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News and Commentary

Apaf-1/cytochrome *c* apoptosome: an essential initiator of caspase activation or just a sideshow?

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Caspase activation in mammalian cells is mediated via two main routes, often referred to as 'the intrinsic pathway' and 'the extrinsic pathway'.[1] The extrinsic pathway of apoptosis is induced by ligand-mediated activation of the tumour necrosis factor (TNF) family of cell surface receptors. A large protein complex, known as the DISC, forms at the cell membrane and recruits and activates the initiator caspase, caspase-8.[1–4] Active caspase-8 directly processes and activates effector caspases, which in turn cleave several hundred caspase substrates to induce the characteristic morphological features of apoptosis. Caspase-8 can also induce apoptosis indirectly by cleaving the BH3-only protein, Bid.[1,2,4] Cleavage of Bid triggers its translocation to the mitochondria to mediate membrane permeabilisation by an unknown mechanism and release of cytochrome *c*. Released cytochrome *c* then facilitates the formation of the apoptosome-containing adaptor Apaf-1 and another initiator caspase, caspase-9.[1–4] Like caspase-8, caspase-9 can directly activate the effector caspase, caspase-3. While this serves as an amplification mechanism during the extrinsic pathway of cell death, damage to mitochondria and subsequent apoptosome-mediated caspase-9 activation is widely accepted as the initiating event in the intrinsic pathway of apoptosis.[1–4] This is perhaps surprising as in *Caenorhabditis elegans*, there is no involvement of mitochondria in the developmentally programmed cell death (PCD). Recent results indicate that release of cytochrome *c* is also not required for the activation of the initiator caspase DRONC in *Drosophila melanogaster*.[5,6]

Why then in mammals does the intrinsic pathway of apoptosis proceed solely via the mitochondria? A recent study demonstrates that, in fact, some stress-induced cell death pathways may not require mitochondria after all.[7] Several other studies also show that during stress signalling caspase-2 activation occurs upstream of mitochondrial damage and the release of cytochrome *c*, suggesting that caspase-9 activation by the Apaf-1 apoptosome is not an initiator of the caspase cascade.[8–10] Using fetal liver cells derived from Apaf-1-deficient and caspase-9-deficient mice to reconstitute the haematopoietic compartments of irradiated donor mice, Marsden *et al*[7] demonstrate that apoptosis occurs normally in the absence of either Apaf-1 or caspase-9. While levels of caspase activation are substantially reduced in Apaf-1 or caspase-9-deficient cells than in their wild-type counterparts, the cells show little defect in their ability to undergo apoptosis in response to physiological and pathological stimuli.[7] Importantly, while Apaf-1 and caspase-9 are dispensable for cell death, BCL-2 does confer a survival advantage to these deficient cells. Thus, in an experimental system where cytochrome *c* has no involvement, BCL-2 can still protect against cell death, arguing against the widely held view that BCL-2 solely protects cell death by preventing the release of apoptogenic factors from mitochondria and maintaining mitochondrial membrane integrity.[4] In addition, the authors show that treating cells with several highly specific broad-spectrum caspase inhibitors produced a similar phenotype as did overexpression of BCL-2, and retarded release of mitochondrial cytochrome *c*.[7]

Thus, the study by Marsden *et al*[7] supports the argument that mitochondria exist as amplifiers of the caspase cascade rather than as initiators of caspase activation.[11,12] This study may explain why BCL-2 can mimic the protective effects of its worm homologue, CED-9 on cell death and also prevent the ectopic cell death that occurs in *C. elegans ced-9* loss-of-function mutants.[13] In analogy to the simple cell death machinery in *C. elegans*, BCL-2 would function to sequester a caspase adaptor protein (CED-4 homologue) preventing its binding to and activating an initiator caspase (CED-3 homologue).[7,11,12]

If the Apaf-1/caspase-9 apoptosome is not essential in the intrinsic cell death pathway, which caspase(s) acts as an initiator caspase? By analogy with CED-3, this upstream caspase could be predicted to contain a CARD. Marsden *et al*[7] show that in an Apaf-1 deficient cell line treated with etoposide, active caspase-1 can be isolated from cell extracts by binding to biotin-DEVD, suggesting that caspase-1 is a candidate for an initiator caspase-activated upstream of and independent of mitochondria. Another species was isolated that could represent caspases-11 or caspase-12.[7]

The work by Lassus *et al*[8] demonstrates that one potential candidate working upstream of mitochondria is caspase-2. Of all CARD-containing caspases, caspase-2 and caspase-9 are the only caspases implicated in cell death, whereas most others, including caspase-1, -4, -5, -11, and -12, are predominantly involved in inflammatory pathways.[2,14–17] Caspase-2 is several orders of magnitude less sensitive to many of the so-called broad-spectrum caspase inhibitors, meaning that its activity may have been undetected in experiments employing these inhibitors. Intriguingly, caspase-2 is most closely related to the worm CED-3 of all the mammalian caspases, and is the most evolutionarily conserved caspase of the family.[18] Perhaps caspase-2, not caspase-9, is the true homologue of CED-3, and its adaptor will be more homologous to CED-4 than Apaf-1. Caspase-2 is also one of the few caspases that is transcriptionally

upregulated in some circumstances.[14,19] Indeed, it shares high homology with DRONC, the only CARD-containing caspase in *Drosophila*, which is transcriptionally upregulated by the steroid hormone ecdysone during fly development.[20,21] Furthermore, consistent with a potential role as an initiator caspase, caspase-2 can autoprocess and undergoes rapid activation in response to apoptosis induced by a variety of agents.[22–24] Unlike most other caspases that are primarily cytosolic, caspase-2 localises to several cellular compartments, including the nucleus,[25] and has the ability to trigger cytochrome *c* release and apoptosis from the nucleus.[26] As discussed in a review by Troy and Shelanski in this issue of *Cell Death and Differentiation*,[17] caspase-2 has been implicated in many cell death pathways, including those initiated by β-amyloid and ischemia.

Although the caspase-2 knockout phenotype is unremarkable,[27,28] arguing against a critical role of caspase-2 in apoptosis, the study by Lassus *et al*[8] demonstrates that caspase-2 is required for DNA-damage-induced apoptosis in E1A-transformed fibroblasts and in some cancer cell lines. Using RNA interference (RNAi) to ablate caspase-2 mRNA, knockdown of caspase-2 in these cells prevents cell death induced by etoposide, cisplatin and UV. The caspase-2 ablation also prevented cytochrome *c* and Diablo release from mitochondria, as well as inhibited the translocation of BAX to mitochondria.[8] Thus, these experiments clearly show that caspase-2 functions upstream of mitochondria, and that an initiator caspase is activated prior to mitochondrial permeabilisation.[8,12] Interestingly, knockdown of caspase-1 had no effect on stress-induced cell death in these cells.[8] It is likely that several upstream caspases become activated in response to stress signals and that different caspases are employed depending on the expression level, type of stimulus and cell type. Perhaps in the case of caspase-2 knockout cells, another initiator caspase is able to compensate for the lack of caspase-2 during development. In fact Troy *et al*[9] have demonstrated that in the brain and sympathetic neurons from the caspase-2-deficient animals, caspase-9 and Diablo are upregulated, suggesting the presence of compensatory parallel pathways.

These recent studies suggesting that initial caspase activation in the intrinsic pathway of apoptosis occurs upstream of mitochondria and that Apaf-1 apoptosome facilitated by cytochrome *c* primarily acts as an amplification mechanism (Figure 1), raise many issues. Clearly, Apaf-1 and caspase-9 are essential in developmental cell death in the central nervous system, as the knockout of either of these molecules generates profound brain hyperplasia in mice.[30] Furthermore, the RNAi results of Lassus *et al* suggest that Apaf-1 is essential, albeit downstream of caspase-2, for apoptosis to occur. Thus, inhibition of developmental PCD in the CNS of Apaf-1 and caspase-9 knockout mice does not rule out a role for a caspase upstream of mitochondria. However, results of Marsden *et al* with haematopoietic cells and MEFs suggest that Apaf-1/caspase-9 pathway is primarily an amplification loop and cell death can proceed without it. How do we then reconcile these apparent discrepancies? The simplest explanation is that the mitochondrial pathway is essential in some cells, whereas in others it simply facilitates the amplification of the caspase-activation cascade

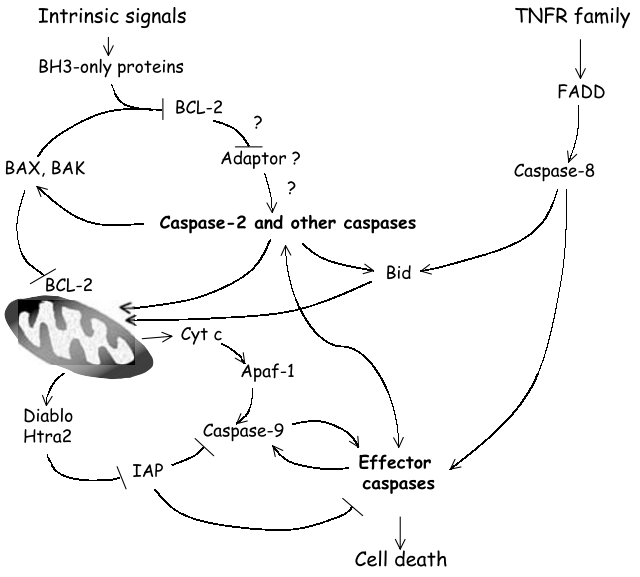

Figure 1 Making sense (or not) of the old and new data. Numerous previous studies have suggested that the mitochondrial cytochrome *c* release is crucial to the initiation of the caspase activation cascade. In this pathway, caspase-9 acts as an initiator caspase. However, recent data[7,8] indicate that the initial caspase activation occurs upstream of the mitochondria and is required for mitochondrial permeabilisation. In this scenario, mitochondrial pathway of caspase activation may simply act as an amplification loop for caspase activation, as it does in the extrinsic pathway mediated via the TNFR family members. See text for more details.

(Figure 1). In any case, it is now clear that apoptosome-mediated caspase-9 activation is unlikely to be the initiator caspase-activation event. As mitochondria seem unimportant for caspase activation in nematode and in fly, it is possible that this pathway evolved much later, perhaps to make the caspase activation more efficient and robust, and to provide more checkpoints for the regulation of caspase activation.

While the recent papers provide much food for thought, many obvious questions remain. How many caspases can act upstream of mitochondria and how are they activated? How do BCL-2 family members regulate the activation of these upstream caspases? Are there undiscovered CED-4-like adaptor molecules that directly bind BCL-2 and regulate caspase activation? Given that Apaf-1 is the only known CED-4 homologue with a demonstrated role in caspase activation, it may be necessary to test all similar proteins in the genome/protein database for their potential function in caspase activation. It is also unclear how the upstream caspases such as caspase-2 cause mitochondrial permeabilisation? The study of Guo *et al*[10] suggests that caspase-2 can directly (or via the cleavage of Bid) cause release of cytochrome *c* from mitochondria. This implies that caspase-2 may be cleaving a mitochondrial membrane protein(s) to effect permeabilisation. What is the identity of this protein? What is the role of BAX-like proteins and where exactly do they lie in the pathway? Do BH3 only proteins solely act by blocking the function of prosurvival BCL-2 upstream of premitochondrial caspase activation, or is their role at the mitochondria equally important? A quick look at the published literature suggests that there is ample evidence to support the mitochondrial model of caspase

activation. Given the strongly held views in both the pro- and antimitochondrial camps, lively debate and some reassessment of the published data are expected to follow in light of the recent findings reviewed here. However, as in most biological systems, truth probably lies somewhere in the middle, and it may be quite a while before appropriate experimental systems are developed to address some of the current controversies.

1. Hengartner MO. (2000) Nature 407: 770–776
2. Nicholson DW (1999) Cell Death Differ. 6: 1028–1042
3. Kumar S (1999) Cell Death Differ. 6: 1060–1066
4. Wang X (2000) Gene Dev. 15: 2922–2933
5. Dorstyn L *et al.* (2002) J. Cell Biol. 156: 1089–1098
6. Zimmermann K *et al.* (2002) J. Cell Biol. 156: 1077–1087
7. Marsden VS *et al.* (2002) Nature 419: 634–637
8. Lassus P *et al.* (2002) Science 297: 1352–1354
9. Robertson JD *et al.* (2002) J. Biol. Chem. 277: 29803–29809
10. Guo Y *et al.* (2002) J. Biol. Chem. 277: 13430–13437
11. Finkel E (2001) Science 292: 624–626.
12. Kumar S and Vaux DL (2002) Science 297: 1290–1291
13. Vaux DL *et al.* (1992) Science 258: 1955–1957
14. Kumar S *et al.* (1994) Genes Dev. 8: 1613–1626
15. Wang L *et al.* (1994) Cell 78: 739–750
16. Kumar S *et al.* (1997) Cell Death Differ. 4: 378–387
17. Troy CM and Shelanski ML (2003) Cell Death Differ. 10: this issue
18. Lamkanfi M *et al.* (2002) Cell Death Differ. 9: 358–361
19. Kinoshita M *et al.* (1997) J. Cereb. Blood Flow Metab. 17: 507–514
20. Dorstyn L *et al.* (1999) Proc. Natl. Acad. Sci. USA 96: 4307–4312
21. Cakouros D *et al.* (2002) J. Cell Biol. 157: 985–995
22. Butt AJ *et al.* (1998) J. Biol. Chem. 273: 6763–6768
23. Colussi PA *et al.* (1998) J. Biol. Chem. 273: 26566–26570
24. Harvey NL *et al.* (1997) J. Biol. Chem. 272: 13134–13139
25. Colussi PA *et al.* (1998) J. Biol. Chem. 273: 24535–24542
26. Paroni G *et al.* (2002) J. Biol. Chem. 277: 15147–15161
27. Bergeron L *et al.* (1998) Genes Dev. 12:1304–1314
28. O'Reilly LA *et al.* (2002) Cell Death Differ. 9: 832–841
29. Troy CM *et al.* (2001) J. Neurosci. 21: 5007–5016
30. Zheng TS *et al.* (1999) Cell Death Differ. 6: 1043–1053