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REVIEW

The Role of Apaf-1 in Programmed Cell Death: From Worm to Tumor

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ABSTRACT. **Apoptosis or programmed cell death is an important process to eliminate unnecessary or hazardous cells. Apaf-1, a mammalian homologue of CED-4 of *C. elegans,* is the essential adaptor molecule in the mitochondrial pathway of apoptosis. Mice lacking Apaf-1 show accumulation of neurons in the developing central nervous system due to reduced apoptosis. Apaf-1-deficient cells are remarkably resistant to various apoptotic stimuli. Apaf-1-mediated apoptosis plays a role in the prevention of tumorigenesis. However, Apaf-1-independent cell death pathways are also indicated. In this review, we will summarize what has been learned about the role of Apaf-1 by biochemical and genetical approaches.**

Key words: Apaf-1/apoptosis/mitochondria/caspase/cytochrome *c*

In the year 2002, three scientists were awarded the Nobel Prize in Physiology or Medicine for their discoveries concerning the genetic regulation of organ development and programmed cell death. Robert Horvitz, one of the three laureates, characterized the key genes controlling cell death by using the roundworm *Caenorhabditis elegans* as an experimental system. He also showed how these genes interacted with each other during the cell death process and that corresponding genes existed in higher species. These discoveries are of significance not only for the understanding of the cell death mechanism on a molecular basis but also for the medical research on the pathogenesis of various diseases.

The body of any multicellular organisms consists of different cell types. In human body, for instance, there are about 10^{14} cells with hundreds of cell types, all of which originated from the single fertilized egg. Thus, during the embryonic and fetal development, large numbers of cells are generated by cell division. Even in an adult human, numerous cells are created every day. In parallel with this process of cell increase, large numbers of cells die through a controlled "suicide process" referred to as programmed cell death (PCD). First observed during amphibian metamorphosis, PCD has been discovered to occur in many developing tissues (for review, see Clarke and Clarke, 1996) and studies have revealed how important PCD is during animal development and in the maintenance of homeostasis. PCD serves many functions including sculpting structures to form the proper shapes of organs by removal of cells, deleting unneeded structures, and adjusting the number of cells that are overproduced. Elimination of dangerous or injured cells, such as virus-infected cells or cells with gene mutation, is also an important function of PCD (reviewed in Jacobson *et al.*, 1997). As one can well imagine, failure of PCD may cause various problems and diseases due to the accumulation of unnecessary and/or harmful cells.

Spectacular progress has been made in past years in understanding the molecular mechanisms of PCD. The role of the mitochondria, which generate cellular energy as a form of ATP by oxidative phosphorylation, in apoptosis came into focus when studies revealed that several mitochondrial proteins, which reside in the intermembrane space of mitochondria, are released and activate the cell death program at the early phase of apoptosis (see the detailed review by Wang, 2001). Amongst these mitochondrial proteins, cytochrome *c* (Cyto *c*), a component of the mitochondrial electron transfer chain, is translocated into the cytoplasm in response to a variety of apoptotic stimuli (Kluck *et al.*, 1997; Yang *et al.*, 1997; Bossy-Wetzel *et al.*, 1998). By reconstitution experiments with cell free extracts, Cyto *c* has been shown to trigger the activation of a group of

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Abbreviations: PCD, programmed cell death; Cyto *c*, cytochrome *c*; CARD, caspase-recruitment domain; ES cells, embryonic stem cells; EF, embryonic fibroblasts; PNS, peripheral nerve systems.

proteases (designated caspases), whose activities are responsible for the morphological changes characteristic of apoptosis (Liu *et al.*, 1996; Kluck *et al.*, 1997). Wang and coworkers in their monumental report then identified Apaf-1, which activates caspases in the presence of Cyto *c*, as the key adaptor molecule in the mitochondrial pathway of apoptosis (Zou *et al.*, 1997). Since the discovery of Apaf-1, biochemical analyses as well as those using genetically modified Apaf-1-deficient (*Apaf-1* knockout) mice (Cecconi *et al.*, 1998; Yoshida *et al.*, 1998) have revealed the critical roles of Apaf-1 in the apoptosis of cells. This review summarizes the recent data on the molecular roles of Apaf-1 in the mitochondrial pathways of apoptosis and physiological roles of Apaf-1-mediated apoptotic program in the development of body and its homeostatic maintenance.

Discovery of Apaf1, the "missing link"

Detailed studies by the Nobel Laureates revealed that specific genes control the cellular death program in the nematode *C. elegans*. They demonstrated that 131 of the total of 1090 cells die reproducibly during development. In a series of sophisticated experiments by Horvitz and colleagues, the first two death genes, *ced-3* and *ced-4*, were identified as prerequisite for the programmed cell death of the worm (Ellis and Horvitz, 1986). Later, another gene, *ced-9*, was cloned as a protector against cell death induced by *ced-3* and *ced-4* (Hengartner *et al.*, 1992). Genetically, *ced-9* exerts its anti-apoptotic effect upstream of *ced-4* and *ced-3*, the latter of which works downstream of the former. Strikingly, it was revealed that *ced-9* is a functional homologue of human proto-oncogene, *bcl-2* (Hengartner *et al.*, 1992). Also, *ced-3* encodes a protein similar to mammalian interleukin-1 beta-converting enzyme (ICE) (Yuan *et al.*, 1993) and is more homologous to *cpp32* (caspase 3) (Xue *et al.*, 1996), a member of a group of cysteine proteases called "caspases" that enzymatically degrade cellular proteins. The identification of the mammalian counterpart of *ced-4* had to wait until the discovery of the apoptotic protease-activating factor 1 (Apaf-1) (Zou *et al.*, 1997) (Fig. 1A).

Apaf-1 was identified from HeLa cell extracts as a Cyto *c*-dependent activator of caspase (Casp) 3. As shown in Fig. 1B, structurally, the NH2-terminal 85 amino acids of Apaf-1 show 21% identity and 53% similarity to the NH2-terminal prodomain of CED-3, that is called caspase-recruitment domain (CARD). The midportion of Apaf-1 has 22% identity and 48% homology to CED-4 and has a nucleotide-binding domain that dATP/ATP binds to. In addition, the COOH-terminal portion of Apaf1 has multiple WD-40 repeats to which Cyto *c* binds. Soon after the discovery of Apaf-1, Wang's group reported that Apaf-1, in the presence of dATP/ATP and Cyto *c*, oligomerizes to form "apoptosomes", which recruit pro-Casp 9 (the inactive form of caspase 9) via the CARD domains resulting in the activation of Casp 9. The activated Casp 9 in turn proteolytically activates downstream caspases including Casp 3 (Fig. 1C) (Li *et al.*, 1997; Zou *et al.*, 1999).

By the discovery of the "missing link" between *bcl-2* and caspases in mammals, all the big pieces of the jigsaw puzzle called "Mitochondrial Pathway of Apoptosis" appeared to be put together. The accumulated results are summarized in Fig. 1C. Once various apoptotic stimuli insult mitochondria, proteins including Cyto *c* are released from the intermembrane space into the cytoplasm. Although the precise mechanism(s) of the release is still controversial (Vander Heiden *et al.*, 1997; Hirsch *et al.*, 1998; Shimizu *et al.*, 1999; Vander Heiden and Thompson, 1999; Shimizu *et al.*, 2001), the involvement of anti- and pro-apoptotic members of the bcl-2 family is beyond doubt. Released Cyto *c* binds to Apaf-1 at the WD-40 repeats and provide for stable binding of dATP/ATP at the nucleotide binding site (Jiang and Wang, 2000), resulting in the conformational change of Apaf-1 followed by its oligomerization (Hu *et al.*, 1998; Srinivasula *et al.*, 1998; Hu *et al.*, 1999; Saleh *et al.*, 1999; Zou *et al*., 1999). The "apoptosome" activates the upstream caspase, Casp 9, and then downstream caspases to degrade cellular substrate proteins, including nuclear lamin, PARP, and DFF40 (Cryns and Yuan, 1998). Irrespective of various differences, the overall framework of cell death is evolutionally conserved from nematode to *Drosophila* (Kanuka *et al.*, 1999; Varkey *et al.*, 1999; Colussi *et al.*, 2000; Kumar and Doumanis, 2000; Zhang *et al.*, 2000) and to mammals.

Apaf-1 knockout mice

One year after the discovery of Apaf-1, two groups including ours independently reported the generation of *Apaf-1* knockout mice (Cecconi *et al.*, 1998; Yoshida *et al.*, 1998). Reflecting the fact that *Apaf-1* is most strongly expressed in the central nervous system (CNS) at the early phase of fetal development (Yoshida *et al.*, 1998), there was virtually no apoptosis detected in the developing CNS and, as a result, huge numbers of neurons accumulated. These accumulated neurons formed protruding masses in the brain of the fetus, and exencephaly, cranioschesis, and spina bifida were observed as a result. Proper formation of lens and retina was also affected. During the development of fetal brain, the cell number increases exponentially (Takahashi *et al.*, 1995a and b). However, those newly produced cells undergo PCD unless they are properly stimulated by survival signals delivered by their target cells (Blaschke *et al.*, 1996; Blaschke *et al.*, 1998). Thus, most of the produced neurons die by apoptosis when they connect to the wrong target cells, and in this process of cell elimination, the Apaf-1-mediated pathway of apoptosis plays critical role for controlled adjustment of the number and proper function of neurons. An alternative, but not mutually exclusive mechanism of cell elimination is suggested by the possible presence of the short form of Apaf-1 that lack WD-40 repeats (Kanuka *et al.*, 1999). The short form of Apaf-1

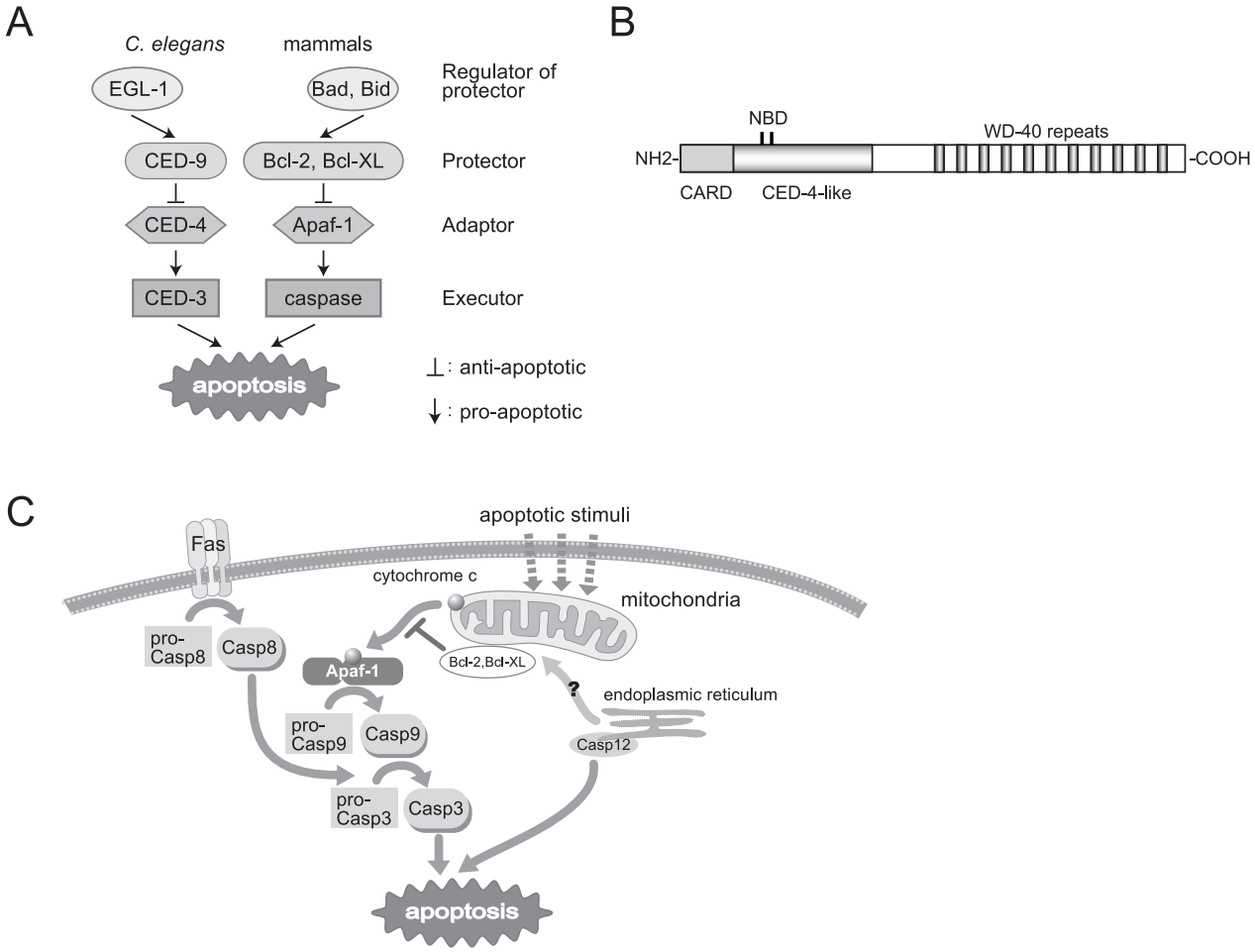

Fig. 1. Apaf-1-dependent apoptotic pathway. (A) Schematic illustration of the apoptotic pathways in *C. elegans* and mammals. (B) Structure of Apaf-1. Amino terminal CARD domain, CED-4-like domain, and WD-40 repeats are shown. NBD, nucleotide-binding domain. (C) Mitochondrial pathways of apoptosis, showing release of cytochrome *c* in response to apoptotic stimuli and the subsequent activation of caspase cascade. Two other apoptotic pathways, Fas-mediated and endoplasmic reticulum (ER) stress-mediated ones, are also shown.

(designated as Apaf-1S), a splice variant of Apaf-1, does not require Cyto *c* for activation of Casp 9 (Adrain *et al.*, 1999; Kanuka *et al.*, 1999). The expression of Apaf-1S, therefore, may eliminate neurons in a stochastic manner. Interestingly, a recent publication has revealed that the Forebrain Outgrowth (*fog*) phenotype in mice, such as forebrain defects, facial defects, spina bifida, and hydrocephaly, is attributed to lower levels of *Apaf1* expression (Honarpour *et al.*, 2001).

Results from Cyto *c* and caspase knockout experiments verified the linearity of the cytochrome-*c*-Apaf-1-caspase 9-caspase 3 pathway that had been biochemically demonstrated. The Apaf-1 protein remained in the monomeric state in Cyto *c*-deficient cells even after various apoptotic stimuli (Li *et al.*, 2000). Casp 9- or Casp 3-deficient mice showed remarkably similar deformity of the fetal brains to Apaf-1-deficient mice due to the accumulation of neurons (Kuida *et al.*, 1996; Hakem *et al.*, 1998; Kuida *et al.*, 1998; Woo *et al.*, 1998). In Apaf-1- or Casp 9-deficient cells, no Casp 3 activation was detected in the presence of apoptotic stimuli even though Cyto *c* was released into the cytosol (Hakem *et al.*, 1998; Yoshida *et al.*, 1998).

Embryonic stem (ES) cells and embryonic fibroblasts (EF) from Apaf-1-deficient mice showed remarkable resistance to various apoptotic stimuli including UV, γ-irradiation, and chemotherapeutic drugs such as VP-16. Apaf-1-deficient thymocytes, either from neonatal Apaf-1 knockout mice or thymocytes reconstituted in *RAG1*$^{-/-}$ mice, also showed more resistance than wild-type thymocytes to treatment with γ-irradiation, UV, and dexamethasone (Yoshida *et al.*, 1998). Thus, Apaf-1-mediated pathway is critically required for apoptosis in response to these stimuli.

Role of Apaf-1-mediated apoptosis in the development of tumors

Elimination of hazardous cells, such as virally infected cells or cells with gene mutation, is an important function of apoptosis. The ability of p53 to induce either cell cycle arrest or apoptosis is critical for its tumor suppressive function. Mouse EF cells, when transformed with *c-Myc*, a mitogenic oncogene, are susceptible to some apoptotic stimuli including serum withdrawal or anoikis (suspension culture). *p53* null, *c-Myc*-expressing EF cells are remarkably resistant to these apoptotic stimuli, indicating the apoptosis induced in these situations is *p53*-dependent. Apaf-1-deficient, *c-Myc*-expressing EF cells are similarly resistant to these p53-dependent apoptosis, implicating the interrelationship between p53 and Apaf-1 (Soengas *et al.*, 1999). Introduction of temperature-sensitive (ts) form of p53 into *c-Myc*-expressing Apaf-1 null EF cells did not restore responsiveness to p53-dependent apoptosis at permissive temperature, demonstrating that Apaf-1 acts downstream of p53 to induce apoptosis. Strikingly, these transformed Apaf-1 null EF cells were as tumorigenic as p53 null EF cells, forming colonies in nude mice, as do p53 null EF cells. Thus, Apaf-1 is an essential downstream molecule of p53 to induce apoptosis, functioning as a tumor suppressor. Lines of evidence revealed that p53 is activated in response to various stresses, such as DNA damage, and induces the expression of various genes including BAX, p53AIP1, and PIGs (Miyashita and Reed, 1995; Polyak *et al.*, 1997; Oda *et al.*, 2000). Proteins encoded by these p53 target genes affect mitochondria to release Cyto *c* and initiate the Apaf-1-dependent apoptotic pathway (Fig. 2). Interestingly, Soengas *et al*. reported some cases of drug resistant malignant melanomas in which the expression of *Apaf-1* was impaired (Soengas *et al.*, 2001). In these cells, anti-cancer drug treatment induced the release of Cyto *c*, but failed to activate Casp 9, reiterating the tumor suppressive roles of *Apaf-1* in tumor development. Others have also reported the involvement of Apaf-1 in tumorigenicity (Yamamoto *et al.*, 2000; Jia *et al.*, 2001; Wolf *et al.*, 2001; Liu *et al.*, 2002).

Role of Apaf-1 in the development of lymphocytes

Failure to delete possibly self-reactive lymphocyte may lead to autoimmune diseases. Impairment of Fas and Fas ligand system, either by gene mutation or gene targeting, results in the accumulation of lymphocytes in the lymph nodes and spleen, and causes systemic autoimmune diseases in mice and in human (for review, see Nagata and Golstein,

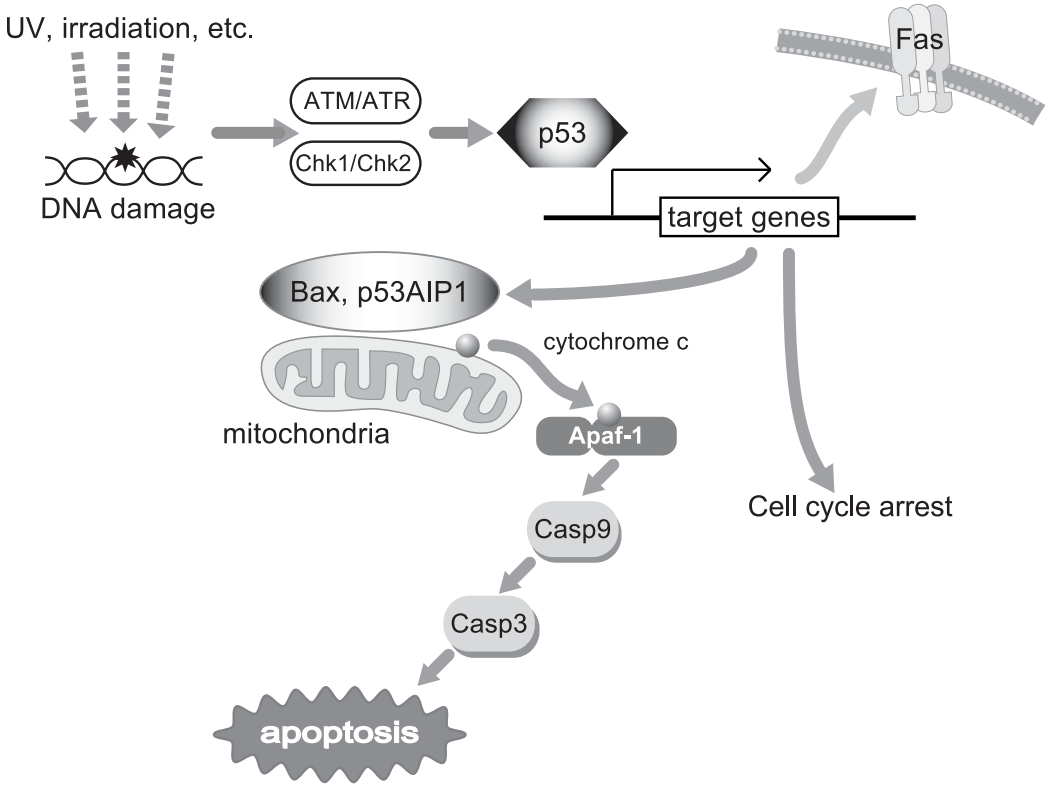

Fig. 2. p53-mediated activation of Apaf-1-dependent apoptotic pathway. DNA damage-induced activation of p53, transcriptional activation of target genes, and release of cytochrome *c* by BAX, p53AIP1, etc. are shown.

1995). Apaf-1-deficiency, however, does not seem to cause such accumulation of autoreactive lymphocytes. No evidence of lymphocyte accumulation was observed in Apaf-1-deficient mice or in *RAG-1*$^{-/-}$ mice reconstituted with Apaf-1-deficient lymphocytes (Yoshida *et al*., 1998). Despite the remarkable resistance to various apoptotic stimuli of Apaf-1-deficient thymocytes, Apaf-1-dependent apoptosis is not required for the negative selection of thymocytes expressing T cell receptors reactive to self-antigens (Hara *et al*., 2002). As stimulation of thymocytes with relevant antigenic peptide induces the release of Cyto *c* and also as Apaf-1-deficient thymocytes showed partial resistance to antigenic stimulation-induced cell death *in vitro*, partial involvement of Apaf-1-mediated apoptosis in negative selection is suggested (Hara *et al*., 2002). This leads us to speculate that there are various mechanisms involved in the process of negative selection, presumably acting in a synergistic and compensatory fashion with each other, to avoid failure of the deletion of self-reactive thymocytes.

Cell death in Apaf-1-deficient cells

Despite the striking brain deformity and the fact that most of Apaf-1-deficient mice died *in utero* (Yoshida *et al*., 1998), of pertinent interest is the fact that most organs in Apaf-1-deficient mice developed normally. There were no obvious macro-anatomical deformities except for the excess of neurons in the brain. Some Apaf-1 null mice (with mild brain phenotype) survived to adulthood without apparent defects other than male sterility (Yoshida *et al*., 1998; Honarpour *et al.*, 2000). These results contrast studies in *C. elegans* where loss of *ced-4* blocked all developmental cell death in the worm (Ellis and Horvitz, 1986). One possible explanation for this discrepancy is that redundant pathways compensate for the loss of Apaf-1 in mammals. However, scrutiny of human genome revealed that *Apaf-1* is the only gene that shows extensive homology to *ced-4* (Aravind *et al.*, 2001). Thus there appear to be other cell death mechanisms that kill cells in an Apaf-1-independent fashion.

Given the striking resistance of Apaf-1-deficient cells to apoptotic stimuli, one would assume that Apaf-1-deficient cells are immortal, but studies using Apaf-1-deficient EF or ES cells revealed that this was not the case. After stimulation that induces apoptosis in wild-type cells, Apaf-1-deficient cells eventually died, albeit at a slower rate. There was virtually no caspase activity detected, and of note, the dying cells showed necrosis-like morphological features, such as swelling of nuclei as well as that of mitochondria. Dysfunction of mitochondria, as revealed by dissipation of mitochondrial transmembrane potential ($\Delta\psi m$) was observed in the dying cells (Haraguchi *et al.*, 2000; Miyazaki *et al.*, 2001). Haraguchi and coworkers demonstrated that overexpression of *bcl-2* suppressed the necrosis-like death in the Apaf-1-deficient cells (Haraguchi *et al*., 2000). It is assumed that mitochondrial dysfunction induced by the apoptotic stimuli, which may follow or coincide with the release of Cyto *c*, eventually leads to cell death that resembles necrosis, at least morphologically. Release of other pro-apoptotic proteins from mitochondria, such as apoptosis inducing factor (AIF) (Susin *et al.*, 1999) and endonuclease G (Li *et al.*, 2001) may also induce Apaf-1-independent cell death. Actually, Apaf-1-independent, but mitochondria-dependent apoptosis has also been implicated (Marsden *et al*., 2002). Consistent with these observations, loss of interdigital cells in the mouse embryo, a process partly dependent on the Apaf-1-mediated pathway of apoptosis, still occurred in Apaf-1-deficient mice, albeit with a delay (Yoshida *et al*., 1998), and morphological features suggestive of necrotic cell death were observed in the dying cells (Chautan *et al.*, 1999). This raises the possibility that Apaf-1-independent (that may mean caspase-independent) necrotic cell death is also a form of “programmed” cell death, an underlying process that takes place simultaneously with canonical apoptosis.

Analyses of compound knockout mice also revealed the presence of Apaf-1-independent cell death. Mice deficient for both Apaf-1 and Bcl-xL die *in utero* due to failure of hematopoiesis (Yoshida *et al*., 2002). Despite the Apaf-1-deficiency, there was massive cell death detected in the fetal liver of double-knockout mice, similar to the case of Bcl-xL-deficient mice. The dying cells are positive for TUNEL staining and showed nuclear condensation. Thus, cell death of the hematopoietic cells in the fetal liver that is prevented by Bcl-xL is Apaf-1-independent. Interestingly, there was no apoptosis in the developing brain of the double-knockout mice. Taken together with the analyses of mice deficient for both Casp 3 and Bcl-xL by Roth *et al*. (Roth *et al.*, 2000), Apaf-1-dependent apoptosis regulates the number of proliferating neurons during development of the brain, while Bcl-xL is important for the maintenance of post-mitotic neurons. Inactivation of the retinoblastoma (Rb) tumor suppressor in the mouse induces massive apoptosis in the CNS, peripheral nerve systems (PNS), skeletal muscles and lens (Vooijs and Berns, 1999). Compound knockout mice lacking both Rb and Apaf-1 revealed that apoptosis in the PNS and skeletal muscles are only partly dependent on Apaf-1 while apoptosis in the CNS and lens is largely dependent on Apaf-1 function (Guo *et al.*, 2001). Thus, Apaf-1 dependent and independent apoptotic pathways are differentially required in different tissues/organs during embryogenesis.

Perspectives

The discovery and analyses of Apaf-1 revealed the essential roles of Apaf-1-mediated apoptotic pathway in embryogenesis. Despite the significant progress made in this field, several questions are raised. One of the most elusive is the molecular mechanism of Apaf-1-independent apoptosis and necrosis-like cell death. A related question is the presence

of Apaf-1-like molecules. The role of Apaf-1-dependent apoptosis in the adult body is largely unknown. Likewise, the role of Apaf-1 in the pathogenesis of certain types of diseases, such as neurodegenerative diseases, has yet to be elucidated. Some of these questions may be answered by taking advantages of organ-specific gene disruption (conditional knockout mice) of Apaf-1 and other players. We may need to develop more sophisticated strategies to address the questions. Only then will we be able to illuminate the roles of Apaf-1 in the whole course of life, from womb to tomb.

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