Menadione-induced apoptosis and its mechanism in plants

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Abstract Menadione can induce apoptosis in tobacco protoplasts. Typical characteristics are detected including the condensation of chromatin, the formation of apoptotic bodies and the degradation of genomic DNA into "DNA ladder". Specific DNase is activated during this process. Ca^{2+} and Mg^{2+} are necessary for its activation, while Zn^{2+} and EDTA (Ethylenediaminetetraacetic acid) can inhibit its activation. The fragmentation of DNA and lamin can be inhibited by DEVD (Ac-Asp -Glu- Val- Asp-aldehyde). The fragmentation of lamin can also be inhibited by PMSF (Phenylmethylsulfonyl fluoride) and CH (Cyclohexinide). These results show that activation of specific DNase and proteases is involved in menadion-induced apoptosis in plants.

Keywords: tobacco protoplasts, menadione, apoptosis, DNA fragmentation, lamin protease.

Apoptosis is genetically controlled programmed cell death and it can be triggered by a variety of intrinsic and extrinsic signals. The common characteristics of apoptosis include the condensation of chromatin, DNA fragmentation and the formation of apoptotic bodies. The cleavage of genomic DNA between nucleosomals is the most typical characteristic and it is closely related with the activation of specific DNase[1—3]. In fact, not only the activation of specific DNase but also the action of proteases was involved in apoptosis. Recent study showed that ICE (Interleukin-1β converting enzyme) protease family plays an important role. This family is cysteinyl proteases, and the amino acids around the cysteinyl acid are conserved. Because this family can cleave the substrates around the Asp site, they were given another name caspase (asparate-specific cysteinyl proteases)[1,4,5]. It is of great interest to know if the similar mechanism is shared by apoptosis in plants.

An important hallmark of the activation of ICE proteases is the specific degradation of their substrates. In specific, lamin and PARP (poly(ADP-ribose) polymerase) are two important substrates which are called "death substrate"[6]. Our results showed that not only the fragmentation of DNA but also the degradation of lamin can be inhibited by the specific inhibitor of cysteinyl proteases, which indicated that the action of ICE proteases may be shared by apoptosis in plants and animals. Other results showed that specific DNase activated during this process requires Ca^{2+} and Mg^{2+}, while Zn^{2+} and EDTA can inhibit its activation. The above results provided more evidence for studying on the pathway during apoptosis in plants. Menadione can also induce apoptosis in animal cells[7]. At present, although there are many reports about apoptosis in plants, few touches upon its mechanism[8—11]. During menadione-induced apoptosis in tobacco protoplasts, we can get well reproductive results and high-rate apoptotic cells, which made it possible for us to study further on its mechanism. Based on this work, we are trying to identify one or a few specific factors during this process.

1 Materials and methods

(i) Cell culture. A tobacco (*Nicotiana tobacum*,cultivar BY-2) cell suspension culture was maintained in MS medium supplemented with 1 mg/L 2,4-D at 25℃. Subculturing was done at 7d intervals.

(ii) Protoplast preparation. Harvested tabacco cells were washed twice with washing buffer

containing 0.2 mol/L mannitol, 0.2 mol/L sorbitol and 5 mmol/L MES. Cells were then treated with 2% Cellulase and 0.5% Macerozyme for 4—5 h in the dark at 25℃. The resulting preparation of protoplasts was sieved through 200 μm nylon meshes, and then was freed from cellular debris by suspending the preparation in 0.6 mol/L sucrose and centrifuging at 100 g for 5 min to remove cellular debris. Viable protoplasts were collected and washed twice with washing buffer before suspended in an MS medium supplemented with 1 mg/L 2,4-D, 5 mmol/L glucose, 0.3 mol/L mannitol and 0.2 mol/L sorbitol.

(iii) Induction of apoptosis in tobacco protoplasts. The tobacco protoplasts were re-suspended at the final concentration of 10^5/m L, and menadione was added to the final concentration of 100 μmol/L, incubated at 22℃ and samples were taken at different time. For the detection of effect of different cations and inhibitors on apoptosis, Ca^{2+}, Mg^{2+} , Zn^{2+}, EDTA, EGTA, DEVD, PMSF and CH were added to 0.5 mL induction system to the final concentration of 5 mmol/L, 10 mmol/L, 2 mmol/L, 5 mmol/L, 5 mmol/L, 0.2 mmol/L, 100 μmmol/L, 5 mmol/L, 0.2 mg/mL respectively. Samples were taken after induction for 8 h.

(iv) DAPI staining. Samples were taken after induction for 4 h, and mixtured with 1/4(*v/v*) DAPI (4,6-diamidinao-2-phenyl-indole diacetate) (0.5 μg/mL). The slide was sealed with glycerol-PBS (1 ∶ 1) and observed under fluorescence microscopy.

(v) *In situ* detection of DNA fragmentation by the TUNEL procedure. Protoplasts were immobilized using polylysine. The TUNEL reaction was then carried out following the manufacturer's description (Boehriger Mannhem).

(vi) DNA analysis. DNA was extracted with phenol-chloroform, and precipitated by two volumes of ethanol. Identical amount of DNA samples were run on a 1.5% (*w/v*) agarose gel at 50 V for 1 h. Oligonucleosomal fragments of DNA were visualized by staining with 0.63 mg/L ethidium bromide.

(vii) Extraction of lamin-like proteins. Protoplasts were suspended in cytoskeleton buffer containing 0.5% Triton X-100 to solubilize membrane system and remove soluble proteins. The isolated proteins were further extracted with high salt buffer containing 0.5% sodium deoxycholate. Microtubules, microfilaments and other structural proteins but not intermediate filaments (IF) in the cytoplasm were removed. The remaining extracts in the form of pellet were digested in the digestion buffer containing DNase I (100 mg/mL). Ammonium sulfate was added to the solution at a final concentration of 0.25 mol/L to precipitate the nuclear matrix-lamina-IF fraction and stripped the chromatin and histones.

(viii) Western blotting. The extracted lamine-like proteins were dissolved in standard SDS sample buffer, SDS- PAGE using 10% gel was then performed. The resolved polypeptides were transferred to a nitrocellulose membrane, which was first incubated for 1 h in a blocking buffer (3% BSA in TBS), then incubated with the primary antibody (monoclonal anti-mouse antibodies against lamin A and C, diluted 1 ∶ 1000) for 3.5 h at 37℃, washed thoroughly with TTBS, then incubated with 1 ∶ 1000 diluted Biotin-conjugated goat-anti-rabbit IgG for 2 h at 37℃. The nitrocellulose membrane was further incubated with 1 ∶ 1000 diluted alkaline phosphotase-conjuguted Avidin for 15 min at room temperature. The antibody labeled bands were visualized by the color development in a solution containing 5-bromo-4-chloro-3-indolyl phosphate-toluidine salt and p-nitro tetrzolium chloride.

2 Results

(i) Morphological changes of nuclei during apoptosis. Nuclear changes are important characteristics of apoptosis. The result of DAPI fluorecence staining showed that the nuclei of tobacco protoplasts induced by 100 μmmol/L menadione underwent dramatic morphological changes. Irregular chromatin condensation was seen in a speckle-like pattern 4 h after the incubation. 8 h later, a more intensive condensation of chromatin was observed. These blocks were then extruded into the cytoplasma as apoptotic-like bodies (fig.1(b)—(d)). These changes were observed in approximately 70% of the protoplasts. As for the negative control, the nuclei remained intact and could be stained all

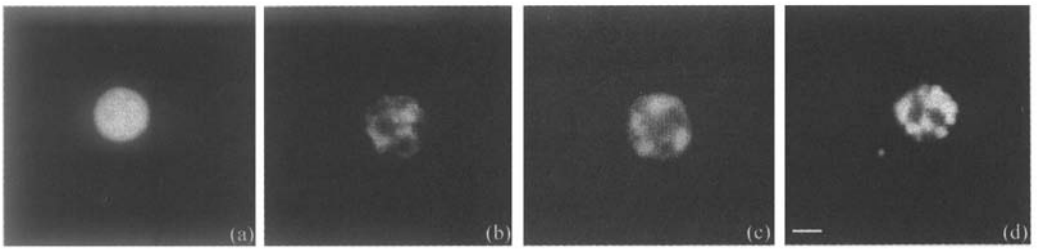

Fig. 1. DAPI staining showing the morphological changes of protoplasts during apoptosis (bar =10 μm). (a) Control; (b) the chromosomes condensed dramatically after induction for 4 h; (c) the margination of the chromosomes appeared after induction for 8 h; (d) the apoptotic bodies came into being after induction for 12 h.

over (fig. 1(a)).

(ii) *In situ* detection of apoptotic cells 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 DAPI staining were observed after incubation by 100 μmmol/L menadione. At the same time, these changes can be labeled *in situ* by the TUNEL method (fig. 2(a), (b)). However, no specific TUNEL fluorescence was seen in the control (data not shown).

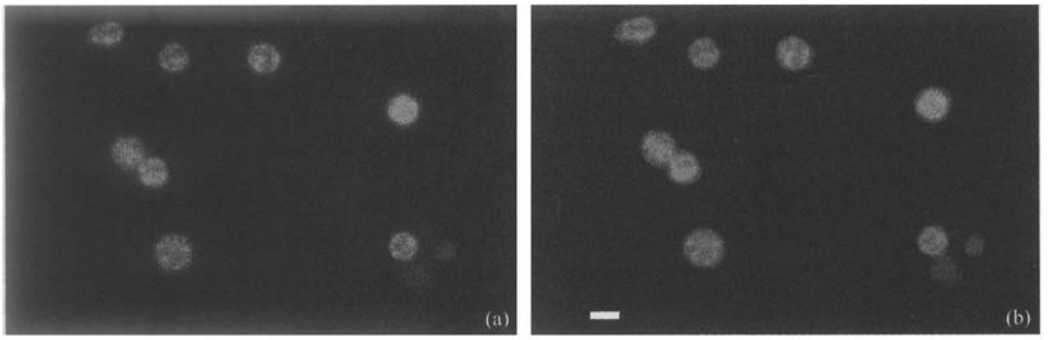

Fig. 2. *In situ* detection of DNA cleavage (bar =10 μm). (a) DAPI staining showing the nucleus chromatin condensation during apoptosis; (b) the same view field, TUNEL assay was used to detect DNA fragmentation.

(iii) Fragmentation of genomic DNA during menadione-induced apoptosis in tobacco protoplasts was accompanied by the activation of specific DNase. The most important hallmark of apoptosis is internucleosomal cleavage of chromatin, giving rise to "ladder" pattern of oligonucleosomal-sized bands corresponding to multiples of 200 bp in agarose gel electrophoresis. The results of agarose gel electrophoresis showed that the genomic DNA was degraded step by step and exhibited "DNA ladder" after induction for 4, 8 and 12 h (fig. 3-1—4). While after induction for 24 h, genomic DNA was degraded almost completely (fig. 3-5). This internucleosomal cleavage of specific DNase can be inhibited by EDTA (fig. 4-7). However, this inhibition can be removed by addition of Ca^{2+} and Mg^{2+} at the same concentration (fig. 4-9). As a chelator of Ca^{2+}, EGTA can inhibit the formation of "DNA ladder" (fig.4-5), while the formation of "DNA ladder" can be recovered by addition of Ca^{2+} at the same concentration (fig. 4-6). If the same concentration of Ca^{2+} was added together with EDTA, the fragmentation of "DNA ladder" can only be partly recovered (fig. 4-8). Most of the apoptosis-related DNase found by now can be inhibited by Zn^{2+}. The result showed that 5 mmol/L Zn^{2+} can inhibit the fragmentation of DNA completely (fig. 4-4). In addition, 100 μmmol/L DEVD and 5 mmol/L PMSF can

Fig. 3. Agarose detection of "DNA ladder". 1, Control; 2, the fragmentation of DNA appeared after induction for 4 h; 3, "DNA ladder" was very clear after induction for 8 h; 4, "DNA ladder" could still be seen after induction for 12 h; 5, DNA was degraded comple -tely after induction for 24 h.

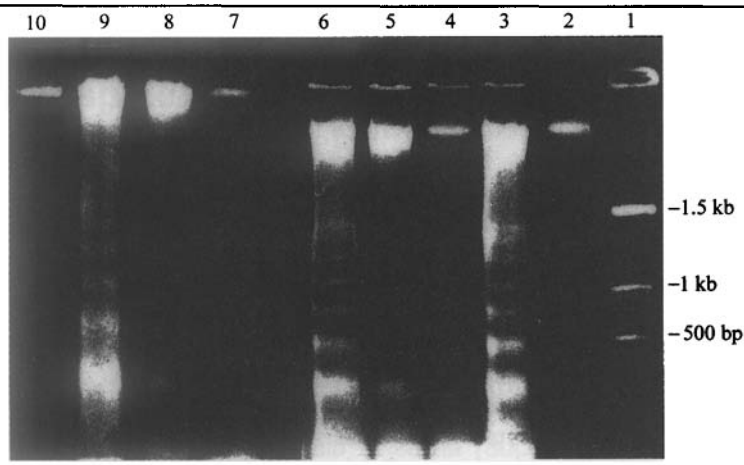

Fig. 4. Agarose gel detection of DNA fragmentation and the influence of divalent cations on the formation of "DNA ladder". 1, DNA marker with 500 bp intervals; 2, control group without inducer; 3, "DNA ladder" detected after induction for 4 h; 4, 5 mmol/L Zn^{2+}; 5, 5 mmol/L EGTA; 6, 5 mmol/L EGTA and 5 mmol/L Ca^{2+}; 7, 5 mmol/L EDTA; 8, 5 mmol/L EDTA and 5 mmol/L Ca^{2+}; 9, 5 mmol/L EDTA and 5 mmol/L Ca^{2+}, Mg^{2+}; 10, 100 μmol/L AC-DEVD-CHO.

also inhibit the formation of "DNA ladder" (fig.4-10; fig.5-3,5). DEVD is the specific inhibitor of caspase-3 and caspase-7, and PMSF is the inhibitor of cysteinyl and serine proteases. Their inhibitive effect on specific DNase showed that the involvement of specific proteases is important for the activation of specific DNase. CH, the inhi- bitor of protein synthesis, has no effect on the formation of "DNA ladder" (fig. 5-4).

(iv) Degradation of lamin during menadione-induced apo- ptosis in tobacco protoplasts. The nuclear lamina is a structure juxtaposed to the nuclear enve- lope that appears to be involved in the structure of the nuclear envelop and chromatin in inter- phase cells. It is composed of up to 4 homolous polypeptides, called lamins A, B1, B2 and C (in order of decreasing m.w). The degradation of lamin during apoptosis facilitates nuclear eve- nts ant thus is an important hallmark of apoptosis. The result of Western blot showed that no degradation was detected after incubation for 2 h, lamin C was degraded into 35 ku fragments after induction for 4 h, and it was degraded almost completely after induction for 8 h (fig. 6-1—4). DEVD and PMSF can inhibit the degradation of lamin completely, while CH can partly inhibit the degradation of lamin (fig. 6-5—7).

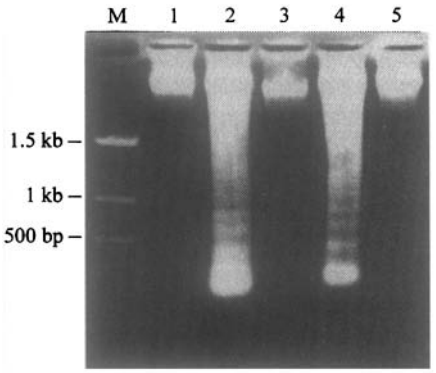

Fig. 5. Agarose gel detection of the influence of different inhibitors on the formation of "DNA ladder". M, DNA marker with 500 bp intervals; 1, negative control; 2, positive control; 3, 100 μmol/L AC-DEVD-CHO; 4, 0.2 mg/mL CH; 5, 5 mmol/L PMSF.

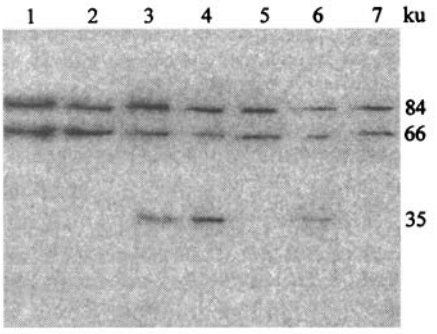

Fig. 6. Western blot showing the degradation of nuclear lamin. 1, Control; 2, no degradation of lamin detected after induction for 2 h; 3, lamin was degraded into 35 ku fragments after induction for 4 h; 4, most of 66 ku lamin degraded after induction for 8 h; 5, 100 μmol/L AC-DEVD-CHO added; 6, 0.2 mg/mL CH added; 7, 5 mmol/L PMSF added.

3 Discussion

Since programmed cell death (apoptosis) in plants was reported in 1994[13], more and more evidence has shown that the characteristics in plants and animals are conserved. Among these characteristics, the fragmentation of DNA was verified to be the most conserved and typical hallmark during apoptosis in plants and animals. Although there are different reports about the length of DNA fragments, this specific but not random cleavage indicated that the activation of specific DNase was involved in the degradation of DNA in plants[8,10,11]. Our study showed that menadione can not only induce typical chromatin condensation formation of apoptotic bodies, but can also induce the degradation of DNA into 200 bp fragments. So, specific DNase-depended internucleosomal cleavage activity appeared during menadione-induced apoptosis in tobacco protoplasts. Further study shows that activation of this DNase depends on Ca^{2+} and Mg^{2+}, while Zn^{2+} and EDTA can inhibit its activity. Taken together, we conclude that the apoptosis-related DNase in plants has similar characteristics to those in animals, and it plays a key role during apoptosis[14].

Cysteinyl proteases are also important for apoptosis in plants. In animal cells, they are also called caspase because of their common activation mechanism and functions[1,5,6]. It has become a focusing point that whether cysteinyl proteases are also involved in apoptosis in plants[1]. Our results showed that the formation of "DNA ladder"and the degradation of lamin can be inhibited by certain concentration of DEVD and PMSF. DEVD is the specific inhibitor of caspase 3/cpp32. PMSF is the inhibitor of cysteinyl and serine proteases. These results showed that the activation of cpp32-like proteases is necessary for apoptosis in plants. Lamin is one of the substrates of caspase during apoptosis in animal cells. The envelop and the condensation of chromatin, which is an important event during apoptosis. Lamin is cleaved by active caspse-6 during apoptosis in animal cells[5,16]. So, we cannot exclude the possibility that there are other kinds of cysteinyl proteases besides caspase-3/cpp32. Moreover, although CH cannot inhibit the formation of "DNA ladder", it can inhibit the degradation of lamin, which indicated that new synthesized protein may be involved in the degradation of lamin.

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