fragmentation (%)
Control	5.5 $\pm$ 1.7	9.1 $\pm$ 2.3
Dex	18.7 $\pm$ 4.3	33.7 $\pm$ 8.6
Dex + cycloheximide (10 μM)	6.3 $\pm$ 1.6	10.0 $\pm$ 1.4
Dex + actinomycin D (0.9 μM)	7.7 $\pm$ 1.7	13.1 $\pm$ 2.8
Dex + aurin (500 μM)	7.2 $\pm$ 2.2	18.3 $\pm$ 4.1
Dex + Zn^{2+} (1 mM)	21.1 $\pm$ 3.4	7.2 $\pm$ 2.8
Control + Zn^{2+} (1 mM)	7.2 $\pm$ 1.2	4.0 $\pm$ 1.2

fluorescence intensity obtained after incubation of the cells with the vital bisbenzimidazole dye Hoechst 33342 (region 1 in Figs. 1a and 1b). The thymocytes in region 1 (Fig. 1b) exhibiting high blue fluorescence have been characterized as apoptotic using a number of criteria, including their size, ultrastructure and DNA ladder pattern on gel electrophoresis [15]. The cells in region 2, with low blue fluorescence, were identified as normal thymocytes. The formation of apoptotic cells was inhibited by (a) cyclo-heximide (Fig. 1c) (an inhibitor of protein synthesis), (b) actino-mycin D (an inhibitor of transcription) and (c) aurintricarboxylic
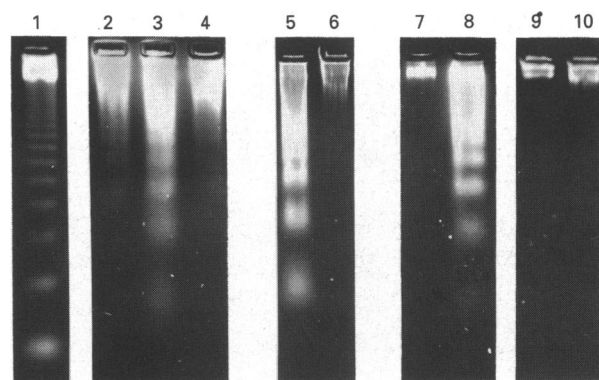

Fig. 2. Zn^{2+} inhibits dexamethasone-induced DNA laddering in thymocytes

Thymocytes (20 x 10^6 cells) were incubated for 4 h either alone or with dexamethasone (0.1 μM) in the absence or in the presence of Zn^{2+} (1 mM), and 1 x 10^6 cells were taken for measurement of DNA laddering by agarose gel electrophoresis. Lane 1 contains molecular size standards of multiples of 123 bp. In unsorted thymocytes, dexamethasone (lane 3) caused an increase in DNA laddering compared with control cells (lane 2). This increase was totally inhibited in the presence of Zn^{2+} (1 mM) (lane 4). Apoptotic cells of high modal density and small diameter were examined by agarose gel electrophoresis after separation by isopycnic centrifugation on discontinuous Percoll gradients following incubation of cells for 4 h with dexamethasone either alone (lane 5) or in the presence of Zn^{2+} (lane 6). Viable thymocytes were sorted by flow cytometry as described in the legend to Fig. 1. In the presence of dexamethasone alone, sorted cells which exhibited high (lane 8) but not low (lane 7) blue fluorescence showed DNA laddering, whereas in the presence of Zn^{2+} cells with neither low nor high blue fluorescence (lanes 9 and 10 respectively) exhibited DNA laddering.

acid (an inhibitor of both endonuclease and protein synthesis) (Table 1). The inhibition of thymocyte apoptosis by these agents is in agreement with many other studies [11,18].

Zinc has also been reported to prevent apoptosis, an effect assumed to be due to the ability of the metal ion to inhibit a Ca^{2+}/Mg^{2+}-dependent endonuclease [9,19]. It was therefore un-expected that Zn^{2+} failed to inhibit the formation of apoptotic cells, as judged by the property of high blue fluorescence (Table 1 and region 1 in Fig. 1d), under conditions where Zn^{2+} almost completely inhibited both DNA fragmentation (Table 1) and laddering induced by dexamethasone (Fig. 2, lanes 3 and 4).

In previous studies, the higher density of apoptotic compared with normal thymocytes has been utilized as the basis for their separation and purification by isopycnic centrifugation on dis-continuous Percoll gradients [7,14]. Using this system, we ob-tained a discrete cell fraction having both a smaller mean diameter and a higher modal density than normal thymocytes following treatment of cells with dexamethasone (0.1 μM), either alone or in the presence of Zn^{2+} (1 mM). These cells were then examined by both electron microscopy and agarose gel electrophoresis. The cells with the lower modal density showed low blue fluorescence when incubated with Hoechst 33342, and the appearance of their euchromatin and heterochromatin was similar to normal thymo-cytes. Following incubation of thymocytes with dexamethasone alone, the high-density cells separated by Percoll gradients exhibited high blue fluorescence with Hoechst 33342. They also showed extensive DNA laddering (Fig. 2, lane 5) and the distinct morphological features of apoptosis (Fig. 3a) described by other groups [1,4,20]. These thymocytes demonstrated the character-istic chromatin condensation and cytoplasmic contraction of apoptosis. They exhibited a condensed cytoplasm containing apparently normal organelles, apart from dilated cisternae of the smooth endoplasmic reticulum, many of which were fused with
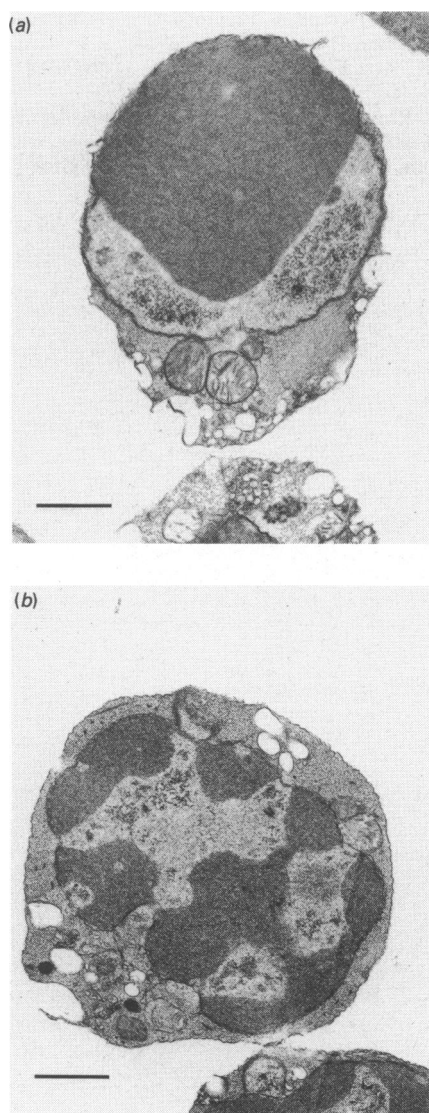
Fig. 3. Cells treated with Zn^{2+} show the earliest morphological changes of apoptosis

Thymocytes treated for 4 h with dexamethasone (0.1 μM) demonstrate the characteristic features of apoptosis (a). Cells treated with this concentration of dexamethasone in the presence of Zn^{2+} showed only the earliest signs of apoptosis (b). Bars represent 1 μm.

the cell membrane. The nuclei were also shrunken, and although the euchromatin retained its normal appearance, the heterochromatin was condensed and usually coalesced against one pole of the nuclear membrane (Fig. 3a).

Strikingly different results were observed in the high-density cells obtained following incubation with dexamethasone in the presence of Zn^{2+}. These thymocytes were also shrunken, with some dilation of the smooth endoplasmic reticulum, but they showed no evidence of DNA laddering (Fig. 2, lane 6). The heterochromatin in these cells was condensed and arranged in several sharply defined clumps which abutted against the nuclear membrane (Fig. 3b). A further clump was present in the centre of many of these nuclei. The cell profiles were mostly regular but the nuclear membrane, although usually intact, was often convoluted. The euchromatin retained its normal density but often included one or more clusters of intensely stained nucleolar remnants. Similar characteristics have been described, in various cell types, as the earliest signs of apoptosis [20,21]. Thus in the presence of Zn^{2+} we appear to have halted the cells at a very early

stage of apoptosis, prior to the effects of the endonuclease. Further support for this conclusion was obtained using cells sorted by flow cytometry. These cells were obtained following treatment with dexamethasone (0.1 μM) in the presence or absence of Zn^{2+} (1 mM) and subsequent incubation with Hoechst 33342.' Normal cells, exhibiting low blue fluorescence, showed no evidence of DNA laddering (Fig. 2, lanes 7 and 9). However, apoptotic cells, exhibiting high blue fluorescence, showed extensive internucleosomal DNA fragmentation following incubation with dexamethasone alone (Fig. 2, lane 8) but not in the presence of Zn^{2+} (Fig. 2, lane 10). The inhibition of DNA laddering by Zn^{2+} may be due to inhibition of endonuclease activity [9,19], but other possible actions of Zn^{2+} cannot be excluded [22].

Thus in the presence of Zn^{2+}, dexamethasone has induced cells which are apoptotic according to a number of criteria including cell density, cell size and morphological features, but which are devoid of DNA laddering. To our knowledge this is the first time, in thymocytes, that a clear dissociation has been observed between the morphological features of apoptosis and DNA laddering. A similar absence has been observed in some cases of programmed cell death, for example in insect metamorphosis and in normal limb development, but marked differences between apoptosis and some other forms of programmed cell death have been emphasized [23]. Originally apoptosis was defined morphologically but it has now begun to be characterized biochemically, in particular by measurement of DNA laddering [4,5,8]. Our results indicate that assessment of apoptosis by biochemical assays alone should be interpreted with caution.

This study supports the hypothesis that the induction of the earliest morphological changes of apoptosis involves enzymes other than the Ca^{2+}/Mg^{2+}-dependent endonuclease and that this endonuclease is involved at later stages, when it is responsible for internucleosomal fragmentation of the DNA [8]. It seems likely that there are several mechanisms responsible for the initiation of apoptosis which may exhibit both cellular and organ specificity [4]. The identification of early key steps in apoptosis is essential to the understanding of this important mechanism of cell death.

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