# More than one way to go

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Dramatic changes accompany the death of cells. Attempts to align these changes into cognate groups and identify the mechanisms responsible for them have led to substantial and sometimes surprising insights into cell biology. One such constellation of structural and functional changes is associated with apoptosis (a death process found in development, atrophy, the targets of cell killing by the immune system, and the response of many cell types to relatively low concentrations of many xenobiotics; ref. 1). Several of the events of apoptosis can be traced to activation of intracellular caspases, a group of cysteine proteases with aspartate-directed cleavage preference (2). Cells, and indeed whole animals, rendered deficient in certain caspases through contrived, germ-line knockout are now available, as are potent, broad-spectrum caspase inhibitors. These resources have revealed that death processes exist that are independent of caspase proteolysis and demonstrate a phenotype unlike that of apoptosis. In a recent paper in PNAS, Sperandio *et al.* (3) present a striking picture of one of these death processes. Their observations are exciting because they may highlight a mechanism of internally programmed cell death that often exists in parallel with apoptosis but may be subject to different controls. In particular, this death process, which the authors name paraptosis, may be relevant to the pathogenesis of central nervous system degenerations.

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Several of the most obvious features of apoptosis are found in the nucleus. Chromatin condenses, initially in a circumferential pattern, but thereafter throughout the whole nucleus, which becomes shrunken and often separates to several spherical bodies. In step with these appearances, DNA is cleaved, first to large chromatin domains of around 50–300 kbp, and then—in many but not all cell types—to oligonucleosomes (4). The nuclear envelope becomes discontinuous and the lamin polymer that normally underlies the nuclear membrane collapses, because of lamin proteolysis. All but the earliest, large-domain cleavage events are usually absent in caspase-deficient cells (4, 5), and all of these events can be reproduced in normal nuclei *in vitro* by exposure to recombinant caspases (2). Two ubiquitous caspases, caspases 3 and 6, appear to be responsible. Among the many intracellular substrates of caspase 3 is a 45-kDa molecular chaperone called ICAD or DFF45 (for inhibitor of caspase-activated DNase or DNA fragmenting factor), whose cleavage results in release of a previously inactive, 40-kDa nuclear-homing nuclease (called CAD or DFF40) that inserts double-strand breaks at internucleosomal sites in DNA (6, 7). Proteins without nuclease activity that nonetheless affect nuclear structure also are activated by caspase 3, including acinus, which causes chromatin condensation (8). Lamin cleavage takes place at an intramolecular site that corresponds exactly to the preferred recognition site of caspase 6 (9), although the rather inconspicuous effects of caspase 6 inactivation relative to caspase 3 suggest that caspase 3 may be important for lamin cleavage, too (10).

These so-called effector caspases are themselves activated through cleavage by other members of the caspase family (4, 9, 11). One well-defined pathway is initiated by ligand binding to receptors of the tumor necrosis factor receptor family on the cell membrane, notably FAS/apo1/CD95. This ligand binding stimulates formation of a protein complex on the cytoplasmic domain of the receptor, in which protein–protein interactions result in activation of caspase 8, an "upstream" caspase with a long N-terminal domain that serves as a regulatory element. A second important pathway is initiated on or near the surface of mitochondria, where caspase 9 is activated through participation in another molecular complex that includes the mitochondrial flavoprotein cytochrome *c*, a protein called Apaf-1, and dATP. The function of both complexes can be greatly modified by inclusion within them of endogenous apoptosis inhibitory proteins, catalytically inert caspase competitors (such as the caspase 8 homologue cFLIP), or members of the heat shock protein family (12, 13). Further partners have been identified in the mitochondrial complex that have proapoptotic properties (14). There are also profoundly influential but poorly understood interactions—both positive and negative—with members of the extended bcl-2 family, which associate with the mitochondrial membrane, or translocate to it during apoptosis (15–17). The CD95-linked caspase 8 pathway and the mitochondrial caspase 9 pathway engage in dialogue through bid, a proapoptotic member of the bcl-2 family. Bid is truncated by caspase 8, and in this form activates bax, a major element in the balance between proapoptotic and antiapoptotic activities on the mitochondrial surface. The mitochondrial procaspase complex has been called the apoptosome (18), but it is probable that other procaspase-containing protein complexes form at several strategically important locations within cells where incoming signals relating to life and death need to be integrated. Thus caspase 12 appears to be activated by calcium-related stress stimuli in endoplasmic reticulum (19), caspase 2 locates on Golgi membranes and within the nucleus (20), caspase 9 also may localize to discrete subnuclear structures (21), and bcl-2 family members associate with endoplasmic reticulum and nuclear membranes as well as with mitochodria (22).

The experiments of Sperandio *et al.* (3), however, indicate the presence of a death pathway that is independent of sequential caspase activation, and correspondingly is not associated with the classical nuclear changes of apoptosis. Confirming the

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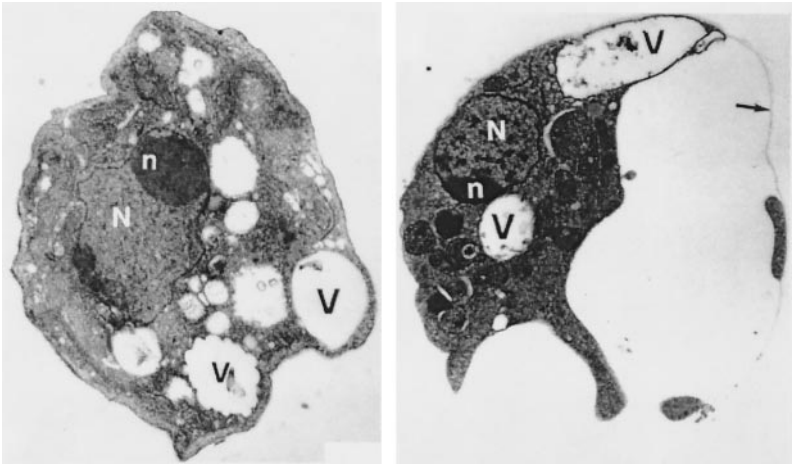


**Fig. 1.** Cell death in *Dictyostelium* cells. (*Left*) The normal control. (*Right*) The dying cell, showing extensive cytoplasmic vacuolation (V) and nuclei (N) with only focal chromatin condensation (n). See ref. 36. [Reproduced with permission from ref. 36 (Copyright 1994, The Company of Biologists).]

results of others, they show first that death can be induced in the 239T fibroblast line through transient overexpression of the insulin-like growth factor receptor, IGF1R. IGF-1 was shown some years ago to be a survival factor for serum-deprived fibroblasts (23). The current experiments reflect a complementary phenomenon: unoccupied IGF-1 receptors deliver death signals. Most of the affected cells, however, lack chromatin condensation, DNA cleavage, or any evidence of caspase activation. Their death is unaffected by synthetic caspase inhibitors or recombinant apoptosis inhibitory proteins. As they die, they round up and lose substrate attachment as apoptotic cells do, but their cytoplasm is unlike that of apoptotic cells, enclosing large vacuoles, apparently filled with fluid. Very similar findings appear in fibroblasts from animals that, through germ-line knockout of Apaf-1, lack the mitochondrial pathway of apoptosis activation. Unexpectedly, caspase 9, but none of the other caspases tested, turns on this type of death in about a third of transfected cells, the others showing apoptosis. Sperandio *et al.* confirmed that their cells contained, intact, all of the machinery required for apoptosis, because another lethal stimulus, bax, produced all of the classical features of apoptosis in both nucleus and cytoplasm. When caspase 9 expression was induced in the presence of caspase inhibitors, however, most of the cell deaths adopted the vacuolated phenotype. Intriguingly, a caspase 9 mutant lacking internal processing sites was just as active as the wild type, confirming that, however caspase 9 exerts its effects in this system, it is not through the classical caspase cascade. Preliminary experiments indicate that the cascade-independent deaths, although requiring RNA and protein synthesis, may involve different patterns of gene expression from those dependent on the full caspase cascade.

Caspase-independent death has been reported before. For example, Bax-expressing thymocytes, treated with caspase inhibitors, die with uncondensed nuclei and fluid-loaded cytoplasm reminiscent of necrosis (24). PC-12 cells, stimulated to die through contact with activated microglia, switch the morphology of death from apoptosis to a similar necrosis-like phenotype when exposed to caspase inhibitors (25). The Bcl-2 family member, BNIP3, a killer protein associated with mitochondria, induces cell death that is independent of caspase activation, Apaf-1, or cytochrome *c* release, and this death also has the necrosis-like phenotype (17). Similarly, in Apaf-1-deficient animals or in the presence of caspase inhibitors, the cells of the interdigital web of murine embryo paws undergo their customary developmentally regulated death approximately on schedule, but with relatively uncondensed nuclei, and dilated mitochondria (26). Thus the morphology of death in all of these caspase-deficient cells appears different from that of apoptosis and may relate to the generalized accumulation of fluid that occurs within endoplasmic reticulum and the mitochondrial matrix in necrosis, where presumably there is nonselective loss of cellular fluid homeostasis.

It is not completely clear whether one or several mechanisms underlie this non-apoptotic cell death. Nonmammalian models may provide a further perspective on this issue. Stalk cells of the primitive eukaryote *Dictyostelium discoides*, whose genome possesses only a remotely related caspase homologue (27), undergo developmentally regulated death (even in the presence of caspase inhibitors), with large cytoplasmic vacuoles and nuclear chromatin that is uncleaved and morphologically nearly normal (28) (Fig. 1). The basis of cytoplasmic vacuolation after cell injury is very poorly understood (29), but the appearance of large new vacuoles, as in the developmental death of *Dictyostelium* cells, and the IGF1R-related deaths described by Sperandio *et al.* (3), suggests a mechanism more complex than mere loss of volume homeostasis. Somewhat similar extensive vacuolation appears in murine neurones undergoing excitotoxic death as a result of kainic acid treatment (30) and in the neurons of *Caenorhabditis elegans* with gain-of-function mutants in *mec4*, *deg1*, and *unc105*, genes encoding subunits of a voltage-gated sodium channel (31, 32). Perhaps the dramatic extension of membrane-lined vacuoles is part of an adaptive response to volume overload that is short-circuited by the subtly efficient caspase mechanism, when it is available. But the observation that this, presumptively archaic, route to death still exists when caspases are silenced is a clear statement that the critical decision to die lies upstream of all these events.

There are only scanty clues as to the molecular nature of this remote, but deeply significant, decision. In addition to cytochrome *c*, mitochondria release another flavoprotein early in the death process, apoptosis initiating factor (AIF) (4). Although caspases are absent from prokaryotes and have not been identified in plants, but appear in primitive metazoans such as *Hydra* (33), AIF homologues are present in animals and plants, eubacteria, and archebacteria (34). Perhaps fundamental endogenous signals for death reside in molecules like AIF or in processes like ion fluxes (35). Once these have been activated, doomed cells may find they have more than one way to go.

1. Kerr, J. F. R., Wyllie, A. H. & Currie, A. R. (1972) *Br. J. Cancer* **26,** 239–257.
2. Earnshaw, W. C., Martins, L. M. & Kaufmann, S. H. (1999) *Annu. Rev. Biochem.* **68,** 383–424.
3. Sperandio, S., de Belle, I. & Bredesen, D. E. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 14376–14381.
4. Susin, S. A., Daugas, E., Ravagnan, L., Samejima, K., Zamzami, N., Loeffler, M., Costantini, P., Ferri, K. F., Irinopoulou, T., Prevost, M. C., *et al.* (2000) *J. Exp. Med.* **192,** 571–580.
5. Johnson, V. L., Ko, S. C., Homstrom, T. H., Eriksson, J. E. & Chow, S. C. (2000) *J. Cell Sci.* **113,** 2941–2953.
6. Nagata, S. (2000) *Exp. Cell Res.* **10,** 12–18.
7. Liu, X., Li, P., Widlak, P., Zou, H., Luo, X., Garrard, W. T. & Wang, X. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 8461–8466.
8. Sahara, S., Aoto, M., Eguchi, Y., Imamoto, N., Yoneda, Y. & Tsijimoto, Y. (1999) *Nature (London)* **401,** 168–173.

9. Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., *et al*. (1997) *J. Biol. Chem.* **272,** 17907–17911.
10. Slee, E. A., Adrian, C. & Martin, S. J. (2000) *J. Biol. Chem.*, in press.
11. Komoriya, A., Packard, B. Z., Brown, M. J., Wu, M. L. & Henkart, P. (2000) *J. Exp. Med.* **191,** 1819–1828.
12. Pandey, P., Saleh, A., Nakazawa, A., Kumar, S., Srinivasula, S. N., Kumar, V., Weichselbaum, R., Nalin, C., Alnemri, E. S., Kufe, D. & Kharbanda, S. (2000) *EMBO J.* **19,** 4310–4322.
13. Mosser, D. D., Caron, A. W., Bourget, L., Meriin, A. B., Sherman, M. Y., Morimoto, R. I. & Massie, B. (2000) *Mol. Cell. Biol.* **20,** 7146–7159.
14. Chau, B. N., Cheng, E. H., Kerr, D. A. & Hardwick, J. M. (2000) *Mol. Cell.* **6,** 31–40.
15. Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B. & Martinou, J. C. (1999) *J. Cell Biol.* **144,** 891–901.
16. Hausmann, G., O'Reilly, L. A., van Driel, R., Beaumont, J. G., Strasser, A., Adams, J. M. & Huang, D. C. (2000) *J. Cell Biol.* **149,** 623–634.
17. Vande Velde, C., Cizeau, J., Dubik, D., Alimonti, J., Brown, T., Israels, S., Hakem, R. & Greenberg, A. H. (2000) *Mol. Cell. Biol.* **20,** 5454–5468.
18. Zou, H., Li, Y., Liu, X. & Wang, X. (1999) *J. Biol. Chem.* **274,** 11549–11556.
19. Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A. & Yuan, J. (2000) *Nature (London)* **403,** 98–103.
20. Mancini, M., Machamer, C. E., Roy, S., Nicholson, D. W., Thornberry, N. A., Casciola-Rosen, L. A. & Rosen, A. (2000) *J. Cell Biol.* **149,** 603–612.
21. Ritter, P. M., Marti, A., Blanc, C., Baltzer, A., Krajewski, S., Reed, J. C. & Jaggi, R. (2000) *Eur. J. Cell Biol.* **79,** 358–364.
22. Lithgow, T., van Driel, R., Bertram, J. F. & Strasser, A. (1994) *Cell Growth Differ.* **5,** 411–417.
23. Harrington, E. A., Bennett, M. R., Fanidi, A. & Evan, G. I. (1994) *EMBO J.* **13,** 3286–3295.
24. Xiang, J., Chao, D. T. & Korsmeyer, S. J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 14559–14563.
25. Tanabe, K., Nakanishi, H., Maeda, H., Nishioku, T., Hashimoto, K., Liou, S.-Y., Akamine, A. & Yamamoto, K. (1999) *J. Biol. Chem.* **247,** 15725–15731.
26. Chautan, M., Chazal, G., Cecconi, F., Gruss, P. & Golstein, P. (1999) *Curr. Biol.* **9,** 967–970.
27. Uren, A. G., O'Rourke, K., Aravind, L., Pisabarro, M. T., Seshagiri, S., Koonin, E. V. & Dixit, V. M. (2000) *Mol. Cell* **6,** 961–967.
28. Olie, R. A., Durrieu, F., Cornillon, S., Loughran, G., Gross, J., Earnshaw, W. C. & Golstein, P. (1998) *Curr. Biol.* **8,** 955–958.
29. Henics, T. & Wheatley, D. N. (1999) *Biol. Cell* **91,** 485–498.
30. Regan, R. F., Panter, S. S., Witz, A., Tilly, J. L. & Giffard, R. G. (1995) *Brain Res.* **705,** 188–198.
31. Hall, D. H., Gu, G., Garcia-Anoveros, J., Gong, L., Chalfie, M. & Driscoll, M. (1997) *J. Neurosci.* **17,** 1033–1045.
32. Garcia-Anoveros, J., Garcia, J. A., Liu, J. D. & Corey, D. P. (1998) *Neuron* **20,** 1231–1241.
33. Cikala, M., Wilm, B., Hobmayer, E., Bottger, A. & David, C. N. (1999) *Curr. Biol.* **9,** 959–962.
34. Lorenzo, H. K., Susin, S. A., Penninger, J. & Kroemer, G. (1999) *Cell Death Differ.* **6,** 516–524.
35. Maeno, E., Ishizaki, Y., Kanaseki, T., Hazama, A. & Okada, Y. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 9487–9492.
36. Cornillon, S., Foa, C., Davoust, J., Buonavista, N., Gross, J. D. & Golstein, P. (1994) *J. Cell Sci.* **107,** 2691–2704.