Apparent caspase independence of programmed cell death in *Dictyostelium*

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During normal development, cell elimination [1,2] occurs by programmed cell death (PCD) [3], of which apoptosis [4] is the best known morphological type. Activation of cysteine proteases termed caspases [5] is required in many instances of animal PCD [6–9], but its role outside the animal kingdom is as yet unknown. PCD occurs during developmental stages in the slime mold *Dictyostelium discoideum* [10,11]. Under favorable conditions, *Dictyostelium* multiplies as a unicellular organism. Upon starvation, a pathway involving aggregation, differentiation and morphogenesis induces the formation of a multicellular fungus-like structure called a sorocarp [12], consisting mainly of spores and stalk cells, the latter being a result of cell death. *Dictyostelium* cell death is similar to classical apoptosis in that some cytoplasmic and chromatin condensation occurs but differs from apoptosis because it involves massive vacuolisation and, interestingly, lacks DNA fragmentation [11]. We examined whether caspase activity is required for *Dictyostelium* cell death. We found that caspase inhibitors did not affect cell death, although some caspase inhibitors that did not inhibit cell death impaired other stages in development and could block affinity-labelling of soluble extracts of *Dictyostelium* cells with an activated caspase-specific reagent. The simplest interpretation of these results is that in *Dictyostelium*, whether or not caspase-like molecules exist and are required for some developmental steps, caspase activation is not required for cell death itself.

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Results and discussion

PCD can be analysed in *Dictyostelium* cells using HMX44 cells derived from the HM44 mutant which differentiate in a monolayer without morphogenesis [13–15]. In this system, cell death is triggered by starvation in the presence of differentiation inducing factor (DIF), a chlorinated cyclohexanone [16,17]. This cell death is assessed through easily discernable morphological lesions (vacuolisation) or by counting cells that are capable of regrowth upon addition of rich medium [11]. *Dictyostelium* HMX44 cells were subjected to the usual sequence of a first period of incubation in starvation medium in the presence of cyclic AMP (cAMP), which does not lead in itself to cell death, then to another period of incubation together with DIF which triggers PCD [11,15]. In some experiments, the caspase inhibitors zVAD-fmk, BOC-Asp-fmk, DEVD-fmk, or YVAD-cmk [18] were added together with DIF to investigate whether caspase activity was required for cell death. Interestingly, the addition of these inhibitors did not block cell death as assessed both by morphological analysis (Figure 1) and by cell counts after regrowth (Table 1, experiments 1,2,4,6). Controlling in part for these observations, cyclosporin A and the general serine/cysteine protease inhibitor TPCK inhibited *Dictyostelium* cell death as assessed by the same criteria (data not shown), in agreement with published data. Absence of inhibition of cell death by caspase inhibitors suggested that *Dictyostelium* cell death is not dependent on caspase activation.

Given the absence of inhibition of cell death by the caspase inhibitors, we investigated whether these inhibitors could affect any stage of *Dictyostelium* development and found two instances where this was the case. Addition of YVAD-cmk, zVAD-fmk or DEVD-fmk to HMX44 cells in the first

Figure 1
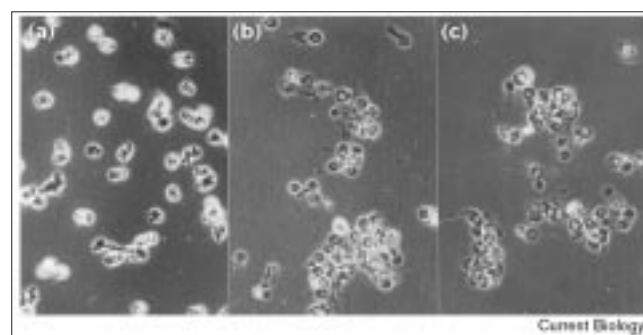
Caspase inhibitors do not block *Dictyostelium* cell death. Morphology of HMX44A cells after incubation in monolayers in SB in the presence of (a) 500 μM DEVD-fmk, or in the presence of (b) 100 nM DIF or (c) 500 μM DEVD-fmk and 100 nM DIF (magnification 400×).

Table 1

Effect of caspase inhibitors on cell survival after DIF addition.
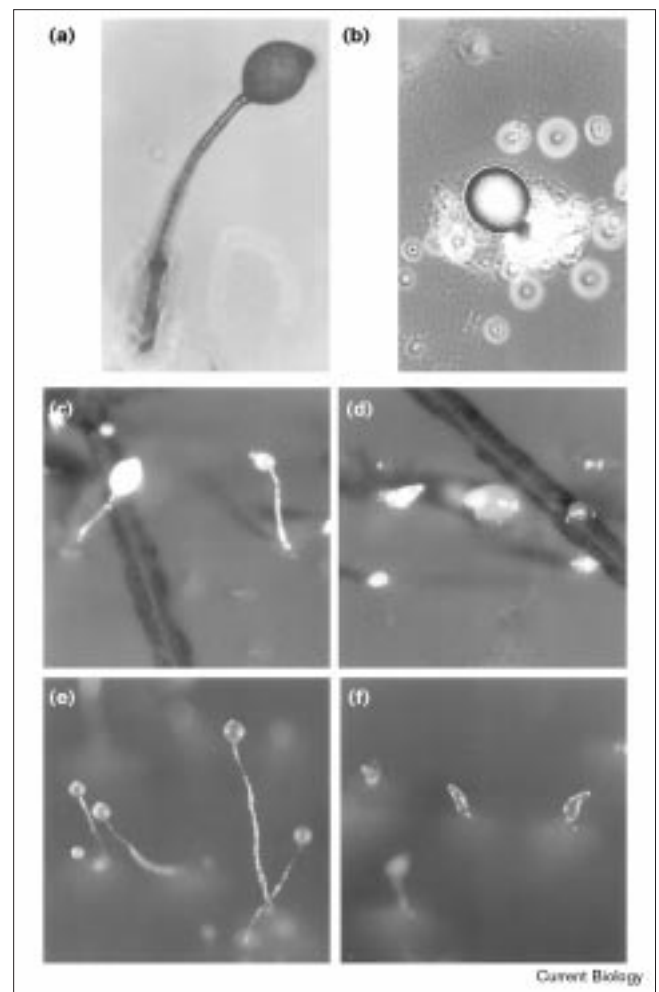
Expt	First incubation SB+cAMP+	Second incubation SB+	Cells/µl	% control +DMSO	% control −DIF
1		DMSO	367	100	
		DMSO+DIF	13	4	4
		DMSO+BAF	413	113	
		DMSO+BAF+DIF	6	2	1
2		DMSO	367	100	
		DMSO+DIF	13	4	4
		DMSO+DEVD	350	95	
		DMSO+DEVD+DIF	21	6	6
3	DMSO	DMSO	174	100	
	DMSO	DMSO+DIF	21	12	12
	DMSO+DEVD	DMSO+DEVD	185	106	
	DMSO+DEVD	DMSO+DEVD+DIF	45	26	24
4		DMSO	367	100	
		DMSO+DIF	13	4	4
		DMSO+zVAD	330	90	
		DMSO+zVAD+DIF	16	4	5
5	DMSO+zVAD	DMSO+zVAD	277		
	DMSO+zVAD	DMSO+zVAD+DIF	77	NA	28
6		DMSO	367	100	
		DMSO+DIF	13	4	4
		DMSO+YVAD	470	128	
		DMSO+YVAD+DIF	18	5	4
7	DMSO	DMSO	516	100	
	DMSO	DMSO+DIF	33	6	6
	DMSO+YVAD	DMSO+YVAD	645	125	
	DMSO+YVAD	DMSO+YVAD+DIF	207	40	32

The effect of caspase inhibitors on the number of cells regrowing after differentiation of *Dictyostelium* HMX44A cells in monolayers. PCD was induced in *Dictyostelium* HMX44A cells (obtained from J.G. Williams, UCL), adding DIF or 500 µM of inhibitor or both. In experiments 1, 2, 4 and 6, inhibitors BOC-Asp-fmk (BAF), DEVD-fmk (DEVD), YVAD-cmk (YVAD) or zVAD-fmk (zVAD) were added together with DIF. In experiments 3, 5 and 7, inhibitors were added together with cAMP before incubation with DIF. After a 24 h incubation at 23°C, cells were checked for morphological signs of PCD, and HL5 rich medium was added to the cultures [11]. After a further 48 h incubation, regrowth in the cultures was determined as cell concentrations (cells/µl) and percentages (% of control sample incubated with DMSO, where the cell concentration in the presence of DMSO is taken as 100%; and % of control sample incubated without DIF, where the cell concentration in the absence of DIF is taken as 100%). Similar results were obtained in a total of 17 experiments. The experiments for the various inhibitors were not necessarily performed in parallel. All groups included DMSO since by itself DMSO led to an increase in the number of cells recovered after regrowth (data not shown).

Figure 2

The caspase inhibitor DEVD-fmk blocks *Dictyostelium* development. Cells from *Dictyostelium* AX2 cultures were washed twice with Soerensen buffer (SB) as starvation medium [11] and inoculated in SB to induce morphogenesis, with or without caspase inhibitor. The morphology of differentiating formations was obtained after incubation (a,c,e) in the presence of DMSO or (b,d,f) in the presence of 500 µM DEVD-fmk. Various growth conditions were also used: (a,b) tissue culture plastic support (magnification 400×); (c,d) cellulose–ester filter support (magnification 80×); (e,f) Durapore filter support (magnification 80×). Similar results were obtained whether standard filter supports or tissue culture plastic flasks were used.

incubation period, when DIF was absent, enhanced regrowth compared with the regrowth obtained when the inhibitors were added only in the second incubation period together with DIF (Table 1, experiments 3,5,7). These results demonstrated that the inhibitors used could affect HMX44 cells and suggested a role for caspase-like molecules in pre-DIF events, but not in DIF-triggered cell death.

We also tested the caspase inhibitors on developmentally competent *Dictyostelium* AX2 cells. These cells were induced by starvation to undergo multicellular development. Caspase inhibitors were added at the onset of starvation.

The presence, number and morphology of differentiating formations were checked after 18 hours and/or after a 24 hour incubation at 23°C. Compared with the dimethyl-sulphoxide (DMSO) control, addition of DEVD-fmk or YVAD-cmk resulted in a sharp decrease in the proportion of complete, stalk-comprising sorocarps, and led to a correlative increase in the percentage of aggregates/culminants and elements that almost completely lack stalks (Figure 2). This effect was dependent on the concentration of DEVD-fmk or YVAD-cmk, whereas addition of zVAD-fmk or BOC-Asp-fmk had no detectable effect at any of the concentrations tested (Figure 3). In many of the formations, no
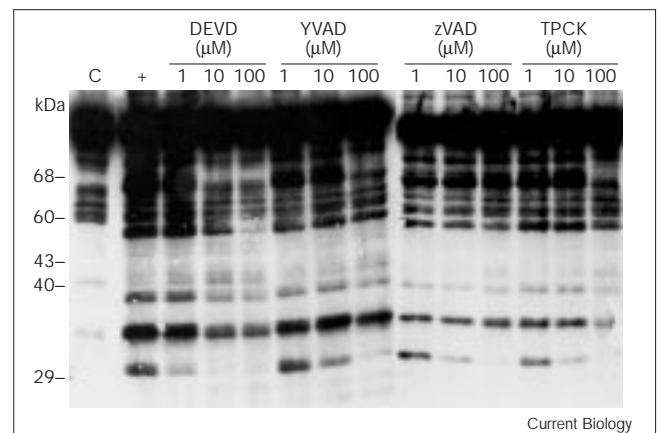
Figure 3
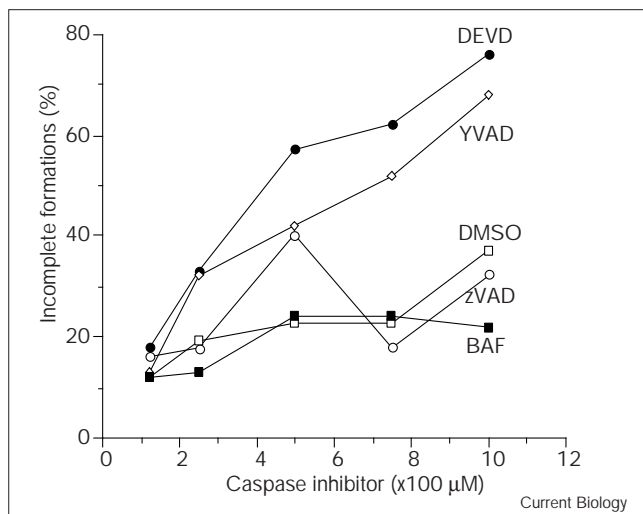
The effect of caspase inhibitors on the development of *Dictyostelium* AX2 cells (expressed as the percentage of incomplete formations observed per flask after incubation in starvation conditions on tissue culture plastic). After a 24 h incubation, the number of complete (having visible stalk) and incomplete (lacking stalk) formations was determined per flask at fixed settings on a stereomicroscope. Results presented are from one out of sixteen (DEVD-fmk), seven (zVAD-fmk and BOC-Asp-fmk) or three (YVAD-cmk) similar experiments that yielded similar results whether counts were performed after 18 h or 96 h. Some of these experiments, as others in this report, were read without knowledge of the nature of the experimental group. The same batches of zVAD-fmk and BOC-Asp-fmk, which did not affect *Dictyostelium*, were at least as effective as DEVD-fmk or YVAD-cmk at blocking nuclear signs of mammalian cell death (data not shown). For DMSO, the scale on the x-axis represents the added volume in µl/ml SB.

Figure 4

Binding of the caspase affinity-labelling reagent z-EK(bio)D-aomk to molecules in HMX44 *Dictyostelium* cell extracts and its inhibition by caspase inhibitors. Extracts were obtained as described [25] from exponentially growing HMX44A cell cultures in HL5 medium, using cell extraction buffer containing PMSF, leupeptin, aprotinin, antipain, chymostatin and pepstatin A. Aliquots were incubated for 15 min at 37°C with 1 µM z-EK(bio)D-aomk. Extracts were then diluted, heated to 95°C for 5 min and cleared by centrifugation, before being subjected to SDS–PAGE on a 10% acrylamide gel. Proteins were transferred to a nitrocellulose membrane, probed with horseradish-peroxidase-coupled streptavidin and visualised using an enhanced chemiluminescence kit. For treatment with inhibitors, samples were incubated with the indicated concentrations of inhibitor for 15 min at 37°C. The samples were then labelled with 1 µM z-EK(bio)D-aomk as described above. The lane marked C represents the unlabelled control, detecting 'background' polypeptides reacting with streptavidin; the lane marked + represents a labelled control sample that had been incubated without inhibitors. Molecular weight markers are indicated on the left.

stalk appeared even upon much longer incubation periods (up to 4 days). The spores formed appeared morphologically normal and were able to germinate upon incubation in rich medium (data not shown). Similar results were obtained using another AX2 strain and a *stkA* (*stalky*) mutant strain (whose development leads to sorocarps with a high proportion of stalk cells), both bearing a *lacZ* reporter construct driven by the *ecmA* promoter [19]. In these cases as well, development was strongly affected in the presence of DEVD-fmk or YVAD-cmk, and expression of the reporter gene was the same as in control groups at comparable developmental stages (data not shown). Altogether, these results showed that DEVD-fmk and YVAD-cmk affected *Dictyostelium* development, apparently between the end of aggregation and the initiation of stalk development. Thus, the same caspase inhibitors that did not detectably affect *Dictyostelium* DIF-induced cell death, as assessed both qualitatively through morphological lesions and quantitatively through ability to regrow, impaired pre-death *Dictyostelium* development.

A possible objection to the conclusion of caspase independence of *Dictyostelium* cell death is that the caspase inhibitors used may not be able to recognise *Dictyostelium* caspases but instead may be able to interfere with functionally important non-caspase molecules. We therefore looked for alternative ways of showing that the inhibitors used were interfering with caspase activity in *Dictyostelium*. Extracts from vegetative *Dictyostelium* cells cleaved caspase substrates, although this cleavage could not be blocked with known specific caspase inhibitors (data not shown), suggesting that a non-specific proteolytic activity may perhaps mask a specific caspase activity. We then turned to direct affinity-labelling with z-EK(bio)D-aomk, a reagent which covalently labels activated caspases [20–22]. Electrophoretic fractionation of z-EK(bio)D-aomk-treated extracts from vegetative HMX44 *Dictyostelium* cells revealed that a number of molecules were labelled, of around 29, 35, 38, 55 and 68 kDa. The labelling of some of these molecules could be inhibited by DEVD-fmk at concentrations as low as 1 µM (Figure 4). For the other inhibitors tested (YVAD-cmk, zVAD-fmk and TPCK), concentrations of 10 to 100 µM had to be used to obtain some degree of inhibition. Further experiments (data not shown) indicated that: some variation in the labelling pattern seemed to occur upon differentiation,

which requires a more thorough analysis; similar labelling and inhibition of labelling was obtained when testing extracts from vegetative AX2 cells; and no labelled bands were observed when testing in isolation the HL5 medium used for vegetative growth. These results reveal the presence in *Dictyostelium* extracts of molecules that could be labelled by a caspase-specific labelling agent.

Altogether, these results show inhibition by some caspase inhibitors, especially DEVD-fmk, of morphogenesis but not of cell death in *Dictyostelium*, and detection of labelled molecules by DEVD-fmk-inhibitable caspase affinity-labelling. An interpretation of these results is that caspase-like proteases are present in *Dictyostelium* and the activation of these proteases is required for morphogenesis at a stage which could be clarified in future work by studying the impact of DEVD-fmk on other molecular markers of *Dictyostelium* development, and by planned cloning and knock-out experiments. Cell death could not be blocked by caspase inhibitors, however, despite these inhibitors having other effects on *Dictyostelium* cells and recognising molecules that react like caspases when treated with a labelling reagent. This strongly indicates that, whether or not caspases exist at all in *Dictyostelium*, they are not involved in *Dictyostelium* cell death. Consistent with the paucity of nuclear effects of *Dictyostelium* PCD ([11] and our unpublished observations), this caspase-independent *Dictyostelium* cell death may be evolutionarily and mechanistically related to described examples of caspase-independent vertebrate cell death (reviewed in [23]) and may therefore provide a genetically tractable model of such death [24]. These results also are consistent with the possibility that caspases, which have been identified so far only in vertebrates and some invertebrates such as *Caenorhabditis elegans* and *Drosophila melanogaster* and not in plants, emerged in evolution earlier, and exist in other kingdoms as well as in the animal kingdom. Early in eukaryote evolution, cell death and caspase pathways may not have been connected, with subsequent diversion of the involvement of caspases to cell death. Compared with *Dictyostelium* (and perhaps plant cells), the completeness of caspase-dependent elimination of dead cells [23] in animals may have been the selective advantage that would have drawn caspase-dependent steps into originally caspase-independent cell death pathways.

Supplementary material
Additional methodological detail is published with this paper on the internet.

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Supplementary material

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Materials and methods

For the experiment shown in Figure 2, cells from exponentially growing *Dictyostelium* AX2 cultures were washed twice with Soerensen buffer (SB) as starvation medium [11] and inoculated at 6.4×10^6 cells/25 cm² flask in 1 ml SB to induce morphogenesis, without or with the indicated inhibitor. Addition of inhibitor did not detectably alter the pH of SB (data not shown). Cultures were incubated slightly tilted. The morphology of differentiating formations formed in the presence of 1000 μM YVAD-cmk was similar to that observed in the presence of 500 μM DEVD-fmk (data not shown). The morphology of differentiating formations formed in the presence of 500 μM zVAD-fmk or 500 μM BOC-Asp-fmk was similar to the morphology observed in DMSO (data not shown). The formation and morphology of spores after incubation of AX2 cells in starvation conditions in the presence of 500 μM DEVD-fmk was similar to the formation and morphology of spores after incubation in SB alone, or in DMSO, 500 μM zVAD-fmk, 500 μM BOC-Asp-fmk, or 1000 μM YVAD-cmk (data not shown). Results were similar using either the indicated standard filter supports, or tissue culture plastic flasks.

For the experiment shown in Figure 4, extracts were obtained as described [25] from exponentially growing HMX44A cell cultures in HL5 medium, using cell extraction buffer containing 50 mM PIPES (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 10 μM cytochalasin B, 1 mM PMSF and 1 μg/ml leupeptin, aprotinin, antipain, chymostatin and pepstatin A (all protease inhibitors from Sigma). As controls, extracts from viable HeLa cells and extracts from apoptotic HeLa cells (HeLa cells incubated for 2 h with 1 μM staurosporine, a time at which cell nuclei did not show an apoptotic morphology) were prepared (data not shown). Aliquots containing 50 mg protein were adjusted to a volume of 20 ml with dilution buffer (10 mM PIPES, pH 7.0, 50 mM NaCl, 5 mM EGTA, pH 8.0, 2 mM $MgCl_2$, 1 mM DTT) and incubated for 15 min at 37°C with 1 mM z-EK(bio)D-aomk (Peptide Institute). Extracts were then diluted with 1/2 volume of 3X sample buffer, heated to 95°C for 5 min and cleared by centrifugation for 10 min at 13 000 rpm, before being subjected to SDS–PAGE on a 10% acrylamide gel. Proteins were transferred to a nitrocellulose membrane, probed with HRP-streptavidin (Amersham), and visualised using an enhanced chemoluminescence kit (Amersham). For treatment with inhibitors, samples containing 50 mg protein were adjusted to 20 ml with dilution buffer, and incubated with either 1, 10 or 100 μM inhibitor for 15 min at 37°C. The samples were then labelled with 1 mM z-EK(bio)D-aomk as described above.