Is internucleosomal DNA fragmentation an indicator of programmed death in plant cells?

Andrea Kuthanova, Zdenek Opatrny and Lukas Fischer*

Charles University in Prague, Faculty of Science, Department of Plant Physiology, Vinicna 5, CZ 128 44 Prague 2, Czech Republic

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Abstract

Specific DNA fragmentation into oligonucleosomal units occurs during programmed cell death (PCD) in both animal and plant cells, usually being regarded as an indicator of its apoptotic character. This internucleosomal DNA fragmentation is demonstrated in tobacco suspension and leaf cells, which were killed immediately by freezing in liquid nitrogen, and homogenization or treatment with Triton X-100. Although these cells could not activate and realize the respective enzymatic processes in a programmed manner, the character of DNA fragmentation was similar to that in the cells undergoing typical gradual PCD induced by 50 μM CdSO₄. This internucleosomal DNA fragmentation was connected with the action of cysteine proteases and the loss of membrane, in particular tonoplast, integrity. The mechanisms of DNase activation in the rapidly killed cells, hypothetical biological relevance, and implications for the classification of cell death are discussed.

Key words: Non-PCD internucleosomal DNA fragmentation, programmed cell death, protease inhibitor, tobacco BY-2 cell line, vacuolar integrity.

Introduction

The process of programmed cell death (PCD) is based on the actively controlled degradation of intracellular components and facilitates removal of unwanted, incorrect, or damaged cells from multicellular organisms. PCD fulfils several essential functions in plant life: terminal differentiation of cells and sculpting tissues in plant development, responses to abiotic and biotic stress (e.g. hypersensitive response), and senescence connected with the reutilization of nutrients. Although individual processes differ in the triggering factors, it was suggested that all share a singular event: the action of the vacuole. Vacuole collapse, releasing sequestered hydrolases, is regulated by the cell itself and probably represents the ‘point of no return’—the moment of cell death (Jones, 2001).

Not only the triggers, but also the executive phase and typical hallmarks of PCD, differ under different occasions. The classification of PCD has proved to be difficult; specialized types of PCD; autophagy, paraptosis or mitotic catastrophe differ from the apoptosis first identified both morphologically and in their mechanisms (Bröker et al., 2005). In animal apoptosis, specific proteases (mainly cytosolic cysteine/aspartate proteases caspases) activate executive enzymes involved in the digestion of cells from the inside (reviewed in Kumar, 2007). One of the first biochemical symptoms/indicators of animal apoptosis to be identified was the specific internucleosomal fragmentation of chromosomal DNA (Wyllie et al., 1980), although it may not be detectable, even in cells which undergo morphologically typical apoptosis (Oberhammer et al., 1993).

Cleavage of genomic DNA during apoptotic PCD is realized in two subsequent steps; an early cleavage into high molecular weight fragments (in sizes consistent with chromatin loop domains) and, later, an intense fragmentation, usually forming oligonucleosomal fragments (Brotner et al., 1995), which can be detected by DNA electrophoresis (in the whole tissue or cell population). In situ excessive DNA fragmentation can be visualized in individual cells by TUNEL reaction (TdT-mediated dUTP nick-end labelling; Gavrieli et al., 1992), however, without the possibility to distinguish between internucleosomal and random cleavage.
Several different DNA endonucleases have proved to be responsible for producing both large and small DNA fragments. The majority of those already identified mainly in animals, for example, DNase I (Oliveri et al., 2001), DNase II (Barry and Eastman, 1993), or CAD/CPAN/DFT40 (Enari et al., 1998) are activated by caspases. However, there are also some DNases, which belong to the caspase-independent biochemical pathway; for example, L-DNase II (Torriglia et al., 1998) or endonuclease G (Li et al., 2001), which are associated with intracellular acidification. Moreover, activation of some plant DNases involved in internucleosomal fragmentation during PCD can also be mediated by serine proteases (Ye and Varner, 1996). Serine proteases were also involved in internucleosomal fragmentation observed during necrotic (non-programmed) death of animal cells (Dong et al., 1997).

In this study, we present to our knowledge, the first description of non-PCD internucleosomal fragmentation of DNA in plant cells exposed to different treatments causing immediate cell death; freezing in liquid nitrogen, homogenization, and treatment with a detergent Triton X-100. Biochemical and cytological analysis of the rapidly killed cells was performed and compared with the cells treated with 50 μM and 1 mM CdSO₄ causing either programmed or non-programmed cell death (Fojtová and Kovařík, 2000; Kuthanova et al., 2004; Yakimova et al., 2006). Based on the results, a mechanism of the non-programmed DNase activation is proposed and our results are discussed with respect to their possible biological relevance and practical consequences for cell death classification.

Materials and methods

Plant material

The tobacco BY-2 cell line (Nicotiana tabacum L., cv. Bright Yellow 2) was maintained in the modified MS medium (Murashige and Skoog, 1962) and subcultured every seventh day according to Nagata et al. (1992). Three-day-old cell cultures (in the exponential phase of growth) were used in all experiments. Tobacco plants (cv. Samsun) were grown on solidified MS medium at 25 °C under a 16/8 h light/dark photoperiod and an irradiance of 120 W m⁻² (leaves of 8-week-old plants were used in the experiments).

Induction of cell death

Freezing in liquid nitrogen: BY-2 cells (either untreated or just after the addition of CdSO₄ to a final concentration of 1 mM) or detached tobacco leaves (from 3-week-old plants) were killed by immediate freezing in liquid nitrogen for 3 min, followed by quick thawing at 60 °C (for 5 min).

Mechanical killing: BY-2 cells, filtered via Nalgene filter equipment (Nalgene Nunc International, Rochester, NY, USA) with a nylon mesh (20 μm) to reduce the culture volume by approximately 20 times, were killed mechanically (in 2 ml Eppendorf tubes) by homogenization with two steel balls in the mixer mill Retsch MM301 (Retsch GmbH, Haan, Germany, 5 min, 25 oscillations s⁻¹, at 25 °C).

Following the treatments mentioned above, the cultures (of dead cells) were incubated at 25 °C for 1–7 d in darkness on a shaker. Frozen and thawed tobacco leaves were kept in a sterile tube with a small amount of MS medium to provide high humidity.

CdSO₄ and Triton X-100 treatment: CdSO₄ (at a final concentration of 50 μM and 1 mM) or Triton X-100 (at a final concentration of 5%) supplemented the cultivation medium. The cultures were further cultivated under standard cultivation conditions for 1–7 d. The viability of cells was determined using fluorescein diacetate staining according to (Wildholm, 1972).

Protease inhibitors

Protease inhibitors supplemented BY-2 cultures just before killing in liquid nitrogen, and DNA fragmentation was assessed 1 d afterwards. Inhibitors used were, inhibitor of cysteine proteases E-64c (in 1% methanol) at final concentrations of 10 μM and 50 μM (Moriyasu and Ohsumi, 1996); serine (cysteine) protease inhibitor PMSF (phenylmethanesulphonyl fluoride, in dimethyl sulfoxide) at final concentrations of 1 mM and 10 mM, and serine protease inhibitor aprotinin (in water) at final concentrations of 0.10 μM and 0.50 μM.

Evaluation of DNA integrity

Genomic DNA was isolated from 250 mg fresh weight of filtered BY-2 cells or tobacco leaves as described by Fojtová and Kovařík (2000).

The presence of oligonucleosomal fragments was evaluated by electrophoresis (at 0.1–0.2 V cm⁻¹) in 1.8% agarose gel in the presence of ethidium bromide.

TUNEL reaction, terminal deoxynucleotidyl transferase (Tdt)-mediated deoxyuridinetriphosphate (dUTP)-nick labelling, was used to detect 3′-OH termini in fragmented nuclear DNA. The procedure was performed according to Jones et al. (2001) using the TMR-red (red fluorescence) in situ cell death detection kit (Roche Diagnostic GmbH, Mannheim, Germany). Cell integrity assessment

The integrity of dying BY-2 cells was evaluated under a confocal microscope TCS SP2 AOBS (TCS NT, Leica, Heidelberg, Germany) equipped with Nomarski optics (DIC). The membrane system was visualized either using transgenic line BY-GV7, stably expressing GFP-AtVam3p fusion protein localized in the vacular membranes (Kutsuna and Hasezawa, 2002) or by an FM4-64 probe (Molecular Probes) used for the endomembrane system. FM4-64 (dissolved in DMSO) was added to the BY-2 culture to a final concentration of 32 μM just before the treatment with CdSO₄ or 24 h before freezing. The vacular integrity was, in all cases, evaluated 24 h after FM4-64 addition, when it should preferentially stain vacuoles according to Kutsuna and Hasezawa (2002). The cells were observed under a confocal microscope TCS SP2 AOBS equipped with ArKr laser and a filter set for TRITC and filter set for FITC. Objective lenses Plan Apo (magnification 63×, numerical aperture 1.2) were used for all observations.

Results

DNA fragmentation

Internucleosomal fragmentation of genomic DNA was studied in tobacco cells subjected to different conditions inducing either rapid or slow cell death. Integrity of the
total DNA at different times after the treatment was analysed by agarose gel electrophoresis and, in selected cases, by TUNEL reaction.

**Cell death induction and electrophoretic detection of internucleosomal fragmentation:** DNA isolated from untreated tobacco BY-2 cells was never fragmented into oligonucleosomal units and formed a single, high molecular weight band (Fig. 1A) even after prolonged cultivation for 2 weeks (data not shown). To induce slow, programmed cell death of the cells, the culture was treated with 50 μM CdSO₄, causing a gradual decrease in cell viability between the second and the fifth days of the treatment (Fojtová and Kovářík, 2000; Kuthanova et al., 2004). Specific oligonucleosomal fragments were clearly detected after 7 d; i.e. approximately from the time when

![Fig. 1. Internucleosomal fragmentation of tobacco genomic DNA during 7 d after different treatments.](image)

(A) Untreated control BY-2 cells in the exponential phase of growth, (B) BY-2 cells treated with 50 μM CdSO₄, (C) BY-2 cells treated with 1 mM CdSO₄, (D) BY-2 cells killed by freezing in liquid nitrogen and further cultivated after thawing at 25 °C, (E) BY-2 cells killed by liquid nitrogen and further cultivated at 25 °C in the presence of 1 mM CdSO₄, (F) tobacco leaves frozen in liquid nitrogen and cultivated at 25 °C, (G) BY-2 cells killed mechanically by homogenization in a mixer mill and further cultivated at 25 °C, (H) BY-2 cells treated with 5% Triton X-100. DNA isolated from the cells (representative samples from 2–5 independent experiments) was separated in 1.8% agarose gel in the presence of ethidium bromide. Arrows indicate positions of oligonucleosomal fragments. Line markers: M, molecular mass marker; C, untreated control leaves; 0.5, 1, 3, 7, d of culture (exposure in case of CdSO₄ and Triton X-100 treatments).
practically all cells in the culture died as determined by fluorescein diacetate staining (Fig. 1B). BY-2 cells treated with 1 mM CdSO₄ died quickly within several hours. DNA isolated from these cells during subsequent cultivation for 7 d remained without or with almost undetectable fragmentation (Fig. 1C). Freezing of BY-2 cells in liquid nitrogen, followed by quick thawing resulted in immediate cell death. DNA isolated from these cells (subsequently cultivated at 25 °C) was fragmented as early as the first day after thawing. The progressive fragmentation continued, yielding shorter oligonucleosomal fragments, during prolonged cultivation (Fig. 1D). When CdSO₄ (at 1 mM final concentration) was added to the culture just before freezing, the progression of DNA fragmentation was partially slowed as compared to the culture frozen without CdSO₄ (Fig. 1E). Fragmentation of DNA isolated from tobacco leaves frozen in liquid nitrogen, thawed, and further cultivated at 25 °C was clearly detectable from the third day of cultivation after the thawing, although particular oligonucleosomal fragments were less evident (Fig. 1F). Mechanical disintegration of BY-2 cells by homogenization in a mixer mill or the addition of Triton X-100 to a final concentration of 5% also resulted in pronounced internucleosomal fragmentation of DNA isolated from the cultures during subsequent cultivation at 25 °C for 1–7 d (Fig. 1G, H, respectively).

**TUNEL reaction:** TUNEL reaction was used in parallel with electrophoretic analysis for *in situ* visualization of fragmented DNA in selected treatments. In rapidly killed cells (by freezing in liquid nitrogen) the fluorescent signal was observed in almost 50% nuclei (referred as TUNEL positive nuclei) by 3 d after freezing (almost 80% of nuclei were malformed after the treatment; Fig. 2). In the culture treated with 50 μM CdSO₄ the frequency of TUNEL-positive nuclei reached about 40% after 7 d (Fig. 2D). Practically no TUNEL-positive nuclei were detected in untreated control cells and in the cells treated with 1 mM CdSO₄ (Fig. 2D; Kuthanova et al., 2004).

**Morphology of the cells**

To assess the impact of structural changes for the induction of DNA fragmentation, the cellular, and in particular vacuolar, integrity were analysed after staining with the membrane probe FM4-64 and using Nomarski differential contrast. To confirm the specificity of FM4-64 staining, the cellular integrity was evaluated in the BY-GV7 transgenic cell line with GFP targeted into the tonoplast (Kutsuna and Hasezawa, 2002). Untreated exponential BY-GV7 culture (as well as the untransformed BY-2 line) was characterized by long files of small isodiametric cells with apparent cytoplasmatic strands and several middle-sized vacuoles (Fig. 3A–D). Treatment with 50 μM CdSO₄ stopped cell division and resulted in typical elongation during the first 3 d of the treatment (Fig. 3E–H). In the cells, few large and numerous small spherical vacuoles/vesicles were visible.
in the cytoplasm (Fig. 3F–H). Treatment of BY-GV7 (BY-2) cells with 1 mM CdSO₄ resulted in cell death within a few hours, characterized by protoplast shrinkage. The internal architecture of the cells was not visually disturbed; vacuoles, cell walls, and plasma membranes looked to be integral, even though the cells underwent plasmolysis (Fig. 3I–L). Cells rapidly killed by either freezing in liquid nitrogen (Fig. 3M–P) or direct mechanical disintegration (data not shown) lost their integrity immediately. Disruption of vacuoles and cell walls, and swelling of membranes was observed just after the treatment. The FM4-64 signal, a membrane-staining probe, was dispersed as well as the GFP signal in the BY-GV7 transgenic cells (Fig. 3N, O). Similar disintegration of membranes was observed after several hours of Triton X-100 treatment (data not shown).

**Protease inhibitors**

Proteases play an important role in PCD and are usually responsible for DNase activation. In order to examine the involvement of different proteases in the internucleosomal fragmentation in rapidly killed cells, specific protease inhibitors were tested. While cysteine protease inhibitor E-64c strongly suppressed DNA fragmentation in a concentration-dependent manner (Fig. 4A), aprotinin, predominantly inhibiting serine proteases, and PMSF, inhibiting serine and, partially, at higher concentrations, cysteine proteases as well (Renier, 2004), had only minor effects on DNA fragmentation in BY-2 cells killed by freezing in liquid nitrogen (Fig. 4B, C, respectively). The solvents (methanol and dimethyl sulphoxide) used for inhibitor stock solutions did not influence internucleosomal fragmentation at all (data not shown).

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**Fig. 3.** Morphology of BY-GV7 cells on 1 d after different treatments. (A–D) Untreated cells, (E–H) elongated cells in the presence of 50 μM CdSO₄ with small rounded vacuoles, (I–L) plasmolysed dead cells in the presence of 1 mM CdSO₄ with preserved vacuolar membranes, (M–P) disintegrated cells with diffusional signal of tonoplast after freezing in liquid nitrogen. (A, E, I, M) Nomarski differential contrast, (B, F, J, N) tonoplast targeted GFP signal, (C, G, K, O) staining with vacuolar membrane probe FM4-64, (D, H, L, P) schematic figure of membrane system. Scale bars=10 μm.
DNA fragmentation into oligonucleosomal units was originally described during apoptosis in animal cells (Wyllie et al., 1980). In plant cells, progressive internucleosomal fragmentation was documented during programmed cell death (PCD) in abiotically stressed tobacco BY-2 cell line cultivated at low temperature (4 °C; Koukalová et al., 1997) or treated with 50 μM CdSO₄ (Fojtova and Kovarík, 2000). Although electrophoretic detection of internucleosomal fragmentation is usually considered as a typical symptom and indicator of certain PCD types, this fragmentation in plant cells which demonstrably did not undergo PCD (Fig. 1D, F, G, H) was clearly documented. Besides, using the TUNEL reaction (Gavrieli et al., 1992) the presence of fragmented DNA was confirmed in situ in the majority of nuclei (Fig. 2). The internucleosomal fragmentation was observed after three different treatments causing rapid cell death, but it did not accompany moderately quick death (within several hours) of cells treated with 1 mM CdSO₄ (Fojtová and Kovarík, 2000; Fig. 1C). Since the presence of 1 mM CdSO₄ itself did not substantially inhibit DNA fragmentation in cells killed by freezing (Fig. 1E), morphological differences between the cells in individual treatments were sought. All three treatments inducing rapid internucleosomal fragmentation were characterized by a loss of integrity of the membrane system, in particular the tonoplast, and this was clearly visible under the confocal microscope using both the membrane staining probe FM4-64 and tonoplast targeted GFP in the BY-GV7 (Kutsuna and Hasezawa, 2002) transgenic cells (Fig. 3M–P). By contrast, in cells treated with 1 mM CdSO₄, killed within several hours, the tonoplast integrity seemed to be preserved (Fig. 3I–L) corresponding with no DNA fragmentation in these cells. Intensive changes in the vacuolar system (splitting of vacuoles) accompanied step-wise induction of PCD in the cells treated with 50 μM cadmium (Fig. 3E–H) and preceded the loss of the cell integrity and internucleosomal fragmentation both observed in the late phases of cell death.

The morphological analysis thus indicated that internucleosomal fragmentation was connected with the loss of vacuole integrity, in agreement with the general assumption that vacuole collapse, releasing sequestered hydrolyses, is a common mechanism of the majority of plant PCD types (Jones, 2001). Regulated vacuolar collapse accompanied by the release of hydrolytic enzymes was repeatedly documented during plant PCD; xylem differentiation (Groover and Jones, 1999; Kuriyama, 1999), death of cells in the aleurone layer (Bethke et al., 1999), somatic embryogenesis (Filonova et al., 2000), and the hypersensitive reaction (Hatsugai et al., 2004). Our results indicate, that the executive phase of PCD, normally induced by regulated (programmed) vacuole collapse, can be mimicked by its artificial violent disintegration.

After artificial vacuole disintegration in our experiments, either active DNases or activating proteases might have been released from the impaired vacuoles. In the case of xylem differentiation, ZEN1 DNase is transported into vacuoles directly in an active form just prior to vacuolar collapse, and played a major role in the subsequent non-specific nuclear DNA degradation (Ito and Fukuda, 2001). By contrast, in typical animal apoptosis, inactive DNase proenzymes, for instance specific CAD nucleases (Enari et al., 1998), DNase I (Oliveri et al., 2001), and DNase II (Barry and Eastman, 1993), are activated by cysteine proteases caspases. Alternative caspase-independent pathways, which involve serine proteases, can also mediate the activation of DNases, as in the case of L-DNase II in animal cells (Torriglia et al., 1999). In plants the mechanism of DNase activation is...
less well understood. Using specific protease inhibitors 
Kusaka et al. (2004) reported the involvement of cysteine
proteases during toxin-induced PCD in plant cells.
Caspases were not identified in plants, but plant-specific
cysteine proteases called vacuolar-processing enzymes
(VPEs) are supposed to substitute their function, as was
demonstrated during the PCD triggered by tobacco mosaic
virus (Hatsugai et al., 2004; Kuroyanagi et al., 2005).
Since cysteine protease inhibitor E-64c effectively
distinct DNA fragmentation accompanied by vacuolar
disintegration (Figs 3, 4), VPEs present in the vacuole
might be good candidates for the DNase activation in our
experiments. However, DNA fragmentation mediated by
VPEs was not internucleosomal during virus-induced
PCD (Hatsugai et al., 2004), suggesting that different
DNases might participate in DNA cleavage in our exper-
iments. Although the precise effectors of DNA cleavage
and DNase activation remain unknown, the results indicate
that the same enzymatic apparatus might be involved in
realization of the internucleosomal fragmentation during
both slow programmed cell death and rapid accidental
depth in our tobacco cells. It contrasts with the situation in
animals, where typical apoptotic internucleosomal fragmen-
tation is mediated by cysteine proteases caspases, while in
necrotically (non-programmed) dying cells, DNases were
activated by serine proteases (Dong et al., 1997).
Plant cells (at least those used in our study) appeared to
be predisposed for quick autolysis, for example, there was
an enzymatic apparatus ready to mediate quick specific
DNA cleavage triggered by either rapid accidental vacuole
disintegration or programmed vacuolar collapse. What
could the reason be for this predisposition? Since me-
chanical damage of plant tissues is relatively common and
such injuries might serve as an entry for many pathogens,
quick autolysis of damaged cells could represent another
component of the plant defense mechanism complemen-
tary to hypersensitive cell death. Although the biological
relevance remains unclear, our results document that
internucleosomal fragmentation in rapidly killed tobacco
cells is hardly distinguishable from that specific for
PCD. Therefore, DNA fragmentation into oligonucleoso-
mal units should not be considered as an indicator of PCD
without parallel evaluation of morphological changes of
intracellular components (in particular, the progression of
the loss of vacuolar integrity) especially in quickly dying
cells. Moreover, our findings should be taken into account
in the interpretation of any results based on the detection
of PCD-related protease or DNase activities in cell-free
extracts.

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