J Mol Med (2000) 78:203–211
Digital Object Identifier (DOI) 10.1007/s001090000099

REVIEW

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Bid, a critical mediator for apoptosis induced by the activation of Fas/TNF-R1 death receptors in hepatocytes

Received: 24 January 2000 / Accepted: 28 March 2000 / Published online: 27 April 2000

Abstract The Bcl-2 family proteins consist of both anti-apoptosis and pro-apoptosis members that regulate apoptosis typically at the mitochondrial level, mainly by controlling the release of cytochrome *c* and other mitochondrial apoptotic events. However, death signals mediated by Fas/TNF-R1 receptors can usually activate caspases directly, bypassing the need for mitochondria and escaping the regulation by Bcl-2 family proteins. Bid is a novel pro-apoptosis Bcl-2 family protein that is activated by Caspase 8 in response to Fas/TNF-R1 death receptor activation. Activated Bid is translocated to mitochondria and induces cytochrome *c* release, which in turn activates the downstream caspases. This Bid-mediated pathway is critical in hepatocyte apoptosis induced by Fas/TNF-R1 engagement, where direct activation of cytosolic caspase cascade seems inefficient. The dependence on Bid, and thus on the mitochondrial cytochrome *c* release, of hepatocyte apoptosis induced by the death receptors also renders it sensitive to the inhibitory regulation by the anti-apoptosis members of the Bcl-2 family proteins, such as Bcl-2 and Bcl-xL. Moreover, the revealing of this death pathway in hepatocytes is important to the understanding of the pathogenesis of a number of hepatic diseases such as hepatitis or endotoxemia-related hepatic failure.

Key words Apoptosis · Bcl-2 family proteins · Hepatocyte · Fas · Tumor necrosis factor

Abbreviations *GalN:* D-Galactosamine · *ICAD:* Inhibitor for caspase activated DNAase · *LPS:* Lipopolysaccharide · *MPTP:* Mitochondrial permeability transition pore · *PCD:* Programmed cell death · *z-VAD-fmk:* Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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Introduction

Programmed cell death (PCD) is an active process of cellular self-destruction with distinctive morphological and biochemical features. The term was initially used by developmental biologists to describe cell death occurring in a temporal and spatial way during development [1]. Cells undergoing PCD assume morphological features of apoptosis, which was first categorized by Kerr et al. [2]. The key features of apoptosis include membrane blebbing, chromatin condensation, and nuclear fragmentation. The intracellular organelles usually are intact, in contrast to necrosis. Not all PCD appears morphologically in the form of apoptosis [3, 4]. However, for simplicity, this review uses these terms interchangeably.

PCD is indispensable in the development and maintenance of homeostasis within all multicellular organisms. Normal morphogenesis often requires remodeling of tissues in such a way that certain specific signals are received to activate the process of PCD in the cells to be eliminated. The molecular machinery of PCD is also important for the regulation of homeostasis during adulthood, which is important for the control of neoplasia and autoimmunity. For example, autoimmune diseases are

largely prevented due to self-tolerance, by which self-responsive cells are destroyed through apoptosis [5, 6]. Immune-privileged sites such as testis and the anterior chamber of the eyes are maintained because of the ability of these tissues to induce apoptosis of invading lymphocytes [7, 8]. On the other hand, damage of cells in autoimmune diseases can also be due to apoptotic death, such as by cytotoxic T cells [9]. Understanding how apoptosis occurs in different situations will help to understand the pathogenesis of a number of human diseases and therefore provide clues to the treatment.

The genetic and biochemical pathways of apoptosis

A genetic pathway that regulates PCD has been defined and appears to be conserved from the nematode *Caenorhabditis elegans* to mammals [10]. Several key groups of molecules have been identified. Caspases are the main effector molecules that are activated and/or regulated by molecules that are responsive to death or survival signals.

Caspases are the central executors of apoptosis

At least 14 caspases have been defined in mammalian cells [11]. Four caspases exist in the nematode *C. elegans*, but only one, CED-3, is essential for all developmentally programmed cell death [12, 13]. Five different caspases have also been found in *Drosophila* [14]. Caspases are synthesized in precursor form and proteolytically processed to form an active heterotetrameric enzyme in response to death signals. The activated proteases have a unique cleavage site after aspartic acid. The substrates of caspases include caspases themselves and other cellular proteins. The activation of one caspase by another constitutes a caspase cascade that not only amplifies the process but also transmits the signals from one compartment to another within cells. Based on the relative substrate specificity, the structure, and the function in the caspase cascade, the commonly encountered caspases may be categorized into three subgroups (Table 1) [11, 15, 16]. Group I caspases are mainly involved in inflammation response, while group II and group III are mostly linked with apoptosis in general. Group III caspases are upstream initiator caspases, and serve mainly to activate downstream group II effector caspases. Effector caspases cleave a variety of cytoplasmic and nuclear substrates, such as the inhibitor for caspase activated DNAase (ICAD) and poly ADP-(ribosyl) polymerase among others. The significance of cleavage of these substrates is not always clear. Some of the target proteins may be death substrates, which upon proteolytic cleavage ensure the inevitability of death, such as ICAD [17]. Caspase inhibitors, such as benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), a broad-spectrum inhibitor of caspases [16], can block apoptosis completely in many cases.

Caspases are activated or inactivated through a series of intracellular steps, or pathways, in response to death or survival signals, which are subject to multiple regulations. There are two major apoptotic pathways defined in mammalian cells, the death receptor pathway and the mitochondrial pathway (Fig. 1).

The death receptor pathway is initiated at the cell surface

The Fas/tumor necrosis factor receptor 1 (TNF-R1) death receptor family is defined based on similar cysteine-rich extracellular domains (Table 2). The most extensively characterized death receptors are CD95 (Fas or Apo1) and CD120a (TNF-R1) [18]. Ligation of Fas either by its ligand, FasL, or its agonistic antibodies triggers the homotrimeric association of the receptors. The clustering of the death domains in the intracellular portion of the receptors recruits the adapter molecule called FADD/Mort1 via the death domain of the latter [18]. The death effector domain of FADD can then interact with a similar domain of pro-caspase 8, which results in its oligomerization. Activation of pro-caspase 8 through self-cleavage leads to a series of downstream events, including activation of pro-caspase 3, cleavage of multiple caspase substrates, and induction of mitochondrial damages. In a similar fashion, binding of TNF to TNF-R1 results in the trimerization of the receptor and recruitment of TRADD, which is thought to be followed by a similar reaction seen in the activation of Fas receptors [18]. The known difference is that TRADD also recruits other molecules, such as TRAF2, which actually activates a protective pathway through the transcription factor nuclear factor κB (NFκB). Thus in many types of cells, TNF induces cell death only in the presence of transcriptional inhibitors.

Table 1 The major mammalian caspases

Group	Caspases	Functional category	Optimal substrate recognition site	Common aldehyde tetrapeptide inhibitors	General inhibitor
I	1, 4, 5	Inflammation	(W/l)EHD	WEHD, YVAD	z-VAD-fmk, CrmA
II	2	Initiator/effector?	DEHD	–	–
	3, 7	Effector	DEVD	DEVD	z-VAD-fmk
III	6	Effector	VEHD	IETD	z-VAD-fmk,
	8, 9, 10	Initiator	LE (T/H)D	IETD	z-VAD-fmk, CrmA

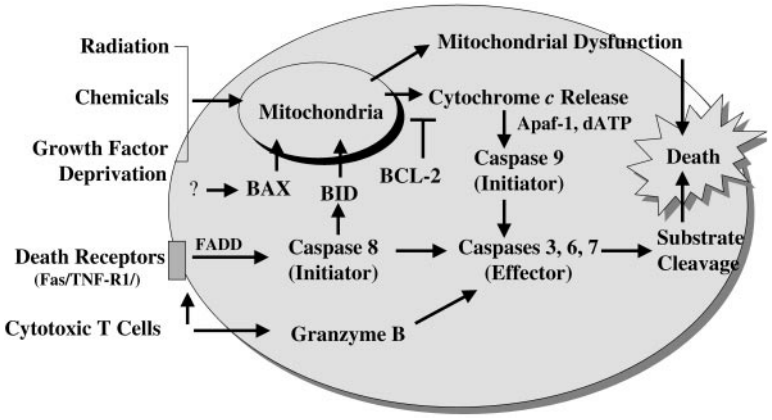

Fig. 1 Two major apoptosis pathways are present in mammalian cells. One is mediated by mitochondria, which release cytochrome *c* in response to death stimuli, which in turn activates Apaf-1 and caspases. Mitochondria may mediate other apoptotic events (mitochondrial dysfunction) that are contributory to final cell demise, such as opening of permeability transition pore. Mitochondrial pathway is subject to the regulation by Bcl-2 family proteins. Death stimuli transmitted through the Fas/TNF-R1 death receptor family or granzyme B are mainly mediated directly by caspase cascades in cytosol. However, in certain types of cells, such as hepatocytes, the effector caspases may not be efficiently activated by Caspase 8 and a mitochondrial pathway mediated by Bid thus becomes critical. Bid is cleaved by Caspase 8 and translocated to mitochondria to induce cytochrome *c* release. In other types of cells, such as lymphocytes, this mitochondrial branch may also play a role in further enhancing the effects of the cytosolic caspase pathway.

Table 2 The major Fas/TNF-R death receptors

Ligand	Receptor
FasL	Fas/Apo 1
TNF	TNF-R1, TNF-R2
?	DR3/Apo 3/TRAMP
TRAIL/Apo 2L	DR4 (TRAIL R1)
	DR5/Apo 2 (TRAIL R2)
	DcR1/TRID (TRAIL R3)
	DcR2 (TRAIL R4)

Mitochondrial apoptosis pathway is initiated within cells

There are, however, a large number of death stimuli that do not seem dependent on the death receptor pathway. In addition, no death receptors have so far been found in the nematode. Although direct activation of pro-caspase 3 by Granzyme B is observed in cytotoxic T cell-induced apoptosis [19], the likely scenario in most cases is that the death signals are relayed to mitochondria, where release of cytochrome *c* is induced [20]. Cytochrome *c* activates Apaf-1, in the presence of dATP, which in turn activates pro-caspase 9 [21] (Fig. 1). Activated Caspase 9 can then cleave downstream effector caspases. Mitochondrial apoptosis pathway is involved in most non-death receptor-mediated death signaling, such as irradiation, DNA damaging, hormone stimulation, and growth factor withdrawal (reviewed in [22]). Although cytochrome *c* release has not been demonstrated in *C. elegans*, the regulation of CED-4, the Apaf-1 homolog, by the mitochondria-localized CED-9 through physical interactions still requires the mitochondria as the main organelle where death signals, such as EGL-1, will target [23].

It must be pointed out that cytochrome *c* release and caspase activation may not be the only effects resulting from the insults on mitochondria [24]. A decrease in mitochondrial transmembrane potential has been observed early in apoptosis [25]. Other changes include increases in free radical generation [25] and changes in membrane integrity [26] and in the pattern of mitochondria distribution [27]. It is not clear how these mitochondrial changes are caused, which may or may not be directly related to caspase effects, or whether they are linked to cytochrome *c* release. Nevertheless, they can significantly contribute to the cellular demise independently of caspase effects [28, 29].

The Bcl-2 family proteins as important apoptosis regulators

Bcl-2 family proteins regulate apoptosis via the mitochondrial pathway

This family of proteins consists of both death antagonists (BCL-2, BCL-X_L, etc.) and death agonists (BAX, Bid, etc.) [30, 31] (Table 3), which are conserved through evolution. Several viruses encode Bcl-2 homologs. The nematode *C. elegans* has its sequence and functional homologs for a death antagonist, CED-9 [32] and a BH3-only death agonist, EGL-1 [33].

The Bcl-2 family members share structural homology in BH1–BH4 domains, although not all members have all domains. Mutagenesis studies have revealed that these domains are important for the function as well as for protein interactions among the family members. The BH1 and BH2 domains of the antagonists are required to heterodimerize with the death agonists and repress cell death [34, 35]. Conversely, deletion analysis has indicated that the BH3 domain of death agonists is required for them to heterodimerize with the death antagonists and to promote cell death [30, 31]. The crystallography study of

Table 3 The Bcl-2 family proteins

Function	Organisms	Members[a]	BH domains
Anti-apoptosis	Vertebrates	Bcl-2, Bcl-xL, Bcl-W, Mcl-1, A1/Bfl-1, NR-13	BH1, BH2, BH3, BH4
	C. elegans	CED-9	BH1, BH2, BH3, BH4
	Virus	E1B-19 K (adenovirus)	BH1, BH2, BH3
		BHRF-1 (EBV), KS-Bcl-2 (HHV8), ORF16 (HSV), LMW5-HL (ASFV)	BH1, BH2
	Xenopus	XR1, XR11	BH1, BH2
Pro-apoptosis	Vertebrates	Bax, Bak, Bok/Mtd, Bcl-xS	BH1, BH2, BH3
		Bad, Bid, Bik/Nbk, Blk, Hrk, Bim/Bod, Nip3/BNIP3, Nix/Bnip3L	BH3
	C. elegans	EGL-1	BH3

[a]Vertebrates in which Bcl-2 family proteins have been defined include human, mouse, rat, and chicken. Boo/DIVA found in mammals is not included, since one investigator found that it is was pro-apoptotic [79], and another found it to be anti-apoptotic [80]

the death antagonist BCL-X_L indicates that these domains assume an α-helical conformation, and that a hydrophobic pocket is created by the close spatial proximity of BH1, BH2, and BH3, which presumably constitutes the structural basis for function and protein interactions [36]. Structural studies of the BH3-only molecule Bid reveal remarkably similar structure mainly of α-helices [37, 38]. Most interestingly, the studies suggest that the BH3 domain of the death agonists must be exposed, either constitutively or through posttranslational modification, for its killing function [31, 37].

Early data support the notion that the ratio of death antagonists to agonists determines whether a cell will respond to apoptotic signals [39]. Competitive dimerization between the pro- and anti-death molecules may result in functional inhibition [34]. However, both genetic and biochemical data [35, 40] suggest that these molecules can work independently of each other. It now seems clear that Bcl-2 family proteins regulate apoptosis mainly via the mitochondrial pathway. Bcl-2, Bcl-xL, Bax, or Bid are found on mitochondria either constitutively or by induction [30, 31]. Bcl-2 family proteins can regulate caspase activation through the regulation of cytochrome *c* release, which is inhibited by death antagonists (Bcl-2 or Bcl-xL), and promoted by death agonists (Bax or Bid) (reviewed in [24, 31]). Bcl-2 family proteins may serve as membrane channels on mitochondria. The channel activities assumed by Bcl-2 and Bax on synthetic lipid membrane have distinct characteristics of ion selectivity and conductivity [24, 30, 31]. It is possible that this channel activity affects mitochondrial permeability and thus regulate either cytochrome *c* release or other mitochondrial functions that are associated with caspase-independent apoptotic processes. Alternatively, it has been shown that direct protein interactions may be responsible for the regulation of caspase activation, particularly in the *C. elegans* system. Competitive dimerization of the pro-apoptotic molecule EGL-1 with CED-9 can disrupt the binding of the latter to CED-4. CED-4 is then released from the mitochondria into the cytosol, where it activates CED-3 and induces cell death [23, 33].

Bid is a unique BH3-only molecule of the Bcl-2 family

The biochemical mechanisms by which BCL-2 family proteins affect mitochondrial functions or cytochrome *c* release are still elusive. It seems that Bid, a BH3-only Bcl-2 family death agonist, may provide some very useful information toward this end. Bid is first cloned from a cDNA expression library based on its interaction with Bcl-2 and Bax [41]. Bid cDNA encodes a 195 amino acid protein with a predicted molecular weight of 21.95 kDa. When *Bid* is overexpressed in an interleukin-3 dependent cell line, it counters the protective effect of Bcl-2. Moreover, overexpression of Bid, either in lymphoid cells or in fibroblasts, induces apoptosis without the need for additional death signals [41]. The BH3 domain is required for its death activity. Furthermore, the death activity of Bid can be inhibited by Bcl-2 or zVAD-fmk, suggesting that Bid induces apoptosis possibly by activating caspases via the mitochondrial pathway.

However, Bid, unlike most other members of the Bcl-2 family, lacks a COOH-terminal membrane-anchoring segment and is largely present in cytosol. How could Bid thus induce caspase activation via mitochondria? One of the possibilities is that Bid must translocate to mitochondria upon the stimulation of a particular death signal to activate the mitochondrial death pathway [41].

Bid is cleaved and translocates from cytosol to mitochondria to induce cytochrome *c* release

In search for such signals, we have recently found that death signals mediated by the death receptors, i.e., TNF-R1 and Fas, can activate Bid in this way [42]. Treatment of an IL-3 dependent cell line, FL5.12, with TNF and cycloheximide for 5 h induced cell death, accompanied by the proteolytic cleavage of Bid. The appearance of the cleaved p15 product was detected first in cytosol, but later on the mitochondria. Protein sequencing analysis of the p15 Bid indicated that it was generated from a proteolytic digestion at Asp-59, the site with a conserved Caspase 8-cleavage sequence (LETD) [15]. Further in vitro analysis

confirmed the role of Caspase 8 in cleaving Bid [27, 42, 43]. Cleavage of Bid was also demonstrated in cells that had been activated through Fas, another major death receptor [27, 43]. Consistent with the hypothesis, Bid was found to induce cytochrome *c* release in vivo after translocation to mitochondria [27, 42, 43]. The fact that the cytoplasm of activated cells depleted of Bid by immunoprecipitation is no longer able to induce cytochrome *c* release [42] indicates that Bid is the only effector molecule downstream of Caspase 8 that has such a capability.

Functionally, truncated Bid is more potent than the full-length form in inducing cell death as well as in interacting with Bcl-xL [27]. Effects of p15 Bid on mitochondria may not be limited to the induction of the cytochrome *c* release but also include other changes in mitochondrial physiology, such as mitochondrial redistribution and loss of mitochondrial $\Delta\psi m$ in the cells [27]. The key question, however, is what is the physiological significance of Bid being activated by Fas/TNF-R1 signaling. Engagement of TNF-R1 or Fas normally activates a cytosolic apoptosis pathway where receptor interaction with adapter molecules leads to the activation of Caspase 8 and the subsequent cytosolic caspase cascade. Why does the connection from the cytosolic pathway to the mitochondrial pathway via Bid exist when Fas/TNF-R1s seem able to effectively activate the caspase cascade in the cytosol and to induce apoptosis all by themselves? It is possible that such a cross-talk can amplify the caspase activation through the mitochondria-activated Caspase 9 and induce additional mitochondrial dysfunction that may be also contributory to cell death. However, the significance of this Bid-mediated pathway may not be limited to a mere amplification of the existing pathway. In certain types of cells, this cross-talk to mitochondria could be unique and therefore irreplaceable to Fas/TNF-R1 induced apoptosis. To assess the different roles of Bid in a pathophysiological context, we decided to employ animal models involving the Fas/TNF-R1 signaling.

The role of Bid in Fas-mediated hepatocyte apoptosis

In vivo administration of anti-Fas antibody induces hepatocyte apoptosis

Activation of Fas-mediated apoptosis in vivo can be achieved by a single injection of an agonist anti-Fas antibody. Mice usually die within 6 h of receiving a lethal dose of anti-Fas antibody, and $Fas^{lpr/lpr}$ mice that do not have functional Fas are completely resistant to this treatment [44]. The major organ affected is the liver and the massive hepatocyte apoptosis is responsible for the animal death [44]. Furthermore, isolated hepatocytes are susceptible to anti-Fas antibody induced apoptosis in vitro, demonstrating the direct cytotoxic effects of Fas activation in this type of cells [45, 46, 47, 48]. Caspase-3/7 activities are detected in liver cells of mice injected with anti-Fas antibodies as well as in hepatocytes incubated with anti-Fas in vitro [46, 47, 49]. A broad-spectrum caspase inhibitor, z-VAD-fmk, could abolish caspase activation, caspase activity and apoptosis of hepatocytes stimulated by anti-Fas in vitro or in vivo [45, 46, 47, 48]. In one study four intravenous injections of z-VAD-fmk, around 1.1 μm in total, given 1 h after anti-Fas injection could also rescue mice from death [46].

It was not clear, however, how caspases are activated in hepatocytes. Conventional wisdom would argue that the cytosolic pathway is the main pathway, as demonstrated in vitro in cells other than hepatocytes. However, the in vivo data actually suggest that a mitochondrial pathway is more likely to be the pathway activated by the Fas signaling in hepatocyte. For example, hepatocytes transgenically overexpressing Bcl-2 are resistant to anti-Fas antibody-induced injury in vivo [50, 51], and even the mouse survival rate can be greatly improved [51]. In addition, when the expression level of Bcl-xL in hepatocytes is enhanced by the treatment of hepatocyte growth factor, mice can be also protected from anti-Fas induced mortality and hepatocyte injury [52]. Such treatment can inhibit group II caspase activities completely. These data support the idea that the toxicity of Fas activation in hepatocytes is mediated mainly via the mitochondrial pathway, in that caspase activation is stimulated by the release of cytochrome *c*, which is inhibited by Bcl-2 or Bcl-xL. We thus hypothesized that it is Bid that is responsible for the induction of cytochrome *c* release and activation of caspase downstream of Fas activation.

Bid is cleaved in hepatocytes in response to anti-Fas antibodies

As the first step, we examined whether Bid can be cleaved in hepatocytes in response to anti-Fas. Indeed, a single intravenous injection of anti-Fas antibody [44] induced cleavage of Bid in liver and caused its translocation to mitochondria [42]. The cleaved p15 Bid was first detected in cytosol 1 h after injection, but was found completely in mitochondria 3 h after injection. We also recently found that Bid was cleaved in isolated hepatocytes cultured in vitro in response to anti-Fas antibodies (Zhao et al., unpublished observation). These studies suggest that Bid could be involved in the pathogenesis of Fas-mediated hepatic cell death.

bid-deficient mice are resistant to anti-Fas induced mortality and hepatocyte injuries

In order to demonstrate the role of Bid in anti-Fas mediated apoptosis, we decided to use *bid*-deficient mice, which were constructed through homologous recombination [53]. These mice are born alive and have no apparent gross development abnormalities. The anti-Fas antibody was given intravenously at a dose of 0.25 μg/g. Wild-type animals were almost universally susceptible to the lethal effect of anti-Fas at this dose, as reported previously [44],

and died within 6 h. The survival rate in the 24-h period was one of eight animals. Strikingly, *bid*$^{-/-}$ mice were resistant to this anti-Fas induced mortality, and 82% (9/11) of *bid*$^{-/-}$ mice survived within the same time period. Upon the injection of the anti-Fas antibodies, the livers of wild-type animals increased dramatically in weight, and histology showed extensive hepatic cell apoptosis and hemorrhaging necrosis, as reported previously. All these changes were much smaller or absent in the livers of *bid*$^{-/-}$ mice. The mean histology grade of wild-type livers scored 2 h after the administration of the antibody was 2.3±1.0, while that of *bid*$^{-/-}$ livers was only 0.3±0.5 (P=0.01) on a scale of 0–3 with zero representing normal morphology and 3 representing the most severely damaged. DNA fragmentation could be clearly demonstrated in wild-type but not in *bid*$^{-/-}$ livers by terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling. Isolated *bid*-deficient hepatocytes were also resistant to anti-Fas antibodies in vitro. These data clearly indicate that *bid*$^{-/-}$ hepatocytes are resistant to anti-Fas induced apoptosis and further suggest that Bid is essential to Fas-mediated hepatocyte apoptosis.

Fas activation normally initiates the cytosolic apoptosis pathway through direct caspase recruitment and activation in cells such as lymphocytes. The much reduced lethality and hepatic apoptosis in *bid*$^{-/-}$ mice suggest that in hepatocytes the main apoptotic pathway initiated by Fas activation is in fact the mitochondrial pathway mediated by Bid. Indeed, immunohistochemistry study on the paraffin-embedded liver sections using an antibody against activated Caspase 3 or 7 showed that wild-type mice receiving anti-Fas antibody treatment had strong signals for activated Caspase 3 or 7 in the livers, which was hardly detectable in the livers of *bid*$^{-/-}$ mice. In contrast, there was no difference in the activities of the upstream initiator Caspase 8, as analyzed by western blot and substrate cleavage assay. Correspondingly, the immunostaining signals of cytochrome *c* were much diminished in hepatocytes of wild-type animals in response to Fas stimulation, implying the release of cytochrome *c* from mitochondria. However, the signals were not changed in *bid*$^{-/-}$ animals, suggesting that Bid is likely the only molecule transmitting the mitochondrial signals in Fas activation. These finding are consistent with the idea that Bid acts downstream of Fas receptor and Caspase 8, but upstream of cytochrome *c* release and effector caspase activation. Moreover, it suggests that in hepatocytes, the mitochondrial pathway mediated by Bid is much more important than the cytosolic pathway in determining caspase activation and the cell fate in response to anti-Fas antibody treatment.

The role of Bid in TNF-R1 initiated hepatocyte apoptosis

TNF mediates the liver injury in murine models of endotoxemia

Since Bid is critical for Fas-signaling induced apoptosis in hepatocytes, it could be equally important to TNF-R1 mediated hepatocyte death. This notion is supported by the finding that Bid can be cleaved and translocated to mitochondria in response to TNF in a lymphoid cell line [42]. The availability of several murine models of TNF-mediated liver injuries makes the test of this hypothesis feasible. The most commonly used in vivo models of TNF toxicity in mice involve endotoxemia-related hepatic failures. In such models microbial cell surface antigens, such as lipopolysaccharide (LPS), are used to initiate the inflammatory response. As rodents are known to be more than 1000-fold less sensitive to LPS than humans, they are often sensitized by a preceding bacterial infection or by a pretreatment with an amino sugar, D-galactosamine (GalN). The use of LPS is to stimulate the production of TNF by macrophages [54, 55]. Alternatively, *Staphylococcus aureus* enterotoxin or concanavalin A can be given to stimulate production of TNF from T cells [56, 57]. GalN is a liver specific transcriptional inhibitor. It is metabolized primarily along the galactose pathway in the liver to produce intermediates, which bind and deplete the available hepatic uridine pool [58]. Neither low-dose LPS nor GalN alone is toxic to mice. However, when administered together, they cause liver-specific damage, which may be the primary factor responsible for the lethality.

The primary hepatotoxic effect of TNF is the induction of apoptosis of hepatocytes. Histological data and biochemical study of DNA fragmentation provide strong support [48, 56, 58]. Furthermore, *tnf-r1*$^{-/-}$, but not *tnf-r2*$^{-/-}$ mice are completely resistant to the endotoxic shock induced by either LPS plus GalN or SEB plus GalN [59, 60], which is consistent with the finding that TNF-R1, but not TNF-R2 is primarily involved in mediating the cytotoxicity of TNF [61]. The hepatic injury caused by TNF also involves necrosis and inflammation. Kinetics studies show that apoptotic bodies are first found in the livers of treated mice, followed by severe necrosis and massive neutrophil infiltration. Release of liver-specific enzymes and erythrocyte agglutination in the hepatic sinusoids are found in the later stage [56]. Neutrophil sequestration in the hepatic sinusoids is actually found in early stage, which is due to activated cell adhesion molecules and cytokines induced by TNF [62]. However, full-blown neutrophilic inflammation and necrosis do not occur until hepatocytes apoptose, which somehow sends the signals to cause neutrophils to transmigrate through sinusoidal endothelium into liver parenchyma [63].

Effector caspases may be activated through mitochondrial pathway via the activity of Bid

The activation of caspases in TNF-mediated hepatocyte death can be readily demonstrated [64]. In vivo treatment with z-VAD-CH_2F in these mice attenuates apoptosis by 80% or more based on histology criteria, suggesting the critical role of caspases in the pathogenesis of TNF-mediated hepatocyte apoptosis [64]. z-VAD-fmk,

as well as Ac-DEVD-CHO, a relatively more specific inhibitor for group II caspases, also blocks apoptosis of hepatocytes treated with TNF plus ActD or an IκB suprasuppressor in vitro [48, 65]. Although z-VAD does not affect the initial inflammatory response, such as the sequestration of neutrophils in sinusoids, it does prevent neutrophil transmigration, apparently due to its inhibitory effect on apoptosis [64]. Thus z-VAD also prevents liver cell necrosis as judged by histology examination and liver enzyme tests [48, 64]. The protective effect of z-VAD on overall mortality of mice in this endotoxin shock model has also been reported [66].

Although the role of caspases in hepatocyte apoptosis induced by TNF has been firmly established, the signals and cellular components involved in the pathway are just being revealed. Caspase 8 is activated at the apical end, and it has been found that cytochrome *c* is released and is responsible for the downstream caspase activation in cultured rat hepatocytes treated with TNF and an IκB suprasuppressor [65]. Consistent with other findings, these results indicate the importance of the mitochondrial pathway in the TNF-induced liver toxicity and strongly imply that Bid is the key mediator between Caspase 8 and cytochrome *c* release. We are now testing this hypothesis by examining the response of *bid*-deficient mice and hepatocytes to TNF. Our preliminary results indicate that Bid is cleaved in mice given LPS and GalN, and that the cleaved p15 Bid is translocated to mitochondria (Zhao et al., manuscript in preparation). Cleavage of Bid is also observed in vitro in cultured hepatocytes treated with TNF and ActD. In both in vivo and in vitro studies it seems that Bid is very likely important to the pathogenesis of TNF-mediated toxicity in hepatocytes.

The molecular mechanism of Bid-induced apoptosis

The mitochondrial apoptosis pathway mediated by Bid is essential for hepatocyte apoptosis induced by Fas/TNF-R1 activation. Truncated Bid induces cytochrome *c* release and thus the activation of effector caspases. How Bid, or other pro-death Bcl-2 family proteins, such as Bax and Bak, induces the release of cytochrome *c* is largely unknown. In addition, Bid, as well as Bax, may induce other mitochondrial changes [27, 28]. The relationship between these activities is not clear, either. The mitochondrial permeability transition pore (MPTP) has been implicated in cytochrome *c* release in a number of systems [24], in addition to its potential role in inducing mitochondrial depolorization and free radical generation [25]. In one study using TNF on in vitro cultured hepatocytes it was found that cyclosporine A, a known MPTP inhibitor, is able to block cytochrome *c* release, effector caspase activation, and hepatocyte death [65]. However, whether this effect on MPTP is mediated by Bid has yet to be demonstrated. It is equally possible that Bid induces cytochrome *c* release independently of MPTP mechanistically. These possibilities are yet to be examined to completely understand the molecular mechanisms of Bid's effects, which may also provide clues to the understanding of other pro-apoptosis Bcl-2 family proteins for their apoptotic mechanisms.

The implication of the role of Bid in the pathogenesis of diseases of liver and other organs

The participation of Bid in the Fas/TNF-R1 mediated pathway suggests that it could be one of the key components in the development of a number of human diseases that are related to the Fas/TNF-R1 activation, particularly the liver failure seen in several clinical scenarios. For example, FasL/Fas interaction has been implicated in the development of viral hepatitis and other types of liver damage [67, 68, 69]. In an animal model of viral hepatitis the hepatitis B surface antigen is transgenically overexpressed on hepatocytes. The transfer of a T cell clone specific for this antigen into these animals results in massive hepatocyte apoptosis, similar to what is found in clinical patients [70, 71]. The killing of the hepatocytes can be blocked by soluble Fas protein, suggesting the importance of this pathway in the disease development [68]. In another scenario, TNF-R1 activation subsequent to septic shock could lead to liver failure as part of the clinical manifestation of endotoxemia. The role of TNF-α in the pathogenesis has been clearly demonstrated in patients [72, 73, 74] as well as in animal models [54, 55, 56, 57]. The current studies on Bid in animal models suggest that this molecule could be essential for the pathogenesis of these diseases in clinic.

Activation of Fas death receptor has also been implicated in human diseases involving other organs, such as pancreas and thyroid [75, 76, 77]. The apoptotic destruction of functional cells in these organs due to either an autoimmune response or injury is thought to be at least in part responsible for the nonobesity diabetes or autoimmune thyroiditis. Whether Bid is essential in the signaling of Fas in these situations has yet to be determined. It must be cautioned, however, that in some systems, such as lymphoid cells and embryonic fibroblasts, activation of the Fas/TNF-R1 pathway leads to a more direct activation of cytosolic effector caspases, so that the role of Bid and the mitochondrial apoptosis pathway may be limited to the amplification of the death signaling [42, 53]. There seems to have two different types of cells, originally defined in vitro based on cell lines, in terms of their dependence on mitochondrial pathway in response to Fas stimulation [78]. Type II cells seem unable to launch a full-scale cytosolic activation of caspases and thus depend on the mitochondrial pathway. Based on our studies, it seems that hepatocytes are one example of type II cells, whereas thymocytes may represent type I cells in vivo. It will be interesting to determine whether other types of cells, such as the β-islet cells, are the type of cells in which activation of Fas relies on the mitochondrial pathway, and therefore Bid, to reach the full effects. These studies will thus promote our understanding of the patho-

genesis of a number of human diseases, which may in turn provide hints for preventional and therapeutic interventions in the future.

Acknowledgements A major part of the work on Bid described in this review was conducted in Dr. S.J. Korsmeyer's laboratory at Washington University, St. Louis, Missouri. The author is indebted to Dr. Korsmeyer for his generous support and to Drs. A. Gross and K. Wang for the fruitful collaboration. X.-M.Y. is in part supported by a NIH grant (NCI, NIH CA74885).

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