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In Vivo Evidence That Caspase-3 Is Required for Fas-Mediated Apoptosis of Hepatocytes

Minna Woo,* Anne Hakem,* Andrew J. Elia,* Razqallah Hakem,* Gordon S. Duncan,* Bruce J. Patterson,‡ and Tak W. Mak2*†

Caspase-3 is essential for Fas-mediated apoptosis in vitro. We investigated the role of caspase-3 in Fas-mediated cell death in vivo by injecting caspase-3-deficient mice with agonistic anti-Fas Ab. Wild-type controls died rapidly of fulminant hepatitis, whereas the survival of caspase-3−/− mice was increased due to a delay in hepatocyte cell death. Bcl-2 expression in the liver was dramatically decreased in wild-type mice following anti-Fas injection, but was unchanged in caspase-3−/− mice. Hepatocytes from anti-Fas-injected wild-type, but not caspase-3−/−, mice released cytochrome c into the cytoplasm. Western blotting confirmed the lack of caspase-3-mediated cleavage of Bcl-2. Presumably the presence of intact Bcl-2 in caspase-3−/− hepatocytes prevents the release of cytochrome c from the mitochondria, a required step for the mitochondrial death pathway. We also show by Western blot that Bcl-xL, caspase-9, caspase-8, and Bid are processed by caspase-3 in injected wild-type mice but that this processing does not occur in caspase-3−/− mice. This study thus provides novel in vivo evidence that caspase-3, conventionally known for its downstream effector function in apoptosis, also modifies Bcl-2 and other upstream proteins involved in the regulation of Fas-mediated apoptosis. The Journal of Immunology, 1999, 163: 4909–4916.

Abbreviations used in this paper: PCD, programmed cell death; Apaf-1, apoptotic protease activating factor-1; DISC, death-induced signaling complex; H&E, hematoxylin and eosin.

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poptosis, or programmed cell death (PCD), is involved in a wide range of biological and pathological processes (1). The fundamentals of the genetic control of apoptosis have been elucidated from studies of the nematode Caenorhabditis elegans, in which developmental cell death involves the regulatory genes ced-3, ced-4, and ced-9 (2). CED-9 is homologous to the mammalian Bcl-2 family of genes, whereas CED-4 is homologous to mammalian Apaf-1 (apoptotic protease activating factor-1) (3). CED-3 shares sequence similarity with a family of at least 14 mammalian cysteine proteases now termed caspases (cysteine aspartate specific protease). Caspases are synthesized as procaspases that are cleaved at specific aspartate sites to generate a tetramer to form the active enzyme. Active caspases share this unique cleavage specificity in that they also cleave their substrates, including other procaspases, at aspartate residues. Caspases are broadly categorized into upstream regulatory caspases and downstream effector caspases. The upstream caspases such as caspase-9 and -8 typically have a long N-terminal prodomain that facilitates interaction with and recruitment of proapoptotic proteins, including other caspases. Downstream caspases, which typically have short prodomains, primarily cleave proteins important for cellular functions, resulting in the execution of the cell (4). Caspase-3, the caspase most closely related in sequence and substrate specificity to CED-3, is thought to be a key apoptotic “executioner” enzyme in mammalian cells because its activation triggers the cascade of enzymatic events that culminates in the death of the cell (5).

Fas, a transmembrane receptor protein belonging to the TNF receptor family, contains a death domain mediating PCD. Fas is constitutively expressed on hepatocytes and has been implicated in many human liver diseases, including autoimmune hepatitis and hepatocellular carcinoma (6, 7). Fulminant hepatitis, an accelerated liver failure that is often fatal, is a complication of many human liver diseases. Injection of an animal with agonistic anti-Fas Ab in vivo causes an immediate and massive apoptotic death of hepatocytes, resulting in fulminant hepatitis and death within hours. The fact that this process is indeed Fas-mediated has been demonstrated by injecting anti-Fas Ab into mice with the lymphoproliferation mutation lpr. The lpr mice lack virtually all functional Fas and are completely resistant to liver failure induced by anti-Fas injection (8).

There is now much in vitro and some in vivo evidence that caspases are involved in the Fas-mediated cell death pathway (9–11). Pretreatment of cultured cells with the general caspase inhibitor benzylxoycarnbonyl-Val-Ala-Asp-fluoromethylketone (Z VAD FMK) has been shown to inhibit anti-Fas-mediated apoptosis (12). Similarly, treatment of hepatocytes with N-acetyl-Asp-Glu-Val-Asp aldehyde (Ac DEVD CHO), an inhibitor that specifically blocks caspase-3-like caspases, also inhibits Fas-mediated apoptosis (13). When mice were injected systemically with caspase inhibitors before treatment with agonistic anti-Fas Ab, the mice failed to show significant apoptosis in the liver and were resistant to hepatic failure (14). In addition, we have previously shown that activated lymphocytes from caspase-3-deficient mice are resistant to apoptosis induced by in vitro treatment with anti-Fas Ab (10). Finally, a recent study of cultured hepatocytes showed that both caspase-3 and -7 are activated upon Fas ligation, implicating them as effector caspases for hepatocyte cell death (15).

During Fas activation, caspase-8 is recruited to the death-induced signaling complex (DISC) on the cell surface through interaction with Fas-associated death domain protein/mediator of
cell death. Mice expressing a liver-specific Bcl-2 transgene are protected from death of the hepatocytes in vivo, and whether caspase-3 is required for either or both pathways, was the subject of this study. Bcl-2 is an important antiapoptotic protein located in mitochondria. Mice expressing a liver-specific Bcl-2 transgene are protected from fulminant liver failure induced by injection of anti-Fas Ab, in vivo evidence that Bcl-2 plays a crucial role in protecting against apoptosis of these proteins and abolishes their antiapoptotic activities (27, 28). Furthermore, the carboxyl-terminal Bcl-2 cleavage product has been reported to trigger cell death in a way similar to that of caspase-3. Bcl-2 and Bcl-xL remain intact and are able to prevent the apoptotic machinery from proceeding. To assess the specific role of caspase-3 in Fas-mediated hepatic cell death, we examined hepatocyte apoptosis in response to agonistic anti-Fas mAb treatment of caspase-3-deficient mice. We show in vivo that, in the absence of caspase-3, the death of animals due to fulminant hepatitis induced by anti-Fas treatment is delayed. Analysis of histological sections revealed that caspase-3 deficiency allowed hepatocytes to remain viable following Fas ligation. Intracellular levels of Bcl-2 did not decrease as in the wild-type hepatocytes and cytochrome c release was impaired in caspase-3−/− hepatocytes. Bcl-2, Bcl-xL, caspase-9, caspase-8, and Bid were all proteolytically processed in wild-type hepatocytes upon Fas ligation but not in caspase-3−/− hepatocytes. These results suggest the presence of a positive feedback loop required for the apoptosis of hepatocytes, which is initiated by caspase-3-mediated cleavage of Bcl-2 and Bcl-xL. In the absence of caspase-3, Bcl-2 and Bcl-xL remain intact and are able to prevent the apoptotic machinery from proceeding. This work provides the first in vivo evidence that caspase-3 plays a critical role in the initial upstream steps of Fas-mediated hepatocyte PCD that lead to fulminant hepatic failure causing death.

Materials and Methods

Mice

The generation of caspase-3 knockout mice has been described previously (10). 129/J/C57BL6/6 chimeric caspase-3−/− mice were backcrossed to C57BL/6 for at least four generations. Although the knockout mice were not born at the expected Mendelian ratio, at least half of those surviving the postnatal stage lived to be healthy adults of more than 6 wk of age. Mice used in this study were adults of between 6 and 12 wk of age.

Anti-Fas mAb injection and histologic examinations

Paraffin-embedded sections were dewaxed in xylene and rehydrated by passing through a graded series of ethanol solutions. Peroxidase blocking was performed in 3% H2O2/H2O for 10 min followed by rinses in dH2O and PBS. Sections were permeabilized with proteinase K (20 μg/ml in 10 mM Tris-HCl, pH 7.4–8.0) at 37°C for 15 min. After washing, sections were stained with fluorescent anti-TdT using the In Situ Cell Death Detection kit from Boehringer Mannheim (Mannheim, Germany). Sections were viewed and photographed using standard fluorescent microscopic techniques.

Immunohistochemistry

Paraffin-embedded sections were dewaxed in toluene for 10 min and rehydrated through a graded series of ethanol solutions, Peroxidase blocking was performed in 3% H2O2/H2O for 10 min followed by rinses in dH2O and PBS. Sections were incubated with anti-Bcl-2 (clone 12-D4; Dako, Glostrup, Denmark) or anti-p53 (clone D07; Novo Castra, Newcastle-upon-Tyne, U.K.) in a moist chamber for 1 h at room temperature (RT). The treated sections were then incubated with biotin-conjugated secondary Ab for 20 min at RT, then streptavidin-HRP for 20 min at RT, followed by development in aminoethylcarbazole (AEC) for 20 min. For cytochrome c staining, sections were dewaxed and rehydrated as described above. Sections were preblocked (3% BSA, 5% goat serum, and 0.3% Tween 20) for 30 min before incubation with cytochrome c Ab (diluted 1:50 in PBS with 3% BSA and 5% goat serum; PharMingen) at RT for 1.5 h followed by washes in PBS. The secondary Ab, an FITC-labeled goat anti-mouse IgG, was from Biotalk (www.biotalk.com, Hamburg, AL) and sections incubated for 45 min at RT. Sections were then washed in PBS and mounted using Vectashield antifade mounting medium (Vector Labs, Burlingame, CA).

Western blotting

Cells were lysed in 1% Nonidet P-40 lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-Cl (pH 8.0), and 1 mM sodium vanadate) supplemented with protease inhibitor mixture (0.1 mM PMSF and 2 μg/ml of leupeptin and aprotinin) for 15 min on ice. Lysates were centrifuged at 10,000 rpm for 5 min at 4°C, and protein concentration was estimated by the Pierce Protein Assay (Pierce, Rockford, IL) using BSA as the standard. Forty micrograms of total protein was loaded onto 14% SDS-PAGE, transferred onto nitrocellulose membranes, and incubated with the appropriate Abs. Abs reactive to Bcl-2 (gift of D. Andrews, Hamilton, Ontario, Canada), Bcl-xL (Calbiochem, La Jolla, CA), Bid (gift of X. Wang, Dallas, TX), caspase-6 and -7 (Santa Cruz Biotechnology, Santa Cruz, CA), caspase-8 and -9 (gift of R. Hakem, Toronto, Canada), or β-actin (Sigma, St. Louis, MO) were used in this study. Western blot analysis was conducted according to standard procedures using enhanced chemiluminescence detection (Amersham, Arlington Heights, IL).

Results

Increased survival of caspase-3−/− mice following anti-Fas mAb injection

Within 2 h of i.p. injection with 10 μg anti-Fas mAb, wild-type mice displayed signs of clinical compromise, including tachypnea, shallow breathing, prostration, and progressive deep hypothermia, consistent with rapid liver failure. Death occurred within 3–6 h. In contrast, the time of death of caspase-3−/− mice was delayed to between 15 and 24 h (Fig. 1). Wild-type mice injected with 100 μg anti-Fas mAb became moribund within 2–4 h, whereas caspase-3−/− mice became ill only much later, at 6–9 h (Fig. 1).
Increased viability of caspase-3−/− hepatocytes compared with wild-type hepatocytes following anti-Fas mAb injection of mice

To examine the induction of hepatocyte apoptosis in vivo, wild-type and caspase-3−/− mice were sacrificed 3 h after administration of 10 μg anti-Fas mAb and the livers were examined by H&E staining. The livers of additional caspase-3−/− mice were examined 6 h after anti-Fas mAb injection. At the 3 h time point, the livers of wild-type mice appeared grossly hemorrhagic. The normal lobular microarchitecture of the liver was maintained but hepatocytes showed characteristic signs of apoptosis, including condensation of chromatin at the nuclear membrane and fragmentation of the cell into subcellular bodies, which accumulated in the sinusoidal lumens. Injury of sinusoidal endothelial cells was also observed, leading to peliosis and panlobular sinusoidal congestion (Fig. 2, B and C). The absence of inflammatory cells was consistent with the noninflammatory nature of apoptotic cell death.

Histological analysis of livers from caspase-3−/− mice 3 h after treatment with 10 μg anti-Fas mAb revealed the presence of far less severe apoptosis than in wild-type mice. The lobular architecture of the liver remained intact and sinusoidal congestion was absent in the majority of caspase-3−/− livers (Fig. 2, E and F). A few mutant livers did show some degree of sinusoidal congestion.

**FIGURE 1.** Caspase-3−/− mice show delayed death following treatment with anti-Fas mAb. All wild-type mice (○) died 2–4 h after injection of 100 μg anti-Fas Jo2 Ab, whereas caspase-3−/− mice (●) survived until 6–9 h after injection. Similarly, all wild-type mice died 3–6 h after injection of 10 μg Jo2 Ab, whereas the caspase-3−/− mice died 15–24 h after injection. Seven mice per group were used for each dose of the experiment.

**FIGURE 2.** Histology of livers from wild-type and caspase-3−/− mice following anti-Fas mAb injection. H&E staining of livers from wild-type (+/+ ) and caspase-3−/− (−/−) mice. Sections of livers from untreated (UT) wild-type (A) and caspase-3−/− mice (D) are shown as controls. B and C, Typical features of apoptosis are observed in the livers of wild-type mice 3 h after the injection of 10 μg anti-Fas Ab, including marked condensation of chromatin and cytoplasm, cellular shrinkage, and the destruction of the parenchymal architecture of the liver; B, low power (magnification, ×100); C, high power (×250). E and F, Livers of caspase-3−/− mice 3 h after injection of 10 μg anti-Fas Ab. No apoptotic features are observed; E, low power (×100); F, high power (×250). G and H, Some apoptosis is observed in livers of caspase-3−/− mice 6 h after injection of 10 μg anti-Fas Ab and some hemorrhaging is visible; G, low power (×100), H, high power (×250).
but with far less apoptosis than was observed in anti-Fas injected wild-type livers. At 6 h post-anti-Fas injection, the livers of caspase-3−/− mice remained intact and most hepatocytes were viable. Minimal apoptosis was observed, although sinusoidal congestion was more prominent (Fig. 2, G and H). No comparison to wild-type littermates was made because very few wild-type mice survived until the 6-h time point.

**Increased TUNEL staining in wild-type hepatocytes compared with caspase-3−/− hepatocytes following anti-Fas mAb injection of mice**

The presence or absence of apoptosis in mouse livers following injection of 10 μg anti-Fas mAb was confirmed by TUNEL staining. No TUNEL staining was observed in either wild-type or caspase-3−/− livers at 90 min after treatment (data not shown). However, at 3 h postinjection, intense fluorescence was observed in livers of anti-Fas mAb-injected wild-type mice, indicating the occurrence of massive apoptosis (Fig. 3, A and B). In contrast, livers from caspase-3−/− mice showed minimal TUNEL staining at 3 h postinjection (Fig. 3, C and D). Even at 6 h postinjection, the livers of caspase-3−/− mice showed less TUNEL staining than livers of wild-type mice at 3 h postinjection (Fig. 3, E and F).

**Dramatic decrease in Bcl-2 expression in wild-type but not caspase-3−/− liver following treatment with anti-Fas mAb**

The cleavage of Bcl-2 by caspase-3 has been shown to abolish the antiapoptotic function of the Bcl-2 molecule and to convert it to a Bax-like proapoptotic molecule (28). Livers of wild-type and caspase-3−/− mice that had been treated with 10 μg anti-Fas mAb were therefore examined for Bcl-2 expression using immunohistochemistry. At 90 min post-anti-Fas mAb injection, no differences in staining pattern were observed between wild-type and caspase-3−/− hepatocytes (data not shown). However, 3 h after anti-Fas mAb treatment, Bcl-2 levels were dramatically decreased compared with the baseline in wild-type liver (Fig. 4, B and C), presumably due to Bcl-2 processing during apoptosis. In contrast, the intensity of Bcl-2 expression did not decrease in livers of injected caspase-3−/− littermates. Instead, Bcl-2 was maintained in mutant cells in a punctate pattern consistent with localization in the mitochondria (Fig. 4, D and G). To ensure that the Bcl-2 expression pattern was not merely a characteristic inherent to livers of caspase-3−/− mice, levels of p53 expression were measured as a control in wild-type (Fig. 4D) and caspase-3−/− (Fig. 5H) livers 3 h after anti-Fas mAb injection. No differences in the p53 expression pattern were noted. These results suggest that Bcl-2 is processed in vivo in response to Fas ligation and that this processing requires caspase-3.

**Attenuation of cytochrome c release in hepatocytes of caspase-3−/− mice following anti-Fas mAb injection**

To determine whether caspase-3 is required for cytochrome c release during Fas-mediated apoptosis in vivo, the localization of cytochrome c in hepatocytes of wild-type and caspase-3−/− mice treated with anti-Fas mAb was determined in liver sections using immunofluorescence. In untreated livers of both wild-type and caspase-3−/− mice, cytochrome c appeared in the cytoplasm in a punctate manner indicative of mitochondrial localization (Fig. 5A). When livers were examined 3 h after injection of 10 μg anti-Fas

**FIGURE 3.** TUNEL staining of wild-type and caspase-3−/− livers after injection of 10 μg anti-Fas Ab. A and B, Section of wild-type liver taken 3 h postinjection of anti-Fas mAb showing the intense TUNEL staining characteristic of massive apoptosis. C and D, Section of caspase-3−/− liver showing a virtual absence of TUNEL staining 3 h postinjection. E and F, Section of caspase-3−/− liver at 6 h post-anti-Fas injection. TUNEL staining has increased compared with the 3-h time point in the mutant but is still less than the amount of staining in wild-type liver at 3 h post-anti-Fas. A, C, and E, Low power (×100); B, D, and F, high power (×250).
mAb, hepatocytes from wild-type mice showed dramatic pan-fluorescence, consistent with a massive release of cytochrome c into the cytoplasm (Fig. 5B). However, most hepatocytes from livers of injected caspase-3−/− littermates did not show this pattern of staining but rather retained the punctate pattern of fluorescence, suggesting that cytochrome c release to the cytoplasm was blocked (Fig. 5C). The punctate pattern of immunofluorescence was still prominent in hepatocytes of caspase-3−/− livers even at 6 h post-anti-Fas mAb injection (Fig. 5D).

**Requirement for caspase-3 in processing of regulatory anti- and proapoptotic proteins**

Studies in vitro have suggested that caspase-3 is a downstream member of the apoptotic protease cascade triggered by either activated caspase-9 or -8 (16, 21). However, in hepatocytes from caspase-3−/− mice injected with anti-Fas mAb, we observed decreased processing of both Bcl-2 and Bcl-xL, considered to be upstream regulatory proteins of apoptosis initiation (Fig. 6A). The processing of caspase-9, caspase-8, and Bid was also decreased in anti-Fas-treated caspase-3−/− mice compared with the wild type (Fig. 6B). Interestingly, cleavage of the downstream effector enzyme caspase-7 was also impaired in the absence of caspase-3. Caspase-6, another downstream effector, was not cleaved in either anti-Fas-treated wild-type or caspase-3−/− hepatocytes (Fig. 6C). These results show that caspase-3 does not function only as apoptotic enzyme downstream of caspase-8 and -9, but rather that caspase-3 also plays an important role at the earliest steps of the initiation of apoptosis, serving to modify crucial upstream caspases and pro- and antiapoptotic regulatory proteins.

**Discussion**

Fas-mediated cell death of hepatocytes is implicated in many human liver pathologies, including hepatitis B virus-related cirrhosis, autoimmune hepatitis, acute liver failure, rejection of transplanted livers, and alcoholic and toxin-induced liver diseases (29–31). Because effective therapies are not yet available for most of these disorders, the elucidation of the mechanism of Fas-mediated hepatocyte PCD could provide valuable insights leading to specific therapeutic options. The present study was designed to address...
whether caspase-3 is required for Fas-mediated hepatocyte apoptosis in vivo and whether an absence of caspase-3 can prolong the survival of the animal.

Caspase-3 is usually thought of as the downstream effector protease most important for the classic nuclear changes associated with apoptosis (10, 32). However, data derived from in vitro studies have indicated that caspase-3 can cleave a number of other substrates. It is now known that cleavage of the anti-apoptotic protein Bcl-2 into a proapoptotic form is a necessary step in Fas-mediated PCD, and that caspase-3 mediates this processing (28).

In this report, we provide direct in vivo evidence that caspase-3 is essential for the initial steps of the massive hepatocyte apoptosis that occurs in response to Fas ligation and leads to the rapid death of wild-type animals. Caspase-3−/− mice were resistant to death induced by injection of agonistic anti-Fas mAb, and caspase-3−/− hepatocytes showed evidence of a block in apoptosis. Bcl-2 expression was maintained at control levels in anti-Fas-treated mutant hepatocytes, and cytochrome c release from mitochondria was limited. Proteolytic processing of the upstream proteins Bcl-2, Bcl-xL, caspase-8, caspase-9, and Bid could not proceed in the absence of caspase-3. The precise mechanism of delayed hepatic cell death remains unclear. However, it is clear that caspase-3 is essential for the initial steps of the massive hepatocyte apoptosis that occurs in response to Fas ligation and leads to the rapid death of wild-type animals. Caspase-3−/− mice were resistant to death induced by injection of agonistic anti-Fas mAb, and caspase-3−/− hepatocytes showed evidence of a block in apoptosis. Bcl-2 expression was maintained at control levels in anti-Fas-treated mutant hepatocytes, and cytochrome c release from mitochondria was limited. Proteolytic processing of the upstream proteins Bcl-2, Bcl-xL, caspase-8, caspase-9, and Bid could not proceed in the absence of caspase-3. The precise mechanism of delayed hepatic cell death remains unclear. However, it is clear that caspase-3 is essential for the initiation of apoptosis in response to Fas ligation. A delay in the initiation of apoptosis of hepatocytes would result in slower deterioration of the liver and prolonged survival of the mutant mice.

From our data, we speculate that the primary role of caspase-3 during Fas-mediated apoptosis in vivo is to cleave Bcl-2 and Bcl-xL, abrogating their ability to block the apoptotic cascade. Although Bcl-2 and Bcl-xL are CED-9 homologues and well established as antiapoptotic proteins, their precise functions during the cell death process are unclear. The structure of the Bcl-xL protein

FIGURE 5. Cytochrome c localization in wild-type and caspase-3−/− livers following anti-Fas mAb injection. Immunostaining of liver sections showing intracellular localization of cytochrome c. A, Similar basal levels of cytochrome c are seen in sections of wild-type and caspase-3−/− livers before treatment with anti-Fas mAb. Fluorescence occurs strictly in a punctate pattern consistent with mitochondrial localization. B, Section of liver from a wild-type mouse 3 h postinjection of 10 μg anti-Fas mAb. Intense but diffuse pan-fluorescence throughout the cell represents significant cytochrome c release from the mitochondria into the cytoplasm. C and D, Sections of liver from caspase-3−/− mice 3 and 6 h postinjection of 10 μg of anti-Fas mAb, respectively. C, At 3 h postinjection, most cells exhibit low levels of fluorescence confined in the punctate pattern. A small proportion of cells show increased and slightly more diffuse fluorescence, indicating limited release of cytochrome c into the cytoplasm. D, At 6 h postinjection, the staining pattern in caspase-3−/− liver remains similar to that observed at 3 h.

FIGURE 6. Proteolytic processing of upstream regulatory proteins is impaired in caspase-3−/− hepatocytes. Western blot analyses of lysates of hepatocytes from wild-type and caspase-3−/− mice following anti-Fas mAb injection. A, Protein levels of intact Bcl-2 and Bcl-xL are reduced in liver lysates of treated wild-type mice compared with untreated (UT) controls. In contrast, processing of Bcl-2 and Bcl-xL is impaired in caspase-3−/− hepatocytes, leading to similar levels of Bcl-2 and Bcl-xL protein in treated and untreated caspase-3−/− cells. Anti-β-actin Ab was used to verify equivalent levels of total protein in each lane. B, Inhibited processing of caspase-8, -9, and Bid is apparent in lysates of caspase-3−/− hepatocytes at 3 h after anti-Fas injection compared with the wild type (+/−). C, The downstream enzyme caspase-6 is not cleaved in lysates of hepatocytes from either treated wild-type (+/−) or caspase-3−/− (−/−) mice, whereas caspase-7 is processed in treated wild-type hepatocytes (+/−), but not in caspase-3−/− (−/−) hepatocytes.
is reminiscent of the pore-forming proteins of bacterial toxins such as diphtheria toxin and the colicins, and it has been hypothesized that Bcl-xL may function as an ion channel regulating the permeability of the mitochondria (33). Such an ion channel could minimze osmotic stress and in so doing prevent the mitochondrial matrix swelling and outer membrane disruption that lead to cytochrome c release. A concise mechanism for Bcl-2 function has yet to be reported. Nevertheless, many laboratories have shown that Bcl-2 is capable of inhibiting cytochrome c release from mitochondria (34). In addition, both Bcl-2 and Bcl-xL have been shown to directly block caspase-8 activity (35), which may also contribute to the prevention of cytochrome c release.

The roles of Bcl-2 and Bcl-xL in Fas signaling have yet to be clarified. Several groups have investigated whether mammalian CED-9 homologues can inhibit Fas-mediated apoptosis, but conflicting data have been obtained. The results vary from no inhibition (36, 37), to partial inhibition (38, 39), to substantial inhibition (33). It has been postulated that the level of caspase-8 activity generated by the Fas DISC complex and the levels of downstream substrates may determine whether Bcl-2 or Bcl-xL can inhibit Fas-mediated death (23). In cell types in which abundant caspase-8 activity is present in the Fas-DISC complex, Bcl-2 and Bcl-xL are capable of inhibiting apoptosis following Fas ligation. However, in cell types that have little caspase-8 activity in the DISC complex, Bcl-2 and Bcl-xL are not able to inhibit apoptosis (23).

We propose a model in which a positive amplification loop is required for Fas-mediated cell death, and that this loop depends on caspase-3 activity. In the presence of caspase-3, Bcl-2 and Bcl-xL are converted to their proapoptotic forms. Caspase-8, caspase-9, and Bid are also processed and activated. Cytochrome c is released from the mitochondria, either because Bcl-2 and Bcl-xL are no longer intact and able to protect the mitochondria from osmotic stress, and/or because caspase-8 and Bid, which promote cytochrome c release, are freed from inhibition by unprocessed Bcl-2 and/or Bcl-xL. In the presence of dATP, Apaf-1 and caspase-9 complex with the released cytochrome c and caspase-9 is activated, triggering the mitochondrial pathway of PCD. The cascade of enzymatic activation that follows includes the cleavage of additional procaspase-3 by activated caspase-9. Thus, a positive feedback loop is created in which activated caspase-3 acts upstream to irreversibly commit the cell to PCD and downstream to trigger its execution.

In the absence of caspase-3, Bcl-2 and Bcl-xL are not processed and remain in the intermembrane space of the mitochondria. Similarly, caspase-8 and Bid are not activated. Both events may effectively block the release of cytochrome c. Without abundant cytochrome c in the cytoplasm, the positive feedback loop is broken, the activation of caspase-9 is impaired, downstream caspses such as caspase-7 are not cleaved, the initial amplification of caspase cascade is aborted, and the cells remain viable. Although our results in caspase-3−/− mice are consistent with published reports of the effects of caspase inhibitors on hepatocyte PCD in vivo (40), they stand in contradiction to a recently published report (41) in which the viability of hepatocytes of wild-type and caspase-3−/− mice did not differ in response to Fas ligation. However, Fas ligation in this case was induced in vitro rather than in vivo in an experimental setting that has been documented to affect the kinetics of apoptosis and the sensitivity of cells to Fas-mediated PCD (15).

Caspase-3 deficiency did not completely rescue the mutant mice from death due to liver failure. There was no evidence of inflammation in mutant livers, a finding that would have suggested cell death by necrosis rather than apoptosis. It is possible that the death of the animals could be due to a slower PCD pathway that depends on other known caspases (12), as yet unknown caspases, or perhaps no caspases at all (29). For example, PCD mediated by the apoptosis-inducing factor (AIF) may operate independently of caspase-mediated pathways (16). The existence of such pathways and their physiological relevance in vivo remain to be determined.

In conclusion, this study provides in vivo evidence that caspase-3 is required for the initial events that occur in hepatocyte cell death following Fas ligation. The data are consistent with a scenario in which caspase-3 modifies Bcl-2 and/or Bcl-xL expression such that their antiapoptotic functions are abrogated, the integrity of the mitochondrial membrane is compromised, and cytochrome c crucial for an irreversible commitment to PCD is released. The caspase-3−/− mice have important clinical implications because fulminant liver failure is a very accelerated process that rapidly leads to death. A means of inhibiting caspase-3 so that liver failure is slowed may gain the physician a critical window of time for interventions promoting hepatocyte regeneration. Thus, the elucidation of the precise roles of caspsases in mouse liver failure may lead to the development of therapeutic drugs designed to alleviate liver damage in humans.

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