Reduced Apoptosis and Cytochrome c–Mediated Caspase Activation in Mice Lacking Caspase 9

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Summary

Caspases are essential components of the mammalian cell death machinery. Here we test the hypothesis that Caspase 9 (Casp9) is a critical upstream activator of caspases through gene targeting in mice. The majority of Casp9 knockout mice die perinatally with a markedly enlarged and malformed cerebrum caused by reduced apoptosis during brain development. Casp9 deletion prevents activation of Casp3 in embryonic brains in vivo, and Casp9-deficient thymocytes show resistance to a subset of apoptotic stimuli, including absence of Casp3-like cleavage and delayed DNA fragmentation. Moreover, the cytochrome c-mediated cleavage of Casp3 is absent in the cytosolic extracts of Casp9-deficient cells but is restored after addition of in vitro-translated Casp9. Together, these results indicate that Casp9 is a critical upstream activator of the caspase cascade in vivo.

Introduction

Apoptosis, or programmed cell death (PCD), is an important mechanism of maintaining homeostasis during development and for responses to external stimuli in multicellular organisms (Glucksmann, 1951; Kerr et al., 1972; Jacobson et al., 1997). Recent studies indicate that the fundamental apoptosis machinery, which consists of distinct effectors, inhibitors, and initiators, has been conserved throughout evolution (Ellis et al., 1991; Yuan et al., 1993; Hengartner and Horvitz, 1994). The key apoptosis effectors in mammals are a family of cysteinecontaining, aspartate-specific proteases called caspases (Alnemri et al., 1996; Nicholson and Thornberry et al., 1997). Caspases exist as dormant proenzymes in healthy cells and are activated through proteolysis. Once activated, caspases cleave a host of cellular substrates, leading to morphological hallmarks of apoptosis including DNA fragmentation and condensation of cellular organelles (for review, see Nicholson and Thornberry et al., 1997).

Six lines of caspase-deficient mice that have been recently generated (Caspases 1, 2, 3, and 11 knockouts) all exhibit cell death abnormalities, indicating that caspases play an important role in apoptosis (Kuida et al., 1995, 1996; Li et al., 1995; Bergeron et al., 1998; Wang et al., 1998; Woo et al., 1998). Interestingly, these knockouts do not exhibit a global suppression of cell death, but rather tissue- and cell type-specific or stimulus-dependent defects of apoptosis. The preferential apoptosis defects suggest that different sets of caspases are involved in separate cell death pathways in vivo. In addition, phenotypes of variable severities were found in two independent lines of Caspase 3-deficient $(Casp3^{-/-})$ mice, in which the majority of homozygous embryos die in utero, while some mutants survive to 4-5 weeks of age (Kuida et al., 1996; Woo et al., 1998). The variability of phenotypes suggests the involvement of additional components, such as the recently reported inhibitor-of-apoptosis (IAP) family of suppressors (Deveraux et al., 1997), in modifying the apoptotic response. One important issue to be elucidated concerns the mechanism of activation of the caspase cascades. A well-understood mechanism is the assembly of a signaling complex at the CD-95 (Fas/Apo-1) receptor, which in turn leads to the autoproteolytic activation of Caspase 8 (Casp8) (Nagata, 1997; Muzio et al., 1998; Yang et al., 1998). Another recently discovered mechanism involves the release of cytochrome c from the mitochondria, which subsequently causes apoptosis by activation of caspases (Kluck et al., 1997; Reed, 1997; Yang et al., 1997). It remains to be clarified whether, or at which step during apoptosis, these two pathways converge.

In a series of biochemical studies on the initiation of Casp3 cleavage in HeLa cells, it was discovered that the apoptotic protease activating factors (Apafs) include cytochrome c released from the mitochondria (Apaf-2), the human homolog of Ced-4 (Apaf-1), and the Caspase 9 proenzyme (Casp9) (Apaf-3) (Liu et al., 1996; Li et al., 1997; Zou et al., 1997). The amino terminal sequences of Casp9 and Apaf1 both contain a caspase recruitment domain (CARD) motif that is indispensible for their interactions (Hofmann et al., 1997; Li et al., 1997). Casp3, which lacks the CARD domain, does not bind to Apaf1. In addition, active site mutations of Casp9 serve as dominant-negative influences on the activation of Casp3 in transfected cells (Li et al., 1997). Together, these data suggest a linear and specific activation cascade between Casp9 and Casp3 in response to cytochrome c released from the mitochondria. Cytochrome c-mediated Casp3 activation may be utilized by a specific and restricted set of external apoptosis stimuli. Alternatively, since many types of cellular insults all damage mitochondria, causing release of cytochrome c into the cytoplasm, and since activated Casp3 can directly initiate certain caspase-activated deoxyribonucleases (Liu et

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Caspase-9

Caspase-3

Apaf-1

al., 1997; Enari et al., 1998), the cytochrome c-mediated caspase activation may serve as an amplification mechanism during most forms of apoptosis.

Although in vitro biochemical studies suggest that Casp9 may be a key element of the cytochrome c-mediated caspase activation, this has not been tested in vivo. To test this hypothesis, we have generated Casp9 null mutation mice by gene targeting. Here we report that the majority of homozygous mutants die perinatally with markedly enlarged cerebrum, caused by a reduction of apoptosis at an earlier stage of brain development. In contrast, the loss of Casp9 does not affect morphological developments of the spinal cord and other nonneural tissues. Despite normal development of the thymus, isolated thymocytes are nevertheless resistant to a subset of apoptosis stimuli and exhibit delayed DNA fragmentation. We show that these Casp9 null mutation phenotypes, which resemble the Casp3 deficiency, are indeed associated with defective activation of Casp3 in vivo. Furthermore, we show that the cytochrome c-mediated cleavage of Casp3 is absent in cytosolic extracts from Casp9-deficient cells but is restored after reconstitution with in vitro-translated Casp9. Taken together, these results indicate that Casp9 is a key activator of the caspase cascade, important for normal brain development and apoptotic responses in thymocytes in vivo.

Results

Null Mutation of Caspase 9 Causes Perinatal Lethality

To generate Casp9-deficient mice, a targeting vector that contains a neo gene cassette flanked by 7 kb of the mouse Casp9 genomic sequence was used to replace a 1.0 kb endogenous fragment encoding the conserved pentapeptide (QACGG) motif common to all caspases (Figure 1A). Linearized construct was electroporated into W9.5 ES cells, and transfectants were selected by G418 and gancyclovir. One homologous recombinant clone, confirmed by Southern blotting analysis, was obtained and injected into C57BL/6 blastocysts to generate chimeras, which were then crossed to C57BL/6 mice to generate heterozygous *Casp9* mutants. Heterozygous mice were crossed to generate homozygous Casp9deficient (Casp9-/-) mice. RT-PCR analysis using total RNA from Casp9-/- mice confirmed the absence of expression of Casp9 mRNA (Figure 1B). Genotyping of more than 500 fetuses and newborn pups derived from interbreeding of heterozygous mice established that the

Table 1. Genotyping of Mice Derived from Caspase 9							
Heterozygous	Breeding						
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Genotype	+/+	+/-	-/-	
E10.5	3	7	3 (23%)	
E12.5	5	3	6 (43%)	
E13.5	10	15	5 (17%)	
E15.5	9	13	6 (21%)	
E16.5	7	19	9 (26%)	
P5-P20	160	246	11 (2.6%)	

The genotype of mice at indicated embryonic (E) or postnatal (P) day were determined by PCR analysis. Wild-type, heterozygous, and homozygous animals are indicated as +/+, +/-, and -/-, respectively.

loss of Casp9 results in perinatal lethality. Although $Casp9^{-/-}$ fetuses were collected at a Mendelian ratio up to embryonic day (E) 16.5, only less than 3% of the total number of genotyped newborn pups were homozygous Casp9 mutants (Table 1). No apparent histological abnormalities were noticed in a survey of various tissues in these few postnatal Casp9 mutants.

Caspase 9 Is Widely Expressed during Mouse Embryogenesis

The observed perinatal lethality indicates that Casp9 plays an important role during development. To test this possibility, we first examined the expression of Casp9 and its potential associating factors, Apaf1 and Casp3, throughout development. Northern blot analysis of poly(A)+ RNA isolated from different stages of wildtype embryos revealed an expression of these apoptosis effectors during early embryogenesis (Figure 1D). Expression of Casp9 mRNA was detected as early as E7, and both Casp3 and Apaf1 transcripts were up-requlated at E11 and continuously expressed thereafter. To examine the tissue distribution pattern of Casp9, we extracted total RNA from different organs of E17.5 fetuses for RT-PCR detection of the expression of Casp9 transcripts (Figure 1C). Casp9-specific bands were detected in all tissues examined, indicating a widespread expression pattern of Casp9 during prenatal development.

Caspase 9 Deficiency Causes Severe Brain Malformation

The most conspicuous phenotype of $Casp9^{-/-}$ individuals is a severe malformation of the brain. At the gross

Figure 1. Targeting Construct and Homologous Recombination at the Caspase 9 Locus and Expression of Caspase 9 mRNA

⁽A) Targeting construct and restriction map of the *Casp9* locus. The restriction map of the wild-type allele, targeting construct, and the mutated allele are shown. The orientation of the *neo* cassette is indicated by an arrow. The diagnostic probe used for Southern blot analysis is shown as a black box.

⁽B) RT-PCR analysis of *Casp9* mRNA. Total RNA was isolated from the kidney of wild-type or *Casp9^{-/-}* mice and reverse-transcribed. The resulting cDNA was used for PCR analysis using specific primers for *Casp9* or for the γ -actin gene. An 860 bp band is amplified by *Casp9*-specific primers, and the 700 bp band represents mRNA for γ -actin. λ HindIII and ϕ X174 HaeIII were used as molecular weight markers (MW). (C) RT-PCR analysis of *Casp9* mRNA expression in various embryonic tissues. One microgram of total RNA from E17.5 fetuses was reverse-transcribed and subjected to PCR analysis using specific primers for *Casp9* or for the γ -actin gene. The specific band for *Casp9* is detected in all tissues examined.

⁽D) Northern blot analysis of *Casp9, Apaf1*, and *Casp3*. Blots containing Poly(A)⁺ RNA isolated from mouse embryos were hybridized with specific probes for *Casp9, Apaf1*, and *Casp3* genes. Expression of *Casp9* mRNA is detected as early as E7, while both *Casp3* and *Apaf1* mRNA are up-regulated at E11 and expressed thereafter.



Figure 2. Gross Morphological Examination of Caspase 9 Heterozygous and Homozygous Fetuses

Embryos were isolated from heterozygotes (left) and knockouts (right) at E10.5 ([A] and [B]), E13.5 ([C] and [D]), and E16.5 ([E] and [F]), respectively. At E10.5 and E13.5, a defect at the midbrain/hindbrain junction is evident in the (-/-) mutants (arrowhead). At E13.5, the retraction of interdigital webbing caused by morphogenetic cell death is completed in both $Casp9^{-/-}$ mutant and control littermates [insets in (C) and (D)]. By E16.5, there is protrusion of the larger and morphologically abnormal brain in knockouts. Bar, 1 mm.

morphological level, a defect of neural tube closure in the hindbrain region was apparent in the E10.5 mutants (Figures 2A and 2B). At E13.5, the hindbrain neural tube remained open, and there was an appreciable expansion of the midbrain region underneath the fetal skin tissue (Figures 2C and 2D). By E16.5, there was prominent expansion and protrusion (exencephaly) of the entire cranial tissues (Figures 2E and 2F). In addition, the protruding brain masses were enlarged and irregular on the surface. No gross malformations are found in other parts of the fetal body. For example, the retraction of the interdigital webbing in the fetal hand plate, a process involving apoptosis, is completed in mutant embryos by E13.5 (insets in Figures 2C and 2D), according to the normal developmental schedule (Kaufman, 1992).

At the histological level, Casp9-/- embryos exhibited prominent and ubiquitous enlargement of the proliferative zone in both the forebrain and midbrain, as well as stenosis of the cerebral ventricles. At E13.5, an expanded proliferative population, indicated by the dark Nissl staining, was evident in the telencephalon, with both the lateral and third ventricles obstructed in the mutants (Figures 3A and 3B). In addition, heterotopias and discontinuities of the proliferative ventricular zone were evident in the mutant telencephalon, leading to invagination and complete interruption of the telencephalic wall (Figures 3C and 3D). By E16.5, the development of the mutant brain was markedly altered, with an expanded midbrain protruding and displacing the telencephalic vesicles (Figures 3E and 3F). In contrast, despite the widespread expression of Casp9 during embryogenesis, the spinal cord and other nonneural organs such as heart and lung were largely normal without morphological alterations in the E16.5 mutants (Figures 3G and 3H). Thus, the absence of Casp9 results in brainspecific malformations.

Brain Malformations in Caspase 9-Deficient Mice Are Caused by Reduced Apoptosis

Apoptosis is a prominent feature during early development of the nervous system (Glucksmann, 1951; Oppenheim, 1991). If the loss of Casp9 prevents the death of early stem cells, this would conceivably lead to the observed enlargement of proliferative zones at a later stage of development. To test this possibility, we used two independent approaches to compare the extent of cell death during brain development between Casp9 homozygous and heterozygous mutants. In the first approach, we used serial toluidine blue-stained semithin sections to compare the incidence of pyknotic clusters in the E10.5 neuroepithelium (Figures 4A-4D). In addition to a greatly enlarged telencephalic neuroepithelial wall, Casp9-/- embryos were characteristically devoid of pyknotic clusters, which are typically observed during normal brain development (Kallen, 1955). For example, pyknotic clusters in the lamina terminalis of the forebrain, which signifies the process of morphogenetic cell death that eventually separates the two cerebral hemispheres, were eliminated in the mutant embryo, with the resulting lamina thicker and increased in the number and density of cells (Figures 4C and 4D). In the second approach, we used TUNEL staining to detect apoptotic cells in the embryonic brain. A nearly 10-fold reduction of the average number of TUNEL-positive cells per section was found in the E12.5 brain in Casp9 knockouts (Figure 4G). Together, these two sets of data indicated a reduction of cell death during early brain development in Casp9^{-/-} mice.

We also used TUNEL staining to compare the extent of cell death during development of the spinal cord, which revealed no apparent structural alterations in the $Casp9^{-/-}$ mutants. At E12.5, only a few TUNEL-positive cells were found in both $Casp9^{-/-}$ and heterozygous



Figure 3. Histological Analysis of Caspase 9 Heterozygous and Homozygous Fetuses

NissI-stained sections are from (+/-) (left) and (-/-) (right) fetuses at E13.5 (A–D), and E16.5 (E and F).

(A and B) Coronal brain sections at E13.5 illustrate the larger diencephalon, obliterated lateral and third ventricles, and the interrupted and invaginated telencephalic wall in knockout fetuses.

(C and D) At higher magnification, the telencephalic wall of the knockout still shows genesis of the superficial postmitotic telencephalic layers, the intermediate zone, and cortical plate.

(E and F) At E16.5 in a caudal brain section, the knockout brain has an increased number of proliferative cells (seen as dark staining), as well as protrusion of the midbrain on top of the brain. The (-/-) section (F) illustrates a proliferative region (star) in this ectopic midbrain.

(G and H) Horizontal sections through the body of the fetuses at the level of the pulmonary artery show that the size and structure of the spinal cord, lungs, and heart remain largely unaffected in the (-/-) fetuses. Bar, 1 mm.

littermate neural tubes (Figure 4G). Although the number of TUNEL-positive cells was reduced in the $Casp9^{-/-}$ spinal cord at E16.5, no obvious histological abnormalities were found (Figures 4E and 4F). Nevertheless, further studies are required to determine whether the reduction of the late onset cell death in the spinal cord may produce an increased number of neurons in $Casp9^{-/-}$ mutants.

Caspase 9 Is Required for Activation of Caspase 3 In Vivo

The brain-specific malformations caused by the reduction of developmental apoptosis in Casp9–deficient mice are strikingly similar to the phenotype of the Casp3 null mutation (Kuida et al., 1996). The similarity of phenotypes indicates that Casp9 and Casp3 may participate in the same biological pathway during brain development.



Furthermore, this is consistent with the scenario that Casp9 is an upstream activator of Casp3 (Li et al., 1997). To test this possibility, we used the recently characterized CM1 antibody, which only recognizes the cleaved 17 kDa subunit and not the 32 kDa proenzyme of Casp3 (A. Srinivasan et al., submitted), for in situ immunodetection of the cleavage and activation of Casp3 in embryonic brains. In E12.5 wild-type and heterozygous embryos, CM1-stained cells were found sporadically in the brain and the ectodermal surface (Figure 5A). Under high magnification, staining for activated Casp3 was present both in the cytoplasm and in the condensed nucleus (data not shown). In contrast, although CM1-stained cells were seen in the meningeal and ectodermal surfaces, no such staining was found in the brain of all Casp9-/- mutants, regardless of the severity of brain malformations (Figures 5B and 5C). These results therefore indicate that Casp9 is required for activation of Casp3 in the developing brain in vivo.

Caspase 9 Is Required for Apoptotic Responses to Defined Stimuli in Thymocytes

To investigate the role of Casp9 in nonneuronal tissues, we focused on the study of the development and apoptotic response of thymocytes. The development of the thymus in Casp9^{-/-} mice appeared to be largely normal, with an equal total number of cells and a comparable ratio of CD4- and CD8-positive thymocytes compared to wild-type mice (Figure 6A). Primary cultures of wildtype and Casp9-deficient thymocytes were then challenged with a variety of apoptotic stimuli and assayed for cell survival by FACS analysis after 6 and 24 hr of treatment (Figure 6B). At both 6 and 24 hr after stimulation, Casp9-1- thymocytes showed increased cell survival in response to application of anti-CD3 plus anti-CD28 antibody (which mimics the physiologic activation of T cell receptors), etoposide, y-radiation, and dexamethasone. In contrast, Casp9^{-/-} and wild-type thymocytes were equally susceptible to Fas-mediated apoptosis.

The preferential resistance to apoptotic stimuli correlated with delayed DNA fragmentation in $Casp9^{-/-}$ thymocytes (Figure 6C). Although DNA fragmentation induced by etoposide, dexamethasone, and γ -radiation was evident at 6 hr and persisted till 24 hr after treatment in wild-type thymocytes, such DNA laddering occurred in $Casp9^{-/-}$ thymocytes only after prolonged (24 hr) treatment with these reagents. In contrast, anti-Fas antibody was equally effective in inducing DNA fragmentation in thymocytes of both genotypes, presumably through a Casp9-independent pathway. Identical results were obtained when DNA cleavage was analyzed by TUNEL/FACS staining (data not shown). Together, these results indicate that Casp9 is selectively involved in the degradation of DNA during apoptosis of thymocytes in response to defined stimuli.

Caspase 9 Is Required for Caspase 3-like Cleavage Activity in Distinct Apoptotic Pathways of Thymoctes

Recent studies have indicated that Casp3 is a direct upstream activator of certain deoxyribonucleases responsible for DNA fragmentation during apoptosis (Liu et al., 1997; Enari et al., 1998). Therefore, the observed resistance to apoptosis and delayed DNA degradation in thymocytes may be caused by the compromised activation of Casp3 in the absence of Casp9. To test this possibility, we analyzed the survival of thymocytes and the cleavage of a DEVD-specific fluorescent substrate after a 6 hr treatment of increasing concentration of etoposide, dexamethasone, γ -radiation, and anti-Fas antibody (Figure 7). Although etoposide, dexamethasone, and γ -radiation all induced a steady dose-dependent increase of cell death and DEVD-specific cleavage in wild-type thymocytes, none of these reagents was capable of initiating an appreciable Casp3-like activity in Casp9^{-/-} thymocytes. In great contrast, anti-Fas antibody induced a linear increase of Casp3-like activity and a concomitant progression in cell death in both wild-type and $Casp9^{-/-}$ thymocytes. Together, these results show that Casp9 is required for Casp3 activation and apoptosis in response to many stimuli, but, notably, that Casp9 is not required for either process in response to Fas.

Caspase 9 Is Essential for Cytochrome c–Mediated Activation of Caspase 3

Our results indicate that Casp9 is an upstream activator of Casp3 in both the nervous system (Figure 5) and in thymocytes (Figure 6) in vivo. This observation is consistent with the recent discovery of the requirement of Casp9 for cytochrome c-mediated activation of Casp3 (Li et al., 1997). To further test the necessity of Casp9 for caspase activation cascades, we analyzed the cleavage of in vitro-transcribed and -translated human CASP3, using 100,000 g cytosolic supernatants (S-100) of the embryonic brain or thymocytes in the presence of dATP, cytochrome c, or both (Figure 8A). In accordance with a previous report using HeLa cell extracts, we confirmed that cytochrome c was indispensable for the cleavage of Casp3 using wild-type embryonic tissue lysates, although in our case the addition of dATP was not necessary for the reaction (Liu et al., 1996). The latter discrepancy is presumably due to a difference of

Figure 4. Decreased Apoptosis in the Embryonic Brain of Caspase 9-Deficient Mice

Controls are from heterozygous embryos (A, C, and E), and homozygous littermates (B, D, and E) are used for comparison.

⁽A and B) Toluidine blue-stained horizontal sections of E10.5 embryos reveal the enlarged neuroepithelium and ventricular stenosis in the mutant (B). The boxed regions in (A) and (B) are shown at higher magnification in (C) and (D), respectively.

⁽C and D) High-power magnification of the lamina terminalis illustrates pyknotic cells (arrowheads) that are found in heterozygous controls (C) but are absent in the knockout (D).

⁽E and F) TUNEL staining of E16.5 thoracic spinal cord indicates a reduced number of apoptotic cells in the mutant (F).

⁽G) Quantitation of TUNEL-positive cells per section, averaged from 3 littermate pairs each, in the E12.5 brain and E12.5 neural tube (NT) and E16.5 spinal cord (SC) in heterozygous controls (black bar) and $Casp9^{-/-}$ individuals (white bar). Asterisk, p < 0.05.



Figure 5. In Situ Immunodetection of Activated Caspase 3 in Embryonic Brains

E12.5 brain tissues from wild-type (A) and homozygous mutant littermates (B and C) were stained with the CM1 antibody specific for the p17 subunit of the activated Casp3.

(A) Positive CM1 staining was found both in the brain and ectodermal surface in the wild-type embryo.

(B and C) Positive CM1 staining was detected only in the ectodermal surface (arrowheads), but not in the $Casp9^{-/-}$ mutant embryos possessing either severe (B) or mild (C) brain malformations.

intracellular dATP concentration between HeLa cells and embryonic tissues. Nevertheless, lysates of $Casp9^{-/-}$ tissues were not capable of initiating the cleavage of Casp3, even in the presence of both dATP and cytochrome c (Figure 8A). Furthermore, the addition of in vitro-transcribed and -translated human CASP9 back into S-100 cytosolic extracts from mutant tissues restored Casp3 processing capability, indicating that all other required elements were present in $Casp9^{-/-}$ cells (Figure 8B). Together, these data establish that Casp9 is required for cytochrome c-mediated activation of Casp3.

Discussion

Although the caspase family of proteases are implicated as essential components of the mammalian cell death machinery, the large number of members of the caspase family, their overlapping tissue distribution, and similar cleavage specificities altogether present great challenges for distinguishing the function of individual caspases in vivo. There are two fundamental guestions regarding the relationship of the caspase family members. First, are caspases functionally redundant? Second, is any single caspase indispensable for the activation of other caspases? The fact that different lines of caspasedeficient mice (Caspases 1, 2, 3, and 11 knockouts) all exhibit preferential apoptosis defects rather than a global suppression of cell death indicates that individual members of the caspase family play a dominant and nonredundant role in apoptosis in a tissue-selective or stimulus-specific manner (Kuida et al., 1995, 1996; Li et al., 1995; Bergeron et al., 1998; Wang et al., 1998; Woo et al., 1998). The present study addresses the second question, regarding the activation cascade of caspases. Our results demonstrate a linear and essential activation cascade from Casp9 to Casp3 in vivo. In addition, it reveals the importance of this caspase cascade in brain development, in apoptosis of thymocytes, and in the cytochrome c-mediated caspase activation.

Caspase 9 to Caspase 3 Cascade

The first indication that Casp9 and Casp3 act along the same biological pathway was the similar phenotypes of mutant mice lacking either of these two caspases. Both Casp9 and Casp3 knockout mice have prominent brain malformations caused by a reduction of apoptosis in proliferative neuroepithelium (Figures 2-4; Kuida et al., 1996). Moreover, similar defects of DNA fragmentation were found in both Casp9- and Casp3-deficient mice (Figure 6C; Woo et al., 1998). One intriguing possibility to account for the similar phenotypes is a linear and obligate proteolytic cascade, since the optimal cleavage motif of Casp9 matches the activation sites of Casp3 (Thornberry et al., 1997). To test this hypothesis, we employed in situ immunodetection of processed Casp3 subunits in embryonic mutant brain (Figure 5) and measurement of Casp3-like activity in thymocytes, using the fluorescent DEVD-specific substrate (Figure 7). Defective activation of Casp3 in vivo was revealed by both approaches. Together, these results establish a linear and essential activation cascade from Casp9 to Casp3 in vivo.

Role of Caspase 9 in Brain Development

Our results indicate that Casp9 may regulate the number of neuroepithelial progenitor cells during brain development. The entire population of cells in the vertebrate telencephalon are derived from neuroepithelial progenitors located in the proliferative ventricular zones. During development, the progeny of these progenitors exit the cell cycle, become postmitotic neurons, and migrate



Figure 6. Reduced Apoptotic Responses to Defined Stimuli in Caspase 9-/- Thymocytes

(A) Flow cytometric analysis of thymocytes in wild-type (WT) and $Casp9^{-/-}$ (KO) mice. One million cells were stained with anti-CD4 and -CD8 antibodies and analyzed by FACS. The percentage of each fraction in a total of five animals was quantified. DN (double negative), CD4⁻CD8⁻; DP (double positive), CD4⁺CD8⁺.

(B) Cell survival analyzed by FACS of FITC-labeled Annexin V and 7-ADD stained thymocytes from wild-type (black bar) and $Casp9^{-/-}$ (white bar) mice after 6 hr or 24 hr of treatment with various reagents in 10% FCS medium. MED, medium alone; CD3/28, 20 µg/ml each of anti-CD3 and anti-CD28 antibody; ETO, 100 µM etoposide; RAD, 1000 rad γ -radiation; DEX, 2 µM dexamethasone; FAS, 1 µg/ml anti-Fas antibody plus 30 µg/ml cycloheximide. $Casp9^{-/-}$ thymocytes are significantly resistant to the challenge of anti-CD28 antibody, dexamethasone, etoposide, and γ -radiation, but equally susceptible compared to controls to Fas-induced apoptosis.

(C) DNA fragmentation in wild-type (WT) and $Casp9^{-/-}$ (KO) thymocytes at 6 hr and 24 hr of the same treatment as in (B). $Casp9^{-/-}$ thymocytes show delayed DNA laddering after application of anti-CD3 plus anti-CD28 antibody, etoposide, dexamethasone, and γ -radiation, but not to application of Fas-antibody.

away from the ventricular zones to form distinct structures of the brain (Rakic, 1988). The size of the progenitor pool in the neuroepithelium, which determines the size of these structures, may be modulated by several mechanisms, including the cell cycle kinetics of stem cells, the rate of exit of the cell cycle (asymmetric division), and programmed cell death of progenitor cells (Caviness et al., 1995; Rakic, 1995; Huttner and Brand, 1997). Classical histological studies have documented the existence of pyknotic clusters within neuroepithelium during early embryogenesis (Glucksmann, 1951; Kallen, 1955), and a reduction of these pyknotic cells is a hallmark of Casp3 null mutation phenotypes (Kuida et al., 1996). Similarly, we observed in the Casp9-deficient embryos





Figure 7. Requirement of Caspase 9 for Caspase 3–like Cleavage Activity in Defined Apoptotic Pathways in Thymocytes Wild-type (filled squares) and $Casp9^{-/-}$ (open circles) thymocytes were treated with various concentration of etoposide (A and E), dexamethasone (B and F), γ -radiation (C and G), and anti-Fas antibody (D and H) for 6 hrs and assayed for cell survival (A–D) and Casp3–like cleavage activity (E–H). Cell survival was assayed by FACS analysis of FITC-labeled Annexin V and 7-ADD stained thymocytes. Casp3–like enzymatic activity was measured as relative fluorescent units (RFU) based on the cleavage of the fluorogenic substrate DEVD-AMC. *Casp9*^{-/-} thymocytes exhibit increased cell survival and concomitant absence of Casp3–like cleavage activity in response to etoposide, dexamethasone, and γ -radiation. but not to Fas-induced apoptosis.

a reduced cell death of neuroepithelial progenitors, indicated by histological analysis of pyknosis and TUNEL staining of apoptotic cells (Figure 4). Therefore, as a consequence of the failure of apoptosis, an abnormal expansion of the proliferative pool may give rise to various malformations in Casp9-deficient animals, such as protrusion of brain mass, stenosis of ventricules, heterotopias, invagination, and interruption of the telencephalic wall (Figure 3).

Although gene disruption of either Casp9 or Casp3 causes brain malformations, the abnormality was far more severe in mice lacking Casp9. Thus, Casp3-deficient fetuses rarely exhibit an overt exencephaly abnormality (Kuida et al., 1996, and unpublished observations), whereas Casp9^{-/-} fetuses regularly show prominent brain malformations. One possibility to account for the difference in the severity of the malformation is that Casp9 may also activate other effector caspases, such as Casp7, in addition to Casp3, in the nervous system. Finally, while the majority of Casp9-deficient animals die prenatally with severe brain malformations, a small subset of mutant mice survive to the postnatal stage without appreciable histological abnormalities, which indicates the existence of substitutive pathways for apoptosis during brain development. The variability of phenotypes, which was also reported in mice with targeted disruption of the p53 or Casp3 gene, may reflect the complexity of the biological mechanisms controlling apoptosis (Sah et al., 1995; Kuida et al., 1996).

Role of Caspase 9 in Thymocytes

Our results suggest that, while Casp9 is not essential for thymic selection, it plays a critical role in apoptosis,

caused by a variety of stimuli in thymocytes. Although the activation of caspases has been implicated to be an important mechanism during thymic selection (Clayton et al., 1997), the fact that the total number and composition of thymocytes is normal in the mutant mice makes it unlikely that Casp9 plays an indispensable role in the negative selection (Figure 6A). In contrast, Casp9deficient thymocytes exhibit an increased resistance to dexamethasone, etoposide, and γ -radiation; no appreciable apoptosis of mutant cells was noticed at 6 hr after treatment, and a significant number of Casp9-/cells were still alive at 24 hr, whereas wild-type thymocytes were all dead by 24 hr. In addition, the resistance to apoptosis is accompanied by an attenuation of DNA fragmentation. This suggests that Casp9, Casp3, and caspase-activated DNA fragmentation factors (ICAD or DFF45) may all be in a linear, nonredundant pathway during acute apoptosis responses (Liu et al., 1997; Enari et al., 1998). Furthermore, since apoptosis and DNA fragmentation are nevertheless found in Casp9^{-/-} thymocytes after prolonged (24 hr) treatment, it is likely that the Casp9-mediated Casp3 and DNases activation pathway may serve as an amplification mechanism to accelerate the apoptotic response.

Interestingly, the protective effect of Casp9 deficiency does not extend to Fas-mediated apoptosis of thymocytes (Figure 6B). It is known that the binding of Fas-Ligand to Fas induces trimerization of the Fas receptor, which then recruits Casp8 through an FADD/MORT1 adaptor (Nagata, 1997). Casp8 and Casp9 both belong to the same subgroup of caspases that may activate effector caspases such as Caspases 2, 3, and 7 (Nicholson and Thornberry, 1997). Therefore, the observed



Figure 8. Absence and Reconstitution of Cytochrome c-Mediated Cleavage of Caspase 3 in Caspase 9-Deficient Cells

(A) Cleavage of ³⁵S-labeled Pro-Casp3 using S-100 cytosolic fractions from E15.5 wild-type and *Casp9^{-/-}* embryonic brains or thymocytes. Addition of dATP (1 mM) and/or cytochrome c (0.2 μ g) is indicated. Cytosolic fractions from *Casp9^{-/-}* cells do not cleave Pro-Casp3 while the wild-type cell fractions are capable of processing Pro-Casp3 (arrowheads).

(B) Reconstitution of in vitro-transcribed and -translated human *CASP9* restores the Pro-Casp3 processing activity in cytosolic extracts of E15.5 *Casp9^{-/-}* brains. The same amounts of dATP and/ or cytochrome c as in (A) were used.

preferential resistance to dexamethasone versus Fasinduced apoptosis implicates a functional diversification of caspase cascades, depending on the external stimulus.

Role of Caspase 9 in Cytochrome c-Mediated Apoptosis

Recent studies indicate that mitochondrial cytochrome c is translocated into the cytosol in cells undergoing apoptosis and activates DEVD-specific caspases (Kluck et al., 1997; Yang et al., 1997; Bossy-Wetzel et al., 1998). In an in vitro biochemical assay using human HeLa cell lysates, it was shown that cytochrome c first binds to the cytosolic protein Apaf1, the human Ced-4 homolog, in the presence of dATP (Liu et al., 1996; Zou et al., 1997). Apaf1 then interacts with Casp9 through the shared CARD domain, and in turn recruits and activates Casp3 through proteolysis (Li et al., 1997). In the present study, we have extended the analysis of cytochrome c-mediated cleavage of Casp3 using embryonic tissue lysates (Figure 8). The absence of Casp3 cleavage in Casp9^{-/-} embryonic lysates, and the correction of the functional defect by reconstitution with the deleted gene, indicates that Casp9 is indeed an essential component for cytochrome c-mediated caspase cascade in vivo. In addition, we found that the cleavage of other effector caspases in the cytochrome c-mediated reactions is also dependent on Casp9 (K. K. et al., unpublished data). Together, these results indicate that Casp9 is a crucial upstream activator of the cytochrome c-mediated apoptotic cascade. These results suggest, therefore, that a deficiency in cytochrome c-mediated and Casp9-dependent apoptosis is responsible for the in vivo phenotypes we report. It is, however, possible that Casp9 can also be activated by other presently unidentified upstream activators.

Release of cytochrome c from the mitochondria has been shown to be an almost universal phenomenon during apoptosis, while it is unclear whether the cytochrome c-mediated caspase cascade is triggered only by a few apoptotic stimuli or serves as a general amplification mechanism to accelerate cell death (Reed, 1997). A growing body of evidence has indicated that caspases are activated not only during developmental programmed cell death but also in a variety of human diseases (Thompson, 1995). If cytochrome c-mediated caspase cascade is involved in the pathogenesis process leading to cell death, Casp9, as the crucial upstream activator of the caspase cascade, should be an appropriate target for future therapeutic intervention.

Experimental Procedures

Generation of Caspase 9-Deficient Mice

Human CASP9 cDNA was amplified by PCR from Jurkat cells using CASP9-specific primers (5'-ATG GAC GAA GCG GAT CGG CGG C-3' and 5'-TTA TGA TGT TTT AAA GAA AAG-3'). 129SV/J genomic library (Stratagene) was screened with the cDNA to obtain a mouse Casp9 genomic clone. An 8 kb HindIII-NotI fragment containing a portion encoding the pentapeptide motif conserved among Caspase family members was subcloned into pBlueScript (Stratagene). The resulting plasmid was digested with Xbal and blunted. EcoRI-HindIII fragment of the neo gene cassette (Nakavama et al., 1993) was also blunted and ligated to the plasmid containing the mouse Casp9 genome. The plasmid was then digested with Xhol and Notl and subcloned into the tk cassette vector (Nakavama et al., 1993). The construct was linearized by Notl and transfected into W9.5 ES cells (a gift from Dr. S. J. Abbondanzo, Roche Research Center), Clones resistant to G418 and gancyclovir were selected, and homologous recombination was confirmed by Southern blotting. One out of 32 clones screened was positive for homologous recombination, and

single integration of the construct was verified by hybridization with the *neo*-specific probe. Chimeric mice were generated by injection of this clone into C57BL/6 blastocysts. The resulting male chimera mice were bred to C57BL/6 females to obtain heterozygous mice. Interbreeding of the heterozygous mice was performed to generate Casp9-deficient mice.

RT-PCR Analysis of Caspase 9 mRNA

Total RNA was isolated from the kidney by Trizol reagent (GIBCO-BRL). Five micrograms of total RNA was reverse-transcribed using the superscript preamplification system (GIBCO-BRL), and the resulting templates were subjected to a PCR reaction with *Casp9*specific primers (5'-GCC ATG GAC GAA GAG GAT CGG CGG-3' and 5'-GGC CTG GAT GAA GAA GAG CTT GGG-3') or primers specific for γ -actin.

Northern Blot Analysis

Poly(A)⁺ RNA blots (Clontech) were hybridized overnight with random primed human *CASP9* cDNA, hamster *Casp3* cDNA (Wang et al., 1996), or human *APAF1* cDNA in Express-hyb buffer (Clontech) at 37°C. The *APAF1* cDNA was amplified from HeLa Marathon-Ready cDNA (Clontech) by PCR using *APAF1*-specific primers (5'-GGG AAG ATG GAT GCA AAA GCT CGA-3' and 5'-CTG GCT GCA ATT CTT CTC TGT AAG-3'). The blots were washed with $2 \times SSC/0.1\%$ SDS for 1 hr at room temperature, followed by $0.2 \times SSC/0.1\%$ SDS for 1 hr at 50°C. The blots were then analyzed with a Fuji BAS-1500 bio-image analyzer.

Histology and Immunocytochemistry

Fetuses fixed in 4% paraformaldehyde were embedded in paraffin and cut in 10 μ m increments. Coronal brain or horizontal body sections were stained with 0.1% cresyl violet. For semithin sections, embryos were fixed in 4% paraformaldehyde and 1.5% glutaraldehyde, embedded in plastic, and serial 1 μ m sections were stained with 1% toluidine blue. For immunocytochemistry, E12.5 day embryos were fixed in Bouin's solution, cryoprotected in 30% sucrose, and then sectioned horizontally. The polyclonal CM-1 antibody specific for the p17 subunit of cleaved Casp3 was generously provided by Dr. Anu Srinivasan (IDUN Pharmaceuticals). Immunopositive cells were revealed by Cy2-conjugated anti-rabbit IgG secondary antibody (Jackson Immunoresearch).

TUNEL Staining

For TUNEL stains to detect DNA fragmentation, 4% paraformaldehyde-fixed E12 heads were cryoprotected in 30% sucrose, frozen, and sectioned horizontally. Frozen 20 μ m horizontal brain sections were incubated in 0.26 U/ml TdT and 1× of supplied buffer (Life Technologies), and 20 μ M biotinylated-16-dUTP (Boehringer Mannheim) for 60 min at 37°C. Sections were then washed three times in PBS (pH 7.4) and blocked for 30 min with 2% BSA in PBS (pH 7.4). The sections were then incubated with FITC-coupled streptavidin (Jackson Immunoresearch) 1:100 in PBS for 30 min, rinsed, and then counterstained with the nuclear stain propidium iodide (1 μ g/ml, Sigma).

Apoptosis and Caspase 3 Cleavage Assay Using Thymocytes

Thymocytes were isolated from wild-type and Casp9^{-/-} mice. One million cells were treated with dexamethasone (Sigma), etoposide (Clontech), anti-Fas antibody (PharMingen) plus 30 µg/ml cycloheximide (Sigma), anti-CD3 plus anti-CD28 antibody (20 µg/ml, PharMingen), γ -radiation (Gammacell), or 10% FCS medium only, for 6 hr or 24 hr. Samples were stained with Annexin V (Boehringer Mannheim) and 7-aminoactinomycin D (7-AAD) (Sigma) to label apoptotic cells for FACS Sort (Becton Dickinson). Thymocytes were also stained with antibody against CD4 and CD8 (PharMingen) and analyzed by FACS analysis. Casp3 activity was measured using ApoAlert CPP32/Casp3 fluorescent assay kit (Clontech). Cleavage of DEVD-AFC was measured by a fluorescent plate reader.

DNA Fragmentation Assay

DNA was isolated using a DNA isolation kit (LeMax Biotech), followed by RNase A treatment. Samples were subjected to electrophoresis on a 2% agarose gel. Gels were stained by ethidium bromide and images were taken by a scanner.

Assay for Cleavage of Caspase 3

Full-length human *CASP3* cDNA was transcribed and translated using TNT quick-coupled transcription/translation system (Promega) and ³³S-methionine (Amersham). To generate the recombinant Casp9, full-length *Casp9* cDNA was translated using the same system. S-100 cytosolic fractions were obtained as described by Liu et al. (1996). An aliquot of in vitro-translated Casp3 was incubated with 20 μ g of S-100 cytosolic fraction in the presence of 1 mM of additional MgCl₂ with or without cytochrome c or dATP at 30°C for 1 hr in a final volume of 20 μ l. For reconstitution of Casp9 activity, 5 μ l of the translated reaction or reticulocyte lysate was added to the reaction. At the end of incubation, 7 μ l of 4× SDS sample buffer was added to each reaction and samples electrophoresed on a 16% SDS-PAGE gel. Gels were dried and exposed to a phosphoimaging plate and analyzed by Fuji BAS-1500 bio-image analyzer.

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