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The Mitochondrial Permeability Transition Augments Fas-induced Apoptosis in Mouse Hepatocytes*

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Tumor necrosis factor-α receptor 1 and Fas recruit overlapping signaling pathways. To clarify the differences between tumor necrosis factor α (TNFα) and Fas pathways in hepatocyte apoptosis, primary mouse hepatocytes were treated with TNFα or an agonist anti-Fas antibody after infection with an adenovirus expressing an IκB superrepressor (Ad5IκB). Treatment with TNFα induced apoptosis in Ad5IκB-infected mouse hepatocytes, as we previously reported for rat hepatocytes. Ad5IκB plus anti-Fas antibody or actinomycin D plus anti-Fas antibody rapidly induced apoptosis, whereas anti-Fas antibody alone produced little cytotoxicity. The proteasome inhibitor (MG-132) and a dominant-negative mutant of nuclear factor-κB-inducing kinase also promoted TNFα- and Fas-mediated apoptosis. Expression of either crmA or a dominant-negative mutant of the Fas-associated death domain protein prevented TNFα- and Fas-mediated apoptosis. In addition, the caspase inhibitors, DEVD-cho and IETD-fmk, inhibited TNFα- and Fas-mediated apoptosis. In Ad5IκB-infected hepatocytes, caspases-3 and -8 were activated within 2 h after treatment with anti-Fas antibody or within 6 h after TNFα treatment. Confocal microscopy demonstrated onset of the mitochondrial permeability transition (MPT) and mitochondrial depolarization by 2–3 h after anti-Fas antibody treatment and 8–10 h after TNFα treatment, followed by cytochrome *c* release. The combination of the MPT inhibitors, cyclosporin A, and trifluoperazine, protected Ad5IκB-infected hepatocytes from TNFα-mediated apoptosis. After anti-Fas antibody, cyclosporin A and trifluoperazine decreased cytochrome *c* release but did not prevent caspase-3 activation and cell-death. In conclusion, nuclear factor-κB activation protects mouse hepatocytes against both TNFα- and Fas-mediated apoptosis. TNFα and Fas recruit similar but nonidentical, pathways signaling apoptosis. The MPT is obligatory for TNFα-induced apoptosis. In Fas-mediated apoptosis, the MPT accelerates the apoptogenic events but is not obligatory for them.

Apoptosis, a morphologically and biochemically distinct form of cell death, is an important physiologic process in both normal development and in pathological processes. Two death factors, Fas ligand and tumor necrosis factor-α (TNFα),[1] bind to their receptors and induce apoptosis, killing the cells within hours (1). Apoptosis controlled by such death receptor pairs can cause tissue destruction (2). Hepatocyte apoptosis, mainly induced by death domain receptor ligands such as Fas ligand and TNFα, is implicated in several experimental and human liver diseases including viral hepatitis, alcoholic hepatitis, acute liver failure, ischemia/reperfusion injury, diseases of the bile ducts, graft-*versus*-host disease, and hepatocellular carcinoma (3).

The TNF receptor family includes Fas, the receptor for Fas ligand, and the two TNFα receptors (TNFR) (1). Upon binding to Fas ligand, Fas forms a complex with the associated protein, Fas-associated death domain protein (FADD), which directly binds and activates caspase-8. Recent studies showed that FLICE-associated huge protein interacts with FADD and caspase-8 (4). FLICE-associated huge protein may control apoptosis at the level of caspase activation. TNFR1 interacts with the adaptor protein TNFR-associated death domain protein (TRADD) that recruits FADD, which again directly activates caspase-8. TNFα also induces other signaling pathways via TRADD including the protein kinase receptor interacting protein and TNF receptor-associated factor 2 (TRAF2). TNFα activates the mitogen-activated protein kinase kinase kinase, NF-κB-inducing kinase (NIK), via either protein kinase receptor interacting protein or TRAF2 (5, 6). NIK in turn phosphorylates and activates the IκB kinase (IKK) complex (7–9). IKKs phosphorylate IκB, targeting it for NF-κB activation (10–12).

Recent studies indicate that NF-κB activation by TNFα protects cells from TNF cytotoxicity (13–15). TNFα binding to the TNF receptor potentially both initiates apoptosis and activates NF-κB, which suppresses apoptosis by induction of NF-κB-responsive genes, including TRAF1, TRAF2, and the inhibitor of apoptosis proteins (16). The expression of the IκB superrepressor by an IκBα (S32A, S36A)-expressing adenovirus (Ad5IκB), which blocks NF-κB activation, sensitizes primary rat hepatocytes to TNFα-mediated apoptosis (17). Furthermore, TNFα-mediated cytotoxicity is enhanced by the addition

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[1] The abbreviations used are: TNF, tumor necrosis factor; TNFR, tumor necrosis factor-a receptor; FADD, Fas-associated death domain protein; TRADD, tumor necrosis factor-α receptor-associated death domain protein; TRAF, tumor necrosis factor-α receptor-associated factor; NF-κB, nuclear factor κB; NIK, NF-κB-inducing kinase; IKK, IκB kinase; AD5IκB, adenovirus expressing IκBα superrepressor (532A, 536A); MPT, mitochondrial permeability transition; CsA, cyclosporin A; HDM, hormonally defined medium; HA, hemagglutinin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; TMRM, tetramethylrhodamine methyl ester; ActD, actinomycin D; m.o.i., multiplicity of infection; TFZ, trifluoperazine; AFC, amino-4-trifluoromethyl coumarin.

of inhibitors of protein or RNA synthesis (cyclohexamide and actinomycin D) (18).

Fas induces NF-κB binding activity in certain, but not all, cell types. Fas can stimulate the DNA binding activity of NF-κB in a variety of tumor cells irrespective of their sensitivity or resistance to Fas-mediated cytotoxicity (19). Another report showed that the activation of NF-κB can induce target gene expression that rescues TNFα- but not Fas-mediated apoptosis in T24 cell lines (13). However, whether NF-κB is activated in Fas-mediated apoptosis in nontumor cells, such as hepatocytes, is not clear. Anti-Fas antibody injection into mice induces severe liver failure with apoptosis of hepatocytes (2). However, anti-Fas antibody alone induces apoptosis in less than 20% of the cultured hepatocytes *in vitro*, whereas all cells were killed by anti-Fas antibody in the presence of actinomycin D or cycloheximide (20, 21). These results suggest that cultured mouse hepatocytes may express protective proteins against apoptosis. Furthermore, Fas-mediated apoptosis was delayed in hepatocytes during liver regeneration in mice (22). This suggests that TNFα may act as one of the protective factors against Fas-mediated hepatocyte apoptosis, because initiation of liver regeneration requires TNFα. Perhaps TNFα-induced activation of NF-κB protects hepatocytes from Fas-mediated apoptosis.

Mitochondria play a key role in the regulation of apoptosis (23–26). Opening of the mitochondrial permeability transition (MPT) pore, which is regulated by members of the Bcl-2 family, causes the release of soluble proteins, such as cytochrome *c* and apoptosis-inducing factor, from the intermembrane space. Inhibitors of MPT pore opening, including cyclosporin A (CsA), block apoptosis in some systems (27, 28). The MPT is an essential component in the signaling pathways in TNFα-mediated cytotoxicity in the L929 line of mouse fibroblast (29) and TNFα-induced apoptosis in rat hepatocytes (17). Anti-apoptotic Bcl-2 family proteins reside in mitochondria and can prevent the MPT. Bcl-xL/Bcl-2 prevented the release of cytochrome *c*, yet other aspects of mitochondrial dysfunction still transpired and cells died (30), suggesting that the release of cytochrome *c* may not be required for cell death. Some studies using nonhepatic cells demonstrate that the translocation of cytochrome *c* from mitochondria to cytosol does not require a mitochondrial transmembrane depolarization (31–33), whereas others show that mitochondrial depolarization accompanies cytochrome *c* release (34). Thus, mitochondrial involvement and the role of cytochrome *c* and MPT in apoptosis are still controversial.

The purpose of this study was to elucidate the differences between Fas and TNFα pathways in hepatocyte apoptosis and the roles of NF-κB activation and MPT in Fas-mediated apoptosis. The results show that NF-κB activation has a protective role in not only TNFα- but also Fas-mediated apoptosis. Furthermore, we show that Fas agonistic antibody induces the MPT, which accelerates apoptosis, but is not essential for it.

MATERIALS AND METHODS

Primary Hepatocyte Cultures—About 8-week-old C57Bl6 male mice were anesthetized with ketamine/acepromazine malate administered by intraperitoneal injection. Hepatocytes were then isolated by a retrograde, nonrecirculating *in situ* collagenase perfusion of livers cannulating through the inferior vena cava by a procedure modified from Moldeus *et al.* (35). Livers were first perfused *in situ* with an oxygenated 0.5 mM EGTA containing calcium-free salt solution (8 ml/min, 37 °C for 5 min), followed by perfusion with solution containing 0.04% collagenase D (Roche Molecular Biochemicals) for 10 min. The liver was then gently minced on a Petri dish and filtered with polyamide mesh (I 003 Y NITEX 3–60/45, TETKO Inc., NY). Hepatocytes were washed two times and centrifuged at 50 × *g* for 2 min. Cell viability was consistently >90% as determined by trypan blue exclusion. Hepatocyte cultures contained less than 1% Kupffer cells and the stellate cells as determined by fluorescein isothiocyanate-labeled latex beads (1 μm, Polysciences, Warrington, PA) and autofluorescence, respectively. 5×10^5 cells were plated on 6-well plates coated with mouse collagen type I in Waymouth's medium containing 10% fetal bovine serum, 0.1 μM insulin, and 0.1 μM dexamethazone. 1.5×10^6, 2.5×10^6, or 8×10^6 cells were plated on a 60-, 100-, or 150-mm dish, respectively. After 2 h, the culture was washed with phosphate-buffered saline and changed to hormonally defined medium (HDM) containing 0.1 μM insulin, 2 mM L-glutamine, 5 μg/ml transferrin, 3 μM selenium, and 10 nM free fatty acids in RPMI basal medium. Cells were infected with recombinant adenoviruses in HDM containing 30 plaque-forming units/cell for 2 h at 37 °C and then changed to HDM containing recombinant murine TNFα (R&D Systems, Minneapolis, MN), Jo-2 (Pharmingen, San Diego, CA), or other treatments. All animals received humane care in compliance with the guidelines of the University of North Carolina.

Adenoviruses—The adenovirus 5 variants Ad5IκB, Ad5LacZ, Ad5ΔFADD and Ad5crmA, expressing HA-IκBα (S32A, S36A), β-galactosidase, a truncated form of FADD, and crmA, respectively, have been described elsewhere (17, 36). The Ad5 vector expressing ΔNIK (Ad5ΔNIK) was constructed by cre-lox recombination as described (37). An insert from pCDNA-HA2101 (deletion of amino acids 1–623, a gift from Dr. G. Natoli) (6) was subcloned into the shuttle vector pAdlox using standard techniques, and the construct was confirmed by restriction digests. Expression of the ΔNIK construct was confirmed with a luciferase reporter gene assay in monkey kidney fibroblasts (COS-7, ATCC-CRL-1651, American Type Culture Collections) as described previously (36) and Western blotting using a mouse anti-HA monoclonal antibody (Babco, Berkeley, CA). Briefly, When COS cells reached subconfluence on 6-well culture plates, the cells were transfected with 3 μg of DNA and 1 μg of (κB)3-Luc, a reporter plasmid containing three copies of the NF-κB binding site (38), using LipofectAMINE (Life Technologies, Inc.). Twenty-four h after transfection, medium was replaced with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum with or without 20 ng/ml of TNFα. After a 5-h incubation, cellular extracts were prepared using enhanced luciferase assay reagents (Analytical Luminescence, San Diego, CA). Some cells were infected with Ad5ΔNIK 24 h after transfection of (κB)3-Luc, were stimulated, and were harvested as described the above.

Measurement of Apoptosis—For quantitation of cell viability (presented as mean ± S.E.), cells were infected and treated as described above. After 17–20 h of TNFα or Jo-2 treatment, cell viability was determined by exclusion of trypan blue. Viable cells were counted in three different 200× power fields, and the percentage of treated viable cells to untreated viable cells was determined as a percentage of control viability. For propidium iodide nuclear staining, cells were fixed in 3:1 methanol/acetic acid, stained with 10 μg/ml popidium iodide, and viewed with an Olympus fluorescence microscope using a rhodamine filter set. Hepatocyte cell death was confirmed as apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (Roche Molecular Biochemicals). TUNEL staining was performed according to the manufacture's instructions. Positive (apoptotic) cells were counted in three different 200× power fields. To assess DNA ladder formation, 2×10^6 cells were digested overnight at 37 °C in 0.5 mg/ml proteinase K, 0.5% sarcosyl in phosphate-buffered saline, treated with 10 μg of RNase for 1 h at 37 °C, gently extracted with phenol and chloroform, and analyzed on 2% agarose gels. Amino-4-trifluoromethyl courmarin (AFC) release assays for caspase-3 and -8 activities were performed using the FluorAce kit (Bio-Rad) according to the manufacturer's instructions. Briefly, whole cell lysates were combined with 25 μM z-DEVD-AFC or IETD-AFC (Enzyme and Systems Products, Livermore, CA) and were incubated 2 h at 37 °C. The change in fluorescence (excitation at 370 nm and emission at 490 nm) was monitored at 1-h intervals, converted to picomoles of AFC released by using a standard curve, and normalized for protein concentration.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay—Nuclear protein extracts were prepared from primary mouse hepatocytes as described previously (17, 36). Protein-DNA binding reactions were carried out for 20 min on ice using 5 μg of nuclear extract and ^{32}P-labeled DNA probes for the NF-κB consensus binding site (39). Complexes were separated by electrophoresis on nondenaturing 5% acrylamide gels and assayed by autoradiography and PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). For supershift assays, 8 μg of antibody against p65 or p50 subunit of the NF-κB complex (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the reaction mixture, and the incubation time was extended for an additional 30 min.

Western Blot Analysis for Cytochrome c—The preparation of cytosolic S100-fractions and Western blot analysis was performed as described previously (17). Briefly, S-100 fractions were prepared from 8×10^6

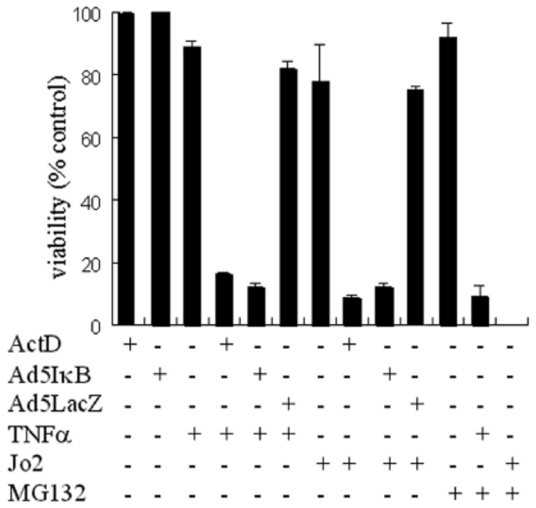

FIG. 1. **The IκB superrepressor sensitizes mouse hepatocytes to TNFα- and Fas-mediated cell death.** Primary mouse hepatocytes were treated with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml) after Ad5IκB infection (30 m.o.i.). Some cells were pretreated with ActD (0.2 μg/ml) or MG132 (20 μM). Cell viability was assessed after 17 h by a trypan blue exclusion test. Data are shown as average percent viability ± S.E. of two to four different experiments.

hepatocytes by differential centrifugation in buffer containing 250 mM sucrose. Lysates containing 25 μg of protein was separated by electrophoresis on 15% acrylamide SDS gels and transferred into nitocellulose membranes (Schleicher & Schuell). Equal loading was confirmed by Ponceau S staining. Cytochrome *c* was detected using primary monoclonal anti-cytochrome *c* antibody (Pharmingen, San Diego, CA) and secondary anti-mouse horseradish peroxidase-conjugated antibody (Santa Cruz Biotechnology). Proteins were detected with ECL detection reagents (Amersham Pharmacia Biotech).

Confocal Microscopy—Cell loading and confocal microscopy were carried out as described previously (40). Briefly, 1–2 × 10^6 hepatocytes plated on collagen-coated 40-mm-diameter glass coverslips were infected with Ad5IκB in HDM supplemented with 50 mM HEPES (pH 7.0) to stabilize pH during the confocal measurements. The cells were loaded with 250 nM tetramethylrhodamine methyl ester (TMRM, Molecular Probes, Eugene, OR) and 1 μM calcein-acetoxymethyl ester (Molecular Probes) in Krebs Ringer-Hepes buffer for 15 min at 37 °C. The coverslips were mounted on a Nikon microscope (Nikon, Melville, NY) in HDM-HEPES containing 100 nM TMRM, and the temperature was maintained at 37 °C. The first image (time point 0) was then recorded. Subsequently, TNFα or Jo-2 was added to the medium, and images were collected at given time-points. Calcein and TMRM fluorescence were excited with an argon laser through a double dichroic reflector at 488 and 568 nm, respectively. TMRM was imaged through a 590-nm-long path emission filter using a Bio-Rad MRC-600 confocal system (Bio-Rad). Calcein fluorescence was collected through a 515–560-nm band path emission filter. A numerical aperture 1.4, 60× objective lens was used, and pinholes were set to 4 in both channels. Laser attenuation and power were set at 0.3% and low, respectively.

RESULTS

The IκB Superrepressor Sensitizes Mouse Hepatocytes to TNFα- and Fas-mediated Apoptosis—Hepatocytes are resistant to TNFα-mediated apoptosis unless they are also treated with an inhibitor of protein synthesis (*i.e.* cycloheximide), RNA synthesis (*i.e.* actinomycin D) (18, 41), or NF-κB activity (*i.e.* an IκB superrepressor) (17). To extend these studies into Fas-mediated apoptosis, we switched to primary cultures of adult mouse hepatocytes, because rat hepatocytes only express low levels of Fas (42). TNFα alone had no cytotoxicity in primary mouse hepatocytes, but actinomycin D (ActD) plus TNFα caused massive cell death (Fig. 1, 16.2 ± 0.4%, % control viability at 17 h after treatment). Also, treatment of Ad5IκB-infected mouse hepatocytes expressing the IκB superrepressor with TNFα induced cell death (12.0 ± 1.1%). Cells expressing IκB superrepressor but not treated with TNFα did not lose

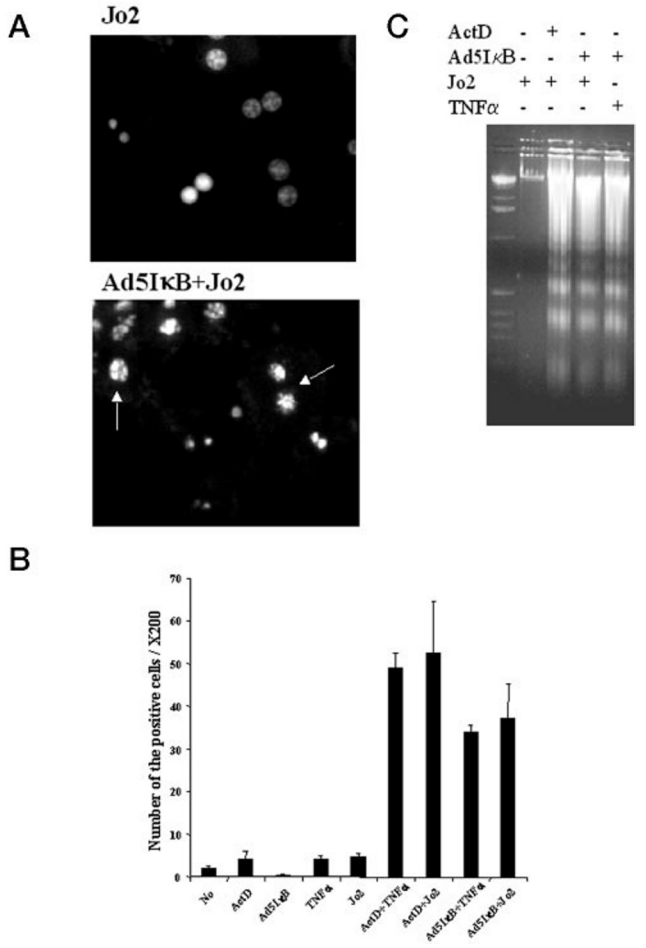

FIG. 2. **The IκB superrepressor sensitizes mouse hepatocytes to TNFα- and Fas-mediated apoptosis.** *A,* propidium iodide-stained images of noninfected (*upper panel*), and Ad5IκB-infected cells (*lower panel*) at 12 h after Jo2 treatment (original magnification of ×600). *Arrows* indicate representative apoptotic nuclei. *B,* TUNEL assay performed at 13 h after treatment. Apoptotic positive cells were counted in three different 200× power fields. Data are shown as average positive cells ± S.E. of two different experiments. *C,* DNA ladder assay from cultured hepatocytes (2 × 10^6 cells) collected 19 h after treatment. Cytosolic DNA was isolated and subjected to 2% agarose gel electrophoresis.

viability. Furthermore, Ad5LacZ had a minimal effect on cell viability after TNFα treatment (82.0 ± 2.5%), compared with noninfected hepatocytes after TNFα.

To study the role of NF-κB activation on Fas-mediated apoptosis, we treated cells with anti-Fas agonist-like antibody Jo2. Ad5IκB plus Jo2 (12.0 ± 1.2%) or ActD plus Jo2 (8.7 ± 0.9%) rapidly induced massive cell death, whereas Jo2 alone had low cytotoxicity (78.0 ± 11.5%). Furthermore, Jo2 did not induce significant cell death in Ad5LacZ-infected hepatocytes (75.0 ± 1.1%). The cytotoxic effects of Jo2 were dose-dependent in mouse hepatocytes expressing the IκB superrepressor (data not shown). The Ad5IκB-infected hepatocytes treated with Jo2 displayed nuclear condensation and fragmentation by propidium iodide staining, characteristic of apoptosis (Fig. 2*A*, *lower panel*), whereas uninfected cells displayed normal nuclear morphology after Jo2 treatment (Fig. 2*A*, *upper panel*). To confirm hepatocyte death as apoptosis, TUNEL assay was performed. Although TUNEL positive cells were minimal after TNFα or Jo2 treatment, significant positive hepatocytes were observed after TNFα or Jo2 treatment in ActD-sensitized or Ad5IκB-infected hepatocytes (Fig. 2*B*). These results were consistent with cytotoxicity determined by the trypan blue extrac-

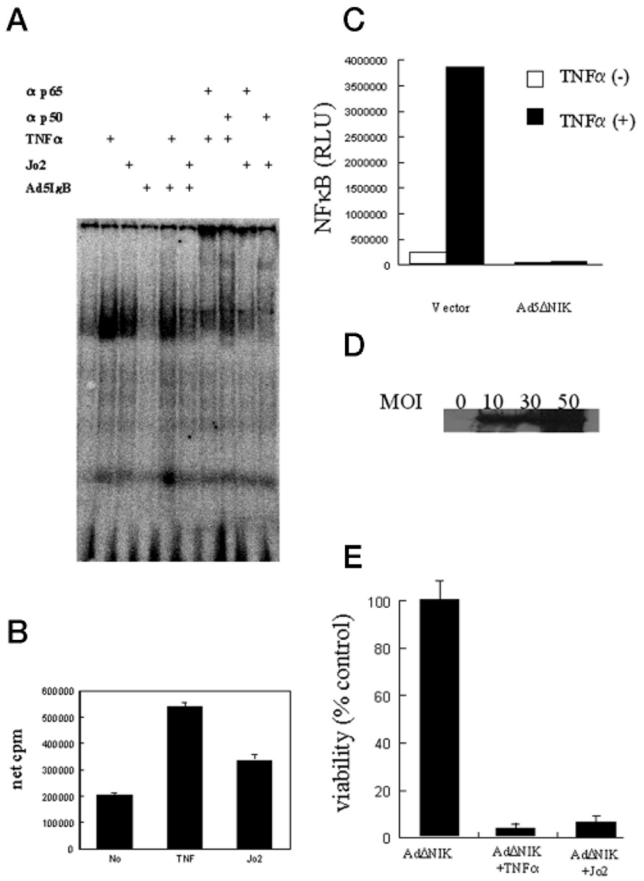

FIG. 3. **TNFα or Fas activates NF-κB in primary mouse hepatocytes and overexpression of dominant-negative mutant NIK sensitizes hepatocyte to TNFα- or Fas-mediated cell death.** *A,* NF-κB DNA binding activity was assessed by an electrophoretic mobility shift assay using NF-κB binding sites as the probe with nuclear extracts prepared after a 30-min incubation with TNFα or Jo2. For supershift assays, 8 μg of antibody against the p65 or p50 subunit of the NF-kB complex were added to the reaction mixture, and the incubation time was extended for an additional 30 min. *B,* band intensity was quantified using phosphoimager analysis. Data are shown as mean of net cpm ± S.E. of three different experiments. *C,* a reporter gene assay was performed using $(\kappa B)_3$-luc. NF-κB activation was induced by a 5-h incubation with 20 ng/ml TNFα. Results from one representative experiment performed in duplicate are shown. *D,* the HA-tagged ΔNIK was detected by Western blotting using anti-HA antibody in whole extracts after Ad5ΔNIK infection. *E,* Ad5ΔNIK-infected hepatocytes were untreated or treated with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml). Cell viability was assessed after 17 h by trypan blue exclusion test. Data are shown as average percent viability compared with uninfected cells (con) ± S.E. of three different experiments.

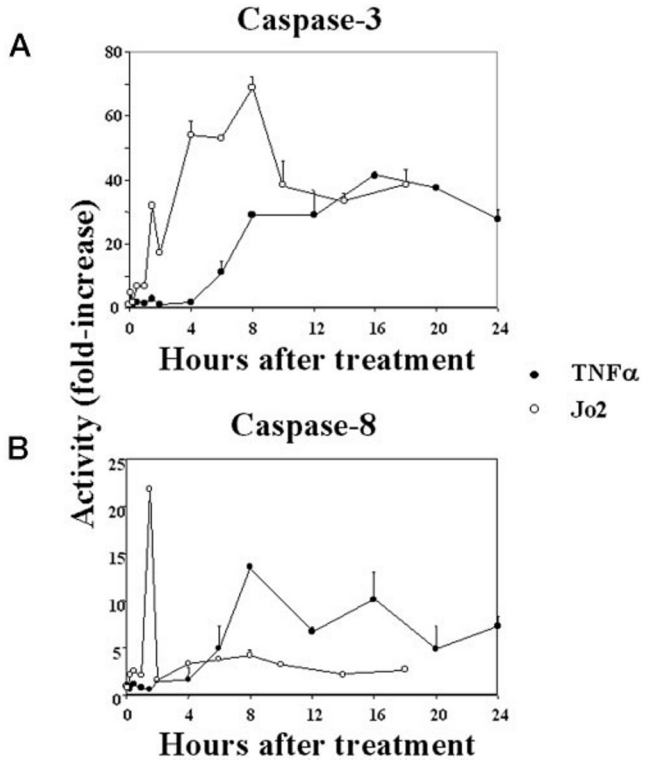

FIG. 4. **TNFα and Jo2 activate caspase-3 and -8 in Ad5IκB-infected hepatocytes with different time courses.** Ad5IκB-infected hepatocytes were treated with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml) and then lysed and assayed for caspase 3 and 8 activity (*A* and *B*, respectively) at 1-h intervals. Data are shown as average fold increases of basal levels without treatment ± S.E. of three different experiments performed in duplicate.

tion test. Furthermore, apoptosis was confirmed by the detection of fragmented chromosome DNA in infected cells after exposure to TNFα or Jo2 (Fig. 2*C*). However, no DNA fragmentation was observed in the uninfected cells after Jo2 treatment. We also documented the role of the IκB/NFκB system in TNFα- and Fas-mediated apoptosis with a proteasome inhibitor, because proteasome inhibitors block IκBα degradation and reduce NF-κB activation (43). MG-132, a potent and specific proteasome inhibitor (Fig. 1, MG-132 alone, 92.0 ± 4.6%), promotes TNFα- and Fas-mediated apoptosis (9.0 ± 3.6% and 0%, respectively).

To examine whether Jo2 directly activates NF-κB, NF-κB DNA binding activity was assessed by electrophoretic mobility shift assay using an NF-κB binding site as probe. TNFα treatment for 30 min induced an increase in NF-κB DNA binding activity (2.2-fold increase, $p < 0.001$, *versus* untreated hepatocytes) (Fig. 3, *A* and *B*). Jo2 treatment also induced NF-κB binding activity (1.5-fold increase, $p < 0.005$, *versus* untreated hepatocytes), although to a less extent than TNFα. This activation was observed even at 15 min after Jo2 treatment with the peak at 30 min after Jo2 (data not shown). The NF-κB complex activated by Jo2 treatment of mouse hepatocytes was composed of p50-p65 dimers, as determined by supershifts (Fig. 3*A*). These results show that TNFα and Jo2 activate NF-κB in mouse hepatocytes and blocking NF-κB sensitizes mouse hepatocytes to TNFα- and Fas-mediated apoptosis.

Overexpression of Dominant-Negative Mutant NIK Sensitizes Hepatocytes to TNFα- and Fas-mediated Apoptosis—NIK has been identified as a TRAF2-interacting protein that signals for NF-κB activation (5). Adenovirus (Ad5ΔNIK)-mediated overexpression of the C-terminal NIK fragment (NIKΔ2101) impaired the induction of NF-κB by TNFα in a reporter gene assay in COS cells (Fig. 3*C*). HA-tagged ΔNIK was expressed in primary mouse hepatocytes by infection of Ad5ΔNIK at 10, 30, and 50 m.o.i. (Fig. 3*D*). Dominant-negative expression of NIK sensitized mouse hepatocytes to TNFα- and Fas-mediated cell death (Fig. 3*E*). Cell death by apoptosis was confirmed by TUNEL assay (31.7 ± 1.7, 45.0 ± 3.2, TUNEL positive cells/200× power field 13 h after TNFα or Jo2 treatment in AdΔNIK-infected hepatocytes, respectively). These results support a protective role for NF-κB activation in TNFα- and Fas-mediated apoptosis and that NIK is required for the activation of NF-κB by TNFα or Fas.

TNFα and Jo2 Induce Caspase Activation in Ad5IκB-infected Hepatocytes with Different Time Courses—To compare TNFα and Fas signaling pathways in hepatocyte apoptosis, time courses for caspase-3 and casapase-8 activation were determined. Ad5IκB-infected hepatocytes were treated with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml) and then lysed and assayed for

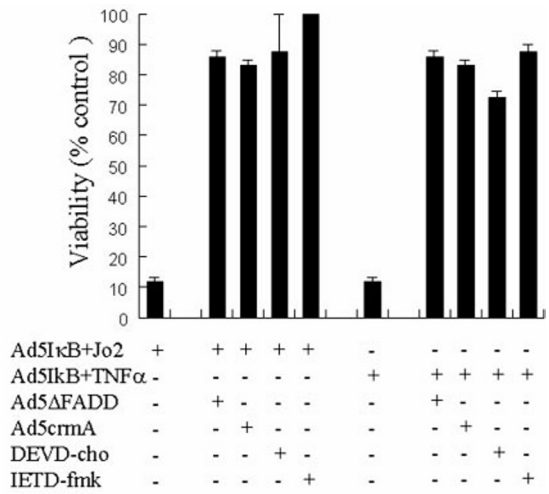

FIG. 5. **ΔFADD, crmA, and caspase inhibitors block TNFα- and Fas-mediated cell death in Ad5IκB-infected hepatocytes.** Primary mouse hepatocytes were infected with Ad5IκB and then treated with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml). Some hepatocytes were infected with Ad5ΔFADD (30 m.o.i.) or Ad5CrmA (30 m.o.i.) or also treated with DEVD-cho (50 μM) or IETD-fmk (50 nM). Cell viability was assessed after a 17-h treatment by a trypan blue exclusion test. Data are shown as average percent viability ± S.E.

caspase-3 and caspase-8. Caspase-3 was activated 11-fold for 6 h after TNFα treatment with the peak of the activity of 41-fold at 16 h after treatment (Fig. 4*A*). Caspase-8 was activated 14-fold at 8 h. In Jo2-treated hepatocytes expressing the IκB superrepressor, caspase-3 was activated 32-fold at 90 min after treatment, with the peak of 69-fold at 8 h after treatment (Fig. 4*B*). Also, a distinct early peak of caspase-8 activation was observed 90 min after Jo2 treatment. At later time points, caspase-8 was induced to higher levels by TNFα than Fas. These results show that these caspases were activated in both TNFα- and Fas-mediated apoptosis but that Fas activated caspases earlier than TNFα.

ΔFADD, crmA, and Caspase Inhibitors Block TNFα- and Fas-mediated Cell Death in Ad5IκB-infected Hepatocytes—To investigate the involvement of apoptotic signals from the complexes of TNFR·TRADD·FADD and Fas·FADD and the role of proximal caspases, primary mouse hepatocytes were infected with Ad5IκB together with Ad5ΔFADD (30 m.o.i.) or Ad5crmA (30 m.o.i.) and then treated with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml). Ad5ΔFADD expresses a truncated, dominant-negative mutant of FADD (44). Ad5crmA expresses crmA, a serpin inhibitor of a subset of caspases including caspases-1 and -8 (45, 46). Adenovirus-mediated expression of ΔFADD and crmA prevents TNFα- and Fas-mediated apoptosis (Fig. 5). To assess the role of apoptotic protease cascade in hepatocyte apoptosis, cells were treated with DEVD-cho (50 μM)(an inhibitor of caspase 3) or IETD-fmk (50 nM)(an inhibitor of caspases-4, -5, and -8) together with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml). Caspase inhibitors, DEVD-cho or IETD-fmk, clearly inhibit TNFα- and Fas-mediated apoptosis (Fig. 5). These results indicate that Fas and TNFR1 appear to utilize similar or at least partially overlapping pathways including FADD, caspase-3, caspase-8, and/or caspase-1.

TNFα and Jo2 Induce the MPT and Mitochondrial Depolarization in Ad5IκB-infected Hepatocytes with Different Time Courses—Primary mouse hepatocytes were treated with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml) after Ad5IκB infection (30 m.o.i.) and then loaded with calcein to monitor the MPT and TMRM to monitor mitochondrial depolarization. Their fluorescence was monitored simultaneously in living cells on a heated platform by confocal microscopy. Before treatment with Jo2, each

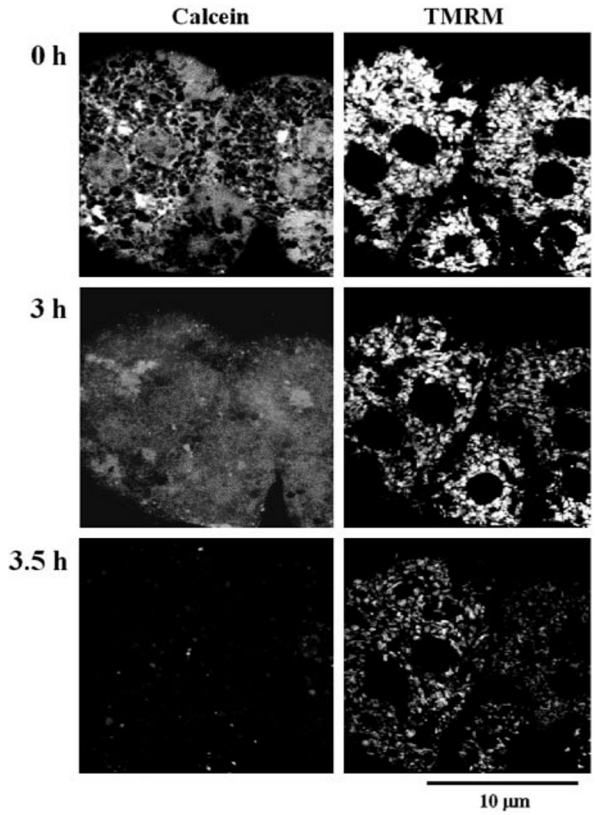

FIG. 6. **Jo2 induces the MPT and mitochondrial depolarization in Ad5IκB-infected hepatocytes.** Primary mouse hepatocytes were treated with Jo2 (0.5 μg/ml) after Ad5IκB infection (30 m.o.i.) and then loaded with calcein (*left panel*) to monitor the MPT and TMRM (*right panel*) to monitor mitochondrial depolarization. Calcein and TMRM fluorescence was monitored simultaneously over time in living cells by confocal microscopy.

TMRM-labeled mitochondrion corresponded to a dark void in the calcein image, showing that the mitochondria were polarized and impermeable to low molecular weight solutes (Fig. 6, *upper left panel*). At 3 h after treatment of Jo2, some mitochondria filled with calcein fluorescence (Fig. 6, *middle left panel*), demonstrating permeabilization of the inner mitochondrial membrane, corresponding to the onset of the MPT. Simultaneously, these mitochondria lost TMRM fluorescence, indicating depolarization (Fig. 6, *middle right panel*). Finally, after 3.5 h of exposure to Jo2, there was hepatotoxicity with extravasation of calcein (Fig. 6, *lower left panel*). In contrast to Jo2 treatment, TNFα treatment induced MPT and mitochondrial depolarization at 8–10 h in mouse hepatocytes (data not shown). This TNFα result was similar to previous studies in primary rat hepatocytes (17). These results show that MPT is induced in both TNFα- and Jo2-mediated apoptosis but at different time courses.

The Combination of CsA and Trifluoperazine (TFZ) Blocks TNFα- but Not Fas-mediated Cell Death in Ad5IκB-infected Hepatocytes—CsA, an immunosuppressive cyclic oligopeptide, specifically blocks the MPT and has been shown to prevent cell injury in several kinds of models (40, 47). TFZ also blocks the MPT and prevents mitochondrial depolarization, ATP depletion, and cell death (48). Hepatocytes overexpressing IκB superrepressor were treated with TNFα or Jo2 in the presence of CsA (5 μM) and TFZ (12.5 μM). Confocal studies showed that TMRM and calcein distributions did not change between 8 (Fig. 7*A*) and 12 h (Fig. 7*B*) in TNFα-treated hepatocytes. Similarly, these distributions did not change between 7 (Fig. 7*C*) and 13 h (Fig. 7*D*) in Jo2-treated hepatocytes. These results indicate

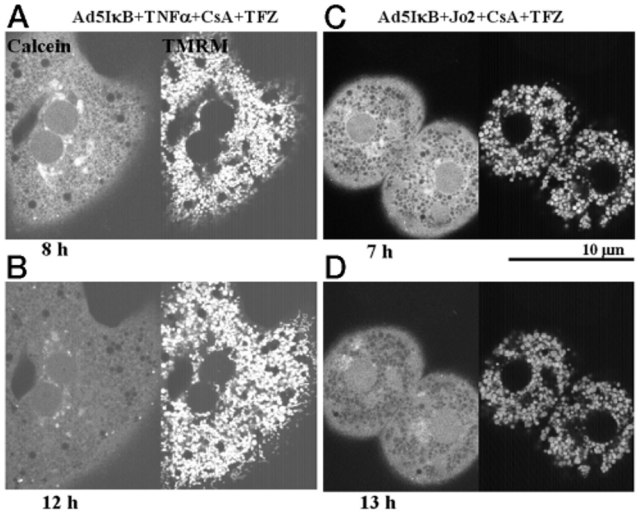

FIG. 7. **CsA plus TFZ blocks the MPT induced by TNFα or Jo2.** Primary mouse hepatocytes were treated with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml) with CsA (5 μM) plus TFZ (12.5 μM) after Ad5IκB infection (30 m.o.i.) and then loaded with calcein to monitor the MPT and TMRM to monitor mitochondrial depolarization. These fluorescences were monitored simultaneously over time in living cells by confocal microscopy. *A* and *B,* 8 and 12 h after TNFα treatment, respectively. *C* and *D,* 7 and 13 h after Jo2 treatment, respectively.

that CsA plus TFZ block mitochondrial depolarization and MPT in TNFα- and Fas-treated hepatocytes.

To test the ability of CsA plus TFZ to protect against TNFα- and Fas-mediated apoptosis, primary mouse hepatocytes were infected with Ad5IκB and then treated with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml) with and without CsA (5 μM) and/or TFZ (12.5 μM). CsA alone partially inhibits TNFα- and Fas-mediated apoptosis (Fig. 8*A*). Furthermore, the combination of CsA and TFZ significantly protects Ad5IκB-infected hepatocytes from TNFα-mediated apoptosis but not from Fas-mediated apoptosis. Although multiple concentrations of CsA (1–10 μM) and TFZ (2.5–25 μM) were tested, the maximal protective effect of CsA plus TFZ on TNFα- and Fas-mediated apoptosis were observed at the above concentrations.

MPT Accelerates Fas-mediated Apoptosis—Our previous study showed that the MPT is required for TNFα-mediated cytochrome *c* release and subsequent apoptosis in rat hepatocytes (17). CsA plus TFZ blocked the TNFα-mediated MPT (Fig. 7, *A* and *B*) and cell death (Fig. 8*A*) in mouse hepatocytes overexpressing the IκB superrepressor. Although CsA plus TFZ blocked the Fas-mediated MPT (Fig. 7, *C* and *D*), cell death still occurred (Fig. 8*A*). Therefore, to assess the relationship between MPT and cytochrome *c* release, S-100 fractions were prepared from TNFα- or Jo2-treated hepatocytes overexpressing IκB superrepressor with or without CsA plus TFZ. Primary mouse hepatocytes were treated with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml) after Ad5IκB infection (30 m.o.i.). S-100 fractions were analyzed for cytochrome *c* content by Western blotting. TNFα induced cytochrome *c* release into the cytoplasm at 6 h after treatment (Fig. 8*B*). Jo2 induced cytochrome *c* release at 2 h and peaks at 4 h after treatment (Fig. 8*B*). Thus, cytochrome *c* release follows the MPT in AdIκB-infected hepatocytes after Jo2 exposure. The treatment of CsA plus TFZ substantially decreased cytochrome *c* release at 2 or 4 h after Jo2 treatment (Fig. 8*B*), whereas CsA plus TFZ completely blocked TNFα-mediated cytochrome *c* release (Fig. 8*B*). However, CsA alone or TFZ alone had no effect on blocking cytochrome *c* release in TNFα- or Fas-mediated apoptosis (Fig. 8*C*). In addition to cytochrome *c* release, caspase-3 activation was suppressed at early time points after Jo2 treatment in hepatocytes treated with CsA plus TFZ (Fig. 9*A*). CsA plus TFZ blocked 70% of TNFα-induced cell death in Ad5IκB-infected hepatocytes even at 24 h after treatment (Fig. 9*B*). Treatment of CsA plus TFZ delayed the time of 50% Fas-mediated cell death from about 8 to about 20 h (Fig. 9*C*). Furthermore, confocal studies demonstrated that bleb formation and cell shrinkage was observed at 16–18 h after Jo2 treatment despite blocking the MPT (Fig. 9*D*), whereas some mitochondria remained polarized. These results indicate that although the MPT is not required for Fas-mediated apoptosis in hepatocytes, the MPT accelerates the progression of apoptotic cell killing.

DISCUSSION

Fas agonistic antibody (Jo2) and TNF-α are potent mediators of hepatoxicity *in vivo* and in cultured cells. In the present study, we demonstrated that (*a*) inhibition of NF-κB activation by the IκB superrepressor by a proteasome inhibitor or by a dominant-negative NIK sensitizes mouse hepatocytes to TNFα- and Fas-mediated apoptosis; (*b*) both TNFα and Fas activates NF-κB in primary mouse hepatocytes; (*c*) TNFα and Fas recruit similar pathways including FADD, the activation of caspase-3 and caspase-8, the MPT, and cytochrome *c* release, but the Fas signaling pathway for apoptosis is more rapid; (*d*) inhibition of the MPT with CsA and TFZ blocks TNFα-mediated apoptosis, but delays rather than prevents Fas-mediated apoptosis; and (*e*) inhibition of MPT markedly decreases cytochrome *c* release and delays caspase-3 activation in Fas-mediated apoptosis. These observations suggest that two pathways contribute to Fas-mediated apoptosis and that the MPT contributes to an early and more rapid pathway to apoptotic cell killing (Fig. 10).

Anti-Fas antibody (Jo2) injection rapidly induces massive hepatocyte apoptosis in mice *in vivo* (2). Nevertheless, many studies using cultured mouse hepatocytes have shown that Fas-mediated apoptosis requires the presence of an inhibitor of translation (cycloheximide), an inhibitor of RNA synthesis (actinomycin D), or a protein kinase inhibitor (H7) (20, 21). Consistent with these reports, anti-Fas antibody alone has very low cytotoxicity in cultured mouse hepatocytes, whereas Act D sensitizes hepatocytes to Fas-mediated apoptosis in this study (Fig. 1). These results suggest that cultured mouse hepatocytes may express protective proteins against apoptosis from Fas-mediated apoptosis. NF-κB activation prevents TNFα toxicity in many cell types (13–15), including hepatocytes (49). Thus we hypothesized that NF-κB may also protect primary mouse hepatocytes from Fas-mediated apoptosis.

NF-κB is sequestered in the cytoplasm by inhibitory proteins, such as IκBα, which mask the nuclear localization signal of NF-κB (50). The phosphorylation of two serines 32 and 36 of IκBα by the IKK complex, which includes IKKα, IKKβ, and IKKγ, triggers polyubiquitination of IκB proteins, which targets them for rapid proteasome-dependent degradation (7, 8, 10–12, 51). The loss of IκB binding allows NF-κB to translocate to the nucleus and activate NF-κB-dependent transcription. In this study we selected three methods for inhibition of NF-κB activation: adenovirus-mediated expression of an IκB superrepressor and a dominant-negative mutant NIK, and a proteasome inhibitor, MG-132. Inhibition of NF-κB activation by any of these methods clearly sensitizes mouse hepatocytes to TNFα- and Fas-mediated apoptosis, whereas these treatments without TNFα or Fas stimulation have no significant cytotoxicity (Figs. 1, 2*B*, and 3*E*). Furthermore, mobility shift assays indicate that Jo2 induces NF-κB DNA binding activity (1.5-fold increase) in cultured mouse hepatocytes, even though this activity was weaker than TNFα-induced NF-κB DNA binding activity (2.2-fold increase) (Fig. 2*B*). This TNFα-induced NF-κB DNA binding activity is comparable to that in myocytes (2.1-fold increase) (52). These results suggest that Fas increases

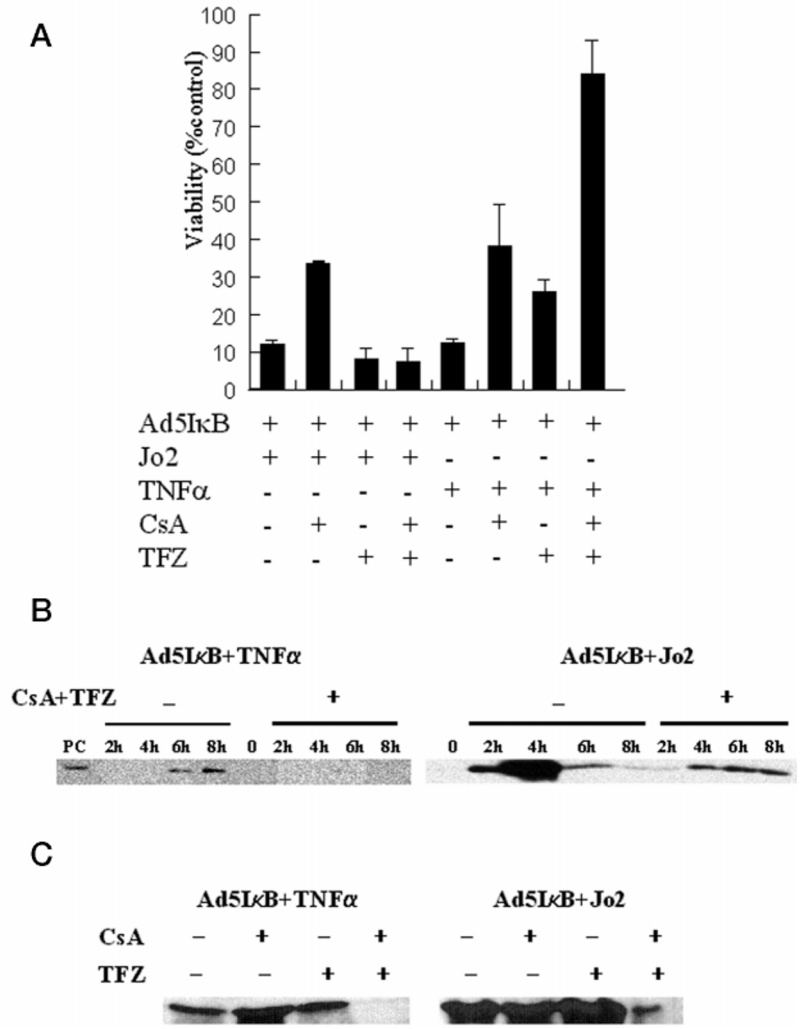

FIG. 8. **The combination of CsA and TFZ blocks TNFα- but not Fas-mediated cytochrome *c* release and cell death in Ad5IκB-infected hepatocytes.** *A,* primary mouse hepatocytes were infected with Ad5IκB and then treated with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml) with or without CsA (5 μM) and/or TFZ (12.5 μM). Cell viability was assessed at 20 h after treatment by a trypan blue exclusion test. Data are shown as average percent viability ± S.E. *B,* primary mouse hepatocytes were treated with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml) with or without CsA (5 μM) plus TFZ (12.5 μM) after Ad5IκB infection (30 m.o.i.). S-100 fractions were prepared at the indicated time points and analyzed for cytochrome *c* content by Western blotting. *C,* primary mouse hepatocytes were treated with TNFα (30 ng/ml) or Jo2 (0.5 μg/ml) with CsA (5 μM) alone, TFZ (12.5 μM) alone, or both after Ad5IκB infection (30 m.o.i.). S-100 fractions were prepared 8 or 4 h after TNFα or Jo2 treatment, respectively, and analyzed for cytochrome *c* content by Western blotting.

NF-κB DNA binding activity, which is mediated by activated NIK, activated IKK, phosphorylated IκB, and subsequent IκB degradation by proteasomes with translocation of NF-κB to the nucleus. A previous study showed that Fas stimulates the DNA binding activity of NF-κB in a variety of cells, irrespective of their sensitivity or resistance to Fas-mediated cytotoxicity (19). However, in our study, NF-κB activation had a protective effect in Fas-mediated apoptosis in cultured mouse hepatocytes.

One of the protective proteins against apoptosis is inducible nitric-oxide synthases. NO prevents apoptosis by suppressing the increase of caspase-3-like activity (53). NO-mediating *S*-nitrosylation of the cysteine-containing enzymes that mediate apoptosis may regulate the balance between apoptosis and necrosis (54). The mRNA of inducible nitric-oxide synthases is regulated by NF-κB, and NO prevents hepatocyte apoptosis initiated by the removal of growth factors or exposure to TNFα or anti-Fas antibody (55–57). Therefore, inducible nitric-oxide synthases might be an NF-κB-inducible protective gene mediating resistance to TNFα and Fas cytotoxicity.

Ceramides have been implicated as a second messenger in signaling pathways leading to apoptosis (58–61). A recent paper showed that primary rat hepatocytes are resistant to ceramide-induced toxicity (62). However, NFκB inactivation or ActD sensitize a rat hepatocyte cell line to ceramide toxicity, suggesting that ceramide may act as a downstream mediator of TNFα toxicity. On the other hand, it has been reported that GD3 ganglioside, a product of ceramide, is required for Fas- and ceramide-induced apoptosis (63), induces the MPT and apoptosis in rat hepatoma cells (64), and directly induces the MPT in isolated liver mitochondria (64, 65). Although significant cell-type-specific differences exist in cell death pathway, further investigations will determine the relationship between Fas, ceramides, and the MPT in hepatocytes.

A recent study defined two pathways for Fas-mediated apoptosis in different cell types (66). In type I cells, caspase-8 is activated within seconds and caspase-3 within 30 min, whereas in type II cells cleavage of caspases is relatively delayed. Both cells showed similar Fas-mediated apoptosis and loss of mitochondrial transmembrane potential, but only in type II cells does overexpression of Bcl-2 or Bcl-xL block caspase-8 and caspase-3 activation as well as apoptosis, indicating type II cells are dependent on mitochondria. Our previous study showed that the MPT is an essential component in TNFα-mediated apoptosis in rat hepatocytes and functions upstream of caspase-3. However, our present study demonstrates differences in signaling pathways between TNFα- and Fas-mediated apoptosis in hepatocytes. The time course in the activation of caspases by Fas was different from those by TNFα. Activation of caspase-3 and -8 was within 2 h after Jo2 treatment (Fig. 4, *A* and *B*), whereas TNFα activated caspase-3 and -8 after 6 h. This result is consistent with the report that the Fas signaling pathway is more rapid and strong in hepatocytes compared with TNFα (67). These results on the activation of caspases by Fas indicate that primary mouse hepatocytes act predominantly as type II cells.

Several pieces of evidence implicate mitochondria in the

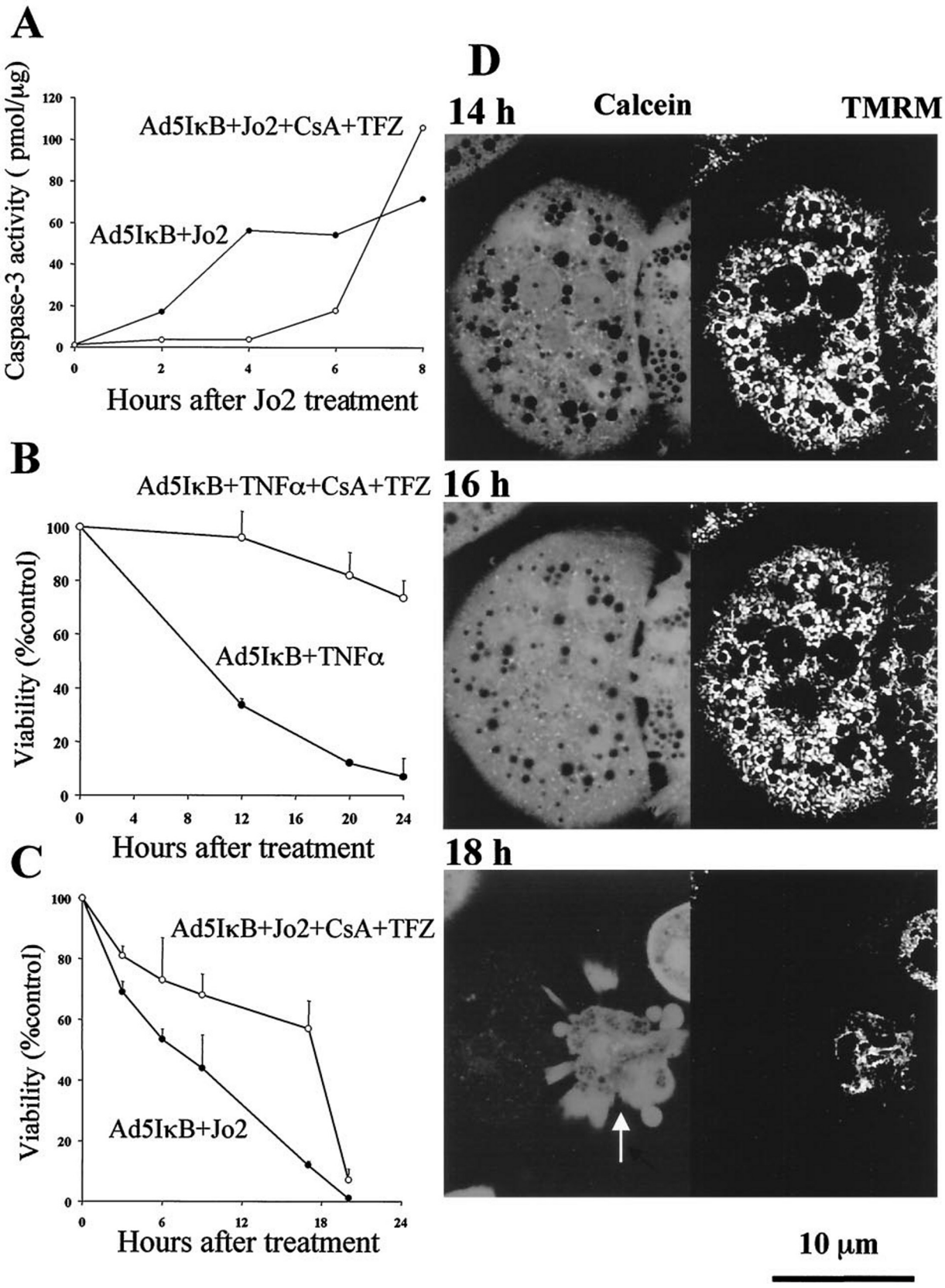

FIG. 9. **MPT accelerates Fas-mediated apoptosis.** *A,* Ad5IκB-infected hepatocytes were treated with Jo2 (0.5 μg/ml) with/without CsA (5 μM) plus TFZ (12.5 μM) and then lysed and assayed for caspase 3 activities. Representative data are presented as pmol/μg protein. *B* and *C,* cell viability was assessed by a trypan blue exclusion test after TNFα (*B*) or Jo2 (*C*) treatment. Data are shown as average percent viability ± S.E. *D,* Fas-induced hepatotoxicity despite blocking the MPT. Primary mouse hepatocytes were infected with Ad5IκB and then treated with Jo2 (0.5 μg/ml), CsA (5 μM), and TFZ (12.5 μM) and then loaded with calcein and TMRM. Calcein and TMRM fluorescence was monitored simultaneously over time in living cells by confocal microscopy. Bleb formation and cell shrinkage were observed in the cell that the *arrows* indicate.

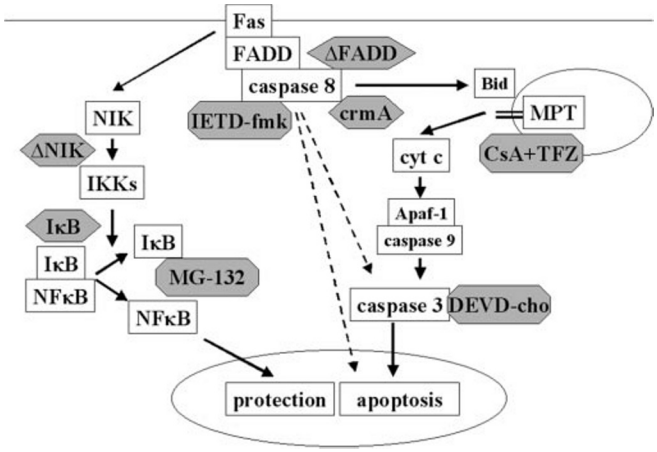

FIG. 10. **Fas signaling pathway in primary mouse hepatocytes.** IκB superrepressor, dominant-negative NIK, or MG-132 (a proteasome inhibitor) sensitized hepatocytes to Fas-mediated apoptosis. FADD, casapse-8, the MPT, and caspase-3 were involved in Fas apoptotic signaling pathway as well as in the TNFα signaling pathway. When the MPT was blocked with CsA plus TFZ, Fas induces caspase-3 and apoptosis via an MPT-independent pathway.

process of apoptosis. Cytoplasmic events including activation of protease cascades and MPT participate in the control of nuclear apoptosis (24, 68, 69). Whether the MPT is essential for cellular apoptosis remains controversial, because some studies claim that cytochrome *c* release during apoptosis occurs without mitochondrial depolarization (68, 70), whereas other studies show the opposite (34). Here we show cyclosporin A plus trifluoperazine prevents the MPT and cytochrome *c* release by TNFα as well as apoptosis in mouse hepatocytes, as described previously in rat hepatocytes (17). These data suggest that mitochondria, especially the MPT, is essential for hepatocyte apoptosis by TNFα in mice and rats.

However, the involvement of the MPT in Fas-mediated apoptosis has remained elusive. Activation of caspase-1 precedes the disruption of the mitochondrial inner transmembrane potential, but caspase-3 activation and nuclear apoptosis only occur in cells in which the mitochondrial transmembrane potential is fully disrupted (71). This indicates that the MPT is essential for apoptosis downstream of caspase-1 and upstream of caspase-3. These human CEM-C7.H2 lymphoma cells seem to be type II cells, as described above (66). In our study cyclosporin A plus trifluoperazine prevented the MPT and markedly decreased cytochrome *c* release by Fas, but apoptosis was not blocked in mouse hepatocytes (Figs. 7, 8*C*, 9*A*, and 9*B*). These results indicate that Fas induces the MPT, which accelerates but is not necessary for apoptosis.

The mechanism by which activated caspase-8 recruits the mitochondria to participate in apoptosis is cleaving and activating Bid. Bid then translocates to the mitochondria to trigger cytochrome *c* release (72, 73). Cytochrome *c* binds to Apaf1, which in turn self-associates and binds procaspase-9. Transactivation of the complexed procaspase-9 then activates downstream caspases. Our results demonstrating Fas-induced MPT are consistent with an apoptotic pathway that includes the mitochondria. However, Fas-mediated apoptosis is intact in Apaf1 knockout T cells (74). Furthermore, while this manuscript was under review, it was reported that Fas-mediated apoptosis was delayed rather than prevented in Bid-deficient hepatocytes (75). These results combined with our study indicate that Fas signaling in hepatocytes activates both a mitochondrial independent pathway and a mitochondrial dependent pathway of apoptosis (Fig. 10).

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