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Mutations to Bid Cleavage Sites Protect Hepatocytes From Apoptosis After Ischemia/Reperfusion Injury

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Abstract

Background—Apoptosis of hepatocytes contributes to many forms of liver pathology and can compromise liver function. Hepatocytes have been shown to require mitochondrial disruption to execute apoptosis, a process that is controlled by members of the Bcl-2 family. Bid is a proapoptotic Bcl-2 family member that is cleaved to its active form, tBid, by caspase 8 and granzyme B. Studies in the Bid-deficient mouse have established that hepatocytes require Bid to undergo apoptosis.

Methods—We generated aspartic acid to glutamic acid mutations in the rat Bid protein, at the caspase 8 and granzyme B cleavage sites, and utilized recombinant adenoviruses to express this protein in hepatoma cells and in the livers of rats.

Results—Cells transduced with recombinant adenoviruses encoding Bid containing mutations to the caspase 8 and granzyme B cleavage sites are significantly protected from both tumor necrosis factor-α–induced and cell-mediated apoptosis. Protection occurs through a mechanism that includes decreased Bid cleavage, caspase activation, and mitochondrial membrane damage. Further, after warm ischemia/reperfusion injury, we show that rats expressing cleavage-resistant Bid in the liver display significantly less hepatocyte apoptosis as compared to control rat livers and this results in improved liver function and survival.

Conclusion—Our results suggest that reagents that prevent the cleavage of Bid would be an effective strategy to inhibit hepatocyte apoptosis and decrease liver injury.

Keywords

Apoptosis; Bid; Liver

Hepatocytes are uniquely sensitive to induction of apoptosis because they express the death receptors Fas, tumor necrosis factor (TNF) receptor 1 (TNFR1), and TNF-related apoptosis-inducing ligand receptor 2 (TRAILR2/DR5) but do not express the antiapoptotic protein, Bcl-2 (1, 2). Indeed, hepatocyte apoptosis is a characteristic of many forms of liver pathobiology, including viral hepatitis, cholestasis, transplant rejection, and ischemia/

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reperfusion (I/R) injury (3–7). Ligation of the death receptors with their respective ligands, FasL, TNFα and TNF-related apoptosis-inducing ligand (TRAIL), activates the extrinsic pathway of apoptosis. In this pathway, death receptor ligation leads to the activation of caspase 8, which then directly activates downstream caspase 3 that culminates in apoptotic cell death (8). However, in some cell types, including hepatocytes, caspase 8 activation alone is insufficient to activate caspase 3 and fully propagate the death signal (9, 10). In this case, the apoptosis cascade proceeds through the intrinsic pathway, where caspase 8 activation of downstream caspase 3 requires amplification through mitochondrial membrane destruction.

Proapoptotic and antiapoptotic members of the Bcl-2 family are critical in the regulation of mitochondrial-dependent pathways of apoptosis. The Bcl-2 family of proteins is defined by the presence of at least one of four homology domains, BH1 to BH4. Bid contains only the BH3 domain and is a 23-kDa, proapoptotic member of the Bcl-2 family (11). Bid is distinguished from other proapoptotic Bcl-2 family members in that it plays a critical role in both receptor- and granule-mediated apoptosis because Bid is a substrate for both active caspase 8 and granzyme B. Active caspase 8 cleaves Bid at aspartic acid residue 60 (D60), while granzyme B preferentially cleaves Bid as aspartic acid residue 76 (D76) (11–13). The cleavage product as processed by either protease, termed truncated Bid (tBid), then translocates to the mitochondria through mechanisms that include selective binding to cardiolipin, a mitochondrial membrane lipid (14). Once at the mitochondria, tBid induces the oligomerization of Bax and Bak, leading to mitochondrial membrane permeability and release of mitochondrial proteins such as cytochrome c and Apaf-1 (15, 16). Cytochrome c and Apaf-1 form a complex with procaspase 9, and together with ATP/dATP form the apoptosome, which activates caspase 9 (17). Active caspase 9 cleaves and activates caspase 3, leading to DNA fragmentation and completion of apoptosis (18).

Cleavage of Bid is a decisive step in hepatocyte apoptosis, as hepatocytes lacking this protein are resistant to Fas-induced apoptosis. Moreover, this resistance confers protection from the lethality of systemic treatment of agonistic anti-Fas antibodies (9, 19). Because Bid is critical in the relay of cell-surface death signals through the intrinsic apoptosis pathway in hepatocytes, Bid cleavage is an attractive target to prevent hepatocyte apoptosis. We generated mutations in the rat Bid protein at the caspase 8 and granzyme B cleavage sites, and utilized recombinant adenoviruses to express this protein in hepatoma cells and in the livers of rats. Our results suggest that disruption of Bid cleavage can prevent liver injury.

MATERIALS AND METHODS

Generation of Cleavage-Resistant Bid and Cell-Free Cleavage Assays

Rat Bid in pET28a was used as a template for site-directed mutagenesis using the QuikChange site-directed mutagenesis kit per manufacturer's instructions (Stratagene, La Jolla, CA). Aspartic acid residues, D60 and D76 were changed to glutamic acid, and assessed by DNA sequencing. Three Bid mutants were constructed: BidD60E, BidD76E, and a double mutant BidD60E/D76E (BidEE). For cell-free cleavage assays, purified, wild-type Bid (wtBid) and Bid mutant proteins (10–20 μg) were incubated with active human

caspase 8 (1 U; Alexis Biochemicals, San Diego, CA) for 1 hr at 37°C or with human granzyme B (0.7 U; Alexis Biochemicals) for 1 hr at 25°C.

Generation of Recombinant Adenovirus

Homologous recombination was used to generate adenoviruses encoding BidEE or the bacterial X-Gal-responsive gene, LacZ (20). Adenoviral recombinants containing BidEE were verified by restriction digest and DNA sequencing. Adenoviruses were packaged in HEK 293 cells, purified over two CsCl gradients, and titered by plaque assay (21).

Apoptosis Assays on FaO Cells

FaO cells are a rat hepatoma cell line derived from the AxC rat strain and have been described as a model to study apoptosis of a hepatocyte-like cell (22, 23). FaO cells readily undergo apoptosis upon treatment with TNFα through a pathway that includes Bid cleavage and mitochondrial membrane damage (24). FaO cells were plated (3×10^5 cells/ml), and infected to >95% with 30–50 multiplicity of infection (MOI) for each virus for 36 hr. After this, cells were treated with rat recombinant TNFα(20 ng/ml) (R&D Systems, Minneapolis, MN) and actinomycin D (0.2 μg/ml; Sigma, St. Louis, MO) for 4 or 6 hr, or with staurosporine (2 μM, Sigma) for 24 hr. Hypodiploid DNA was quantitated by staining with propidium iodide followed by flow cytometry on a FACScan (BD Biosciences, La Jolla, CA) and analyzed using Cell Quest software (BD Biosciences). Apoptotic FaO cells were visualized by adding Hoeschst 33342 (1 μM; Molecular Probes, Eugene, OR) at 25°C for 15 min after treatment. Nuclei were visualized through an ultraviolet filter on an inverted microscope.

Mitochondrial Membrane Potential Analysis

Loss of mitochondrial membrane potential was measured using dihexyloxacarbocyanine iodide ($DiOC_{6,}$ Molecular Probes) followed by flow cytometry. After apoptosis induction, cells were harvested washed once in fluorescence-activated cell sorting buffer (0.5% fetal calf serum and 0.1% of sodium azide in phosphate-buffered saline) and incubated with 40 nM $DiOC_6$ for 15 min at 37°C. Data are presented as a percentage loss of mean fluorescence intensity compared to untreated control cells.

Immunoblotting and Caspase Activation Assays

After treatment with TNFα(20 ng/ml) and actinomycin D (0.2 μg/ml), FaO cells were lysed in lysis buffer (50 mM Tris buffer pH 7.4, 1% NP-40, 0.5% deoxycholic acid, 150 mM NaCl, 0.5 mM ethylenediamine tetraacetic acid, 2 mM PMSF, 0.5 mg/ml aprotinin, 0.5 mg/ml leupeptin, 0.05 mg/ml pepstatin), and lysates (100 μg) separated by 12% SDS-PAGE. Proteins in the gel were transferred to nitrocellulose and immunoblotted with anti-rat Bid antibodies (5 μg/ml or 8 μg/ml, as indicated) or anti-β-actin antibodies (1:1000 dilution; Sigma), followed by horseradish peroxidase (HRP)-goat anti-rabbit or HRP-goat anti-mouse secondary antibodies (Jackson Immunoresearch). AdBidEE could be distinguished using our polyclonal anti-Bid antibodies since the N-terminal Flag tag on AdBidEE increases its size by approximately 1 kDa, a difference which can be resolved using SDS-PAGE. Thus, we are able to monitor the expression of AdBidEE relative to the endogenous Bid expressed by

FaO. Immunoblots were visualized using ECL chemiluminescence (Amersham). Lysates were analyzed for caspase activation by utilizing colorimetric caspase 8, caspase 9, and caspase 3 activation assays (Alexis Biochemicals) as per manufacturer's instructions.

Natural Killer Cell Apoptosis Assay

FaO cells (0.4×10^6 cells/ml) were infected with AdBidEE or AdLacZ (50 MOI) for 36 hr. Infected FaO target cells were labeled with ^{3}H-thymidine (5 μCi/ml; Perkin-Elmer, Boston, MA) for 24 hr prior to use (25). RNK-16 effector cells were added to FaO targets in the presence or absence of EGTA (2.5 mM final concentration) at the indicated E:T ratios for 4 hr, harvested, and intact DNA measured on a beta plate reader (Wallac, Gaithersburg, MD). Apoptosis was calculated using $((S-E)/S)\times100$, where S was retained DNA without effectors and E was retained DNA with effectors.

Warm Ischemia/Reperfusion Injury

Wistar rats underwent warm I/R injury, 48 hr after infection with AdLacZ or AdBidEE, through clamping of the thoracic aorta for 30 min followed by restoration of blood flow for an additional hour, or clamping of the portal vein, hepatic artery and hepatic inferior vena cava (total vascular exclusion) for 60 min followed by restoration of blood flow for one hour (26). Animals were sacrificed immediately after treatment unless otherwise indicated and livers were harvested from the rats after sacrifice. Paraffin-embedded sections were analyzed for apoptotic hepatocytes by terminal UDP nicked-end labeling (TUNEL) using the ApoTag peroxidase in situ apoptosis kit, per manufacturer's instructions (Chemicon International, Temecula, CA). Sections were counterstained in methyl green for 20 min at 60°C. TUNEL$^+$ hepatocytes were quantitated by a blinded counting of 20 fields for each animal (n=3 for each group) at 20× magnification. Hepatic injury was determined by serum aspartate aminotransferase (AST) and serum aminotransferase (ALT) and these analyses were performed by the Department of Comparative Medicine at Stanford University. All experimental procedures were performed in compliance with the guidelines set forth by Stanford's Administrative Panel for Laboratory Animal Care.

Statistical Analysis

Statistical significance was calculated using the paired Student's t test for cell cycle analysis with FaO cells, colorimetric caspase activation assays, $DiOC_6$ staining, cytotoxicity assays and quantification of TUNEL$^+$ hepatocytes. In the warm I/R injury model, survival was measured through 7 days postinjury and significance between the AdBidEE and AdLacZ treatment groups was calculated using the log-rank Test.

RESULTS

Mutations to Bid Cleavage Sites Protect Bid from Cleavage by Caspase 8 and Granzyme B

The importance of Bid in hepatocyte apoptosis has been clearly demonstrated by the lack of apoptosis, after treatment of hepatocytes with agonist anti-Fas antibodies or TNFα, in Bid$^{-/-}$ mice (19, 27). To prevent the generation of the active tBid, we altered the sites of cleavage on Bid. Using site-directed mutagenesis, we replaced the aspartic acid residues at the sites of cleavage, for caspase 8 and granzyme B, with glutamic acid, generating mutations at the

caspase 8 cleavage site, D60E, or the granzyme B cleavage site D76E, as well as mutations at both sites, BidEE (Fig. 1A) (28, 29). Whereas wild type Bid is cleaved to tBid by caspase 8 in a cell-free cleavage assay, the BidD60E mutant is completely protected from cleavage by caspase 8 (Fig. 1B, n=3). In contrast, BidD60E and wtBid are cleaved by granzyme B, as indicated by the disappearance of full-length BidD60E. (Fig. 1C, n=3). Conversely, the BidD76E mutant is protected from granzyme B cleavage, but not from caspase 8 cleavage (Fig. 1B, C). BidEE, containing mutations at both cleavage sites, is protected from cleavage by caspase 8 as well as granzyme B (Fig. 1B, C). Hence, the introduction of mutations to Bid cleavage sites can block the generation of tBid by active caspase 8 and granzyme B.

FaO Cells Expressing AdBidEE Are Protected From Receptor-Mediated Apoptosis

We tested if BidEE could protect cells of hepatic origin from apoptosis. We generated recombinant adenoviruses that express BidEE (AdBidEE), and observed expression (n=2) in FaO cells over a wide range of multiplicities of infection (15–1000 MOI; Fig. 2A).

FaO cells were infected with 50 MOI of AdBidEE for 36 hr, followed by treatment with TNFα and actinomycin D, and apoptosis measured by cell-cycle analysis. Figure 2B is a representative of four independent experiments in which we observed hypodiploid DNA, indicating apoptosis in 39.2% of FaO cells expressing AdLacZ, whereas only 4.8% of FaO cells expressing AdBidEE were apoptotic after 6 hours of treatment with TNFα and actinomycin D. In four independent experiments, summarized in Figure 2C, we observed that FaO cells expressing AdBidEE had a significant reduction in apoptosis in (3.7±1.6%) compared with AdLacZ-expressing FaO cells (33.1±7.2%, $P<0.005$) after 4 hours of treatment with TNFα and actinomycin D. Similarly, we observed a significant decrease in apoptosis after six hours of treatment in AdBidEE-expressing FaO cells (5.4±1.1%) compared to AdLacZ-expressing FaO cells (51.9±3.6%, $P<0.005$; Fig. 2C). In contrast, staurosporine, a protein kinase inhibitor and potent apoptosis inducer that does not rely on Bid cleavage, triggered similar amounts of apoptosis in AdLacZ- and AdBidEE-expressing FaO cells (Fig. 2C). Further, we assessed if adenoviral infection itself could induce apoptosis by infecting FaO cells with AdLacZ and AdBidEE for 96 hr, and measuring cellular viability using both trypan blue exclusion and cell cycle analysis. We observed that infection at 50 MOI of each virus had no affect on either the viability or growth of FaO cells over the 96-hour infection period (data not shown). We noted that BidEE provides protection from apoptosis even when not overexpressed and in the presence of endogenous Bid (Fig. 2A). In fact, protection from apoptosis is similar at higher MOI (data not shown); thus, we utilized the lowest MOI that is effective (50 MOI) in all subsequent experiments.

In addition to DNA fragmentation, apoptosis induces several morphological changes that are readily visualized. Using Hoechst 33342 to stain DNA, we detected brightly staining nuclei in AdLacZ-infected control cells after 6 hr of treatment with TNFα and actinomycin D (Fig. 2D, top left panel, arrowheads, a representative of two experiments is shown), indicating that these cells had undergone the chromatin condensation typically observed during apoptosis. In contrast, FaO cells expressing AdBidEE have intact, less-brightly staining nuclei. Phase contrast photos (Fig. 2D, right panels) show more rounded and detached cells in the AdLacZ-infected cells than in the AdBidEE-infected cells, providing further evidence

that the AdBidEE-expressing FaO cells are protected from apoptosis. Thus, expression of AdBidEE protects FaO cells from TNFα-mediated apoptosis and this protection can be observed by both a reduction in the morphological changes consistent with apoptosis, and the generation of hypodiploid DNA.

FaO Cells Expressing AdBidEE Display Less Bid Cleavage and Caspase Activation

Having demonstrated that FaO cells expressing AdBidEE are protected from TNFα-mediated apoptosis, we further examined the biochemical events of the intracellular apoptosis pathway. We prepared lysates from AdLacZ-and AdBidEE-infected FaO cells that were untreated, or treated with TNFα and actinomycin D for 6 hours, and measured caspase 8 and caspase 3 activation. AdLacZ-expressing FaO cells treated with TNFα displayed a 59.2±1.6% average increase in active caspase 8 over media alone, compared with only 18.9±3.2% in AdBidEE-expressing FaO cells treated with TNFα(Fig. 3A; n=4). Moreover, AdLacZ-expressing FaO cells showed a 193.4±9.2% average increase active caspase 3 over media alone, compared to 53.3±9.2% in AdBidEE-expressing FaO cells (Fig. 3B; n=4). In AdLacZ-infected FaO cells, we detected a decrease of the full-length Bid by Western blot, indicating that Bid is cleaved in these cells, whereas AdBidEE-expressing FaO cells retained full-length Bid upon treatment with TNFα(Fig. 3C; n=3). Bid cleavage products are not reliably detected in FaO cells undergoing apoptosis, possibly due to the small quantities of tBid that are generated. However we do detect Bid cleavage products concomitantly with a decrease in full-length bid as shown in our cell-free experiments (Fig. 1B).

The extent of mitochondrial membrane permeabilization in AdLacZ- and AdBidEE-expressing FaO cells was examined by labeling with $DiOC_6$, which is released from the mitochondria upon membrane damage. In four independent experiments, we observed that AdLacZ-transduced cells had a significant loss of $DiOC_6$ fluorescence after six hours of treatment with TNFα(36.2±4.9%, $P<0.001$), compared with AdBidEE-transduced FaO cells (6.8±3.0%; Fig. 3D, n=4). These results indicate that AdLacZ-expressing FaO cells sustain significantly more damage to the mitochondrial membrane than do AdBidEE-expressing FaO cells. Based on these observations, we conclude that expression of cleavage-resistant Bid protects FaO cells through a pathway that includes decreased activation of caspases, Bid cleavage, and mitochondrial membrane damage.

FaO Cells Transduced With AdBidEE Are Protected From Cell-Mediated Apoptosis

Effector cell induction of hepatocyte apoptosis has been implicated in a variety of liver pathologies. Natural killer (NK) cells are an abundant population in the liver, comprising up to 50% of total lymphocytes in normal liver (30, 31). NK cell-mediated death of hepatocytes has been observed during the immune response to viral infections such as cytomegalovirus and hepatitis B, as well as during graft rejection (31–34). We determined if expression of AdBidEE would protect FaO cells from NK cell-mediated cell death by measuring DNA fragmentation of ^{3}H-thymidine labeled FaO cells that have been incubated with the rat NK-cell line, RNK-16. We observed significantly less ($P<0.01$: n=4) cell death in FaO cells expressing AdBidEE compared to AdLacZ-infected FaO cells at every effector to target (E:T) ratio tested (Fig. 4A). We further investigated the mechanism of this partial protection by assessing if RNK-16-mediated lysis was dependent on granule release. When the

apoptosis assays were performed in the presence of EGTA, a calcium chelator that blocks the calcium-dependent NK cell granule release, we observed a nearly complete blockage of cell death, suggesting that RNK-16-mediated lysis is granule dependent (Fig. 4B, n=4). These data indicate that expression of cleavage-resistant Bid provides partial protection against NK cell-mediated cell lysis.

Expression of AdBidEE Protects Rats From Ischemia/Reperfusion Injury

Prevention of Bid cleavage protects cells from apoptosis in vitro, thus we determined if expression of AdBidEE would protect hepatocytes from apoptosis during liver injury. AdBidEE was expressed in Wistar rats, which were then subjected to one of two models of warm ischemia/reperfusion (I/R) liver injury: a nonlethal 30-minute ischemia model or a lethal 60-minute ischemia model. We have demonstrated that 30 min of warm ischemia induced by clamping of the thoracic aorta followed by 1 hour of warm reperfusion as blood flow is restored to the liver induces significant hepatocyte apoptosis in Wistar rats (data not shown).

Expression of AdBidEE in Wistar rat liver lysates is detected 48 hr after intravenous injection with 5×10^9 pfu/ml of AdBidEE (Fig. 5A: n=2). Forty-eight hours after injection with either AdBidEE (n=3) or control AdLacZ (n=3), rats underwent a nonlethal I/R injury. Apoptotic hepatocytes were identified by in situ TUNEL staining of liver sections obtained after induction of I/R injury. We observed significantly less hepatocyte death in the livers of animals expressing AdBidEE (Fig. 5B, right panel) after I/R injury as compared to animals expressing control AdLacZ (Fig. 5B, left panel). AdLacZ-expressing rats (n=3) contained an average of 11.9±1.5 $TUNEL^+$ hepatocytes per field after I/R injury, whereas AdBidEE-expressing rats (n=3) contained an average of 2.4±1.4 $TUNEL^+$ hepatocytes/field, suggesting that these animals have significantly more viable hepatocytes after I/R injury (Fig. 5C, $P<0.05$). We investigated the biochemical pathway underlying this protection by measuring liver lysates from AdBidEE and AdLacZ animals, after nonlethal I/R injury, for activation of caspase 9 and 3. We observed significantly less active caspase 9 and caspase 3 in liver lysates from AdBidEE-expressing rats as compared to liver lysates from AdLacZ controls after I/R injury (Fig. 5D and E, n=3), suggesting that expression of BidEE protects hepatocytes from the cytochrome c and ATP-dependent pathway of apoptosis (Fig. 5D, E).

Serum obtained from rats 1 hour after I/R injury was measured for aspartate aminotransferase (AST) levels. We observed eightfold higher levels of serum AST in the AdLacZ-expressing control rats (17362±6636, n=3) compared with animals expressing AdBidEE (2198±1121 IU/L, $P<0.05$, Fig. 5F). Similarly, the serum alanine transferase (ALT) levels were higher in the AdLacZ-expressing control rats (18815 ± 8507, n=3) compared with animals expressing AdBidEE (2935±2848 IU/L). The lower serum AST/ALT levels in the AdBidEE rats compared with the AdLacZ-expressing controls correlates with the decreased $TUNEL^+$ hepatocytes and caspase activation in AdBidEE-expressing animals.

The efficacy of AdBidEE expression to protect hepatocytes and preserve liver function was examined in a more stringent model of warm I/R injury to the liver. In this model, ischemia is induced for 60 min by clamping of the hepatic artery, the portal vein, and the hepatic

inferior vena cava (IVC) followed by reperfusion for an additional hour. Similar to the 30-min ischemia model, we found that AdBidEE-expressing rats had significantly ($P<0.05$; n=5) fewer apoptotic hepatocytes (22.8±8.9) compared with AdLacZ-expressing rats (61.6±18.7) as detected by TUNEL staining immediately after this treatment. Importantly, rats expressing AdBidEE (n=8) had significantly improved survival after I/R injury compared with AdLacZ-expressing rats ($P=0.0079$, n=6, Fig. 5G). One week after I/R injury, all AdBidEE-treated rats tested had normal ALT levels (352±320 IU/L; n=3). Normal levels of ALT are 116–384 IU/L in the rat. AST levels were similarly decreased to close to normal levels at 1 week after I/R injury All untreated and LacZ-expressing animals died within the first 24 hr after I/R injury, whereas all AdBidEE-expressing animals survived at least 2 days, with five of eight rats (62.5%) surviving the 7-day study period. These results indicate that BidEE expression protects hepatocytes from apoptosis in vivo and leads to decreased liver damage and survival after ischemia/reperfusion injury.

DISCUSSION

Studies using Bid-deficient mice have clearly established the importance of Bid in Fas induced apoptosis of hepatocytes. We have shown for the first time that a non-cleavable form of Bid protects FaO cells from death-receptor and cell-mediated apoptosis, even in the presence of endogenous Bid. Further, we show that blockade of Bid cleavage protects hepatocytes from warm I/R injury, protects from liver damage, and improves survival.

AdBidEE expression protects hepatocytes from apoptosis that occurs during warm ischemia/reperfusion (I/R) injury to the liver. Warm I/R injury is common during cardiac arrest, liver surgery and resection, and liver transplantation and has been shown to result in apoptosis of hepatocytes (5, 35). Studies by Bilbao et al. indicate that adenoviral expression of anti-apoptotic Bcl-2 in mouse liver protects cells from apoptosis, and preserves liver function after cold ischemia/reperfusion injury (36). Further, a recent study demonstrates that administration of a pancaspase inhibitor, IDN-6556, abrogates apoptosis and results in lower serum AST and ALT levels after cold ischemia/reperfusion injury (37, 38). Together with our findings, these studies confirm the importance of the intrinsic apoptosis pathway in hepatocytes during I/R as occurs during organ preservation and surgery, thereby establishing the feasibility of targeting Bid cleavage in the design of therapeutics to prevent hepatocyte apoptosis and preserve liver function.

Previous studies have demonstrated that $Bid^{-/-}$ hepatocytes are resistant to Fas-mediated apoptosis, and biochemical analysis revealed similar levels of active caspase 8 in both the $Bid^{+/+}$ and wild type hepatocytes (19). In contrast, we observed significantly less caspase 8 activation in AdBidEE-expressing FaO cells compared with AdLacZ-expressing controls after TNFα-induced apoptosis, suggesting that blockade of Bid cleavage can affect the activation of apical caspase 8 as well as downstream caspase 3.

Interestingly, our data show that expression of AdBidEE protects FaO cells from apoptosis despite the presence of endogenous, wild-type Bid. We propose that the presence of BidEE is sufficient to block active caspase 8 from cleaving endogenous Bid, thereby functioning phenotypically like a dominant-negative mutant. Our biochemical analysis in AdBidEE-

expressing FaO cells supports this interpretation, as we do not detect Bid cleavage after TNFα treatment. We have not conducted biacore studies to determine if mutant Bid is better able to bind active caspase 8 compared to wild-type Bid; however, by changing the aspartic acid cleavage residues to glutamic acid, we have made a minor structural change that would not, according to the published structure of Bid, affect binding of active caspase 8 (39). In fact, it is possible that the extended side chain of glutamic acid chain compared with aspartic acid might improve caspase 8 binding to Bid, while preventing cleavage. We propose that since BidEE is so structurally similar to Bid, it is able to function as a dominant-negative because of its ability to bind caspase 8 without allowing cleavage.

NK cells have been shown to play a role in viral immunity, where NK cell secretion of perforin and granzyme B is critical for the control of cytomegalovirus in the liver and the secretion of IFNγ has been shown to play a role in hepatocyte damage during hepatitis B infection (32, 33, 40). We have shown that expression of AdBidEE partially protects FaO cells from NK cell-mediated cytotoxicity, suggesting that Bid-independent apoptosis pathways may be engaged, utilizing other lytic proteins in the granule. In support of this, we have shown that when cytotoxicity assays were performed in the presence of EGTA, a calcium chelator that blocks NK cell release of granules, we observed a nearly complete blockage of cytotoxicity.

Recently, there has been an increased focus on the use of donors after cardiac death (DCD) in liver transplantation as a means of increasing the number of donor livers available for transplantation. The overwhelming limitation to the success of using DCD is prolonged warm ischemia, resulting in nonfunction of the transplanted liver (41). By blocking cleavage of Bid in this setting, hepatocytes may be protected from apoptosis, thereby significantly decreasing the incidence of primary nonfunction after liver transplantation. Finally, our observations support the conclusions that Bid cleavage is a decisive event in hepatocyte apoptosis, and that prevention of Bid cleavage protects hepatocytes from apoptosis and decreases liver damage.

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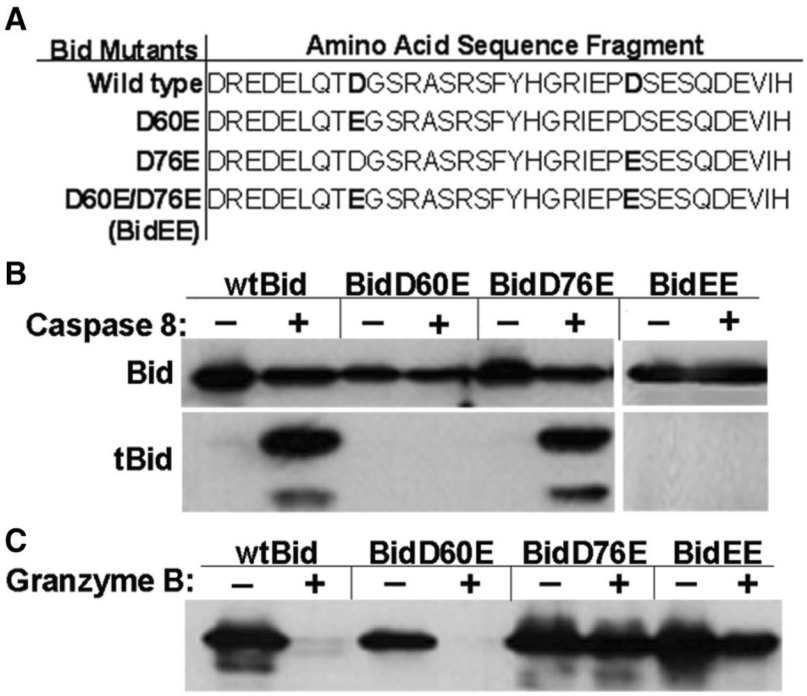

FIGURE 1.

Mutations to cleavage sites protect Bid from caspase 8 and granzyme B cleavage. (A) Aspartic acid (D) cleavage sites (D60 and D76) were mutated to glutamic acid (E). Shown are protein sequence fragments from wild-type Bid (wt Bid), Bid with a mutation at the caspase 8 cleavage site (BidD60E), Bid with a mutation at the granzyme B cleavage site (BidD76E), Bid with mutations to both cleavage sites (BidD60E/ D76E or BidEE). (B and C) Wild-type Bid protein (wtBid) and Bid mutant proteins (BidD60E, BidD76E, and BidEE) were incubated with 1 U of active caspase 8 (B) or with 0.7 U of active granzyme B (C). Cleavage reactions were immunoblotted with anti-Bid (5 μg/ml) antibodies. Shown is a representative of three experiments.

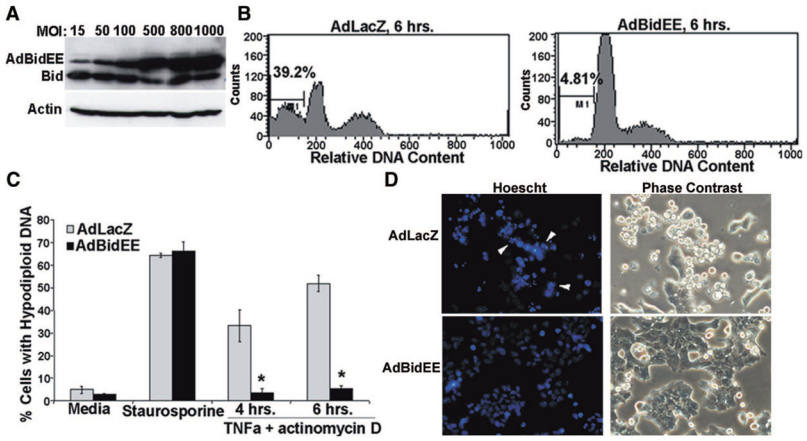

FIGURE 2.

AdBidEE expression protects FaO cells from TNFα-mediated apoptosis. (A) FaO cells were infected with the indicated MOI of AdBidEE for 36 hr. Lysates were measured for BidEE expression by immunoblot with anti-Bid antibodies (5 μg/ml). (B) FaO cells were infected with AdLacZ or AdBidEE (50 MOI) for 36 hr followed by treatment with recombinant rat TNFα(20 ng/ml) and actinomycin D for 4 or 6 hours. Representative histograms of the percent of cells with hypodiploid DNA is shown. (C) The percentage of apoptotic cells was determined in AdLacZ- and AdBidEE-infected FaO cells that were left untreated (media), or treated for 4 or 6 hours with staurosporine (2 μM) or TNFα and actinomycin D, by flow cytometry of the cell cycle (n=4, *P<0.005). (D) AdLacZ- and AdBidEE-expressing FaO cells after treatment with TNFα and actinomycin D for 6 hours were visualized by phase contrast (right) or after Hoechst 33342 (1 μM) staining through an ultraviolet filter (left), at 20× magnification. Experiments shown are representative of two to four separate experiments.

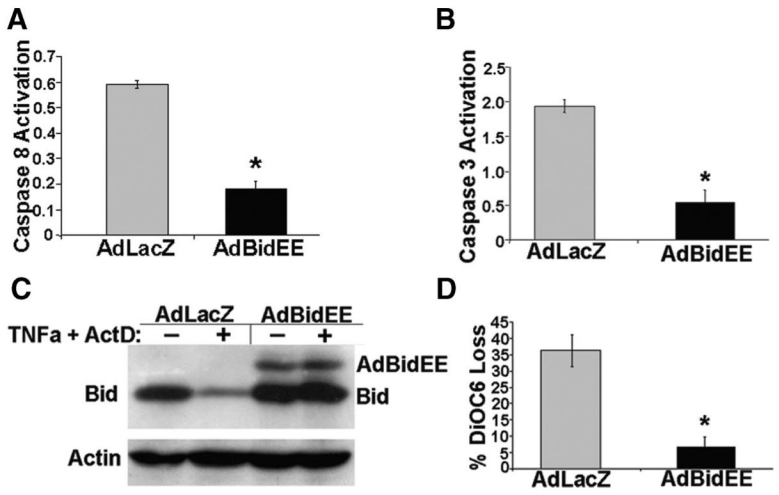

FIGURE 3.

FaO cells expressing AdBidEE demonstrate decreased caspase activation, Bid cleavage, and mitochondrial damage. FaO cells were infected with 50 MOI AdLacZ or AdBidEE for 36 hr followed by treatment for 6 hr with TNFα(20 ng/ml) and actinomycin D (0.2 μg/ml). Lysates were prepared and measured for caspase activation using a pNA-tagged caspase 8-specific substrate (A) or caspase 3-specific substrate (B). Data are represented as the average (n=4) fold increase in caspase 8 and 3 activation over media control. *P<0.001. (C) Lysates were immunoblotted with anti-Bid (5 μg/ml), and anti-β Actin antibodies. Shown is a representative of three independent experiments. (D) Mitochondrial membrane disruption was measured by staining with $DiOC_6$ and analysis by flow cytometry. $DiOC_6$ staining is graphed as a percent loss of mean fluorescence intensity (MFI) relative to media control after treatment with TNFα(n=4, *P<0.001). Shown is a representative of four independent experiments.

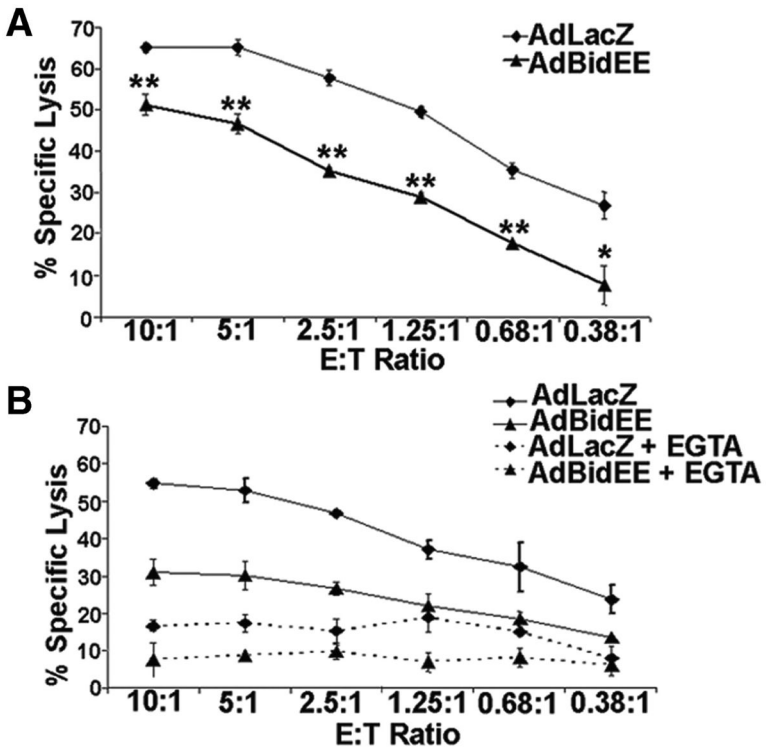

FIGURE 4.

FaO cells expressing AdBidEE display significantly less NK cell-mediated lysis. (A) FaO cells were infected with 50 MOI of AdLacZ (diamonds) or AdBidEE (triangles) for 36 hr and used as targets in a JAM assay, with RNK-16 effector cells at the indicated E:T ratios. Data are represented as a percent loss of intact DNA over FaO cells incubated in media alone. (**P<0.005, *P<0.05). (B) Cytotoxicty was abrogated when RNK-16 effector cells were incubated with AdLacZ- or AdBidEE-expressing FaO target cells in the presence (hatched line) of the calcium chelator EGTA. Shown is a representative of four independent experiments.

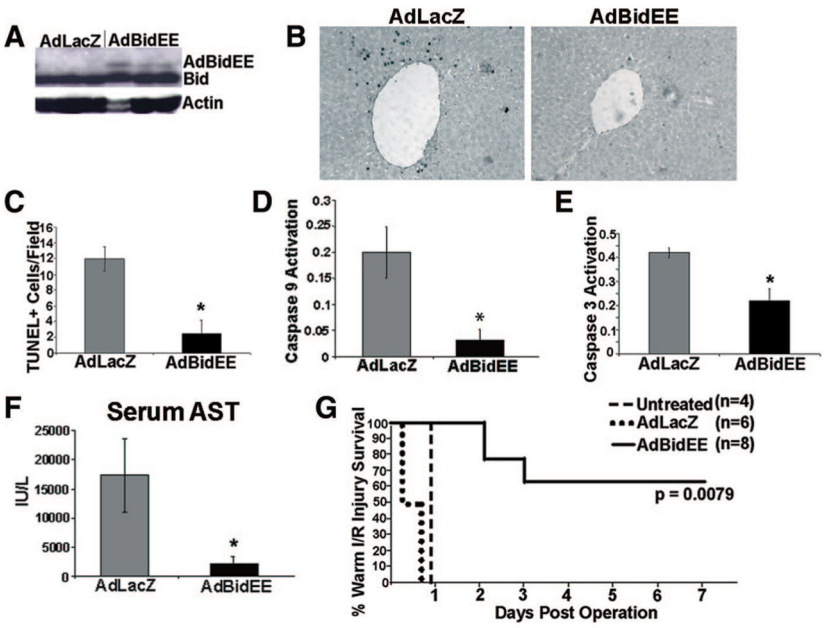

FIGURE 5.

Expression of AdBidEE protects the liver from ischemia/reperfusion (I/R) injury. (A) Wistar rats were infected with AdLacZ or AdBidEE (5×10^9 pfu/ml) for 48 hr and AdBidEE expression in the liver was measured by immunoblot with anti-Bid antibodies (8 μg/ml). Shown is a representative of two independent experiments. (B) I/R injury (ischemia time=30 min, reperfusion time=60 min) was induced in rats expressing AdLacZ or AdBidEE and hepatocyte apoptosis was visualized using in situ TUNEL stain on liver tissue sections obtained after sacrifice. (C) Apoptotic hepatocytes were quantitated by blinded counting of $TUNEL^+$ hepatocytes. Data are represented as the average of $TUNEL^+$ cells/field, (*P<0.05, n=3). (D and E) Liver lysates were measured for caspase 9 and caspase 3 activation by colorimetric activation assay. Data are represented as average absorption (405 nm) for each group of rats (n=3, *P<0.05). (F) Serum from rats was measured AST levels. Mean AST levels for AdLacZ=17361.6 IU/L, AdBidEE=2197.5 IU/L. (G) Kaplan-Meyer plot of survival of untreated (n=4) AdLacZ (n=6) and AdBidEE (n=8) rats after induction of lethal I/R injury (ischemia time=60 min, reperfusion time=60 min). The P value (P=0.0079) was calculated using the log-rank test.