Letter to the Editor

Differential requirement for Apaf1 and Bcl-X_L in the regulation of programmed cell death during development

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Dear Editor,

Studies using gene-targeting in mice have delineated the molecular framework of the mitochondria-dependent pathway of programmed cell death (PCD),¹ which has been conserved over species. While Bcl-X_L, a CED-9 homologue, prevents apoptosis, Apaf1, the mammalian homologue of CED-4, promotes cell death in the developing nervous system^{2–7} by activating caspases.^{8–11} Despite recent elucidation of the mitochondria-dependent pathway of apoptosis on molecule bases, it is as yet to be clarified whether these molecules exert their anti- and pro-apoptotic effects in the same linear fashion in different tissues during development.

To address this issue, we generated *apaf1* and *bcl-x* double mutant mice and looked for rescue of phenotypes of respective single mutant mice. Bcl-X_L-deficient (*bcl-x*^{-/-}) mice die around embryonic day (E) 13 with massive apoptosis of both brain cells and hematopoietic precursors in fetal liver.^{2,12} In contrast, Apaf1-deficient (*apaf1*^{-/-}) mice die at a later stage (E16.5 or later) with an accumulation of neurons in the brain.^{6,7} As shown in Table 1, no *apaf1*^{-/-}/*bcl-x*^{-/-} mice were present among 215 live-born pups born to intercrosses between heterozygotes for the *apaf1* and *bcl-x* mutations (*apaf1*^{+/-}/*bcl-x*^{+/-}). Furthermore, normal *apaf1*^{-/-}/*bcl-x*^{-/-} embryos were not observed beyond E13.5, indicating that *apaf1*^{-/-}/*bcl-x*^{-/-} embryos died around E13. Similarly, all *bcl-x*^{-/-} embryos died by E13, but *apaf1*^{-/-} embryos survived beyond this stage. Thus, all embryos lacking Bcl-X_L (with or without Apaf1) died in utero by E13.5. These data show that deletion of *apaf1* cannot rescue the embryonic lethal phenotype of Bcl-x deficiency.

The *apaf1*^{-/-} embryos examined in this study showed morphological abnormalities at E12.5 and beyond that were consistent with previous reports.^{6,7} In particular, *apaf1*^{-/-} embryos were subject to exencephalus (Figure 1A; K/W, arrowhead). Interestingly, *apaf1*^{-/-}/*bcl-x*^{-/-} embryos showed morphological abnormalities similar to those of *apaf1*^{-/-} embryos, including exencephalus (Figure 1A; K/K, arrowhead), while no such abnormalities were observed in any *bcl-x*^{-/-} or wild-type embryos. Histological analyses revealed hyperplasia of the brain in both *apaf1*^{-/-} and *apaf1*^{-/-}/*bcl-x*^{-/-} embryos (Figure 1B; K/W and K/K). Thickening of the cerebral cortex with supernumerary cells and narrowing of the ventricles were also observed in *apaf1*^{-/-} embryos, phenotypes that were not rescued by mutation of the *bcl-x* gene. These data indicate that Apaf1 regulates the apoptosis of neurons in a pathway that operates independently of Bcl-X_L.

To further examine the effects of Apaf1 and Bcl-x deficiency on the PCD of the developing brain, we performed TUNEL assays on brain tissue E12.5 wild-type and mutant embryos. In wild-type embryos, TUNEL-positive cells were observed in the hindbrain and dorsal root ganglia (DRG) (Figure 1C–E, W/W; 24 ± 5.8 in the hindbrain and 57 ± 29.0 in three DRG). In *apaf1*^{-/-} embryos, far fewer TUNEL-positive cells were detected in the same regions (Figure 1C–E, K/W; 8.6 ± 0.9 and 6 ± 1.6 in the hindbrain and three DRG, respectively). In contrast to wild-type and *apaf1*^{-/-} embryos, *bcl-x*^{-/-} embryos showed a dramatic increase in TUNEL-positive cells in the intermediate zones of the ventral midbrain (Figure 1C; He/K; number not determined), in hindbrain (Figure 1D; 511.7 ± 47.2) and in DRG (Figure 1E; 292.3 ± 62.7). However, very few TUNEL-positive cells were detected in *apaf1*^{-/-}/*bcl-x*^{-/-} embryos, no more than in *apaf1*^{-/-} embryos (Figure 1C–E, K/K; 9.6 ± 1.9 and 7 ± 1.8 in the hindbrain and three DRG, respectively). These results indicate that Apaf1 deficiency can counter the increased apoptosis of postmitotic immature neurons caused by Bcl-X_L deficiency.

Although the most striking feature of *apaf1*^{-/-}/*bcl-x*^{-/-} embryos was their brain abnormality, these embryos died in utero around E13, just like Bcl-X_L-deficient mice and considerably earlier than Apaf1-deficient mice. This result demonstrates that the lethality at E13 was conferred by Bcl-x_L deficiency. We have previously reported that massive cell death takes place in immature hematopoietic cells of *bcl-x*^{-/-} embryos, and that Bcl-X_L is required for the prevention of apoptosis during the final stages of erythropoiesis.^{2,12} In *apaf1*^{-/-}/*bcl-x*^{-/-} embryos as well as in *bcl-x*^{-/-} embryos, the liver was pale and appeared anemic (data not shown), suggesting that the hematopoietic phenotype in the liver of *bcl-x*^{-/-} embryos was not mitigated by mutation of *apaf1*. Histopathological analysis revealed scattered TUNEL-positive cells in wild-type fetal livers (Figure 1F, W/W; 254.6 ± 38.8 per field), reflecting the physiological PCD that occurs during the maturation of hematopoietic cells. As reported previously,² increased numbers of TUNEL-positive cells were detected in *bcl-x*^{-/-} fetal liver (Figure 1F, He/K; 1125 ± 110.3 per field) compared to wild-type liver. In *apaf1*^{-/-}/*bcl-x*^{-/-} liver (Figure 1F, K/K), the number of TUNEL-positive cells was significantly greater than in the wild-type and almost the same as that in *bcl-x*^{-/-} liver (990 ± 85.6 per field). In contrast, the number of TUNEL-positive cells in *apaf1*^{-/-} embryos (Figure 1F, K/W) was only slightly increased over that in wild-type liver (386 ± 89.4 per field).

Figure 1 Histopathological examination of *apaf*^{-/-}, *bcl-x*^{-/-} and *apaf*^{-/-}/*bcl-x*^{-/-} embryos. (A) Macroscopic views of E12.5 embryos. Arrowheads indicate exencephalus. For all figures: W/W, wild-type; K/W, Apaf1-deficient mice; K/K, Apaf1- and Bcl-x-double deficient mutant mice; He/K, Bcl-x-deficient mice. (B) Microscopic views of the E12.5 embryos shown in (A). Uteri from plugged females at E12.5 were isolated in ice-cold phosphate-buffered saline (PBS), fixed overnight in 4% paraformaldehyde in PBS at 4°C, dehydrated, and embedded in paraffin. Sections (6 μm in thickness) were cut and stained with hematoxylin and eosin. Arrowheads and asterisks indicated exencephalus and thickening of the cortex, respectively. (C) Apoptosis in the brain was detected by the terminal deoxy-nucleotidyltransferase-mediated UTP end-labeling (TUNEL) method using the *In situ* Cell Death Detection kit (Boehringer Mannheim) according to the manufacturer's directions. (D) High magnification (×200) views of areas marked by rectangles in (C). (E) Detection of apoptosis in dorsal root ganglia by TUNEL staining. (F) Detection of apoptosis in fetal liver. Magnification, ×200. (G) Electron microscopic examination of fetal liver. Fetal livers were fixed with 1× fixing buffer (2.5% glutaraldehyde, 0.1 M sucrose and 3 mM CaCl₂ in 0.1 M cacodylate buffer (pH 7.4)) on ice and processed through 1% OsO₄ for 1 h at 4°C, dehydrated, and embedded in Epon 812 resin. Thin sections were stained with 5-uranyl acetate and lead acetate. Arrows show condensed nuclei in macrophages, and arrowheads indicate degenerated cells within macrophages

Table 1 Genotype of embryos and live-born mice from intercrosses of *bcl-x*^{+/-}/*apaf1*^{+/-}^a

	E12.5 n=64	E13.5 n=32	E14.5 n=21	Live-born n=215
<i>bcl-x</i> ^{+/+} / <i>apaf1</i> ^{+/+}	4	5	2	29
<i>bcl-x</i> ^{+/+} / <i>apaf1</i> ^{+/-}	6	6	1	79
<i>bcl-x</i> ^{+/+} / <i>apaf1</i> ^{-/-}	5	0	4	2
<i>bcl-x</i> ^{+/-} / <i>apaf1</i> ^{+/+}	14	4	1	34
<i>bcl-x</i> ^{+/-} / <i>apaf1</i> ^{+/-}	13	6	8	70
<i>bcl-x</i> ^{+/-} / <i>apaf1</i> ^{-/-}	7	3	1	1
<i>bcl-x</i> ^{-/-} / <i>apaf1</i> ^{+/+}	5	1 (1) ^b	1 (1) ^b	0
<i>bcl-x</i> ^{-/-} / <i>apaf1</i> ^{+/-}	6	6 (6) ^b	1 (1) ^b	0
<i>bcl-x</i> ^{-/-} / <i>apaf1</i> ^{-/-}	4	1 (1) ^b	3 (3) ^b	0

^aGeneration of *apaf1*- and *bcl-x*-deficient mice were described in Results and Discussion. To generate mice with mutations for both *bcl-x* and *apaf1*, double heterozygotes (*bcl-x*^{+/-}/*apaf1*^{+/-}) mice were intercrossed. Embryos from E12.5 to E14.5 and live-born mice were genotyped by PCR analysis. ^bNumbers in the parentheses indicate dead embryos

Electron microscope examination of wild-type fetal liver revealed the presence of macrophages containing several condensed apoptotic nuclei (Figure 1G; W/W). These inclusions in macrophages reflect the process of terminal maturation and enucleation of erythrocytes. Greater numbers of macrophages with condensed nuclei were detected in *bcl-x*^{-/-} liver (Figure 1G; He/K) compared to the wild-type (data not shown). Of note, in both *bcl-x*^{-/-} and *apaf1*^{-/-}/*bcl-x*^{-/-} liver samples, many degenerated cells (in addition to condensed nuclei) were observed within macrophages (Figure 1G, arrowheads). This finding implies that the macrophages had phagocytosed other types of apoptotic cells (but did not physiologically enucleate them). In *apaf1*^{-/-}/*bcl-x*^{-/-} liver (Figure 1G; K/K), the condensed nuclei within macrophages were morphologically indistinguishable from those observed in *bcl-x*^{-/-} liver. These results, together with the results of the TUNEL assays, indicated that Apaf1-mediated pathways play little, if any, role in physiological PCD during hematopoiesis in fetal liver. Moreover, Apaf1 does not appear to affect apoptotic events caused by Bcl-X_L-deficiency in hematopoietic cells.

Our study of double mutant mice lacking both Apaf1 and Bcl-X_L indicates that Apaf1 has a dominant role in regulating apoptosis in the developing brain, and that this role is independent of Bcl-X_L. Roth *et al.* have described the effects of Bcl-X_L and Casp3 double deficiency and have demonstrated that Casp3 mediates apoptosis of both founder and postmitotic neurons, while Bcl-X_L regulates apoptosis in postmitotic neurons.¹³ Considered in this light, our data strongly suggest that Apaf1 and the Apaf1-mediated pathway of apoptosis play a critical role in the execution of PCD in the developing brain. We have also demonstrated that the massive PCD of hematopoietic cells in fetal liver is entirely independent of Apaf1. Bcl-X_L is required for the maintenance of hematopoietic precursors.¹² During the development of hematopoietic (erythroid) cells, nuclear condensation (and probably enucleation) is dependent on DNase II.¹⁴ Significantly, Bcl-X_L-deficient embryos die by E13.5 whereas DNase II-deficient embryos survive until E17.5 or later,¹⁴ demonstrating that Bcl-X_L exerts its anti-apoptotic function before nuclear condensation dependent on DNase II takes place.

Studies in *C. elegans* have shown that molecules involved in PCD in this organism, CED-9, CED-4 and CED-3, function in a linear, epistatic manner. In mammals, however, our and others' data indicate that the mammalian counterparts of these apoptosis-related molecules, Bcl-2/Bcl-X_L, Apaf1 and caspases differentially regulate PCD depending on the tissue and/or stage of development/differentiation. This difference may be accounted for by the fact that multiple family members of related function exist in mammals.^{15,16} Each of these family members could be uniquely involved in PCD in a different type of tissue or cell. In addition, mammals possess molecules not found in *C. elegans*, such as apoptosis-inducing factor (AIF)¹⁷ and endonuclease G.¹⁸ The complex web, in which all these players contribute to the regulation of mammalian PCD, and the issue of whether these molecules act independently of or interdependently with Bcl-X_L and Apaf1 in mitochondria-mediated apoptosis, remain to be completely resolved. Our work offers the clarification that Apaf1 and Bcl-X_L have distinct functions in regulating the development PCD of neurons and hematopoietic precursors.

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