Hepatocyte Fas-associating Death Domain Protein/Mediator of Receptor-induced Toxicity (FADD/MORT1) Levels Increase in Response to Pro-apoptotic Stimuli

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EXPERIMENTAL PROCEDURES

Materials—Williams Medium E, penicillin, streptomycin, 1-glutamine, and HEPES were purchased from Invitrogen. Insulin was purchased from Eli Lilly (Indianapolis, IN). Low endotoxin calf serum was from HyClone Laboratories (Logan, UT). Pan-caspase inhibitor benzylxycarbonyl-Val-Ala-Asp fluoromethylketone (Z-VAD-fmk) and caspase-8 inhibitor Ac-Ile-Glu-Thr-Asp-CHO were from Alexis Corp. (San Diego, CA). Stock solution of Z-VAD-fmk was prepared at 100 mM in dimethyl sulfoxide, and stock IETD-CHO was prepared in water at 100 mM. Mouse recombinant TNFα was obtained from R&D Systems (Minneapolis, MN). Antibodies used for this study were purchased from Cell Signaling Technology (Beverly, MA) and Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF406779.

We examined the regulation of Fas-associating death domain (FADD) protein as an important adaptor molecule in apoptosis signaling and hypothesized that the regulation of FADD could contribute to hepatocyte death. FADD/mediator of receptor-induced toxicity (MORT1) is required for activation of several signaling pathways of cell death. In this study we report the interesting and unexpected result that actinomycin D increased the expression of FADD protein, and we demonstrate that other cellular stresses like ultraviolet irradiation or heat shock could also increase FADD levels in hepatocytes. In cells treated with actinomycin D, FADD levels were elevated homogeneously in the cytoplasm. The increase in cytoplasmic FADD protein by actinomycin D or FADD overexpression alone both correlated with cell death, and specific antisense inhibition of FADD expression consistently diminished ~30% of the cell death induced by actinomycin D. These data indicate that FADD protein expression can increase rapidly in hepatocytes exposed to broadly cytotoxic agents.
were purchased from Sigma (for α-actin), Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (for TRADD), and Stressgen (Victoria, British Columbia, Canada) (for FADD, caspase-3, and caspase-8). To ensure the specificity of the antibody used to detect FADD, the rat FADD gene was cloned (GenBank accession no. AF406779) and expressed by transient transfection of 293 human kidney cells (Invitrogen). A 29-kDa rat FADD protein was detected by Western blotting with the anti-mouse FADD antibody (data not shown). Rabbit polyclonal antibody for BID was a generous gift from Dr. Xiao-Ming Yin. Bicinchoninic acid (BCA) protein and Supersignal chemiluminescent protein detection reagents were from Pierce. Unless otherwise indicated, other reagents were purchased from Sigma.

Preparation of Primary Hepatocytes and Cell Culture—Primary hepatocytes were isolated and purified from male Sprague-Dawley rats (Harlan, Indianapolis, IN) or C57BL/6 mice (Charles River Laboratories, Wilmington, MA) by a collagenase perfusion method as described previously (18, 19). Highly purified hepatocytes (>98% purity and >95% viability by trypan blue exclusion) were suspended in Williams’ E medium supplemented with 10% calf serum, 1 μM insulin, 2 mM l-glutamine, 15 mM HEPES, pH 7.4, 100 units/ml penicillin, and 100 μg/ml streptomycin. The cells were plated on collagen-coated tissue culture plates at a density of 2 × 10^6 cells/ml in 12-well plates for cell viability analysis, or 5 × 10^5 cells/ml/10-cm dish for Western blotting assays. Cells were cultured overnight at 37 °C in 5% CO₂. Adenoviral particles were obtained by incubating 293 cell culture medium containing 2000 units/ml TNFa and 200 ng/ml Act D for 8–12 h. Hepatocytes were then scraped off the plates and centrifuged. For cytosolic lysates, cells were washed with cold phosphate-buffered saline (PBS) and resuspended in 5-fold volume of hypotonic buffer A (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml pepstatin, and 10 μg/ml leupeptin). After three cycles of freezing and thawing, cell debris was removed by centrifugation at 13,000 × g at 4 °C for 20 min. The supernatant was used as a cytosolic lysate for Western blotting analysis. Protein concentration was determined using BCA assay (Pierce) with bovine serum albumin as standard.

Animal Studies—All procedures in this experiment were performed according to the guidelines of the Council on Animal Care at the University of Pittsburgh and the National Research Council’s Guide for the Care and Use of Laboratory Animals. Sterile Act D (15 μg/kg) or saline was injected via the dorsal pen vein into Sprague-Dawley rats under brief isoflurane anesthesia, and the livers were harvested 24 h later. Serum samples were obtained at the time of death, and the liver function tests were determined for aspartate aminotransferase and alanine aminotransferase using the Randox ASR (Randox Laboratories, Bayer Co., Tarrytown, NY). Liver samples were immersed by cryostat and prepared as for cultured hepatocytes. Isolated hepatocyte protein lysates were assessed by Western blotting and probed for FADD protein.

Immunofluorescent Staining—Acid-cleaned, 3-aminopropyltriethoxysilane, 0.25% glutaraldehyde, collagen-1-coated (50 μg/ml) or Sytox stain (green; Molecular Probes, Eugene, OR) was loaded as assessed by quantitation with UV spectrophotometry and neutral density fields.

Immunoblotting Analysis—Forty micrometers of protein were separated on 13% SDS-PAGE and transferred onto a nitrocellulose membrane. Loading of equal protein amounts was assessed by staining of nitrocellulose membranes with 0.1% Ponceau S (Sigma) in 5% acetic acid. Nonspecific binding was blocked with PBS-T (14 mM sodium phosphate (monobasic, monohydrate), 88 mM dibasic sodium phosphate (anhydrous), 100 mM NaCl, and 0.1% Tween 20) containing 5% nonfat milk for 1 h of incubation with rat 37 °C. After drying, cells were lysed with 1% sodium dodecyl sulfate (SDS) solution, and dye uptake was measured at 550 nm using a 96-well microplate reader. Cell viability was calculated from relative dye intensity of the mean for duplicate samples and presented as percentages relative to untreated samples. Cell viability is presented with the representative Western blotting results from the same hepatocyte harvest. Immunoblotting Analysis—Forty micrometers of protein were separated on 13% SDS-PAGE and transferred onto a nitrocellulose membrane. Loading of equal protein amounts was assessed by staining of nitrocellulose membranes with 0.1% Ponceau S (Sigma) in 5% acetic acid. Nonspecific binding was blocked with PBS-T (14 mM sodium phosphate (monobasic, monohydrate), 88 mM dibasic sodium phosphate (anhydrous), 100 mM NaCl, and 0.1% Tween 20) containing 5% nonfat milk for 1 h of incubation with rat 37 °C. After drying, cells were lysed with 1% sodium dodecyl sulfate (SDS) solution, and dye uptake was measured at 550 nm using a 96-well microplate reader. Cell viability was calculated from relative dye intensity of the mean for duplicate samples and presented as percentages relative to untreated samples. Cell viability is presented with the representative Western blotting results from the same hepatocyte harvest. Immunoblotting Analysis—Forty micrometers of protein were separated on 13% SDS-PAGE and transferred onto a nitrocellulose membrane. Loading of equal protein amounts was assessed by staining of nitrocellulose membranes with 0.1% Ponceau S (Sigma) in 5% acetic acid. Nonspecific binding was blocked with PBS-T (14 mM sodium phosphate (monobasic, monohydrate), 88 mM dibasic sodium phosphate (anhydrous), 100 mM NaCl, and 0.1% Tween 20) containing 5% nonfat milk for 1 h of incubation with rat 37 °C. After drying, cells were lysed with 1% sodium dodecyl sulfate (SDS) solution, and dye uptake was measured at 550 nm using a 96-well microplate reader. Cell viability was calculated from relative dye intensity of the mean for duplicate samples and presented as percentages relative to untreated samples. Cell viability is presented with the representative Western blotting results from the same hepatocyte harvest.
RESULTS

Act D Induced Hepatocyte Death and Increased Levels of FADD Protein—Previous studies have shown that hepatocyte death cannot be induced by TNFα unless used in conjunction with sensitizing agents such as Act D (25). We tested the hypothesis that Act D influenced cell death by altering FADD protein expression. Act D alone induced a concentration-dependent decline in hepatocyte viability that was associated with a dose-dependent increase in FADD protein levels (Fig. 1A). FADD levels were also up-regulated by Act D in a time-dependent manner, which inversely correlated with viability (Fig. 1B). The increase in FADD levels was observed as early as 4 h after Act D (200 ng/ml) treatment, with up to 5-fold increase in FADD protein levels that seemed to plateau after 8 h. Exposure to TNFα alone had minimal effects on hepatocyte viability, but when added with Act D, TNFα further reduced viability from 49 ± 5.2% to 37 ± 11.2% (Fig. 1C). TNFα alone did not significantly increase FADD levels and did not alter the Act D-induced increases in FADD. As expected, cell death by Act D was associated with a decrease in procaspase-8 and increase in caspase-8 and caspase-3 cleavage bands (Fig. 1D). Rat hepatocytes were treated with Act D (200 ng/ml) for 6 h and harvested for total RNA. RNA was probed by Northern blotting with rat FADD cDNA, stripped, and re-probed with β-actin cDNA. Act D decreased FADD and β-actin mRNA levels. 28 and 18 S ribosomal RNA was assessed to measure equal loading of total RNA.

TNFα with the transcriptional inhibitor d-galactosamine has been reported to increase FADD mRNA and protein levels in hepatocytes in vivo (26). To determine whether Act D would increase FADD in vivo, FADD protein levels were measured in
Actinomycin D Up-regulates FADD/MORT1

**Fig. 2. Subcellular localization of rat FADD in hepatocytes stimulated with Act D.** A, light microscopy of unstimulated rat hepatocytes plated at $10^5$ cells/ml and cultured overnight appear as a confluent, sinusoidal monolayer. B, hepatocytes treated with Act D (200 ng/ml) for 12 h contract and detach from the cell plate with characteristics of apoptosis (black arrows). Approximately 35% of the plate surface was covered by viable cells. C and D, immunofluorescent microscopy of FADD (red) in untreated rat hepatocytes (C) and hepatocytes treated with Act D (200 ng/ml) for 6 h (D). Hepatocyte nuclei are stained with Hoechst dye (blue). FADD protein stains homogeneously throughout the cytoplasm. Act D increases the intensity of FADD staining in the cytoplasm. E, immunofluorescent analysis of hepatocytes infected with Ad-EGFP (green) at m.o.i. 10 demonstrates >90% infectivity of primary hepatocytes. F–H, immunofluorescent staining of rat hepatocytes for FADD expression (red) and nuclear staining (green). Cells were mock-infected with medium (F), control virus that expresses β-galactosidase (Ad-LacZ, G), or Ad-FADD-WT (H) at m.o.i. of 10 for 4 h. Twenty-four hours later, overexpression of FADD in hepatocytes resulted in apoptotic morphology with condensed nuclei (yellow arrow). J, higher magnification of hepatocyte 6 h after treatment with Act D (200 ng/ml) and immunostained for FADD (red) and Sytox for nuclei shows FADD localizing to the plasma membrane (yellow arrow). K and L, hepatocytes treated with Act D for 6 h showed more TUNEL-positive nuclei (L, green) than cells treated with medium alone (K). Scale bar = 50 μm.

Liver lysates from rats injected intravenously with Act D (15 μg/kg). Fig. 1D demonstrates a marked increase in FADD levels 24 h after Act D injection. At this time point, no changes in liver morphology or serum liver enzymes were observed (data not shown).

To assess whether Act D also affected FADD mRNA levels, Northern blots were performed on total RNA isolated from hepatocytes treated with Act D. We used the 720-bp rat FADD cDNA coding region as a probe for Northern blotting. In contrast to protein levels, hepatocyte FADD mRNA levels were decreased 6 h after treatment with Act D (200 ng/ml; Fig. 2). This result suggested that FADD protein up-regulation occurred at the post-transcriptional level. β-Actin levels were also decreased in hepatocytes treated with Act D consistent with the action of Act D as a transcriptional inhibitor, but 28 and 18 S RNA levels in each lane showed equivalent total RNA loading.

**Subcellular Localization of FADD in Hepatocytes**—To determine whether the up-regulation of FADD was associated with changes in the subcellular localization of FADD, light and immunofluorescent microscopic imaging studies of the cells were undertaken. Unstimulated hepatocytes remained adherent to collagen-coated plates as a semiconfluent monolayer (Fig. 2A). Hepatocytes stimulated for 12 h with Act D displayed characteristics of apoptotic cell death, including cell shrinkage and nuclear condensation, and detached from the plate (Fig. 2B). Unstimulated hepatocytes analyzed by immunofluorescent staining for FADD protein exhibited a cytoplasmic distribution of FADD protein (Fig. 2C). A homogeneous increase in FADD immunofluorescent staining was observed in the cytoplasm in hepatocytes treated with Act D for 6 h (Fig. 2D) that was consistent with the increase in FADD protein observed by Western blotting (Fig. 1A). These localization studies demonstrated that the increase in cytoplasmic FADD levels did not involve translocation of protein from a sequestered, intracellular source.

These studies demonstrated that Act D treatment of hepatocytes would result in increased FADD expression, but the role of elevated FADD protein in the activation of cell death pathways in primary hepatocytes was unclear. FADD overexpression has been shown to induce death in the B lymphoma cell line BJAB and MCF-7 breast cancer cells, as well as embryonic fibroblasts (7, 27). To evaluate whether overexpression of FADD protein alone would activate death pathways in primary hepatocytes, we constructed a replication-deficient adenovirus that expressed wild type mouse FADD (Ad-FADD-WT). Fluorescent analysis of hepatocytes infected with a control virus that expressed enhanced green fluorescent protein (Ad-EGFP) at m.o.i. of 10 resulted in >90% infectivity without induction of cell death (Fig. 2E). Ad-FADD-WT infection of hepatocytes resulted in an increase in FADD immunofluorescent staining in the cytoplasm and condensed, apoptotic nuclear and cellular morphology (Fig. 2F) compared with infection with control virus (Fig. 2F) or medium alone (Fig. 2G). Higher magnification of hepatocytes treated with Act D showed nuclei with condensed apoptotic morphology (Fig. 2J). To assess for DNA fragmentation, cells treated with Act D (Fig. 2L) showed an increase in TUNEL-positive staining compared with...
untreated hepatocytes (Fig. 2K). These results demonstrate that Act D treatment or overexpression of FADD could induce an apoptotic cytoplasmic and nuclear morphology in hepatocytes.

FADD Overexpression Induced Cell Death in Hepatocytes, and FADD Antisense Expression Blocks Hepatocyte Death by Act D—To assess the molecular effects of FADD overexpression, we examined the cleavage of procaspase-8 and Bid, which are molecules immediately downstream of FADD in the apoptotic signaling pathway. Eight hours following Ad-FADD-WT infection at m.o.i. 10, FADD protein expression was significantly increased in hepatocytes, as detected by Western blotting (Fig. 4A). Cleavage products of caspase-8 and BID (tBID) were seen 18 h after Ad-FADD-WT infection but not after Ad-EGFP infection (Fig. 3A). To quantitate the cell death induced by FADD overexpression, infection of hepatocytes with Ad-FADD-WT resulted in a dose-dependent decrease in viability of hepatocytes (Fig. 3B), which was similar to results after treatment with Act D (Fig. 1A). Ad-FADD-WT infection at m.o.i. 10 induced a reduction in cell viability to ~58% compared with controls (Fig. 3C, lane 3). As expected, death by Ad-FADD-WT infection was inhibited by co-culture with pan-caspase inhibitor Z-VAD-fmk (100 μM) or by caspase-8 inhibitor IETD-CHO (100 μM) (Fig. 3C, lanes 5 and 6, respectively; n = 4, ANOVA p < 0.05). TNFα alone did not induce death in hepatocytes, nor did it augment the cytotoxic effects of Ad-FADD-WT at m.o.i. of 10.

To determine whether the hepatotoxicity induced by Act D was dependent on FADD protein expression, an adeno viral vector expressing FADD antisense mRNA (Ad-FADD-AS) was constructed. Infection of hepatocytes with Ad-FADD-AS decreased endogenous FADD protein levels at day 3 after infection (Fig. 4A), which demonstrated the efficacy of the Ad-FADD-AS construct to abrogate FADD expression. Cell viability was not significantly changed in cells treated with Ad-FADD-AS at an m.o.i. of 10 (Fig. 4B, lane 3). Ad-FADD-AS infection inhibited cell death by TNFα/Act D (Fig. 4B, lane 7; n = 4, ANOVA p < 0.05) or Act D alone (Fig. 4B, lane 6; ANOVA p < 0.05) by ~30%. These results suggested that death induced by Act D alone was at least partially FADD-dependent.

Ultraviolet Irradiation and Extreme Heat Shock Increased FADD in Hepatocytes—We wanted to determine whether other cytotoxic stimuli could also affect FADD expression levels. Ultraviolet (UV) irradiation has been shown to induce apoptosis in several cell types via a FADD-dependent mechanism that involves cross-linking of death receptors such as Fas or TNF receptor (28, 29). To assess whether FADD levels also correlated with UV exposure in hepatocytes, FADD protein levels were assessed after UV treatment and found to be increased as soon as 6 h after exposure to 200 mJ/cm² UV (Fig. 5A). Hepatocytes exhibited a dose-dependent increase in FADD protein measured at 8 h after exposure to UV (Fig. 5B). The lowest exposure associated with a rise in FADD levels (50 mJ/cm²) was associated with a 50% decrease in cell viability by 8 h after UV exposure.

UV and Act D might have induced hepatocyte death by activation of FADD-mediated pathways of apoptosis, but cells can also respond to stress by the activation of cytoprotective mechanisms that include heat shock proteins (30). We postulated that the level of stress would dictate whether hepatocytes activated pro-death pathways through FADD or pro-survival mechanisms. This hypothesis was tested in cultured hepatocytes exposed to heat shock, a stimulus that, depending on severity, is known to induce either the stress response or death (31). After modulating the duration of 42 °C heat exposures from 20 to 120 min, induction of the cytoprotective protein Hsp70 was assessed as a marker of the heat shock response in hepatocytes exposed to varying degrees of stress (32, 33). Hsp70 has been shown to block recruitment of caspase-9 to
Apaf-1 (34), and elevation of Hsp70 by nitric oxide in hepatocytes has been shown to inhibit apoptosis (32). Cells treated for up to 120 min with heat shock showed no immediate change in viability by morphology or crystal violet staining (data not shown). FADD levels, however, were markedly elevated after 120 min of heat shock (Fig. 5C). After cells were cultured at 37 °C for 18 h, minimal changes in viability were seen with up to 40 min of initial heat shock, although 120-min exposures to heat shock resulted in global cell death. Coincident with the minimal mortality of the 40-min heat exposure was the absence of early FADD expression (Fig. 5C) and the marked up-regulation of Hsp70 at 18 h (Fig. 5D). In contrast, 120-min heat exposure was associated with a marked early up-regulation of FADD protein (Fig. 5C), failure to up-regulate Hsp70 (Fig. 5D), and ~93% cell death at 18 h. The kinetics of FADD protein elevation observed as a response to extreme heat shock was notably more rapid than the responses observed in hepatocytes exposed to UV (Fig. 5A) or Act D (Fig. 1B). These experiments showed that FADD protein could be increased by stresses known to induce cell death through ligand-independent mechanisms including ultraviolet irradiation and thermal injury. Moreover, at levels of heat shock that led to cell death, up-regulation of the cytoprotective protein hsp70 was replaced by significant elevations in FADD protein.

Finally, to assess whether other metabolic inhibitors could also increase FADD protein levels, hepatocytes were treated with cycloheximide (CHX), a translational inhibitor. CHX has been used to sensitize hepatocytes to cell death via receptor ligation, and CHX can induce hepatocyte death in vitro. We found that CHX (100 μM) also increased FADD in a time-dependent manner (Fig. 5E). Taken together, these studies demonstrate that levels of FADD protein were increased by several inducers of hepatocyte death.

**DISCUSSION**

The essential role of FADD in the initiation of many apoptotic signaling pathways led us to examine the role of changes in FADD protein levels in apoptosis. In these studies we have demonstrated that FADD levels were modulated under conditions associated with increased apoptosis and that cell death could be controlled by intracellular changes in FADD levels. Constitutive expression of FADD has been shown in several different cell types (9). However, it was unknown whether basal levels are adequate to initiate ligand-dependent apoptosis and whether elevation in endogenous FADD levels represents a mechanism to activate apoptotic signaling. In the studies reported here, FADD levels correlated with hepatocyte death following treatment with Act D (Fig. 1), CHX (Fig. 5E),
ultraviolet radiation (Fig. 5A), or heat shock (Fig. 5C). These data indicate that FADD can be rapidly induced in cells exposed to a broad range of cytotoxic stimuli. Furthermore, similar increases in specific FADD expression in hepatocytes induced caspase-dependent cell death in the absence of other external insults. Conversely, depletion of FADD protein by expression of antisense FADD diminished cell death induced by Act D alone or by TNFα in combination with Act D by ~30%. Unlike FADD protein expression, steady-state levels of FADD and actin mRNA were decreased by 6-h treatment with Act D (Fig. 1E). It is likely that hundreds of proteins are decreased in expression by the transcriptional inhibitor Act D. These data, in conjunction with the observation that Act D up-regulated FADD protein, indicate that increased FADD levels contributed significantly to Act D-induced death. More importantly, changes in FADD levels could serve as a mechanism to activate cell death signaling pathways following exposure to significant stress.

Nevertheless, the mechanisms for the up-regulation of FADD are not yet clear. We have treated HepG2 hepatocellular carcinoma and A549 lung cancer cells with Act D (200 ng/ml), and we did not observe an increase in FADD protein expression above baseline for these cells (data not shown). This raises interesting questions because these tumor cell lines responded differently from primary hepatocyte cultures. One possibility is that immortalized cells lose the ability to up-regulate FADD that exists in primary cells in culture. Tumor necrosis factor-α alone does not readily induce hepatocyte death in vitro or in vivo unless used in conjunction with Act D or β-galactosamine (5). Transcriptional inhibitors β-galactosamine and α-amanitin also increased FADD protein levels in cultured hepatocytes (data not shown). Others have shown in HeLa and SHEP neuroblastoma cells that Act D decreased short-lived inhibitors of apoptosis like FLIP and XIAP (35, 36). We did not observe changes in FLIP or IAP protein levels in hepatocytes exposed to Act D (data not shown). Taken together, these findings lend support to the idea that up-regulation of FADD by transcriptional inhibitors may also act to sensitize hepatocytes to cell death by TNFα, and we postulate that another rapidly metabolized factor exists that may regulate FADD protein expression.

Other investigators have reported the appearance of “death effector filaments” in HeLa cells induced for apoptosis via death receptors (37, 38). We did not observe cytoplasmic filaments in treated or untreated hepatocytes immunostained for FADD. No change in subcellular localization was observed when hepatocytes were treated with Act D alone, but FADD levels were higher in the cytoplasm. Although expression levels of ligands and death receptors were not manipulated or measured in this study, it would appear that FADD could act to precipitate apoptosis in the absence of direct receptor ligation and that this process becomes more efficient as FADD levels increase. Chen et al. (39) have shown that Ras-dependent apoptosis appears to signal through FADD via a receptor-independent mechanism, and chemotherapeutic agents induce cell death in U937 monocytes via FADD-dependent, ligand-independent mechanisms. However, whether FADD up-regulation occurs in these situations is not known and warrants further investigation. Our findings suggest that elevations in cytoplasmic FADD expression by cellular stresses may play an important regulatory role in the induction of apoptosis.

Because hepatocytes are difficult to transiently transfect in vitro and in vivo, to express FADD wild-type and antisense genes, we generated adenoviral vectors that readily infect primary hepatocytes. Both the full length and truncated forms of FADD have been shown to mediate apoptotic and necrotic cell death pathways. Expression of dominant-negative FADD induced non-apoptotic death in NIH3T3 cells treated with TNFα (40). In Jurkat T cells and L929 fibroblasts, necrotic death was induced by signaling through Fas receptor that appeared to be FADD-dependent (41–43). We found that Act D induced cell death with apoptotic morphology in hepatocytes. Our results in conjunction with other studies suggest that FADD may be involved with multiple death pathways including caspase-dependent apoptosis as well as caspase-independent necrosis (44). Therefore, in our studies, we used crystal violet staining of adherent cells as a simple and accurate viability assay for hepatocytes. Surprisingly, the dominant-negative, mutant FADD protein has been shown to retain some pro-death activity in normal prostate cells (45). For these reasons we justified the use of antisense technology to prevent murine FADD protein expression and inhibit cell death instead of expressing the dominant-negative human FADD protein. Preliminary results indicate that Ad-FADD-AS may protect against death induced by heat shock or UV in hepatocytes, but it was unclear in those experiments whether adenoviral infection itself introduced additional hepatotoxicity. We decided to pursue that question in a physiologically and biologically relevant model; we have demonstrated in human keratinocytes, as in hepatocytes, that UV increases FADD protein levels and induced apoptosis which was inhibited by antisense inhibition of FADD protein expression by Ad-FADD-AS.

Our findings of endogenous augmentation in FADD expression support the pursuit of future studies to determine its biological relevance. FADD expression was absent in PLC/PRF/5 hepatocellular carcinoma cells (46), and also shown to be decreased 10-fold in mantle cell lymphomas (47). Therapeutically, activation of FADD/MORT1 function by gene therapy has been used to induce cell death in cancers of the brain (48, 49). In our studies, severe cellular stress in the form of heat shock increased FADD protein levels that correlated with decreased cell viability (Fig. 5C). By contrast, mild to moderate amounts of stress was associated with increases in the protective protein Hsp70. FADD levels were not increased immediately after 60 min of heat shock (Fig. 5D). Because of the stress induced by heating cells, hepatocyte death by heat shock is likely more complex and complete than death induced by the other agents like Act D. In preliminary experiments, death induced by heat shock was not blocked by caspase inhibitors (data not shown) and may represent death by necrosis. In the setting of moderate heat, we expect additional unknown, pro-necrotic mecha-

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2 P. K. M. Kim, R. Weller, and T. R. Billiar, submitted for publication.
nisms other than FADD up-regulation would also influence cell death.

These data lend support to a model of a "severe stress sensor" whereby the cell has a mechanism to detect and respond to the degree of stress to which it is exposed. Cells exposed to mild injury appear to respond with elevations of cytoprotective proteins like hsp70 that might inhibit cell death by various mechanisms and allow cellular repair (34); after exposure to lethal stresses, FADD may be preferentially up-regulated to ensure eradication of incapacitated or mortally injured cells (Fig. 6). This reciprocal expression of the two proteins raises the interesting possibility that Hsp70 may regulate FADD expression, a hypothesis that is not yet tested. Because this response mechanism to toxic stresses may modulate cell death after severe injury, we and others have proposed the manipulation of FADD-mediated hepatocyte death as an alternative modality to protect the liver from fulminant hepatic failure in vivo (1, 50). Finally, because FADD can effectively mediate multiple death pathways, one should not be surprised to discover that other human diseases may be caused, modified, and treated by inducing changes in the expression of this apoptotic signaling molecule (51, 52).

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