Differential involvement of DNases in HeLa cell apoptosis induced by etoposide and long term-culture

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Abstract

We have applied to human HeLa cells two different stimuli of apoptosis: the antitumoral drug etoposide, and a more 'physiological' death condition, obtained by growing cells in the same medium for long time periods, for up to 10 days. Analysis of different parameters demonstrated that in both experimental systems the same apoptotic features are visible. However, the DNA degradation pattern appeared to be different, suggesting the involvement of different DNases. In this view, we have analyzed the activity and expression of Ca^{2+} - Mg^{2+} -dependent and acid DNases. We have observed that DNase I is not modulated during apoptosis. In contrast, the acid L-DNase II (derived from Leukocyte Elastase Inhibitor by post-translational modification), recently identified in our laboratory, is mainly active in the apoptotic pathway induced by long term-culture. Furthermore, we have provided evidence that while caspase 3 is activated by both inducers, caspase 1 is essential only for the etoposide-induced apoptosis.

Keywords: apoptosis; DNases; caspases; PARP proteolysis; etoposide

Abbreviations: BSA, bovine serum albumin; CAD, caspase activated DNase; CHO, chinese hamster ovary cells; CPAN, caspase activated nuclease; DAPI, 4',6-diamidino-2-phenylindole; FCS, fetal calf serum; ICAD, caspase activated DNase inhibitor; ICE, interleukin 1 β -converting enzyme; LEI, leukocyte elastase inhibitor; PARP, poly(ADP-ribose) polymerase; PBS, phosphate buffered saline; PFGE, pulsed field gel electrophoresis; SDS, sodium dodecyl sulfate; TAE, tris-acetate EDTA buffer; TCA, trichloroacetic acid; TNF α , tumor necrosis factor

Introduction

Apoptosis is a prevalent phenomenon in complex multicellular organisms. It is characterized by the deletion of scattered cells

of a living tissue without disturbance of the continuity of tissue function. Apoptosis is accomplished by a process with a conserved phenotype, that strongly suggests a common underlying effector mechanism (for a review, see Vaux and Strasser¹). This phenomenon presents a multistep DNA cleavage characterized by appearance of high molecular weight DNA molecules^{2,3} and oligonucleosomal fragments.⁴ The typical ladder was firstly described by Hewish and Burgoyne⁵ who demonstrated the Ca^{2+} , Mg^{2+} -dependent autodigestion of DNA in isolated liver cell nuclei. Based on this observation, it was widely considered that the endonuclease responsible for the internucleosomal DNA cleavage was Ca^{2+} , Mg^{2+} -dependent.

Since, several attempts have been made to identify the DNase(s) involved in apoptosis. A number of cation-dependent endonucleases acting at neutral pH have been characterized, such as in thymocytes, NUC 18/cyclophilin A,⁶ DNase I,⁷ DNase γ ,⁸ and the new 92 KDa DNase.⁹ In human myeloid cell lines, Mg^{2+} -dependent, Ca^{2+} -independent endonucleases have been identified.^{10,11} However, Barry and Eastman¹² described in CHO cells a different endonuclease able to play a role in the apoptotic DNA digestion, that is the acid and non cation-dependent DNase II. This enzyme is activated by intracellular acidification which occurs during apoptosis.^{13,14}

DNA is not the only macromolecule degraded during apoptosis. In fact, several proteins are cleaved by serine and cysteine proteases, which display their activity in the induction or in the execution phases of apoptosis.⁵ Among the targets of cysteine proteases, recently renamed 'caspases',¹⁶ the best described is the nuclear enzyme poly(ADP-ribose) polymerase (PARP), which is converted from the 116 KDa form to 89 and 24 KDa fragments.¹⁷ PARP proteolysis is carried out by caspase 3 family, including CPP32, apopain, Yama, and probably caspase 7 (for a review, see Cohen; Thornberry and Lazebnik^{18,19}). Synthetic peptides inhibiting these caspase activities are widely used to implicate different caspases in different apoptotic pathways.²⁰ For instance the synthetic DEVD peptide is currently used to inhibit caspase 3, while YVAD or ZVAD are commonly used to inhibit the caspase 1 family of proteases, including ICE (interleukin 1 β -converting enzyme). Very recently, a relationship between caspases and endonucleases has been reported, suggesting the existence of a caspase-activated DNase (CAD),²¹ regulated by a specific inhibitor, ICAD.²² A novel caspase-activated nuclease (CPAN) has been also described in Jurkat cells.²³

We have reported that DNase II is implicated in apoptotic DNA degradation occurring during chick lens cell differentiation.²⁴ By a further characterization of this DNase II, we have recently shown that this 27 KDa-peptide is derived, by a post-translational modification, from a precursor of 42 KDa. This precursor, in its native form, is identical to the Leukocyte Elastase Inhibitor (LEI). The anti-

protease activity of LEI is lost during its conversion to active DNase II. As this DNase II is different from other DNases II already described,^{25,26} and it is derived from LEI, we named it L-DNase II and we have demonstrated its involvement during apoptosis.²⁷

The aim of this study was to compare different morphological and biochemical parameters of apoptosis, including DNase activation in cells driven to apoptosis by different stimuli. To do this, we have used the human tumoral HeLa cells either treated with etoposide, a drug inhibiting topoisomerase II and widely used in cancer therapy, or long term-cultured with no medium change, allowing growth factor depletion.

We have evaluated DNase activity and expression. From our data, two pathways of genome cleavage can be distinguished. L-DNase II and Ca^{2+} - Mg^{2+} -dependent DNases, other than DNase I, appeared to be involved in the induction of apoptosis by long term-culture but not in etoposide-induced apoptosis. DNase I contributes for a minor account to DNA cleavage in both apoptotic systems. Moreover, the induction of apoptosis seemed to follow different pathways to activate DNases, L-DNase II being activated by an ICE-independent pathway. However, experiments carried out with the inhibitor of caspase 1 revealed that this enzyme is essential only for the etoposide-induced apoptosis. To investigate the involvement of caspases in HeLa cell apoptosis, we examined PARP proteolysis as a marker of caspase activity. Since the best PARP-degrading enzymes belong to group 3, we argue that caspase 3 is activated during HeLa cells apoptosis.

Results

Morphology of apoptotic cells

The morphology of etoposide-treated and of long term-cultured HeLa cells was analyzed by DNA staining with the fluorochrome Hoechst 33258. Results of a typical experiment are illustrated in Figure 1. After 3 h of exposure to 100 μM etoposide followed by culture in drug-free medium for 3 h (recovery), chromatin fragmentation and condensation were evident in about 8% of cells (Figure 1B). The number of cells with an apoptotic phenotype was increased after a longer recovery period reaching a maximum of 31% (24 h; Figure 1C).

In long term-culture experiments, we found that cells showed a normal nuclear morphology after 4 days of culture (Figure 1D), while typical apoptotic features were observed in 12% of cells after 7 days in the same medium (Figure 1E). This phenomenon was more evident in cell cultures maintained for up to 10 days in the same culture medium (Figure 1F, 43%). As an effect of etoposide treatment followed by 24 h of recovery and of 10 days culture, a fraction of cells detached from the Petri dish and floated. Most of floating cells exhibited apoptotic features, while still adherent cells were characterized by about 5% of apoptosis (not shown). No morphological difference has been observed in apoptotic cells with respect to the inducer of apoptosis.

Figure 1 Analysis of nuclear morphology of HeLa cells treated with etoposide or long term-cultured. Nuclear morphology was analyzed in each different sample by staining with Hoechst 33258. (A) Control cells. (B) Cells treated with 100 μM etoposide for 3 h and incubated with fresh medium for 3 additional hours. (C) Cells treated with 100 μM etoposide for 3 h and further incubated with fresh medium for 24 h. (D–F) HeLa cells cultured without changing of the medium for up to 4 days (D), 7 days (E) or 10 days (F)

Flow cytometry

Cells showing a DNA content lower than the 2C, which is typical of the G_1 peak, were defined as cells in the sub G_1 - A_0 region. Figure 2 illustrates the cell cycle distribution of control cells (Figure 2A), of cells treated for 3 h with 100 μM etoposide and further incubated for 24 h in fresh medium (Figure 2B) and of cells cultured up to 9 days without changing of the medium (Figure 2C). The effect of drug-treatment analyzed by flow cytometry revealed the appearance of propidium iodide-stained DNA in the sub- G_1 region, corresponding to cells showing DNA fragmentation or condensation. No evidence of material typical of necrotic cells was observed. As indicated in panel B, the A_0 region accounted for about 40% of total DNA.

As the age of the culture increased from 4 to 9 days, the percentage of cells in A_0 phase was considerably

Figure 2 Flow cytometry analysis of HeLa cells treated with etoposide or long term-cultured. DNA was stained with propidium iodide and its content determined by flow cytometry. Data are expressed as the percentage of cells in each phase and represent the average of two independent experiments. (A) Control cells. (B) Cells treated with 100 μ M etoposide for 3 h and incubated with fresh medium for 24 additional hours. (C) HeLa cells cultured in the same medium for up to 9 days

higher, reaching about 31% at 7 days (not shown) and 55% at 9 days (C). The presence of cells in the sub-G₁ region indicates that apoptotic DNA degradation and/or condensation occurred in long term-cultured cells as well as in cells incubated with etoposide. The fraction of cells in the A₀ region is higher than the number of cells with apoptotic phenotype; this discrepancy can be explained by an overestimation of cells in the A₀ region. In fact, in the A₀ peak early apoptotic cells (which cannot be easily visualized under light microscopy) as well as fragmented cells may be included. The comparison between the DNA content profiles obtained from etoposide-treated and long-term cultured cells, showed, in both samples, a marked increase of cells in the sub G₁ region. However, it is evident that the topoisomerase inhibitor interferes with the cell cycle by inducing an accumulation of cells in the S/G₂ phases. In contrast, growth factor deprivation caused a clear cell loss from the G₁ and G₂ compartments. Although the number of cells present in the S-phase was not significantly different from control samples, these cells are also committed to die, as indicated by their decrease in light scatter (not shown).

DNA fragmentation

To compare whether two different apoptotic stimuli could induce, in HeLa cells, the same pattern of DNA fragmentation, we analyzed DNA size, using pulsed field (PFGE) and conventional gel electrophoresis.

HeLa cells were treated with 100 μ M etoposide for 3 h and harvested either immediately or after 3 and 24 h of recovery in fresh medium. DNA was analyzed by PFGE and, in parallel, by conventional gel electrophoresis. As shown in Figure 3A, a 3 h-etoposide treatment was sufficient to produce high molecular weight DNA molecules ranging from 600 to 50 Kb (lane 2). These molecules were still present, even at lower extent, in samples harvested after a 3 h-recovery in drug-free medium (lane 3) and disappeared after prolonged recovery periods (24 h, lane 4). DNA extraction from etoposide-treated cells revealed a ladder (Figure 3A, lanes 5–8) whose intensity was lower in

cells harvested immediately after drug administration (lane 6), while it increased proportionally with recovery time periods (lanes 7 and 8, corresponding to 3 and 24 h, respectively).

We extended DNA analysis to cells maintained in culture for increasing times for up to 10 days in the same medium (Figure 3B). In this case, PFGE analysis showed detectable high molecular weight DNA fragmentation with a kinetics different from etoposide. As illustrated in Figure 3B, DNA molecules of high molecular weight were slightly visible after 7 days of cell culture (lane 2) and were followed later on by the appearance of DNA molecules of size lower than 50 Kb (lane 3). In the same cultures, the pattern of internucleosomal DNA fragmentation, visible as fragments multiple of about 180 bp appeared after 7 days in culture (lane 6). The intensity of DNA ladder was clearly increased after a longer treatment (9 days), suggesting a time-dependent DNA degradation (lane 7).

PARP proteolysis and activity

Poly(ADP-ribose) polymerase (PARP) is the best described target of proteolysis during apoptosis.¹⁸ Extracts from drug-treated or long term-cultured cells have been analyzed by Western blot with the monoclonal antibody C-2-10 directed against an epitope located within the catalytic domain and the DNA binding domain, which is able to recognize the intact PARP and its 89 KDa fragment. The analysis has been performed on still viable, adherent cells, and on floating cells harvested both after etoposide treatment and long term-culture. The analysis of attached and floating cells by microscopy after DAPI staining showed a significant difference ($P < 0.05$) in the number of apoptotic cells. Apoptotic cells were mostly seen in the floating fraction (Table 1).

A typical result of Western blot for PARP is shown in Figure 4. It is evident (Figure 4A) that in the attached cell population only the 116 KDa molecule is visible. However, when the analysis was performed on the floating apoptotic cell fraction, harvested after etoposide treatment or long

Figure 3 Analysis of high molecular weight DNA fragments and internucleosomal DNA cleavage. **(A)** HeLa cells treated with etoposide. Lanes 1–4, DNA analyzed by PFGE; lanes 5–8: conventional agarose gel electrophoresis. Lanes 1 and 5: control cells, lanes 2 and 6: cells treated with 100 μ M etoposide for 3 h; lanes 3 and 7: cells treated with 100 μ M etoposide for 3 h followed by 3 h of recovery in fresh medium; lanes 4 and 8: cells treated with 100 μ M etoposide for 3 h followed by 24 h of recovery in fresh medium. **(B)** Long term-cultured HeLa cells. Lanes 1–3, DNA analyzed by PFGE; lanes 4–6: conventional agarose gel electrophoresis. Lanes 1 and 5: cells incubated in the same medium for 4 days; lanes 2 and 6: cells incubated in the same medium for 7 days; lanes 3 and 7: cells incubated in the same medium for up to 9 days. Lane 4: molecular weight standards. PFGE MW markers: 21 successive concatamers of lambda DNA. The lowest band represents 48.5 Kb. Ladder MW markers: molecular weight marker IV DNA, the lowest band represents 180 bp

term-culture, intact PARP was no longer evident, being converted to its 89 KDa fragment.

The autoribosylation capacity of PARP analyzed by activity gel revealed that the intact protein displays a very high activity (Figure 4B, attached cells). The full conversion of PARP in floating cells was also proved by the faint autoradiographic band shown by the activity gel autoradio-

Table 1 Apoptotic index of attached and floating HeLa cells, treated with etoposide or cultured without changing the culture medium

Treatment	Cell number (%)		Apoptotic index (%)	
	Attached cells	Floating cells	Attached cells	Floating cells
Etoposide+recovery				
3 h + 3 h	90.8 $\pm$ 1.0	9.1 $\pm$ 1.0	4.3 $\pm$ 0.2	10.1 $\pm$ 0.6
3 h + 24 h	75.3 $\pm$ 1.0	24.7 $\pm$ 1.0	26.0 $\pm$ 1.5	49.1 $\pm$ 1.9
Long term culture (days)				
5	90.0 $\pm$ 1.0	9.5 $\pm$ 1.0	2.4 $\pm$ 0.3	5.6 $\pm$ 0.4
6	77.3 $\pm$ 1.7	22.7 $\pm$ 1.7	2.5 $\pm$ 0.4	7.5 $\pm$ 0.4
7	68.3 $\pm$ 3.3	31.7 $\pm$ 3.3	8.8 $\pm$ 0.9	23.3 $\pm$ 1.4
8	30.0 $\pm$ 2.4	70.0 $\pm$ 2.3	27.0 $\pm$ 0.7	73.0 $\pm$ 2.7

HeLa cells were treated for 3 h with 100 μ M etoposide and further incubated for 3 or 24 h in fresh medium. For long term-culture experiments, cells were grown in the same medium for increasing periods of time, for up to 8 days. Attached and floating cells were recovered separately and counted by a hemocytometer. To evaluate the amount of apoptotic cells, samples were stained with Hoechst 33258. The number of apoptotic cells over the total cell number represents the apoptotic index which is expressed as percentage. Statistical analysis was applied to the data from three independent experiments

graphy (Figure 4B). These data strongly support the involvement of caspase 3 and/or 7 in both apoptotic conditions, leading to the complete proteolysis and, by consequence, to the inactivation of PARP.

DNase activation

We have shown that in HeLa cells, different apoptotic stimuli led to DNA degradation. To verify if the same DNase was implicated, we measured the activity of DNases which have already been reported to play a role during chick lens apoptosis, e.g. DNases Ca^{2+} - Mg^{2+} -dependent, acid DNases, and Mg^{2+} -dependent DNases (Figure 5A). In etoposide-treated cells, we observed an overall decrease in the enzyme specific activity. In contrast, in apoptosis induced by long term-culture we found an increase of both Ca^{2+} - Mg^{2+} -dependent and acid DNases. For Ca^{2+} - Mg^{2+} DNases, a maximum of activity is observed at day 6, while the activity of acid DNase is still increasing (not shown). To further elucidate this phenomenon, we analyzed separately the two cell populations resulting from the apoptosis induction: the attached cells, which were enriched in living cells, and the floating cells, which were enriched in apoptotic cells (Figure 5B). In both populations, we found an increase of the DNase activities starting at 4 days of culture and reaching a plateau until day 8. In floating cells, the activity exhibited a maximum at day 6.

DNase expression

To further investigate the activation of specific DNases in apoptosis, we followed the expression of two DNases using immunochemical techniques. Ca^{2+} - Mg^{2+} -dependent DNases were investigated using a commercial available antibody directed against DNase I. DNase I from HeLa cells was not recognized in Western blot (not shown). Acid endonucleases were followed using a polyclonal antibody to L-DNase II prepared in our laboratory.²⁴ Anti-L-DNase II showed a major band of about 25 KDa (Figure 6), the expected size for human DNase II, which presents an apparent molecular weight lower

than porcine DNase II, used as control. The intensity of this 25 KDa band did not change in etoposide-treated cells. In contrast, it was increased in long term-cultured cells, reaching a peak after 6 days of culture. It is interesting to note the presence of an immunoreactive band of 42 KDa in samples harvested at day 4 by trypsinization (attached cells). The molecular weight of this immunoreactive band corresponds to the molecular weight of leukocyte elastase inhibitor, the precursor of L-DNase II, which we had recently cloned.²⁷

The expression and subcellular localization of these enzymes, were further assessed by immunocytochemistry. Anti-DNase I and anti-L-DNase II reaction was developed using an anti-rabbit IgG-rhodamine isothiocyanate-conjugated. In the same samples, HeLa cell nuclear morphology was visualized by DAPI staining (Figure 7). No difference was observed in the expression of DNase I or L-DNase II between living and apoptotic cells (arrows) when apoptosis was induced by the topoisomerase inhibitor, etoposide.

Figure 4 Analysis of PARP proteolysis by Western blot (A) and of PARP autoribosylation by activity gel (B). For each experimental point, from HeLa apoptotic cell cultures two fractions were harvested: adherent cells (A) and floating cells (F). To induce apoptosis, cells were treated for 3 h with 100 μ M etoposide and further incubated for 24 h in fresh medium (*etoposide*) or cultured for up to 9 days in the same medium (*long term*). The bands of 116 and 89 KDa represent intact and the proteolytic fragment of PARP, respectively

Figure 5 DNase activity of HeLa cell induced to die with etoposide (A) or by long term-culture (B). One μ g of ³H-labeled DNA was incubated in the presence of HeLa cells extracts for 30 min at 37°C. Different buffers were used to measure different DNases: 10 mM Tris-HCl, pH 7.4 containing 10 mM CaCl₂, 10 mM MgCl₂ (Ca²⁺, Mg²⁺-dependent DNases); 10 mM Tris, 10 mM EDTA, pH 5.5 (acid DNases); 10 mM Tris-HCl, 5 mM MgCl₂, 2 mM EGTA, pH 7.6 (Mg²⁺-dependent DNases). Undigested DNA was TCA-precipitated and measured with a β scintillation counter. Etoposide-treated cells were incubated with 100 μ M etoposide for 3 h and further incubated in drug-free medium for 3 h. For long term-cultured cells, adherent and floating cells were analyzed separately. DNase specific activity was expressed as ng of DNA digested/minute/mg of proteins

Figure 6 Western blot analysis of L-DNase II in HeLa cells induced to apoptosis by etoposide (100 μ M, 3 h, followed by 3 h of recovery) (A) or long term-culture (8 days) (B). Adherent (A) and floating (F) cells were recovered and analyzed separately. Proteins from HeLa cells (100 μ g) were separated using 12% acrylamide slab gel, transferred to Immobilon P and developed using anti-L-DNase II. L-DNase II was used as a positive control. Purified DNase I was also loaded on the gel as a negative control. White arrow indicates the 25 kDa active L-DNase II. Grey arrow indicates the 42 kDa precursor of L-DNase II

Similar results were obtained for DNase I, when apoptosis was induced by long term-culture. In contrast, the induction of apoptosis by long term-culture showed apoptotic cells strongly labeled by anti-L-DNase II. Notably, the labeling was not confined to the cytoplasm but was extended also to the nucleus of the cells. Taken together, these results indicate that DNase II and Ca^{2+} - Mg^{2+} -dependent DNases, other than DNase I, are activated in HeLa cells when apoptosis is induced by long term-culture but not when it is induced with etoposide.

Effect of caspase inhibitors

To address whether the different expression of DNases might reflect the activation of two different apoptotic pathways or whether different enzymes might be recruited by the same activating pathway, we investigated the effect on apoptosis induced by etoposide or long term-culture of the synthetic peptide YVAD, used as an inhibitor of group 1 caspases. The expected effect of YVAD on TNF- α -induced apoptosis has been verified (not shown). Drug-induced apoptosis was very sensitive to 20 μ M YVAD (Figure 8A), while apoptosis induced by long term-culture was resistant to this inhibitor, even when higher concentrations of YVAD (60 μ M) were used (Figure 8B). It is worth noting that in the presence of 60 μ M YVAD the percentage of apoptotic cells decreased slightly. However, higher concentrations of YVAD (up to 100 μ M) did not enhance significantly this phenomenon (not shown). This result might reflect the activation of different pathways, leading to the activation of different endonucleases.

To verify this hypothesis, we measured the DNase activity of apoptotic floating cells after 6 days of culture in the presence or in the absence of 60 μ M YVAD (Figure 8C). In the absence of YVAD, both Ca^{2+} - Mg^{2+} -dependent

and acid endonucleases are activated. In contrast, in the presence of YVAD, only Ca^{2+} - Mg^{2+} -dependent endonucleases are inhibited but acid DNases are not sensitive to this inhibitor.

To investigate the correlation between caspase 1 and 3 activities, we have evaluated PARP proteolysis in both etoposide-treated and long term-cultured cells treated with YVAD. Western blot analysis revealed that PARP proteolysis occurs independently of caspase 1 inhibition (not shown).

Discussion

It is currently believed that different stimuli activate a common apoptotic pathway leading to the activation of degradative enzymes which kill the cell.^{28,29} These activities are presumed to be identical for all cell lines and all apoptotic stimuli. This assumption is based on morphological results which revealed similar features for apoptotic cells independently on the inducer. This is also the case of DNA ladder visualized by agarose gel electrophoresis or of cellular DNA content analyzed by flow cytometry.

Human cultured cells have been widely used to elucidate relevant features of the apoptotic process induced by several chemicals.^{30,31} To analyze the pattern of death induction by different stimuli in the same cell line, we have performed a set of experiments on HeLa cells induced to die by two ways: (i) etoposide administration, which is widely described apoptogenic treatment^{32,33} and (ii) long term-culture.^{34,35}

Microscopical observation indicated that, during apoptosis induction by different ways, cells tend to detach from the solid support, to float in the medium and to show typical apoptotic markers. Morphological studies and internucleo-

somal DNA degradation analysis revealed common features in etoposide-treated and long term-cultured HeLa cells. These features are not dependent of the cell cycle period in which apoptosis is induced as demonstrated by flow cytometry. Moreover, caspases 3 and 7 are activated in both systems, as demonstrated by PARP proteolysis. Remarkably, cleaved PARP is present almost exclusively in

floating cells, which are, by consequence, devoid of PARP activity. We can state that this cell population, isolated both after etoposide administration and long term-culture as an effect of loss of cell adhesion,³⁶ is enriched in apoptotic cells.

The study of DNA fragmentation by pulsed field gel electrophoresis showed in long term-cultures a degradation

etoposide

anti-DNase I

anti-DNase II

long term-culture

anti-DNase I

anti-DNase II

Figure 7 Immunocytochemistry of HeLa cells induced to apoptosis with etoposide (100 μ M, 3 h, followed by 3 h of recovery) or long term-culture (8 days), using polyclonal antibodies to DNase I or L-DNase II. Cells were seeded on glass coverslips, cultured and induced to die. Nuclei were stained with DAPI. Anti-DNase I and anti-L-DNase II were localized using a secondary antibody coupled to rhodamine-isothiocyanate. Scale bar=15 μ m

Figure 8 Effect of YVAD on HeLa cell apoptosis and DNase activity. HeLa cells were induced to apoptosis with etoposide (**A**) or long term-culture (**B**), in the presence or in the absence of YVAD. Apoptotic and living cells were counted after DAPI staining. (**A**) control: apoptotic index of control cells, YVAD: apoptotic index of cells incubated with 20 μ M YVAD. *Etoposide*: cells cultured in the presence of etoposide (100 μ M, 3 h, followed by 3 h of recovery). *Etoposide+YVAD*: cells preincubated for 30 min with 20 μ M YVAD, cultured in the presence of both 100 μ M etoposide and 20 μ M YVAD and maintained in the presence of YVAD for further 3 h. (**B**) HeLa cells were cultured in the same medium for up to 8 days in the presence or in the absence of different concentrations of YVAD. (**C**) Activity of DNases in HeLa cells induced to die by long term-culture (6 days) in the presence or in the absence of 60 μ M YVAD. Extracts from floating cells were incubated with 1 μ g of ³H-labeled DNA for 30 min at 37°C in 10 mM Tris-HCl, pH 7.4 containing 10 mM CaCl₂, 10 mM MgCl₂ (Ca²⁺, Mg²⁺-dependent DNases); or in 10 mM Tris, 10 mM EDTA pH 5.5 (acid DNases). Undigested DNA was TCA-precipitated and measured with a β scintillation counter. DNase specific activity was expressed as ng of DNA digested/minute/mg of proteins. Results of a representative experiment are shown

pattern different from etoposide-treated cells, the latter system showing DNA fragments of 600 Kb certainly produced by the inhibition of topoisomerase II itself. The different degradation pattern may be ascribed to the activation of distinct endonucleases. To verify this hypothesis, we measured DNase activity and expression in both the experimental systems. During etoposide-induced apoptosis, we found no activation of the DNases to date believed to be implicated in apoptosis,^{6-12,24} whereas we observed that the induction of apoptosis by long term-culture activated both Ca²⁺-Mg²⁺-dependent DNases and acid DNases. No increase in DNase I expression was noted, suggesting that Ca²⁺-Mg²⁺-dependent DNases other than DNase I are implicated in this phenomenon, as already reported by other authors.³⁷ In contrast, as clearly visible in Western blot, the level of the active form of the acid L-DNase II (25 KDa) was greatly enhanced. This observation is in agreement with the increase in enzyme activity observed in extracts from cells maintained in culture for up to 6 days. These results are also supported by the immunocytochemical data which show a strong increase in L-DNase II labeling in long term culture-induced apoptosis. Note that cells not showing an apoptotic morphology are also labeled. In fact, Western blot analysis revealed also that after 4 days of cell culture, the attached cells synthesize the precursor of L-DNase II,²⁷ a protein of 42 KDa, which is not visible in floating apoptotic cells showing all the precursor molecules converted to the active DNase.

Under these experimental conditions, the induction of apoptosis by long term-culture may occur in individual cells by different stimuli: metabolic stress produced by deprivation of nutrients and of growth factors, or synthesis of cell death molecules, like TNF- α . It has been already shown that in different cell lines, including HeLa, the induction of apoptosis by TNF- α activates Ca²⁺-Mg²⁺-dependent DNases.³⁸ This event is inhibited by the caspases inhibitor YVAD.³⁹ Under our experimental conditions, the use of this tetrapeptide strongly inhibits etoposide-induced apoptosis but, when used at the same concentration (20 μ M), has little effect on apoptosis induced by long-term culture. With higher concentrations of YVAD, up to 100 μ M, a slight inhibitory effect can be noted. This phenomenon might reflect the existence of different pathways of apoptosis activated in different individual cells, depending on the nature of the apoptotic stimulus.

The activity of the Ca²⁺-Mg²⁺-dependent DNase is strongly inhibited in YVAD-treated cells while L-DNase II activity is not affected. This indicates that long term-culture activates in different individual cells different pathways. Likely, a pathway promoting the activation of Ca²⁺-Mg²⁺-dependent DNases is mediated by group 1 caspases while another, leading to the activation of L-DNase II, is independent on caspase 1.

The existence of distinct endonucleases activated by different stimuli has been already shown, as alternative pathways, in cells defective in one of them.⁴⁰ Our results show that the same cell line is able to activate different apoptotic pathways even if it is not defective in one enzyme, according to the apoptotic inducer.

From our data, caspase 3 and 7 seem to be activated in etoposide-treated and long-term cultured cells, while caspase 1 activity seems not to be involved in long term-culture induced apoptosis, at least in the cells that activate L-DNase II. However, caspase 3 is considered to be activated downstream of caspase 1.^{41–44} This suggests that this pathway is still activated in the cell but it is not the pathway that actually kills the cell, perhaps because it is not sufficient to mediate apoptosis in this metabolic state, as it has already been suggested in other cell lines.⁴⁵ Alternatively, caspase 3 may be activated by a caspase 1-independent pathway, also involved in the activation of L-DNase II. This question is under current investigation.

Taken together, our results suggest the existence in the same cell line of diverse apoptotic pathways which can be achieved by different stimuli. These observations argue against the model of a single central apoptotic pathway within the cell. Moreover, our data demonstrate the involvement of L-DNase II in cell death by apoptosis occurring under 'physiological' conditions.

Materials and Methods

Materials

Etoposide was obtained from Bristol Italiana; low melting agarose, 4',6-diamidino-2-phenylindole (DAPI) and Hoechst 33258 were purchased from Sigma; RNase A and RNase T₁, proteinase K and DNA molecular weight marker VI were from Boehringer. Lambda DNA ladder PFGE marker was from New England Biolabs. Agarose for conventional and pulsed-field gel electrophoresis were from Pharmacia and Sigma, respectively. Nitrocellulose and pre-stained molecular weight markers for electrophoresis were from BioRad. The scintillation liquid was Luma Safe from Packard. Immobilon P and GF/C filters were obtained from Millipore. TRITC-conjugated and peroxidase-conjugated goat anti-rabbit IgG were from Biosys. Alkaline phosphatase-conjugated goat anti-mouse IgG and BCIP-NBT revelation system were purchased from Promega. Tumor necrosis factor α (TNF α) was obtained from Genzyme. The tetrapeptide ICE-inhibitor, Ac-YVAD-CHO was from Calbiochem. Purified DNase I and II were from Sigma and Worthington, respectively. Polyclonal antibody against DNase I was from Rockland. Polyclonal antibody against L-DNase II was prepared in our laboratory²⁴ as well as the labeled calf thymus DNA (Pharmacia) used in DNase activity assay. Monoclonal antibody C-2-10 against poly(ADP-ribose) polymerase, which recognizes an epitope located between the DNA binding domain and the automodification domain, was provided by Dr. Guy Poirier, Université Laval, Québec. Deoxy [8-³H] adenosine 5' triphosphate, ammonium salt (code TRK 34, 777 GBq/mmol) was from Amersham, ³²P-NAD (800 Ci/mmol) was a NEN-Life Science product.

Cell culture

HeLa S3 cells were grown as monolayer in Dulbecco's modified Eagle's medium (D-MEM, GIBCO-BRL) supplemented with 10% fetal calf serum (FCS; HyClone, Logan, UT, USA), 4 mM glutamine, 2 mM sodium pyruvate, 100 U/ml penicillin and 0.1 mg/ml streptomycin (all from GIBCO-BRL). Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂.

Cell treatment

HeLa cells were seeded at a concentration of 3×10^5 cells/ml, maintained in culture for 3 days and then treated for 3 h with 100 μ M etoposide. Thereafter, cells were washed with Phosphate Buffered Saline (PBS) to remove the drug, and harvested immediately or maintained in culture with fresh medium for different time periods (3 h and 24 h). For long term-culture experiments, HeLa cells were trypsinized and seeded at 1×10^5 /ml, maintained in culture without changing the medium and harvested at different times up to 10 days. The fraction of cells detached from the solid support at the end of each treatment was recovered and further analyzed separately from still adherent cells.

Analysis of cell morphology

Apoptotic cells were identified through microscopic observation of samples stained with Hoechst 33258. The analysis was performed directly on HeLa cells previously seeded on glass coverslips. For the fraction of HeLa cells detached at the end of the treatments, a further step of cytocentrifugation on glass slips at 500 r.p.m. for 3 min in PBS containing 10% FCS at a concentration of 10^6 cells/ml was applied. Cells were then fixed for 10 min with ice-cold 70% ethanol and then washed several times with ice-cold PBS. DNA was stained with 0.1 μ g/ml Hoechst 33258 for 10 min at room temperature. The number of apoptotic cells was evaluated through fluorescence observation using a Leitz Orthoplan microscope equipped with a 50X objective. For each sample, 500 cells were counted. Pictures were obtained using Kodak Tmax (400 ASA) b/w film.

For immunofluorescence experiments, HeLa cells were seeded on coverslips at a concentration of 10^5 cells/ml, maintained in culture for 3 days and treated as above. In some experiments, cells still adherent to the solid support and floating cells were harvested separately. Floating cells were fixed in paraformaldehyde and spread on a glass coverslip before immunological analysis.

Flow cytometry

Flow cytometric detection of DNA fragmentation was performed on cells fixed in 70% ethanol. Samples were stained for 30 min with 50 μ g/ml propidium iodide in 10 mM Tris-HCl, pH 7.4 containing 100 U/ml RNase A. Measurements and further analysis were performed with a Coulter Epics XL (Coulter, CO, USA) flow cytometer. Ten thousand cells were measured for each sample.⁴⁶ Percentage of cells in each phase was calculated by using the software provided with the instrument.

Analysis of DNA fragmentation

Cells were rinsed twice in cold PBS containing 5 mM EDTA. To analyze high molecular weight DNA fragmentation, pulsed-field gel electrophoresis was applied.⁴⁷ 2.5×10^6 cells were embedded in agarose plug as previously described.³⁵ The run was performed by a CHEF-DR11 electrophoresis system (BioRad, Hercules, USA) at 14°C in $0.5 \times$ TAE buffer with ramped switch time from 20–40 s over 20 h. Constant voltage (160 volts) was applied throughout the total run time. Gel was stained with 0.5 μ g/ml ethidium bromide in $0.5 \times$ TAE and DNA was visualized by UV transillumination. Twenty-one successive concatamers of lambda DNA were used as marker, the lowest band corresponding to 48.5 Kb. To visualize internucleosomal DNA fragmentation, genomic DNA was extracted from 10^7 cells as described by Herrmann *et al.*⁴⁸ with minor modifications⁴⁹ and analyzed by conventional gel electrophoresis.

Analysis of PARP proteolysis and autoribosylation

Extracts from still adherent and floating cells were prepared according to Shah *et al.*⁵⁰ and were further used for the analysis of the structural and catalytic properties of PARP, by Western blot and activity gel respectively.

For Western blot analysis, an equal amount of cells was loaded onto a 7.5% SDS-polyacrylamide gel and electrophoresed under standard conditions. Proteins were transferred onto a nitrocellulose membrane according to Towbin *et al.*⁵¹ The incubation with C-2-10 monoclonal antibody against PARP, diluted 1:10 000 in PBS containing 10% newborn calf serum and 0.2% Tween-20, was carried out for 3 h at r.t. and was followed by the incubation with the secondary antibody conjugated with alkaline phosphatase. Visualization of the immunoreactive peptides was performed using a BCIP/NBT color development substrate according to the manufacturer's protocol.

The PARP activity gel procedure was carried out on the same extracts analyzed by Western blot. The protocol implies SDS-PAGE, *in situ* renaturation of proteins, incubation of the gel with ³²P-NAD, removal of non-incorporated substrate and autoradiography.^{52,53} The radioactive band at 116 KDa represents automodified PARP.

Preparation of HeLa extracts for DNase activity measurements

HeLa cells (6×10^7) were homogenized in ice in 2 ml of 10 mM Tris-HCl, pH 7.4 containing 1 M NaCl, using a Potter homogenizer, and disrupted in a sonication bath for 15 min. The homogenate was then centrifuged ($10\,000 \times g$, 4°C) for 30 min. The supernatant was dialyzed overnight against 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 at 4°C, overnight. Protein concentration was evaluated with the BCA method (Pierce), using bovine serum albumin (BSA) as standard.

Measurement of DNase activities

Acid DNase II activity was measured in 200 μ l of 10 mM Tris-HCl buffer containing 10 mM EDTA, pH 5.75. Ca^{2+} - Mg^{2+} -dependent DNase activity was measured in 200 μ l of 10 mM Tris-HCl, pH 7.4 containing 10 mM CaCl_2 and 10 mM MgCl_2 , while Mg^{2+} -dependent DNases were measured in 10 mM Tris, 2 mM EGTA, 5 mM MgCl_2 . The reaction, started with 1 μ g ³H-DNA, was allowed to proceed for 30 min at 37°C, then stopped by precipitation for 1 h at 0°C with 200 μ l of 10% trichloroacetic acid (TCA) containing 1% sodium pyrophosphate (PP_i). The non digested DNA was then collected on a GF/C filter previously embedded in 1% PP_i , rinsed five times with 2 ml of 5% TCA containing 1% PP_i , twice with 2 ml of 100% ethanol and air dried. Radioactivity was quantified in a beta scintillation counter.

Immunoblotting detection of DNases

Protein samples for electrophoresis were prepared according to Laemmli.⁵⁴ Gel electrophoresis was conducted at a constant current of 20 mA using 12% polyacrylamide slab gels. Prestained high molecular weight markers (Biorad) were used for gel calibration. Transfer of proteins to Immobilon P membranes was carried out electrophoretically.⁵¹ A constant voltage of 40 V was applied for 16 h. Immediately after blotting the membranes were soaked for 2 h at 37°C in PBS containing 5% fat free dried milk. Thereafter, the blot was incubated for 1 h at room temperature with anti-DNase I antibody (80 μ g/ml) or anti-L-DNase II antibody diluted (1/150) in PBS containing 0.5% fat free dried milk (PM buffer). Controls are incubated with an equivalent dilution of rabbit preimmune serum. The binding of the antibodies was visualized after two washings (10 min each) with PM buffer, followed by 1 h incubation with peroxidase-conjugated goat

anti-rabbit IgG (1 μ g/ml) in PM buffer. The sheets were then rinsed successively with PM buffer (2×5 min), PBS containing 0.1% Tween 20 (2×5 min) and finally with PBS (2×5 min). Peroxidase activity was detected using the ECL method (Amersham).

Immunohistochemical analysis of DNases

Cells were fixed in 3% paraformaldehyde for 10 min, washed in PBS and permeabilized with 0.3% Triton X-100 in PBS for 30 min. After washing with BPS, samples were saturated for 30 min at room temperature with PBS containing 1% milk and then incubated for 1 h at room temperature with the specific antisera (diluted 1/150). This incubation was followed by five washings for 5 min in PBS-milk. The immunological reaction was visualized using a TRITC-conjugated goat anti-rabbit IgG diluted 1/200 in PBS-milk (1 h, room temperature). Cells were then washed five times for 5 min in PBS and nuclei were stained with the fluorescent nuclear stain 4',6-diamidino-2-phenylindole (DAPI). Coverslips were then mounted with PBS containing 50% glycerol. Fluorescence was examined under a Leitz Aristoplan microscope equipped with an epi-illuminator HBO and filters for Rhodamine and DAPI fluorescence. Samples were photographed using an Ilford HP5 film (400 ASA).

Inhibition of apoptosis with YVAD

Cells were pre-treated with 20 μ M YVAD for 30 min before etoposide administration (etoposide, 100 μ M for 3 h) in the presence of 20 μ M YVAD. After drug removal, fresh medium was further supplemented with 20 μ M YVAD. Twenty-four hours later, cells were harvested and apoptotic features were quantified by DAPI or Hoechst staining. For long term-culture experiments, increasing concentrations of YVAD (20–100 μ M) were added to the culture medium from day 4 to day 8. As a control of YVAD inhibition of apoptosis, cells were treated in independent experiments with 0.1–50 ng/ml $\text{TNF}\alpha$ for 72 h.

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