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Research Article

Menadione-induced apoptosis and the degradation of lamin-like proteins in tobacco protoplasts

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Abstract. Detection of stereotypic hallmarks of apoptosis during cell death induced by menadione, including DNA laddering and the formation of apoptotic bodies, is reported. Comet assay and the TdT-mediated dUTP nick end labelling (TUNEL) procedure were also performed to detect DNA fragmentation. Inhibition of DNA fragmentation by Ac-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) and phenylmethylsulfosyl (PMSF) implicated the involvement of caspase-like proteases in menadione-induced apoptosis in plants. We further studied the cleavage of lamin-like proteins during apoptosis in menadione-treated tobacco protoplasts. In animals, it has been reported that the solubilization of nuclear lamina and lamin degradation occurs during apoptotic cell death. However, little is known about the fate of lamins in apoptotic plant cells. Our study provided evidence that lamin-like proteins degraded into 35-kDa fragments in tobacco protoplasts induced by menadione, and this preceded DNA fragmentation. The results thus indicated that proteolytic cleavage of nuclear lamins was also conserved in programmed cell death in plants.

Key words. Apoptosis; DNA fragmentation; lamin cleavage; menadione; tobacco protoplasts.

Apoptosis, a form of genetically programmed cell death, is crucial in normal development, the maintenance of homeostasis and response to stress [1]. In recent years, there is a growing body of evidence that apoptosis occurs in plant cells. However, study on apoptosis in plants is still at an initial stage. In plants, apoptosis was found to play a critical role in growth and developmental events such as differentiation of xylem tracheary elements, sloughing of root cap cells, deletion of the suspensor and alearone cells, aerenchyma formation upon hypoxia and so on [2, 3]. Apoptosis can be triggered by biological and physical signals, such as radiation, free radicals, infection of virus and so on [1]. It was found that menadione, a member of a homologous group of naturally occurring 1,4-naphthoquinones, induces apoptosis in animal cells, yet the mechanism is not very well defined [4]. In plants, we reported previously that menadione induces apoptosis in carrot cells [5]. In this study, we provide new evidence that menadione is an effective inducer of apoptosis in tobacco protoplasts. The degradation of lamins was found to be an important event during apoptosis in animal cells, playing an essential role in chromatin condensation and breakdown of the nuclear envelope [6]. In plants, lamin-like proteins were found to occur in nuclei. It is of interest to ask whether the degradation of lamin-like proteins takes place and relates to the cell death program during apoptosis in plants. Our results indicated that the degradation of lamin-like proteins is an integral part of the apoptotic pathway. We also

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discussed a possible caspase-like activity in plants based on our studies. Caspases are asparate-specific cysteinyl proteases. This family has been identified as being a key mediator of apoptosis in mammalian cells [1].

Materials and methods

Cell culture. A tobacco (*Nicotiana tobacum*, cultivar BY-2) cell suspension culture was initiated from cotyledon explants and maintained in Murashige-Skoog (MS) medium supplemented with 1 mg/l of 2,4-D at 25 °C. Subculturing was done at weekly intervals.

Preparation of protoplasts. Harvested tobacco cells were washed twice with washing buffer which contained 0.2 M mannitol, 0.2 M sorbitol, and 5 mM 2-[N-morpholino]-ethanesulfonic acid (MES). Cells were then treated with 2% cellulase and 0.5% macerozyme for 4–5 h in the dark at 25 °C. The resulting preparation of protoplasts was sieved through 200-μm nylon meshes, and then were freed from cellular debris by suspending the preparation in 0.6 M sucrose and centrifuged at 100*g* for 5 min to remove cellular debris. Viable protoplasts were collected and washed twice with washing buffer before being suspended in an MS medium supplemented with 1 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D), 5 mM glucose, 0.3 M mannitol and 0.2 M sorbitol.

Treatment with menadione. Menadione was added into 1 ml of suspended tobacco protoplasts (10^5/ml) at different final concentrations (80, 100, 300 and 600 μM, respectively). Treated protoplasts were left at 25 °C for 12 h. For detecting DNA laddering and lamin-like protein degradation, 100 μM menadione was added into suspended tobacco protoplasts, and samples were taken from 0–18 h at 6-h intervals. For detecting inhibition, 100 μM Ac-DEVD-CHO (Sigma) and 5 mM PMSF, respectively, were added into the inhibition system.

Protoplast death test. To test cell death, protoplasts were treated with 0.5% Trypan Blue for 1 min on slides, and observed under a light microscope. Protoplast death was counted based on Trypan Blue exclusion. For detecting apoptotic protoplasts, 4,6-diamidino-2-phenyl-indole diacetate (DAPI) was dissolved in water at 0.1 mg/ml and used at a working concentration of 5 μg/ml. Nuclear changes were observed using a fluorescence microscope (Lieca, DMRB). The protoplasts showing nuclear condensation and distortion were counted as apoptotic protoplasts.

Neutral comet assay. For detecting nuclear DNA fragmentation, neutral comet assay was performed on microscopic slides by the method of Fairbairn et al. [7] as modified by Jiang et al. [8]. In brief, protoplasts were suspended in low-melt agarose gel. After gel polymerization, protoplasts were lysed for 30–50 min in neutral lysis buffer (30 mM EDTA, 0.5% SDS, pH 8), and were then subjected to electrophoresis at 30 V/cm for 5 min. Gels were stained with 0.05 μg/ml DAPI for 30 min DNA comets were then observed and counted under a fluorescence microscope.

DNA analysis. DNA was extracted from tobacco protoplasts following the method of Ryerson et al. [10]. Identical amounts 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 of ethidium bromide.

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 (Boehringer Mannheim).

Extraction of lamin-like proteins. Extraction of lamin-like proteins was carried out according to the method of Fey et al. [9] as modified by Wang et al. [11]. In brief, protoplasts were suspended in a cytoskeleton buffer containing 0.5% Triton X-100 to solubilize the 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 (IFs) in the cytoplasm were removed. The remained extracts in the form of a pellet were digested in the digestion buffer containing DNase

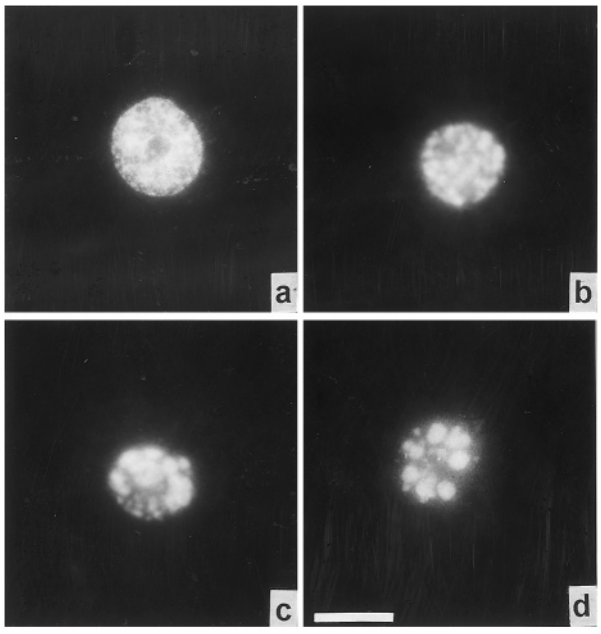

Figure 1. Morphological changes of protoplasts treated with menadione (bar, 10 μm). (*a*) Normal cells; (*b*) nuclear condensation; (*c*) serious nuclear condensation; (*d*) collapse of the nucleus into apoptotic-like bodies.

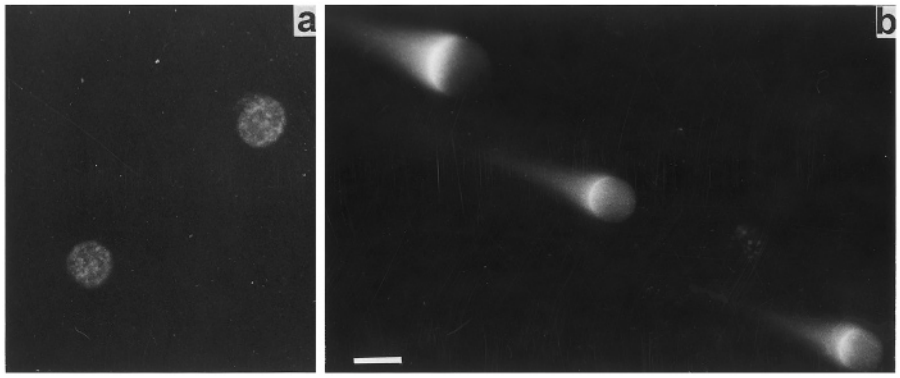

Figure 2. Fluorescence photomicrographs of protoplasts stained with DAPI after neutral comet assay (bar, 10 μm). (*a*) Normal tobacco protoplasts; (*b*) apoptotic tobacco protoplasts induced by menadione.

I (100 mg/ml). Ammonium sulfate was added to the solution at a final concentration of 0.25 M to precipitate the nuclear matrix-lamina-IF fraction and strip the chromatin and histones.

Western blotting. The extracted lamin-like proteins were dissolved in standard SDS sample buffer. SDS-polyacrylamide gel electrophoresis (PAGE) using 10% gel was then performed. The resolved polypeptides were transferred to a nitrocellulose membrane; the nitrocellulose membrane was first incubated for 1 h in a blocking buffer (3% bovine serum albumin (BSA) in tris-HCl balance solution, 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 °C, washed thoroughly with triton-added tris-HCl balance solution (TTBS), then incubated with 1:1000 diluted biotin-conjugated goat-anti-rabbit immunoglobulin G for 2 h at 37 °C. The nitrocellulose membrane was further incubated with 1:1000 diluted alkaline phosphatase-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 tetrazolium chloride.

Results

Nuclear changes in menadione-treated tobacco protoplasts. As shown in figure 1, nuclear changes including nucleus condensation and disintegration were observed in protoplasts treated with 100 μM menadione. Since the culture used in our experiments was not synchronized, it is not surprising that different stages of nuclear changes from complete nucleus to the appearance of distinct apoptotic-like bodies were observed at the same time.

DNA fragmentation detected by comet assay. In the comet assay, protoplasts embedded in agarose were lysed and subjected to electrophoresis. The broken DNA strands migrated through the gel displaying comet-like images. As shown in figure 2, nuclear DNA in protoplasts treated with menadione showed 'comets' whereas protoplasts in control nuclei maintained their normal shape.

Correlation between incidence of comets and percentage of apoptotic protoplasts. Detection of apoptotic protoplasts was carried out by 4,6-deamidino-2-phenyl-induced diacetate (DAPI) staining which displayed nuclear changes during apoptosis. Comet assay, on the other hand, was based on DNA strand breaks which

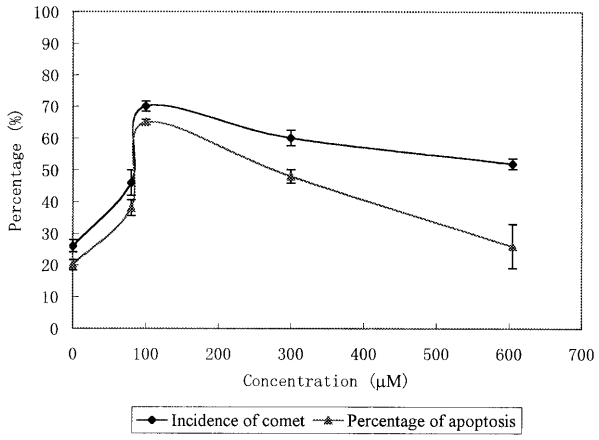

Figure 3. Correlation between incidence of comets and percentage of apoptotic protoplasts. Values are the mean of three individual experiments. Error bars indicate standard deviation.

took place during apoptosis. It is worthy of mention that the neutral comet assay used in our study is a nondenaturing assay which shows sensitivity mainly to apoptotic, but not necrotic DNA damage. Thus, it is conceivable that a relatively close correlation was observed between incidence of comet assay percentage of apoptotic protoplasts induced by menadione (fig. 3).

Effect of menadione on cell death and apoptosis in tobacco protoplasts. As illustrated in figure 4, percentage of cell death detected by Trypan Blue exclusion shot up with the increase of menadione concentration. On the other hand, the percentage of apoptotic protoplasts increased sharply when the menadione concentration was in the range of 0–100 μM. As illustrated in figure 4, 100 μM menadione caused up to 65% of the protoplasts to undergo apoptosis, but only 18% dead cells were observed. This is probably due to the fact that cells undergoing apoptosis induced by menadione at lower concentrations may still be alive and maintain the ability to exclude Trypan Blue. As the concentration of menadione increased, nonapoptotic cell death such as necrosis may take place and became the major form of cell death. In this case, the general appearance of the nuclei was utterly different and was thus easy to distinguish from nuclei in apoptotic cells.

Detection of DNA ladders. DNA laddering resulting from DNA degradation into internucleosomal fragments is a crucial hallmark of apoptosis. DNA ladders were detected in protoplasts treated with 100 μM menadione. It is striking that production of oligonucleosomal DNA fragmentation was augmented with the increase of time of menadione treatment. No DNA laddering was detected in protoplasts treated with menadione for 6 h, whereas when menadione treatment lasted for 10 h, clear-cut DNA laddering displayed as shown in figure 5. To test whether any proteolysis is involved in apoptotic cell death, inhibitors of proteases including the caspase-3- and caspase-7-specific inhibitor Ac-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) and PMSF (phenylmethylsulfonyl), a cysteine and serine inhibitor, were used to treat tobacco protoplasts together with 100 μM menadione. DNA was extracted from the treated protoplasts and analyzed by conventional agarose electrophoresis. It was found that DNA laddering induced by menadione was completely inhibited by 100 μM Ac-DEVD-CHO and 5 mM PMSF (fig. 6). It is noteworthy that in our study the concentration of Ac-DEVD-CHO was the same as that used in camptothecin-induced apoptotic osteosarcoma cells. And the PMSF concentration was comparable with that used in a poly (ADP-ribose) polymerase (PARP) cleavage assay using apoptotic osteosarcoma cell extracts [12]. The results implied involvement of caspase-like cysteine protease in menadione-induced apoptosis.

In situ detection of DNA cleavage by the TUNEL procedure in tobacco protoplasts. Application of the TUNEL procedure enables in situ detection of fragmented DNA based on visualizing DNA 3′–OH nicks in the nuclei.

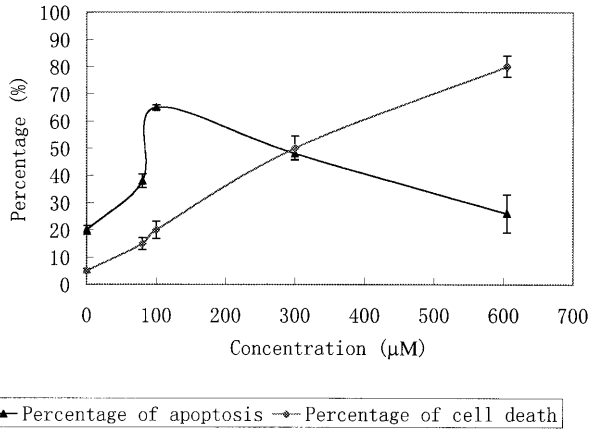

Figure 4. Effect of menadione on cell death and apoptosis in tobacco protoplasts. Values are the mean of three individual experiments. Error bars indicate standard deviation.

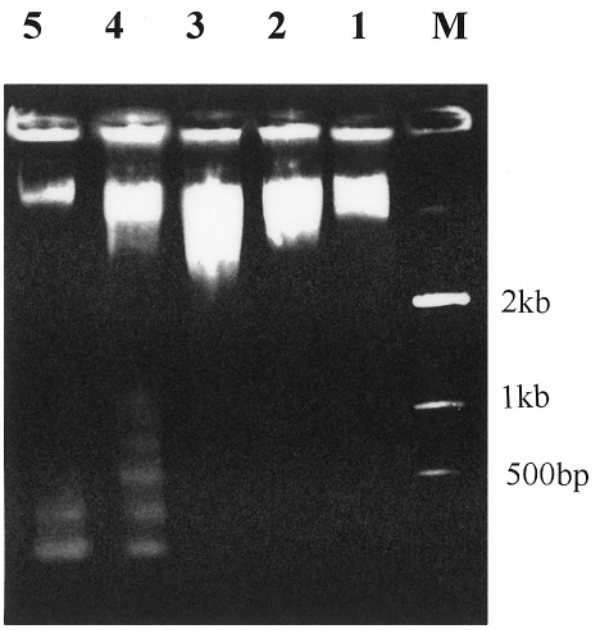

Figure 5. DNA laddering induced by 100 μM menadione in tobacco protoplasts. Lane M, low molecular weight DNA marker (Boehringer Mannheim). Lane 1, control; lane 2, 6 h treatment; lane 3, 8 h treatment; lane 4, 12 h treatment; lane 5, 24 h treatment.

After the TUNEL procedure, protoplasts were counterstained with DAPI, so that DNA cleavage and localization of total DNA were viewed in the same protoplasts with a fluorescence microscope at different ultraviolet (UV) wavelengths.

As illustrated in figure 7c,d, DAPI staining revealed that nuclei of the protoplasts subjected to menadione treatment maintained a similar size and shape but showed nuclear condensation as compared with the control (fig. 7a). At the same time, these protoplasts were found to be TUNEL-positive, exhibiting bright yellow-green fluorescence in the nuclei, whereas no DNA cleavage was observed in control protoplasts (fig. 7b).

Degradation of lamin-like proteins. It was found that lamin C-like proteins degraded into 35-kDa fragments during apoptosis induced by menadione in tobacco protoplasts, as shown in figure 8. The effect of menadione on the degradation of lamin-like proteins occurred in a dose-dependent manner. The degradation of lamin-like proteins was found when 100 and 300 μM menadione were used; when the menadione concentration reached 600 μM, lamin-like proteins degraded completely. More strikingly, when a fixed concentration of menadione (100 μM) was used, and the treatment lasted for different time periods (0, 6, 10 and 14 h), different extents of lamin-like protein degradation were found. At zero

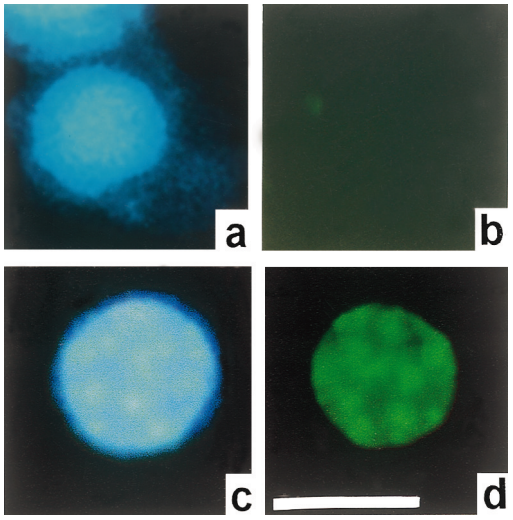

Figure 7. In situ detection of DNA cleavage in menadione-treated tobacco protoplasts. (*a*, *b*) Control protoplasts; (*c*, *d*) protoplasts treated with 100 μM menadione for 12 h. In *a* and *c*, DAPI was used to stain the DNA. In *b* and *d*, the TUNEL assay was used to detect DNA fragmentation. Bar in *d* = 20 μm.

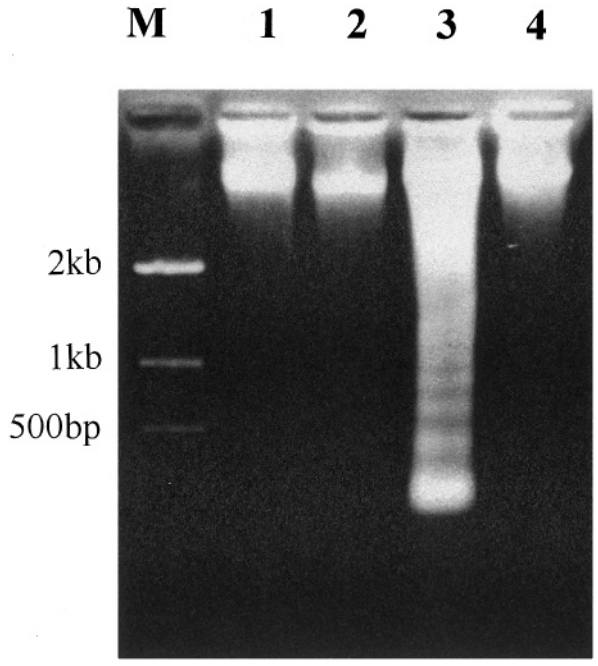

Figure 6. Effect of protease inhibition on DNA laddering. Protoplasts were incubated with 100 μM menadione and inhibitors for 12 h. Lane M, low molecular weight DNA marker (Boehringer Mannheim). Lane 1, control; lane 2, menadione plus 100 μM Ac-DEVD-CHO; lane 3, menadione alone; lane 4, menadione plus 5 mM PMSF.

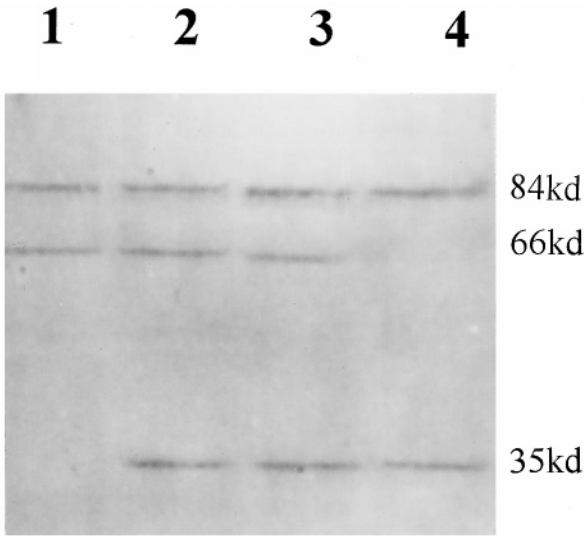

Figure 8. Effect of menadione concentration on the degradation of lamin-like proteins as detected by immunoblotting. Lane 1, control; lane 2, protoplasts were treated with 100 μM menadione for 12 h; lane 3, protoplasts were treated with 300 μM menadione for 12 h; lane 4, protoplasts were treated with 600 μM menadione for 12 h.

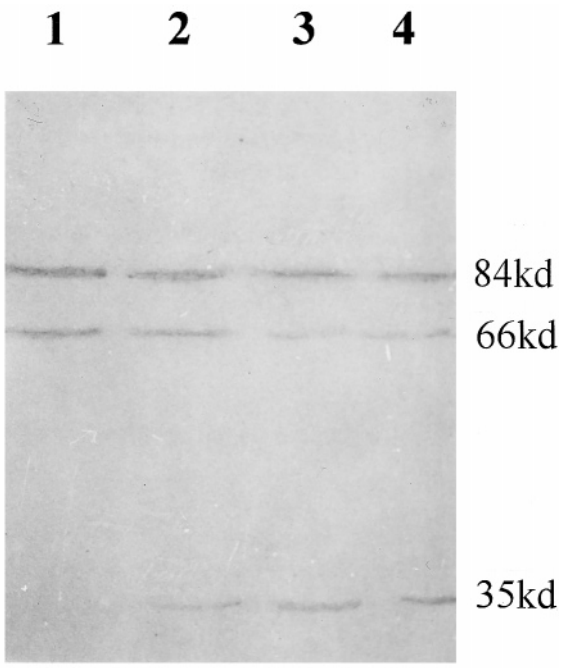

Figure 9. Time course of menadione-induced lamin-like protein degradation as detected by immunoblotting. Lane 1. control; lane 2, protoplasts were treated with 100 μM menadione for 6 h; lane 3, protoplasts were treated with 100 μM menadione for 10 h; lane 4, protoplasts were treated with 100 μM menadione for 14 h.

time, no degradation was detected. When protoplasts were treated with menadione for 6 h, a light band of the 35-kDa fragments was seen. If the menadione treatment lasted for 10 or 14 h, most of the lamin-like proteins had degraded, as shown in figure 9. When a comparison of the time course was made between DNA fragmentation and degradation of lamin C-like proteins, it was clear that degradation of lamin-like proteins preceded oligonucleosomal DNA fragmentation in menadione-treated tobacco potoplasts.

Discussion

Apoptosis, a genetically controlled program in animals, is characterized by chromatin condensation, DNA degradation and the formation of apoptotic bodies [1]. Apoptosis has also been found in plants in recent years. Previous studies demonstrated that the fundamental elements of apoptosis characterized in animals are conserved in plants [2, 3]. In some animal and human cells, it was found that menadione inhibits growth and induces apoptosis [4, 14]. The results reported in this paper indicate that menadione also triggers apoptosis in tobacco protoplasts. This was evidenced by detection of a series of stereotypic hallmarks of apoptosis, including the formation of apoptotic bodies and DNA laddering. TUNEL assay and neutral comet assay were also used to detect the DNA strand breakage. However, the mechanism by which menadione triggers apoptosis in animals and plants is not well defined as yet. The action of menadione, a quinone that undergoes redox cycles leading to the generation of noxious oxygen species, was proposed to be responsible for inducing apoptotic cells. More recently, it was suggested that apoptosis may be induced by menadione through direct arylation of cellular thiols causing depletion of glutathione and inactivation of sulfhydryl-dependent proteins. Elucidation of the mechanism of menadione induction of apoptosis awaits further studies [13].

Interestingly, 5 mM of PMSF, a cysteine and serine protease inhibitor, was found to inhibit DNA fragmentation in menadione-treated tobacco protoplasts. The results suggested a possible involvement of protease in menadione-induced apoptosis. Furthermore, we found that Ac-DEVD-CHO, a potent competitive inhibitor of caspase-3 and caspase-7 in animal cells, completely prevented DNA laddering induced by menadione in tobacco cells. The results implied that caspase-like cysteine protease may also play a critical role in the apoptotic pathway of plant cells [15].

In animal cells, lamins were found to be degraded during apoptosis as an earlier event. Lamin degradation and thus lamina dissolution play a direct role in chromatin condensation and breakdown of the nuclear envelope, which takes place during apoptosis. In addition, caspase-6 was reported to catalyze lamin A degradation during apoptosis in animal cells [16–19]. In plants, there are reports indicating that lamin-like proteins occur in nuclei [10]. In our study, we provided evidence that lamin C-like proteins degraded during apoptosis in plant cells and that this may play an identical role in the apoptotic pathway in plant cells. Our results of the degradation of lamin-like proteins in addition to the above-mentioned data regarding the effect of Ac-DEVD-CHO DNA laddering suggest the possible involvement of caspase-like cysteine protease in programmed plant cell death.

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