# Apoptosis of mouse liver nuclei induced in the cytosol of carrot cells

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**Abstract We report here the apoptosis of mouse liver nuclei induced in the cytosol of carrot cells by cytochrome *c*. Several typical characteristics of apoptosis, such as chromatin condensation, margination and apoptotic bodies, were detected. The result of DNA gel electrophoresis showed that DNA was degraded into nucleosomal fragments. The terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labelling procedure was also performed to detect the breakage of 3′-OH ends of a DNA strand. Furthermore, we found that nuclear lamins were degraded from 88 kDa and 66 kDa to 37 kDa and 47 kDa fragments. The DNA fragmentation could be inhibited by AC-DEVD-CHO and AC-YVAD-CHO. The results indicate that the apoptosis in plant cells may share some similar pathways to apoptosis in animal cells.**



*Key words:* Apoptosis; Carrot cell; Cytosol; Nucleus; Lamin

## 1. Introduction

Programmed cell death is vital for both the normal development and maintenance of many tissues in multicellular organisms [1]. A form of programmed cell death, apoptosis, proceeds with morphological and biochemical characteristics that include chromatin condensation, margination, apoptotic bodies formation and DNA fragmentation [2,3]. Previous studies indicated that the morphological changes were due to series of biochemical reactions and caspases play a key role in this procedure. As an important component, cytochrome *c* is essential for the activation of caspase [4–6].

Present studies have shown that apoptosis also occurs in plants, which can be induced by many agents and environmental changes [7–9]. In the process of a hypersensitive reaction and stress reactions, apoptosis has been observed frequently [10,11]. However, the study on apoptosis in plants is still at its initial stages and the underlying mechanism remains unclear. One fundamental but maybe the most important question is whether apoptosis in plant cells shares a similar pathway with apoptosis in animal cells. It involves the possible signal transduction pathways which decide which cell is committed to die and the possible apoptotic execution machine, which leads to the process of apoptosis. In animal cells, these mechanisms have been studied thoroughly [12–14], while the progress in plant cells lags far behind. The main reason is that it is difficult to obtain large quantities of apoptotic cells from a plant [15]. Thus, it will be very useful if we can have an in vitro system just like that in animals which has promoted the study on the mechanism of apoptosis in animal cells dramatically [4,5,16–19]. In 1993, Lazebnic et al. introduced a cell-free system in the study of apoptosis firstly [20]. We have also developed a cell-free system of apoptosis based on the egg extracts of *Xenopus laevis* in our previous work [21]. In the present study, a high rate of apoptosis has been induced in mouse liver nuclei. The present paper employed a new cell-free system based on the cytosol of plant cells to study apoptosis. The cytosol extract was prepared from suspensions of a carrot cell culture. By using cytochrome *c*, nuclei of mouse liver cells were induced into apoptosis. The typical changes of apoptosis, such as chromatin condensation, margination, apoptotic bodies and a DNA ladder, were detected. A terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labelling (TUNEL) assay was used to detect the breakage of DNA in apoptotic nuclei. Further study showed that nuclear lamins were degraded into 37 kDa and 47 kDa fragments. On the other hand, the appearance of the DNA ladder can be inhibited by two specific inhibitors of caspases, *N*-acetyl-Asp-Glu-Val-Asp-Ala (AC-DEVD-CHO) and *N*-acetyl-Try-Val-Ala-aspartinal (AC-YVAD-CHO). The results suggested that the apoptosis in plants may share some similar pathways with apoptosis in animal cells.

## 2. Materials and methods

### 2.1. Preparation of carrot protoplasts and cytosol extraction CS-100

About 5 g of 5–7 days suspension cultured carrot cell (grass weight) was added to 10 ml enzymatic buffer (2% (w/v) cellulase, 0.5% macerozyme, 5 mmol/l 4-morpholinethansulfonsaure (MES), 6.8 mmol/l $CaCl_2$, 11 mmol/l $KH_2PO_4$, 0.6 mol/l mantiol, 0.4% polyvinylpyrrolidone, pH 5.8), incubated in a shaker incubator with an orbiting speed of 50–100 rpm at 28°C for 2 h, it should be kept in darkness. The protoplasts were collected by centrifugation at $120\times g$ for 5 min. The sediment was washed twice with washing buffer (0.2 mol/l sorbitol, 0.2 mol/l mannitol, 0.05 mol/l MES, pH 5.8–6.0) and then re-suspended with 0.6 M sucrose buffer. The floating protoplasts were centrifuged at $120\times g$, washed twice with washing buffer and suspended in buffer A (20 mmol/l HEPES-KOH, 10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT). The protoplasts were homogenized appropriately, aprotinin and leupeptinin were added to the buffer at a final concentration of 6 mg/ml and 8 mg/ml, respectively. After centrifugation at $100\,000\times g$ (Beckman TLS-55) at 4°C for 2 h, the soluble cytosol was collected and named cytosol CS-100.

### 2.2. The preparation of interphase nuclei of mouse liver

The preparation and purification of interphase mouse liver nuclei was conducted with the instructions of Blobel [22]. The nuclei were suspended in nuclei reserve buffer (10 mmol/l PIPES, 80 mmol/l KCl,

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*Abbreviations:* TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labelling; AC-DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp-Ala; AC-YVAD-CHO, *N*-acetyl-Try-Val-Ala-aspartinal; MES, 4-morpholinethansulfonsaure; EDTA, disodium ethylene diamine tetracetate; EGTA, ethylene glycol-bis(β-amino ethyl ether)-*N*,*N*′-tetra acetic acid; DTT, dithiothreitol; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)/1,4-piperazine-diethanesulfonic acid; DAPI, 4′,6-diamidino-2-phenylindole; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ICE, interleukin-converting enzyme

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20 mmol/l NaCl, 250 mmol/l sucrose, 5 mmol/l EGTA, 0.5 mmol/l spermidine, 0.2 mmol/l spermine, 50% glycerol) after being centrifuged at 124 000×*g* (Beckman TLS-55) and counted before storing in a freezer at −80°C.

*2.3. Induction of apoptosis and the detection of apoptotic nuclei*

For the induction of apoptosis, cytochrome *c* (product of Sigma) was introduced into the CS-100 cytosol of carrot cells at the concentration of 2 μmol/l. Then, about $1\times10^5$ mouse liver nuclei were added to 50 μl CS-100, the system was incubated at 23°C.

*2.3.1. Morphological observation of apoptotic nuclei.* Samples were taken at different times, mixed with 2.5% (v/v) glutaraldehyde and 0.5% μg/ml DAPI for fixation and staining, slides were sealed with glycerol and PBS (1:1, v/v) and then observed using a fluorescent microscope (Leica DMRB). Photos were taken at the same time.

*2.3.2. The degradation of apoptotic nuclei DNA.* To samples induced for a certain time was 400 μl buffer D (100 mmol/l Tris-HCl, pH 8.0, 5 mmol/l EDTA, 0.2 mol/l NaCl, 0.4% SDS, 0.2 mg/ml proteinase K) added, incubated overnight at 37°C, extracted with 1:1 phenol-chloroform, precipitated by two volumes of ethanol and the DNA preparations were electrophoresized in 1.2% agarose gels in TBE buffer. DNA was visualized by ethidium bromide staining.

*2.3.3. Labelling of apoptotic nuclei using the TUNEL method.* TUNEL is a widely used method for the labelling of the 3′-OH end of broken DNA strands in apoptotic cells. Samples were spotted on the slides with polylysine, fixed with 4% formaldehyde at room temperature. Slides were washed three times with PBS (pH 7.2), then incubated in 0.1% Triton X-100, 0.1% citric acid on ice for 2 min, washed three times with PBS, 50 μl TUNEL reaction buffer (45 μl nucleotide mixture and 5 μl terminal deoxynucleotidyl transferase, Boehringer Mannleim) was then added on the surface of these slides and incubated in a wet box at 37°C for 60 min. Samples were washed three times with PBS (pH 7.2) and stained with DAPI at the same time. Slides were examined by a fluorescent microscope (Leica DMRB).

*2.4. Detection of lamins degradation in nuclei*

CSK buffer (10 mmol/l PIPES, 100 mmol/l KCl, 300 mmol/l sucrose, 3 mmol/l $MgCl_2$, 1 mmol/l EGTA, 1.2 mmol/l PMSF, 1% Triton X-100, pH 6.8) was added to samples and incubated at room temperature for 10 min, then centrifuged at 800×*g* for 5 min. The sediment was re-suspended in RSB-magic buffer (42.5 mmol/l Tris-HCl, 8.5 mmol/l NaCl, 2.6 mmol/l $MgCl_2{\cdot}6H_2O$, 1.2 mmol/l PMSF, 1% Tween-40, 0.5% sodium deoxycholate, pH 8.3), incubated at 4°C for 10 min, centrifuged at 800×*g* for 5 min. The sediment, which mainly consisted of lamina-intermediate filament-nuclear matrix, was mixed with one volume of sample buffer. For Western blotting, 20 μl of each sample was separated by electrophoresis using 12% SDS-PAGE gels. Gels were electrotransferred onto Immobilon-P (Whatman) membranes. The blots were blocked in PBS/0.05% Tween 20 containing 5% fetal bovine serum and incubated with the appropriate antibody: anti-lamin A and lamin C antibody C23. Antibodies C23 were kindly provided by Edward R. Kuczmarski (Ward Building 7-342, 303 East Chicago Avenue, Chicago, IL, USA).

*2.5. Inhibition of apoptosis in carrot cytosol*

Different concentrations of AC-YVAD-CHO (Sigma) and AC-DEVD-CHO (Sigma) were used to examine their effect on our in vitro apoptotic system. The samples were incubated at 23°C for 4 h. DNA was extracted and electrophoresized on a 1.2% agarose gel.

## 3. Results

*3.1. Apoptotic changes were induced in mouse liver nuclei*

For the development of an in vitro plant system that can duplicate the apoptosis conveniently and faithfully, a 100 000×*g* cytosolic supernatant (CS-100) from suspension-cultured carrot cells was prepared and supplemented with 2 μmol/l cytochrome *c*.

Purified nuclei, which were added to this cocktail CS-100 induction system, underwent dramatic morphological changes. Irregular chromatin condensation was seen in a speckle-like pattern, 30 min after the incubation (Fig. 1b), 1–2 h later, a more intensive condensation of chromatin occurred and the chromatin shrank to blocks (Fig. 1c). The margination of chromatin was observed at the same time (Fig. 1d). These blocks were then extruded into the cytosol as apoptotic-like bodies (Fig. 1e). Intact nuclei cannot be seen in this system and granular bodies (apoptotic-like bodies) filled in the view after 4 h (Fig. 1f). As for the negative control, the nuclei remained intact and could be stained all over (Fig. 1a).

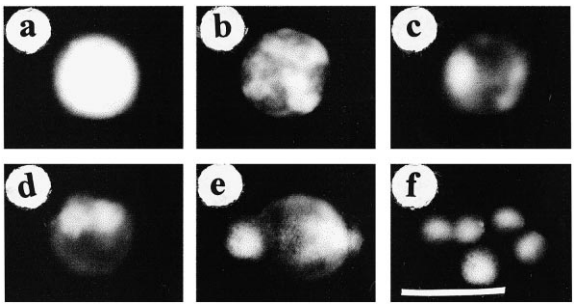


Fig. 1. Purified mouse liver nuclei were added to the CS-100 induction system, in which 2 μmol/l cytochrome *c* was supplemented. Incubated at 23°C, apoptotic changes were induced in nuclei. (a) The control nuclei were added in the CS-100 system without inducer, cytochrome *c*. (b) After 30 min, irregular chromatin appeared. (c) After 1–2 h, further condensation of chromatin occurred and the chromatin shrank into blocks. (d) Moreover, the typical margination of chromatin could also be observed. (e) The blocks in nuclei were extruded into the cytosol as apoptotic-like bodies. (f) After 4 h, intact nuclei could not be seen in the induction system and apoptotic-like bodies filled the view field. Bar=10 μm.

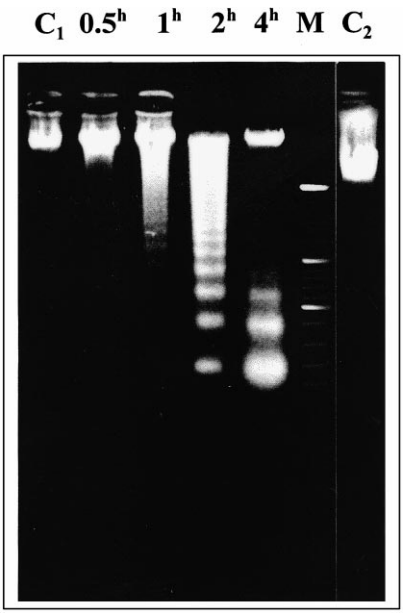


Fig. 2. The specific degradation of DNA into oligonucleosomal fragments in the CS-100 induction system. The results of electrophoresis showed that the degradation of DNA didn not occur 0.5 h after induction by cytochrome *c*, but the degradation of DNA could be seen on the electrophoresis gel after being induced for 1 h. After 2 h, the clear cut DNA ladder could be seen. Furthermore, the most typical DNA ladder appeared after 4 h. $C_1$: negative control, nuclei in CS-100 without cytochrome *c*. M: DNA molecular weight marker (Boehringer Mannheim). $C_2$: control, nuclei in reserve buffer with cytochrome *c* but without CS-100.

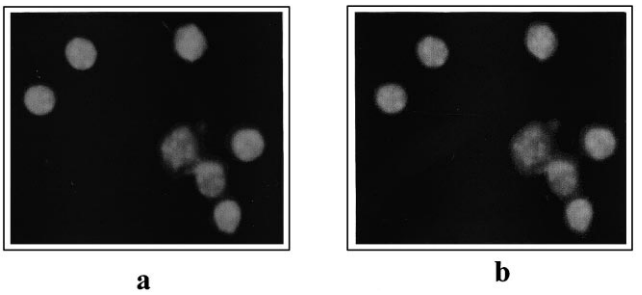


Fig. 3. The 3′-OH DNA strand breakage of apoptotic nuclei was labelled by using the TUNEL method. (a) DAPI staining shows the chromatin condensation and margination. (b) The same view of TUNEL-labelled nuclei showing bright yellow-green fluorescence.

### 3.2. Specific DNA degradation into oligonucleosomal fragments

To examine the validity of this new in vitro apoptotic system, we assessed whether the cleavage of chromatin DNA occurred in the nuclei incubated in this system. It is thought that the cleavage of DNA into oligonucleosomal fragments is typically considered to be a hallmark of apoptosis [3].

Our results showed that the morphological changes in nuclei occur simultaneously with the fragmentation of chromatin DNA. The degradation of DNA occurred about 1 h after incubation. Within 2 h, a clear cut DNA ladder could be seen. The most typical DNA ladder appeared after 4 h. Almost all the DNA was degraded into low molecular DNA fragments after 8 h and the DNA ladder could also be seen even at that time. The chromatin DNA of nuclei, which were added into nuclei reserve buffer with cytochrome *c* but without CS-100, was not detected during the process of the experiment (Fig. 2).

### 3.3. In situ detection of apoptotic nuclei using the TUNEL method

The TUNEL method is specifically used to detect the 3′-OH DNA strand breakage of apoptotic cells. The chromatin condensation and margination of mouse liver nuclei can be visualized by DAPI staining after the induction by cytochrome *c* in the CS-100 induction system. At the same time, these changes can be labelled in situ by the TUNEL method. (Fig. 3a and b; (a) DAPI staining shows the chromatin condensation and margination, (b) the same view of TUNEL-labelled nuclei shows bright yellow-green fluorescence). However, no specific TUNEL fluorescence was seen in the control.

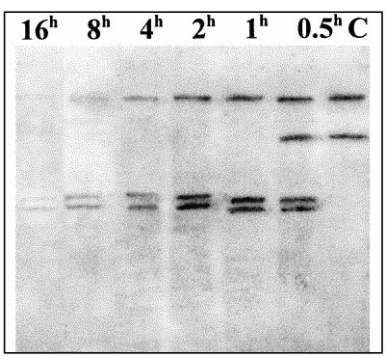


Fig. 4. Induced by cytochrome *c* in the CS-100 induction system, the lamin of nuclei was degraded from 88 kDa and 66 kDa to 37 kDa and 47 kDa fragments. The degradation was time-dependent. Induced for 0.5 h, lamin began to degrade to two fragments and lamins were almost degraded completely after induction for 16 h.

### 3.4. Degradation of nuclear lamin in mouse liver nuclei during apoptosis

Lamin degradation occurs commonly during apoptosis in animal cells [23,24]. Our results showed that lamins were degraded from 88 kDa and 66 kDa to 37 kDa and 47 kDa fragments during the apoptosis in mouse liver nuclei induced by cytochrome *c* in CS-100. Further study showed that this degradation was time-dependent and lamins were completely degraded after 16 h (Fig. 4).

### 3.5. Inhibition of apoptosis in CS-100

Previous studies from many laboratories have shown the involvement of cysteine protease from the CED-3/ICE family in the apoptotic process [25]. To determine whether such proteases were involved in the carrot system, we tested the effects of aldehyde-based tetrapeptide derivatives as specific inhibitors of caspases. The compound AC-DEVD-CHO has been proved to inhibit caspase 3 preferentially, while AC-YVAD-CHO is more selective for the ICE family. Our results showed that the degradation of DNA was partly inhibited by 10 μmol/l AC-DEVD-CHO and 0.5 μmol/l AC-YVAD-CHO and the formation of the DNA ladder could be completely inhibited by 50 μmol/l AC-DEVD-CHO and 5 μmol/l AC-YVAD-CHO (Fig. 5, $C_1$ was the positive control and $C_2$ was the negative control). These results implied that an ICE-like protease ac-

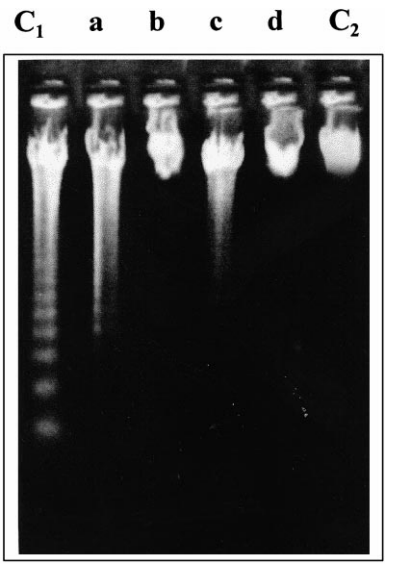


Fig. 5. The formation of the DNA ladder, induced by cytochrome *c* in the CS-100 induction system, could be inhibited by Ac-DEVD-CHO and Ac-YVAD-CHO. The degradation of DNA could be partly inhibited by 10 μmol/l Ac-DEVD-CHO and 0.5 μmol/l Ac-YVAD-CHO, and 50 μmol/l Ac-DEVD-CHO and 5 μmol/l Ac-YVAD-CHO could completely inhibit the formation of the DNA ladder. (a) The degradation of DNA was partly inhibited by 10 μmol/l Ac-DEVD-CHO. (b) The degradation of DNA was inhibited by 50 μmol/l Ac-DEVD-CHO. (c) The degradation of DNA was partly inhibited by 0.5 μmol/l Ac-YVAD-CHO. (d) The degradation of DNA was inhibited by 5 μmol/l Ac-YVAD-CHO. $C_1$: The positive control, which was induced by cytochrome *c* in the CS-100 induction system. $C_2$: The negative control, which was incubated in the CS-100 system without inducer. All samples were incubated at 23°C for 4 h and were induced by cytochrome *c*, except C2.

tivity, rather than a caspase 3, is required for apoptosis in the carrot cell-free system.

## 4. Discussion

The cell-free system described here caused isolated nuclei to undergo apoptosis in several respects with a much higher degree of synchrony than is available in vivo. First, similar morphological changes occurred in nuclei. Chromatin was condensed into discrete regions and the nuclei were eventually fragmented and destroyed. Second, the fragmentation of chromatin DNA into the DNA ladder form was observed and this process was inhibited by caspases-specific inhibitors. Finally, degradation of lamins occurred in the process of apoptosis and lamins were degraded into fragments with specific lengths. It is thought that PCD in plants is very important during the development and stress reactions [7,8]. But the understanding of the apoptosis (PCD) in plants is only at its initial stage. The main reason for this is the lacking of ideal materials, which can provide large amounts of apoptotic cells. There are many advantages when considering the carrot cytosol as induction system: for example, the condition is easy to control and it is also convenient to search for a single factor. It is also easy to get a high rate of apoptotic cells and about 90% of the nuclei could be induced to apoptosis.

The results also indicated that cytochrome *c* could induce apoptosis in carrot cytosol and the stereotypical DNA ladder could be observed. Cytochrome *c* can be used as an apoptosis inducer only recently [4–6]. Liu et al. isolated and sequenced the cytochrome *c* when they studied apoptosis in a Hela extract cell-free system [4]. We found that cytochrome *c* could successfully induce apoptosis of mouse liver nuclei in the carrot cytosol accompanied by morphological changes and the formation of a DNA ladder. The apoptosis was also detected by the TUNEL method in mouse nuclei in CS-100. All together, these results indicated that cytochrome *c* could not only induce apoptosis in animal cells, but also in plant cells.

In the cytosol of Hela cells, it was found that in vitro apoptosis was initiated by dATP/dADP or sometimes by ATP. Further studies demonstrated that cytochrome *c* and dATP were required for the formation of the Apaf-1/caspase 9 complex. However, in the apoptotic cell-free system developed from egg extracts of *X. laevis* (unpublished data). It is highly possible that in some cases, such as in oocytes, there is enough dATP/ATP stored to be ready for the very fast DNA replication upon fertilization. In our cell-free system prepared from carrot cells, the independence of exogenous dATP/ATP may due to that the endogenous dATP or ATP concentration is high enough to cooperate with cytochrome *c* to form the Apaf-1/caspase 9 complex (if there exists such a complex in the plant cell) or due to the different apoptotic signal pathway. However, our results suggested that there is a caspases-like protease activity in the cytosol of plant cells. It could be activated by cytochrome *c* and inhibited by caspase-specific inhibitors.

Lamins degradation precedes the degradation of DNA and plays an essential role during apoptosis [23,24]. Previous studies showed that the degradation of lamins was followed by the breakdown of the nuclear envelope and chromatin condensation. Caspase 6 has been proved to be the key protease during the degradation of lamins [25]. Our results indicated that lamins were degraded into 37 kDa and 47 kDa fragments, which implied that a similar pathway might exist in plant cells.

AC-DEVD-CHO is an inhibitor of caspase 3 while AC-YVAD-CHO is an inhibitor of ICE [14,26]. Our studies have provided evidence that AC-DEVD-CHO and AC-YVAD-CHO can inhibit the formation of a DNA ladder, this indicated that caspase-like proteases which act as the apoptotic execution machines also existed in plant cells. The apoptosis in plant cells may share partly similar pathways to apoptosis in animal cells.

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