Bcl-2–regulated apoptosis and cytochrome c release can occur independently of both caspase-2 and caspase-9

Vanessa S. Marsden,1 Paul G. Ekert,1,2 Mark Van Delft,1 David L. Vaux,1 Jerry M. Adams,1 and Andreas Strasser1

1The Walter and Eliza Hall Institute of Medical Research, Melbourne, Victoria 3050, Australia
2Murdoch Children’s Research Institute, Melbourne, Victoria 3052, Australia

Apoptosis in response to developmental cues and stress stimuli is mediated by caspases that are regulated by the Bcl-2 protein family. Although caspases 2 and 9 have each been proposed as the apical caspase in that pathway, neither is indispensable for the apoptosis of leukocytes or fibroblasts. To investigate whether these caspases share a redundant role in apoptosis initiation, we generated caspase-2−/−/H11002/9−/−/H11002 mice. Their overt phenotype, embryonic brain malformation and perinatal lethality mirrored that of caspase-9−/−/H11002/11002 mice but were not exacerbated. Analysis of adult mice reconstituted with caspase-2−/−/9−/−/H11002/11002 hematopoietic cells revealed that the absence of both caspases did not influence hematopoietic development. Furthermore, lymphocytes and fibroblasts lacking both remained sensitive to diverse apoptotic stimuli. Dying caspase-2−/−/9−/−/H11002/11002 lymphocytes displayed multiple hallmarks of caspase-dependent apoptosis, including the release of cytochrome c from mitochondria, and their demise was antagonized by several caspase inhibitors. These findings suggest that caspases other than caspases 2 and 9 can promote cytochrome c release and initiate Bcl-2–regulated apoptosis.

Introduction

Apoptosis, which is critical for development and tissue homeostasis, is executed by caspases (Adams, 2003). The 10 or so mammalian caspases include both “effectors” (3, 6, and 7), which efficiently digest vital proteins, and “initiators” (e.g., 2, 8, and 9), which proteolytically activate the effectors. Many cell “stress” stimuli, e.g., cytokine deprivation and genome damage, and developmental cues, trigger a common pathway of caspase activation regulated by the Bcl-2 protein family (Adams, 2003). Until recently, the sole apical initiator in that pathway was assumed to be caspase-9, which is activated in a complex termed the “apoptosome” by the scaffold protein Apaf-1 and its cofactor cytochrome c. Evidence that the Bcl-2 family regulates permeabilization of mitochondria argued that cytochrome c release and the ensuing caspase-9 activation were central to the “stress” response. For some neuronal cells, this model is supported, as mice lacking Apaf-1 or caspase-9 die perinatally with brain overgrowth caused by a defect in neuronal apoptosis (Adams, 2003).

The apoptosome is not, however, universally essential for Bcl-2–regulated apoptosis, because certain neuronal (Honarpour et al., 2001), hematopoietic, and fibroblastoid cells (Marsden et al., 2002) lacking Apaf-1 or caspase-9 readily undergo apoptosis in response to diverse insults and, at least in lymphocytes, that apoptosis requires caspase activity (Marsden et al., 2002). Hence, there must be apoptotic pathways regulated by the Bcl-2 family that require the activation of caspases other than caspase-9 (Adams, 2003).

Evidence is also accumulating that certain caspases can contribute to mitochondrial damage and hence may be activated before apoptosome formation (Guo et al., 2002; Lassus et al., 2002; Marsden et al., 2002; Robertson et al., 2002). In particular, caspase-2 has been implicated in cytochrome c release (Guo et al., 2002; Lassus et al., 2002; Robertson et al., 2002) and seems to be necessary for cellular demise in some transformed cell lines (Lassus et al., 2002). However, because apoptosis is not markedly impaired in caspase-2–deficient mice (Bergeron et al., 1998; O’Reilly et al., 2002), caspase-2 cannot have a major nonredundant role in apoptosis.
These discordant findings might be reconciled if caspase-2 acts redundantly with caspase-9, each activating distinct but converging pathways. If so, loss of both caspases should markedly attenuate apoptosis. We address that hypothesis here by studies on mice lacking both caspases 2 and 9.

Results and discussion

To generate mice lacking both caspases 2 and 9, we first intercrossed animals deficient in caspase-2 (O’Reilly et al., 2002) with caspase-9/H11001/H11002 mice (Kuida et al., 1998). As expected from the severe caspase-9/H11002 phenotype (Hakem et al., 1998; Kuida et al., 1998), intercrosses of the resulting caspase-2/H11001/H11002/9/H11001/H11002 mice yielded no weaned progeny lacking caspase-9, irrespective of caspase-2 status (67 progeny genotyped). Mice of all other genotypes appeared at the expected Mendelian ratios and were healthy and fertile (unpublished data).

To investigate whether caspase-2 deficiency exacerbated the neuronal overgrowth characteristic of the caspase-9 deficiency (Hakem et al., 1998; Kuida et al., 1998), intercrosses of the resulting caspase-2/H11002/9/H11002 mice yielded no weaned progeny lacking caspase-9, irrespective of caspase-2 status (67 progeny genotyped). Mice of all other genotypes appeared at the expected Mendelian ratios and were healthy and fertile (unpublished data).

To investigate whether caspase-2 deficiency exacerbated the neuronal overgrowth characteristic of the caspase-9 deficiency (Hakem et al., 1998; Kuida et al., 1998), embryos from the intercrosses were examined at E14.5, when all genotypes appeared in the expected ratios. Brain overgrowth resembling that previously described and observed in caspase-2/H11002/9/H11002 littermates (Fig. 1) appeared in 6/11 caspase-2/H11002/9/H11002 and 2/5 caspase-2/H11001/9/H11002 embryos but never in those expressing caspase-9 (n = 47). All other organs appeared normal. Although the brain abnormalities cannot be quantified, we conclude that caspase-2 loss does not substantially exacerbate the brain phenotype due to caspase-9 deficiency and that other organs develop normally to at least E14.5 without either caspases 2 or 9.

Bcl-2–regulated apoptosis, which is critical for the physiological death of hematopoietic cells (Marsden and Strasser, 2003), can occur independently of caspase-9 (Marsden et al., 2002). To study how caspase-2/H11002/9/H11002 hematopoietic cells respond to the physiological death cues in healthy mice, C57BL/6-Ly5.1 mice were reconstituted with fetal liver–derived hematopoietic stem cells. 10 wk later, the thymocytes were all derived from donor (Ly5.2) cells, irrespective of donor genotype (Fig. 2 A). The absence of caspases 2 and 9 did not augment cell numbers or perturb cell subset composition in the thymus, spleen, lymph nodes, or bone marrow (Fig. 2, B–D; and not depicted). Western blot analysis on reconstituted organs (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200312030/DC1) confirmed the absence of caspase.
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Dying caspase-9–deficient cells display the hallmarks of apoptosis (Marsden et al., 2002). Concomitant absence of caspase-2 did not prevent apoptosis. Two of its classic features, exposure of phosphatidylserine and DNA fragmentation, appeared in caspase-2−/−9−/− thymocytes subjected to γ irradiation (Fig. 4, A and B). It is likely that effector caspases contributed to their death, because the well-characterized caspase substrates ICAD, spectrin, and gelsolin (Fig. 4 C, Fig. S3, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200312030/DC1) were all processed appropriately, albeit to a lower extent than in dying wild-type cells. ICAD is thought to be processed only by caspases 3 or 7 (McIlroy et al., 1999); caspase-3 is required to generate the 41-kD product of gelsolin (Slee et al., 2001), and although either caspases or calpains can generate the 150-kD fragment of spectrin, only caspases give the 150-kD fragment (Methot et al., 2004). In dying caspase-2−/−9−/− cells, all three substrates yielded the expected caspase-dependent products (Fig. 4 C; Fig. S3, A and B). Moreover, the gelsolin processing was inhibited in irradiated caspase-2−/−9−/− thymocytes treated with either of two structurally unrelated caspase inhibitors, IDN-1965 (Fig. S3 C) or Q-VD-OPh (not depicted). Thus, caspases are strongly implicated in the death of these cells.

Processing of synthetic as well as physiological caspase substrates is lower in cell lysates lacking caspase-9 (Marsden et al., 2002). Similarly, with the fluorogenic caspase substrate DEVD-aminomethylcoumarin, dying caspase-2−/−9−/− or caspase-9−/− thymocytes had only ~10–20% of the DEVDase activity of dying wild-type or caspase-2−/− lysates, but the activity was completely blocked by the caspase inhibitor zVAD-fmk (Fig. S3 D). It was unaffected by ALLN, a potent inhibitor of calpains and cathepsins, arguing against any contribution of these proteases in processing this caspase substrate.

To further explore whether the death of caspase-2−/−9−/− cells requires caspases, we examined the ability of four chemically distinct caspase inhibitors to impair their death (Fig. 4 F). Three of them, Q-VD-OPh (Caserta et al., 2003), IDN-1965, and IDN-6275 (Wu and Fritz, 1999), delayed apoptosis substantially up to 24 h after dexamethasone treatment of T cells (Fig. 4 D). zVAD-fmk had a smaller inhibitory effect, probably due to its reportedly inferior stability, membrane permeability, and performance in culture (Nicholson, 1999).

Non-caspase proteases, in particular calpains and cathepsins, have been proposed to contribute to apoptosis in cer-
tain circumstances (Jaattela and Tschopp, 2003). To determine whether either participated in the apoptosis of caspase-2/9 cells, we tested six inhibitors reported to impair apoptosis under certain conditions: the calpain inhibitors z-VF-CHO and PD150606 (Squier and Cohen, 1997), a cell-permeable peptide of the natural calpain inhibitor calpastatin (Altznauer et al., 2004), the dual calpain and cathepsin inhibitors ALLM and ALLN (Ding et al., 2002), and the selective cathepsin inhibitor z-FG-NHO-Bz-pOMe. In contrast to the caspase inhibitors, none of these inhibitors had any anti-apoptotic activity at doses in the range where others have reported efficacy (Fig. 4 D), and none cooperated with IDN-1965 to enhance its antagonism of apoptosis (not depicted). Hence, it appears unlikely that either calpains or cathepsins act in tandem with the caspase cascade to cause apoptosis in these cells.

Cytochrome c release in thymocytes seems to depend on caspase activity (Marsden et al., 2002), and caspase-2 has been implicated in mitochondrial disruption in certain cells (Guo et al., 2002; Lassus et al., 2002; Robertson et al., 2002). Hence, we examined whether caspase-2 was the sole caspase responsible for mitochondrial damage in thymocytes. Western blotting of fractionated cell lysates revealed that cytochrome c release from mitochondria did not require caspase-2 (Fig. 5 A). Furthermore, mitochondrial transmembrane potential in dying thymocytes was lost normally in the absence of caspase-2, or both caspases 2 and 9, although its loss in wild-type thymocytes was attenuated by a caspase inhibitor (Fig. 5 B), as shown previously (Bossy-Wetzel et al., 1998). Hence, both the release of pro-apoptotic molecules from mitochondria and the loss of mitochondrial transmembrane potential can occur independently of caspases 2 and 9.

Our results strongly implicate caspases in the apoptosis of caspase-2/9 cells and thus imply that there is a Bcl-2-regulated and caspase-mediated pathway that does not require either caspase-2 or -9. Which other caspases might be regulated by Bcl-2? As discussed elsewhere (Adams, 2003), caspases 1, 11, and 12 in mice (or caspases 1, 4, and 5 in humans) are attractive candidates, because, like caspases 2 and 9, their NH2-terminal CARD domain could interact with a cognate scaffold to form an apoptosome-like complex. For example, caspase-12, which is implicated in apoptosis induced by ER stress (Nakagawa et al., 2000) and in cytochrome c-independent apoptosis (Morishima et al., 2002; Rao et al., 2002), forms a large complex on serum starvation (Kilic et al., 2002). In other systems, caspase-11 (Hisahara et al., 2001; Kang et al., 2002) or caspase-1 (Hilbi et al., 1998; Marsden et al., 2002; Rowe et al., 2002) have been implicated in apoptosis. Hence, in different circumstances, vari-
uous combinations of caspases 1, 11, and 12, and perhaps also caspase-8, might act redundantly with caspases 2 and 9 to initiate apoptosis.

Whereas our results with primary lymphocytes and fibroblasts implicate caspases in addition to caspases 2 and 9 in the initiation of apoptosis, the accompanying paper, Ekert et al. (2004) shows that the hallmarks of apoptosis fail to appear when myeloid progenitor cell lines lacking both caspases 2 and 9 were deprived of growth factor, but that programmed cell death still prevented their clonogenic survival. These findings and those with transformed human cell lines (Lassus et al., 2002) can be reconciled with our conclusion that caspases 2 and 9 are not essential for loss of viability per se, if some cell types but not others use these caspases to accelerate apoptotic cell demoli-
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Author/s:
Marsden, VS; Ekert, PG; Van Delft, M; Vaux, DL; Adams, JM; Strasser, A

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