Programmed cell death in *Dictyostelium*

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**SUMMARY**

Programmed cell death (PCD) of *Dictyostelium discoideum* cells was triggered precisely and studied quantitatively in an in vitro system involving differentiation without morphogenesis. In temporal succession after the triggering of differentiation, PCD included first an irreversible step leading to the inability to regrow at 8 hours. At 12 hours, massive vacuolisation was best evidenced by confocal microscopy, and prominent cytoplasmic condensation and focal chromatin condensation could be observed by electron microscopy. Membrane permeabilization occurred only very late (at 40-60 hours) as judged by propidium iodide staining. No early DNA fragmentation could be detected by standard or pulsed field gel electrophoresis. These traits exhibit some similarity to those of previously described non-apoptotic and apoptotic PCD, suggesting the hypothesis of a single core molecular mechanism of PCD emerging in evolution before the postulated multiple emergences of multicellularity. A single core mechanism would underly phenotypic variations of PCD resulting in various cells from differences in enzymatic equipment and mechanical constraints. A prediction is that some of the molecules involved in the core PCD mechanism of even phylogenetically very distant organisms, e.g. *Dictyostelium* and vertebrates, should be related.

Key words: cell death, *Dictyostelium*, evolution

**INTRODUCTION**

Cell death occurs as a normal component of the development of multicellular eukaryotes. Classical examples of developmental cell death are embryonic tissue deletions required for proper morphogenesis (Saunders, 1966; Hinchliffe, 1981; Zakeri et al., 1993; Garcia-Martinez et al., 1993), elimination of neurons failing to establish adequate trophic connections at the appropriate time (Oppenheim, 1991; Johnson and Deckwerth, 1993), and negative selection of lymphocytes bearing inappropriate antigen receptors (Cohen, 1991; Golstein et al., 1991). Developmental cell death is programmed, not only as to its occurrence in the organism, but also as to its course within the dying cell (Golstein et al., 1991). We shall use throughout this paper the term programmed cell death (PCD; Lockshin and Zakeri, 1991) in its developmental biology sense (Schwartz and Osborne, 1993) encompassing both programmed occurrence and programmed course of cell death. The term apoptosis (Kerr et al., 1972) usually refers to a morphological type often observed in PCD, associating cytoplasmic and nuclear condensation and fragmentation, DNA fragmentation (Wyllie, 1980) and sometimes a requirement for macromolecular synthesis (Cohen and Duke, 1984; Wyllie et al., 1984). In most of the models studied so far, PCD follows an apoptotic pattern; however, other PCD morphological types have also been described (Clarke, 1990; Schwartz et al., 1993; Vaux, 1993; Schwartz and Osborne, 1993).

Which molecules directly effect cell death? We are referring here not to molecules involved in the transduction of a cell death signal, nor to control molecules such as those encoded by certain oncogenes or viral genes, nor to molecules modifying the morphological aspect of a dying cell, but to ‘core death molecules’ more directly involved in the putative first irreversible event leading to death (Golstein et al., 1991). *ced-3* and *ced-4* involved in developmental cell death in the nematode *Caenorhabditis elegans* (Hedgecock et al., 1983; Ellis and Horvitz, 1986; Ellis, 1992), the *ced-3* equivalent ICE cysteine protease in mouse (Miura et al., 1993), the several molecules preferentially expressed in dying thymocytes (Owens et al., 1991), the recently described *reaper* molecule involved in the death of a large number of cells during embryonic development in *Drosophila* (Abrams et al., 1993; White et al., 1994), or inappropriately expressed cell cycle molecules (Shi et al., 1994) are candidates for being such core death molecules.

*Dictyostelium discoideum*, which multiplies as a unicellular protist under favorable conditions, undergoes, when starved, cell differentiation and morphogenesis leading within 24 hours to a multicellular fungus-like structure called sorocarp (Raper, 1935, 1984). The sorocarp ultimately includes two main cell
populations, on the one hand viable spores, and on the other hand stalk cells. Differentiation to stalk cells seems to result from the sequential action of at least two factors, i.e cyclic AMP promoting, in particular, cell aggregation, and a factor called DIF promoting, in particular, the differentiation of starved cAMP-subjected cells to stalk cells (Town et al., 1976; Town and Stanford, 1979; Sobolewski et al., 1983; Morris et al., 1987). Stalk cells are vacuolated (Raper and Fennell, 1952; Maeda and Takeuchi, 1969; George et al., 1972; de Chastellier and Ryter, 1977; Quiviger et al., 1980; Schaap et al., 1981) and are considered non-viable according to the criterion of non-regrowth in culture medium (Whittingham and Raper, 1960).

The choice of Dictyostelium to study PCD is appealing for several reasons. First, phylogenetically, this organism can be considered as one of the probably several (Kaiser, 1986) evolutionary attempts at multicellularity. If cell death is a corollary to multicellular development, as suggested long ago (Weismann, 1890), it would seem of interest to characterise the type of death observed in the development of this ‘transitional’ organism. Also, Dictyostelium is one of the most anciently diverged currently surviving eukaryotes (Christen et al., 1991; Field et al., 1988): a demonstration of a common cell death mechanism between this organism and some of the higher eukaryotes would be a strong argument for the generality of this mechanism. The second reason is ontogenetic: the relatively simple pattern of development of Dictyostelium should facilitate the study of cell death occurring during this development. The third reason, which builds on the previous one, is that methods exist to trigger, in vitro, differentiation without morphogenesis (Town et al., 1976; Kopachik et al., 1983; Sobolewski et al., 1983; Kay, 1987), and thus to obtain isolated dying Dictyostelium cells, potentially easier to identify and study. Finally, another reason is genetic: the genome of Dictyostelium, about 100-fold smaller than that of higher eukaryotes, is haploid, which might make it feasible to generate and select mutants of death-associated genes, and to identify the latter, especially using recently developed genetic tools (Loomis, 1987; Kuspa and Loomis, 1992). Because of the temporal separation between vegetative growth and development, such developmental mutants can be propagated under vegetative conditions, thus behaving as conditional mutants (Loomis, 1987).

We describe here programmed cell death in Dictyostelium, under in vitro conditions mimicking developmental circumstances. These in vitro conditions are more amenable than previous in vivo experiments on stalk cell death (Whittingham and Raper, 1960) to direct experimental investigation and to further genetic manipulations. Owing to the difficulties of unambiguously defining cell death, we made use of several distinct experimental end points for death: staining with fluorescent dyes reflecting cell membrane permeability changes, checking vacuolisation, checking the state of nuclear DNA by gel electrophoresis, checking morphological changes by electron microscopy, and quantifying reversibility to vegetative growth of surviving cells upon returning to favorable growth conditions. Death could thus be described as a developmentally induced sequence of intracellular events, including an early irreversible step, subsequent massive vacuolisation, cytoplasmic condensation, focal chromatin condensation, and very late membrane lesions. No early DNA fragmentation could be detected. These results show that, while PCD in Dictyostelium has some traits of its own, it is also akin to apoptotic and non-apoptotic morphological aspects of cell death described in higher eukaryote cells, a similarity leading to the hypothesis of a single core molecular mechanism of PCD.

**MATERIALS AND METHODS**

**Cells and culture conditions**

Dictyostelium discoideum AX2, an axenic mutant (Watts and Ashworth, 1970) derived from NC4, and obtained from M. Hildebrandt (Pasteur Institute, Paris), was used to calibrate culture and differentiation conditions. HMX44, an axenic mutant derived from V12M2, not producing DIF but responding to exogenously added DIF, was obtained from J. G. Williams (Imperial Cancer Research Fund Laboratories, Clare Hall). The subclone HMX44A was derived and used in this laboratory. AX2 and HMX44A cells were cultured in HL5 medium (Sussman, 1987) with the following modifications: bactopeptone (Oxoid, Basingstoke, Hampshire, England) 14.3 g/l; yeast extract (Difco Laboratories, Detroit, MI) 7.15 g/l; maltose (Sigma Chemical Co., St Louis, MO) 18 g/l; Na2HPO4, 3.6 mM; KH2PO4, 3.6 mM; source water. Cultures were in 50 ml flasks (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) kept horizontally, and subcultured twice a week in 10 ml of culture medium; 250 ml and 750 ml flasks (Falcon) were used to provide sufficient numbers of cells for large experiments. Cultures and experiments were at 22-23°C in a water-saturated atmosphere.

DO.11.10, a murine T cell hybridoma line (Shimonkevitz et al., 1983) and mouse Balb/c thymocytes were processed and cultured in RPMI 1640 (Gibco/BRL, Grand Island, NY) plus 5% FCS (D. Deutscher, Brumath, France) for DO.11.10 cells, or 10% FCS for thymocytes. Incubations and culture procedures were at 37°C in a water-saturated 8% CO2 atmosphere.

**Differentiation conditions**

Reagents and differentiation media were cAMP (adenosine 3′,5′-cyclic monophosphate; Sigma; 30 mM sterile stock solution in water, aliquoted and stored at −20°C), DIF as DIF-1 (1-(3,5-dichloro-2,6-dihydroxy-4-methoxyphenyl)-1-hexanone; Molecular Probes, Inc.; Eugene, OR; 10-7 M stock solution in absolute ethanol, kept at −20°C), and SB (Soerensen buffer; 50 mM Na2HPO4, 735 mM KH2PO4; 1×: 17 mM phosphate, final pH 6.0). Experiments were done in 50 ml plastic flasks or on 18 mm-diameter coverslips in wells of 6-well plates (Falcon). We followed a protocol by Kay (1987) with only slight modifications. Briefly, logarithmic growing cells were washed twice with SB and were incubated for 8 hours in SB in the presence of 3 mM cAMP at a density of 105 cells/cm2 and a concentration of 104 cells/ml (2.5 ml per flask, 1.25 ml per well). This first incubation was followed by one careful wash with twice the volume of SB used for the first incubation and by a second incubation in SB alone (control) or in SB in the presence of 100 nM DIF (experimental), for the indicated lengths of time. Differentiated cells were then usually examined by fluorescence staining. To ensure maximum differentiation under every experimental condition described in this report, we used DIF at a concentration of 100 nM for the whole duration of the differentiation periods tested. At this concentration, DIF affected neither vegetatively growing Dictyostelium cells, nor the mouse hybridoma cell line DO.11.10, and from another point of view ensured differentiation at 48 hours even when incubated with cells for only 15 minutes (not shown).

**Fluorescence staining**

For fluorescence microscopy, fluorescein diacetate (FDA) and propidium iodide (PI) (both from Sigma; stock solutions at 10 mg/ml in acetone and 8×10-6 M in sterile water, respectively) were added
directly to Dictyostelium-containing flasks to final concentrations of 0.05 mg/ml and 4 µg/ml, respectively. After a 10 minute incubation, the flasks were carefully washed with twice the volume of SB used in differentiation and were ready for observation using an inverted Axiovert 35 microscope (C. Zeiss, Oberkochen, Germany).

For confocal observation, Dictyostelium-bearing coverslips were overlaid with 400 µl of a mixture of FDA and PI solutions in SB at the final concentrations indicated above. After a 10 minute incubation, the coverslips were carefully washed in 5 ml of SB and mounted cells-down in a home-made 400 µl chamber accommodating living cells. These were observed with a TCS 4 D confocal microscope based on a Leica DMR stand and equipped with a mixed gas argon/krypton laser and 3 detectors (Leica Laser Technik, Heidelberg, Germany; Humbert et al., 1993). FDA and PI were excited simultaneously using the argon 488 nm line and the krypton 568 nm line. Simultaneous confocal excitation and detection were performed through a double dichroic mirror and appropriate band-pass filters allowing the recording of the green FDA fluorescence from 520 to 540 nm and the red PI fluorescence above 590 nm. The corresponding electronic images were merged and printed as described (Humbert et al., 1993).

**Electron microscopy**

Cultured cells were rinsed in PBS, fixed in 2.5% glutaraldehyde in 2.5% sucrose in 0.2 M cacodylate buffer, pH 7.4, at room temperature, rinsed twice and postfixed with 1% OsO4. After dehydration in graded ethanol and embedding in araldite (Fluka), sections were obtained with a Reichert Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate and examined with a JEOL 200C electron microscope.

**Re-growth experiments**

‘Re-growth’ experiments checked the ability of differentiating cells to revert to a vegetative state. First, a differentiation experiment was done in flasks as described above. Second, at various times after DIF addition, per flask a volume of 7.5 ml of HL5 culture medium was added to the 2.5 ml of DIF-containing SB. Cells were counted after 2 days of further incubation.

**Checking the state of DNA**

Agarose plugs containing about 5×10⁶ vegetative or 5-10×10⁶ differentiating HMX44A cells were prepared as described (Cox et al., 1990) except that LMP agarose (Gibco/BRL) and 100 µl rectangular molds were used instead of 25 µl ones, with the corresponding adjustments as to final cell concentrations and amounts of solutions. Agarose plugs containing 5×10⁶ Balb/c thymocytes were prepared as follows. Cells were washed twice in PBS and mixed with an equal volume of lukewarm 2% LMP agarose/PBS. Aliquots were pipetted on ice into molds. After cooling, the plugs were incubated in 0.5 M EDTA, pH 8.0/1% sarcosyl/2mg/ml proteinase K (Appligene, Pleasanton, CA) at 8.0°C for 7 hours). For some plugs, the cells had been treated previously with 0.05 mg/ml and 4 µg/ml herring sperm DNA. HMX44A or DO.11.10 probes were random-labeled (Feinberg and Vogelstein, 1984) using 32P-labeled DT. For probing HMX44A Southern blots, probes were prepared with HMX44A total DNA extracted as published (Welker et al., 1985) with some modifications: nuclei were lysed at room temperature in 20 ml of a 0.2 M EDTA, pH 7.2/2% sarcosyl solution, and after a cesium chloride gradient centrifugation the isolated DNA band was diluted with 1 volume of low TE buffer (10 mM Tris-HCl, pH 7.5/0.1 mM EDTA) before ethanol precipitation. A labeled probe was made with 100 ng of total DNA. For thymocyte Southern blots, probes were prepared from one DO.11.10 agarose plug. Briefly, a 100 µl plug containing 5×10⁶ DO.11.10 cells was allowed to melt at 65°C and mixed with 300 µl of distilled water. Probes were prepared using 25 µl of this mix according to standard protocols (Feinberg and Vogelstein, 1984) except that annealing was at 37°C instead of 4°C. Labelled probes were added to membranes in 40 ml of prehybridization buffer. After overnight hybridization at 65°C, 3 or 4 washes were as follows: (1) 2x SSC, 5 minutes, RT, (2-3) 2x SSC, 0.1% SDS, 15 minutes, 65°C, (4) 0.2x SSC, 0.1% SDS, 15 minutes, 65°C. Filters were autoradiographed at −80°C for 5-14 days.

**RESULTS**

We wished to establish a system in which isolated Dictyostelium cells could be triggered to differentiate into stalk cells, facilitating a quantitative study of the death process. Dictyostelium strain V12M2 could be induced by DIF to differentiate in vitro to stalk cells much more efficiently than strain NC4 (Town et al., 1976), probably because of less interference of cAMP with DIF-induced effects (Berks and Kay, 1988). HMX44, a derivative of V12M2 producing little or no DIF but still sensitive to it (Kopachik et al., 1983, 1985), allows precise timing of initiation of differentiation upon addition of exogenous DIF. HMX44 (kindly provided by J. G. Williams, ICRF, Clare Hall) is an axenic derivative of HM44. We derived from HMX44 by serial limiting dilution cloning (with a cloning efficiency close to 100%, not shown) the substrain HMX44A, used in all subsequent experiments. The doubling time of HMX44A under vegetative growth conditions was 8 hours (not shown). The sequence of starvation/DIF addition used in previously described in vitro systems (Kay, 1987) led to the differentiation of these HMX44A cells within loose clumps and as isolated cells, allowing individual cell examination. Accordingly, our standard procedure included, starting from vegetatively-growing cells, a first incubation in SB in the presence of an excess of cAMP for 8 hours, followed by one wash and by a second incubation in SB in the presence of DIF for various lengths of time. This addition of DIF triggered the chain of cell death events studied in this report.

**Massive vacuolisation and late membrane lesions**

Fluorescein diacetate (FDA), a hydrophobic molecule, enters the cell in a passive way and is then cleaved by cytoplasmic enzymes, becoming as a result both fluorescent in the green spectrum and charged, unable to leave the cell. This stain thus labels cells with an intact plasma membrane. Propidium iodide (PI) can only enter cells with an altered plasma membrane and then stains, in particular, nucleic acids, re-emitting mostly red fluorescence. Vegetative HMX44A cells were all labelled by FDA and not by PI.
Cells were allowed to differentiate according to the standard protocol and were treated with both FDA and PI at various times after addition of DIF. As shown in the photographs of Fig. 1 and quantitatively in Fig. 2, at 8 hours almost all the cells were FDA-labelled, fluorescing green. Only a few vacuolated cells could already be seen among these green cells. With time, the percentage of vacuolated cells increased, reached 50% at about 12 hours, peaked at 16-24 hours and then decreased, while vacuolated PI-labelled red cells appeared and increased (Fig. 2). Most of these red cells contained a small dense red mass, most probably the nucleus, which was not obviously altered at this scale even at 48 hours. Only very few non-vacuolated PI-labelled cells could be seen. In three separate experiments, 50% of the cells were vacuolized after 11-14 hours in DIF, and 50% were PI+ after 40-60 hours in DIF.

Confocal microscope fluorescence micrographs of *Dictyostelium* cells subjected to DIF for 24 hours and stained with both FDA and PI are shown in Fig. 3. There are significant morphological differences between even successive optical slices of the same cells. Also of note is the massive vacuolisation of most cells and the presence of a number of heavily vacuolated PI-stained cells. Isolated red-labeled apparently intact nuclei could be seen (Fig. 3), and RNase-treated PI-labeled cells contained morphologically intact nuclei (not shown). No cell was detected which, immediately after staining, fluoresced red and green at the same time. However, some ‘non-specific’ red fluorescence, limited to the cell...
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Surface, was sometimes seen on green cells. Also, lengthy microscopic examination often led to some PI labelling of initially ‘green’ cells (unpublished experiments), resulting in a proportion of green-yellow cells by confocal microscopy (Fig. 3).

The control groups in these experiments were incubated in SB without DIF. Compared to the experimental groups with DIF, most of the cells fluoresced green throughout the experiment, with only a few cells fluorescing red at all times; most strikingly, only very few vacuolated cells appeared (Figs 1, 2). These patterns in control groups and in groups with DIF were very reproducible. In more than 50 differentiation experiments, only one gave results at variance with the description above. This could be traced to an alteration of the quality of the water used to prepare the SB solution, leading to the use of source water for the preparation of all media.

In conclusion for this type of experiment, the addition of DIF led to an early and marked increase in the proportion of vacuolated cells, and later to PI-labelling of a significant proportion of these cells. These results are consistent with the interpretation that DIF induces within a few hours vacuolisation in almost all cells, of which only some (within 4 days) will, significantly later, develop alterations of their plasma membrane.

Early irreversible impairment of re-growth

In experiments on in vivo differentiation of D. purpureum, Whittingham and Raper (1960) studied as an indicator of cell death the inability of cells released from stalks to grow in vegetative culture conditions. We took advantage of in vitro culture conditions to reassess the ability to grow after differentiation, with more precise time kinetics and in conjunction with other indicators of cell death. After incubation in SB and cAMP, Dictyostelium discoideum cells were incubated in the presence of DIF for variable lengths of time, then received an excess of HL5 medium and were incubated for a further 48 hour period. These experimental cells and also control cells were then counted. Vegetative cells grown in the same proportions of SB and HL5 in the presence of DIF did not differentiate and went on multiplying at their usual rate (not shown).

The flasks with cells incubated for 24 hours in DIF, and further incubated for 48 hours in the presence of HL5 medium, contained about 10-20% of the cells in control flasks not subjected to DIF (Fig. 4). These regrowing cells were vegetative-looking and multiplying (not shown). Assuming a similar doubling time for vegetative cells that had never been subjected to differentiation conditions and for cells having survived these conditions, these results indicated that 80-90% of the cells subjected to differentiation conditions for 24 hours were irreversibly altered and unable to re-grow. Longer incubation times in the presence of DIF (48-96 hours) did not significantly change the proportions of regrowing cells (not shown). Of note, the duration of incubation with DIF required for the non-regrowth of 50% of the cells was about 8 hours (shown in three distinct kinetics experiments, Fig. 4A-C), thus a few hours less than that required for vacuolisation of the cells (Fig. 2). A direct comparison in the
same experiment of the kinetics of induction of non-regrowth on the one hand and of vacuolisation on the other hand is shown in Fig. 4C. If non-regrowth means that an irreversible event has occurred in the differentiating cell, then clearly this irreversible event occurred significantly before any morphological alteration detectable by the techniques above.

Absence of early DNA fragmentation
Apoptotic cell DNA shows fragments of 50-300 kbp and often of 200 bp and multiples thereof (see references in the Discussion). We wondered whether DNA in differentiating Dictyostelium cells would be similarly fragmented. Cells incubated with or without DIF were harvested at different times, inserted in agarose plugs, proteinase K-treated, and subjected to classical electrophoresis to assess the existence of small fragments of DNA (200 bp), and to pulsed field electrophor-

Fig. 2. Kinetics of HMX44A cell differentiation quantified on a per cell basis. After FDA/PI staining, cells were examined for fluorescence and the presence of large vacuoles. Three areas of each flask were scored for each point of the graphs. At least 100 cells were examined per area. The results were pooled and expressed as percentages. (A) in SB alone. (B) in SB plus DIF. ■, Non-vacuolated FDA+ cells. □, Vacuolated FDA+ cells. Initially all cells were FDA+, without vacuoles; with time, almost all of these cells became vacuolated, and some became PI+. ○, PI+ cells. Almost all PI+ cells were non-vacuolated in SB alone and vacuolated in SB plus DIF (in 4 experiments the average percentage of non-vacuolated cells in SB plus DIF at 48 hours of differentiation was around 3%).

Fig. 3. Confocal fluorescence micrographs of Dictyostelium cells after 24 hours of incubation in DIF. Three pictures are shown for each of two groups of cells, above and below, respectively. For each group, two views 2.8 µm apart (middle and right) and 8 stacked views (maximum detections) over a total thickness of 19.5 µm (left) are shown. Individual FDA and PI images were superimposed numerically. Pseudocolours mimic FDA or PI staining, as previously described (Humbert et al., 1993).
After 8-12 hours of culture in SB without DIF, spots of condensed chromatin were visible within the nucleus, with significant cytoplasmic condensation (Fig. 6B and C). Cytoplasmic vacuoles were neither more abundant nor more developed than in vegetative cells. After 8-12 hours of culture in the presence of DIF (Fig. 7), the cell contours were smoothened, with loss of microvilli. The cytoplasm was dense and the nucleus contained patches or foci of dense chromatin, however, not associated with the nuclear membrane. The most obvious difference with groups in SB without DIF was the presence of large cytoplasmic vacuoles, either appearing empty or containing residual material (Fig. 7).

Thus, perhaps surprisingly, cells incubated in SB with or without DIF showed similar aspects of chromatin and cytoplasmic condensation, different from those of vegetative cells. Also, cellulose walls were prominent after incubation for 24 hours in SB with, as well as without, added DIF in studies using Calcofluor (not shown). Since HMX44A cells do not immediately die in SB without DIF, the results above indicated that neither the observed levels of cytoplasmic and chromatin condensation, nor cellulose walls, are enough to ensure PCD in Dictyostelium.

**Programmed induction of Dictyostelium cell death**

Cultures of HMX44A *Dictyostelium* cells were subjected to serial additions of cAMP or DIF (not shown). cAMP, known to be necessary in the *Dictyostelium* differentiation pathway, is produced by the cells themselves. We, however, systematically added an excess of cAMP to reduce aggregation. No developmental death was observed in complete HL5 medium or in the absence of DIF. Both starvation and the presence of DIF were required for cell death. Since DIF did not induce the death of vegetative cells, it follows that DIF can induce *Dictyostelium* death only when added to cells that have already reached a certain level of differentiation. More generally, in agreement with previous work (Kay, 1987), under our experimental conditions cell death occurred only when a given program of successive extra-cellular signals, i.e. starvation and DIF, was applied to the cells (not shown).

**DISCUSSION**

We have studied cell death in *Dictyostelium discoideum*, which is phylogenetically distant from currently used model organisms, has a relatively simple developmental pattern, and lends itself well to genetic analysis. In an in vitro system in which death could be induced without inducing morphogenesis, thus facilitating quantitative studies, *Dictyostelium* cell death was shown to be programmed as to both circumstances of occurrence and course within the dying cell (Fig. 8). This PCD in *Dictyostelium*, occurring in vitro upon addition of physiological inducers, seems equivalent to developmental cell death in vivo. The programmed course of death in vivo includes the appearance in dying cells of very large vacuoles, while in vivo all stalk cells are similarly vacuolated. The terms...
Fig. 5. Absence of massive early DNA fragmentation in Dictyostelium PCD. Cells were included in agarose plugs, which were treated to release DNA and inserted in gels run as classical gel electrophoresis (A) or as pulsed field gel electrophoresis (B and C). Cells were vegetative HMX44A Dictyostelium cells (Veget.), vegetative HMX44A cells treated for 3 or 5 days with 20 µg/ml neomycin (Neo 3d or 5d), HMX44A cells incubated for the indicated number of hours either in SB alone as a control, or in SB plus DIF to induce PCD (SB or DIF), and thymocytes either untreated or treated for 6 hours with 10^{-6} M dexamethasone (Thym. and Thym.+ Dex). Size markers were λ phage cut with HindIII (λHindIII), phage λ multimers (polyλ) and yeast chromosomes (yeast).
Fig. 6. Electron microscopy of HMX44A *Dictyostelium* cells, either vegetative or after incubation in SB. (A) A cell during vegetative growth, showing an irregular shape with a highly developed vacuolar system (V); the nucleus (N) contains a large nucleolus (n) associated with the nuclear membrane; the chromatin is homogeneously dispersed. (A') Detail of the nucleus in A. (B and C) After 12 hours of incubation in SB without added DIF, some spots of condensed chromatin can be seen within the nucleus; vacuoles are neither more abundant nor more developed than in vegetative cells. (C') Detail of the nucleus in C. Bars: 1 μm for A, 1.5 μm for B, and 0.5 μm for A', C and C'.
Fig. 7. Electron microscopy of HMX44A Dictyostelium cells after incubation in SB with added DIF. After 8 (A,B) or 12 (C,D) hours in the presence of DIF, cell contours are smoothened with loss of microvilli, the cytoplasm is dense with large vacuoles including some residual material, and the nucleus contains marked patches or foci of dense chromatin. Arrows, cellulose walls. Bars, 0.5 µm. V, vacuolar system; N, nucleus; n, nucleolus.
vacuoles is not just a reflection of an autophagic process (Maeda and Takeuchi, 1969; de Chastellier and Ryter, 1977; Quiviger et al., 1972; George et al., 1972; de Chastellier and Ryter, 1977; Schaap et al., 1980; Clarke, 1990). The presence of large vacuoles may be related to autophagy (George et al., 1972; de Chastellier and Ryter, 1977; Quiviger et al., 1980; Schaap et al., 1981). The inability to regrow occurs on average a few hours earlier than detectable vacuolisation. Electron microscopy showed that the inability to regrow occurs on average a few hours earlier than detectable vacuolisation.

Steps in programmed occurrence and programmed course of Dictyostelium HMX44A cells death. The times indicated after addition of DIF are the average times in hours at which 50% of the cells present the described characteristic.

Fig. 8. Steps in programmed occurrence and programmed course of Dictyostelium HMX44A cells death. The times indicated after addition of DIF are the average times in hours at which 50% of the cells present the described characteristic.

‘stalk’ or ‘vacuolated’ cells would thus seem to correspond to dead or dying cells.

**Dictyostelium** cell death, apoptotic and non-apoptotic morphological aspects of cell death

In *Dictyostelium* PCD, the first detectable irreversible event defining cell death, strictly speaking, was the inability to regrow. Using this criterion, under our experimental conditions about 50% of the cells die within 8 hours and 90% of the cells die within 24 hours after addition of DIF. Death, defined by inability to regrow, did not coincide in time with death defined with fluorescent dyes, or with any other criterion. For instance, membrane lesions, as detected by permeability to PI, occurred very late compared to regrowth inability. Thus, the irreversible lesion (‘death’) detected through inability to regrow leads to detectable morphological alterations only with significant delay. Such an irreversible event must exist also in bona fide apoptosis (see discussion in Golstein et al., 1991), where it has not been better molecularly characterized so far.

A major component of cell death in *Dictyostelium* is vacuolisation. Vacuolisation could be triggered independently of cell death by the same sequence of extracellular events, and might then be related to differentiation towards an aspect reminiscent of that of plant cells or fungi. Alternatively, vacuolisation may be directly related to cell death, either as a cause or as a consequence; the latter seems more likely, considering that the inability to regrow occurs on average a few hours earlier than detectable vacuolisation. Electron microscopy studies indicated that in developing *Dictyostelium* vacuoles may be related to autophagy (Maeda and Takeuchi, 1969; George et al., 1972; de Chastellier and Ryter, 1977; Quiviger et al., 1980; Schaap et al., 1981). The presence of large vacuoles is not just a reflection of an autophagic process secondary to the incubation in starvation medium (as observed in some mutant strains of yeast cells; Takeshige et al., 1992), since HMX44 cells in SB without DIF do not show large vacuoles.

Although vacuolisation seems a peculiar trait of *Dictyostelium* cell death, it may not be that different from events occurring in PCD in other organisms. Vacuolisation has been reported in cell death in *C. elegans* (Robertson and Thomson, 1982) and in higher eukaryotes in at least some cell types (Wyllie et al., 1980; Clarke, 1990). Also, vacuolisation in *Dictyostelium* may be related to the cytoplasmic condensation observed in all types of PCD studied in detail so far. In the present studies of Dictyostelium cell death, cytoplasmic condensation was prominent. A pronounced decrease in the cytoplasmic volume of differentiating *Dictyostelium* cells has been described previously (de Chastellier and Ryter, 1977). Cytoplasmic condensation may result from a loss of cytoplasmic water. This could manifest itself differently in various higher eukaryote cells or in *Dictyostelium* due to the individual constraints in each cell type, leading to spherical surface protuberances in apoptosis (Kerr et al., 1972) and to membrane wrinkling in non-apoptotic death of *Manduca sexta* muscle cells (Schwartz et al., 1993). While many cell types have minimal membrane constraints, *Manduca* muscle cells are attached to the segmental boundaries of the abdomen (Schwartz et al., 1993). In the case of *Dictyostelium* and perhaps of plant cells, the early synthesis of cellulose during differentiation is a major limitation to the modification of shape and the surface ‘boiling’ seen in other cells losing water, and might favor displacement of water into a vacuole localised within the cell.

In apoptosis, early ultrastructural nuclear lesions at a high level of chromatin organisation lead to the appearance of large DNA fragments (300 and/or 50 kbp) revealed by pulsed field gel electrophoresis (Walker et al., 1991; Brown et al., 1993; Oberhammer et al., 1993; Tomei et al., 1993). This is often followed with lower level DNA fragmentation (Wyllie, 1980) resulting in a gel electrophoresis ladder pattern of fragments of 180-200 base pairs and multiples thereof. No clear-cut ladder pattern of DNA fragmentation was to be expected in *Dictyostelium* death, since the nucleosomal organisation of *Dictyostelium* chromatin is irregular (Blumberg et al., 1991). If DNA fragmentation occurred in *Dictyostelium* PCD, assuming a similar chromatin organisation in *Dictyostelium* and in higher eukaryotes one would expect smears in low molecular mass regions and/or in the 50 kb region, both in ethidium bromide-stained agarose gels and in autoradiograms of the corresponding blots probed with radiolabelled total genomic DNA. No such result was obtained with DIF-treated dying *Dictyostelium* cells, while, in control groups, smears appeared in *Dictyostelium* preparations treated with neomycin, and the classical ladder pattern plus a 50 kb band were obtained with thymocytes treated with dexamethasone. Thus, we found no evidence for early alteration of DNA in *Dictyostelium* PCD.

By electron microscopy, we found that PCD in *Dictyostelium* included only limited and focal chromatin condensation. This was strikingly similar to the focal condensation found in the nuclei of *Manduca sexta* muscle cells (Schwartz et al., 1993) and of some nerve cells (quoted by Schwartz et al., 1993) considered undergoing non-apoptotic death. Interestingly, no early DNA fragmentation was detected during
death of *Manduca sexta* muscle cells (Zakeri et al., 1993). In only partial contrast, apoptosis includes as one of its most characteristic traits (Duvall and Wyllie, 1986; Kerr et al., 1987; Wyllie, 1988) the condensation of chromatin and its marginalisation, leading to the formation of chromatin masses with sharply defined edges lining the nuclear membrane. Perhaps similarly, by electron microscopy, differentiating protophloem sieve elements in roots of wheat were shown to undergo cell death morphological pattern, though not its core molecular layers of complexity might have been added during evolution. Multicellular organisms. To this core mechanism successive lar eukaryote, and have been used to achieve PCD in emerging the emergences of multicellularity, in, say, an early unicellu - it might have appeared perhaps only once in evolution, before the same core molecular mechanism in all cases. Elements of emergence of multicellularity. Alternatively, PCD may follow perhaps paralleling or closely following the repetitive events do not necessarily lead to cell death. Altogether, the most remarkable conclusion to us from this and other studies comparing various cell death morphological patterns is perhaps the absence of sharp boundaries between these patterns. An irreversible event must happen in all cases. DNA fragmentation occurs neither in non-apoptotic death nor in *Dictyostelium*, but neither is it found in some instances of apoptosis. Chromatin condensation is prominent in apoptosis but there is some focal condensation in both non-apoptotic death and in *Dictyostelium* PCD. Membrane alteration is a late event in all cases.

**Evolutionary considerations**

When in evolution did developmental cell death emerge? Developmental cell death has apparently been found each time it was looked for in multicellular eukaryotes. This might indicate that its emergence is at least as ancient as the emergence of multicellularity, more than 0.7 billion years ago (Field et al., 1988). Interestingly, however, ‘the evolutionary step from single to multicellular organisms appears to have been taken many times’ (Kaiser, 1986 and references therein). This raises the question of whether developmental cell death emerged before multicellularity, presumably leading to only one core molecular mechanism of PCD in present-day living organisms, or whether it emerged after multicellularity, possibly leading to more than one such mechanism.

PCD can apparently be typically apoptotic (in most vertebrate cells) or non-apoptotic (for instance vacuolisation in *Dictyostelium*, PCD of invertebrate muscle cells). These apparently distinct cell death aspects suggest two interpretations related to the evolutionary argument above. The first interpretation is that there is a plurality of cell death core mechanisms among multicellular organisms. PCD would result from different basic molecular mechanisms, which may have appeared at distinct and multiple times during evolution, perhaps paralleling or closely following the repetitive emergence of multicellularity. Alternatively, PCD may follow the same core molecular mechanism in all cases. Elements of it might have appeared perhaps only once in evolution, before the emergences of multicellularity, in, say, an early unicellular eukaryote, and have been used to achieve PCD in emerging multicellular organisms. To this core mechanism successive layers of complexity might have been added during evolution. Cell death morphological pattern, though not its core molecular mechanism, might then differ somewhat, as a function of the organism (e.g. mouse vs *Dictyostelium*) and/or as a function of the cell within a given organism (e.g. thymocyte vs liver cell, or perhaps muscle cell), or more generally as a function of the enzymatic equipment of, or the mechanical constraints on, the cell that dies.

We are thus led to the hypothesis of a single and therefore ancient core molecular mechanism of PCD. A prediction of this hypothesis is that some genes at play in PCD should be homologous in very diverse organisms. This may be tested through the analysis of molecules at play in phylogenetically remote organisms. Already consistent with this hypothesis, the PCD-controlling ced-3 and ced-9 molecules isolated from *C. elegans* both have structural and functional counterparts in vertebrate cells (Vaux et al., 1992; Miura et al., 1993). In *Dicytostelium*, the identification of some of the genes involved might take advantage of the small haploid genome and of insertion mutagenesis procedures.

We thank J. G. Williams (ICRF Laboratories, Clare Hill) and M. Hildebrandt and M. Veron (Pasteur Institute, Paris) for *Dictyostelium* strains and for very helpful discussions, M. Fathallah for help with the confocal microscope, M. Fraterno for help with electron microscopy and J.-F. Brunet and A. H. Greenberg for comments on the manuscript. This work was supported by institutional grants from INSERM and CNRS, and by an additional grant from the Association pour la Recherche contre le Cancer.

**REFERENCES**


cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. J. Pathol. 142, 67-77.


(Received 23 March 1994 - Accepted 2 June 1994)