

Hepatocytes Sensitized to Tumor Necrosis Factor- α Cytotoxicity Undergo Apoptosis through Caspase-dependent and Caspase-independent Pathways*

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Hepatocytes can be sensitized to tumor necrosis factor (TNF)- α toxicity by repression of NF- κ B activation or inhibition of RNA synthesis. To determine whether both forms of sensitization lead to TNF- α cytotoxicity by similar mechanisms, TNF- α -induced cell death in RALA255-10G hepatocytes was examined following infection with an adenovirus, Ad5I κ B, that blocks NF- κ B activation or following cotreatment with actinomycin D (ActD). TNF- α treatment of Ad5I κ B-infected cells resulted in 44% cell death within 6 h. ActD/TNF- α induced no death within 6 h but did lead to 37% cell death by 24 h. In both instances, cell death occurred by apoptosis and was associated with caspase activation, although caspase activation in ActD-sensitized cells was delayed. CrmA and chemical caspase inhibitors blocked Ad5I κ B/TNF- α -induced cell death but did not inhibit ActD/TNF- α -induced apoptosis. A Fas-associated protein with death domain (FADD) dominant negative decreased Ad5I κ B/TNF- α and ActD/TNF- α -induced cell death by 81 and 47%, respectively. However, downstream events differed, since Ad5I κ B/TNF- α but not ActD/TNF- α treatment caused mitochondrial cytochrome *c* release. These results suggest that NF- κ B inactivation and inhibition of RNA synthesis sensitize RALA255-10G hepatocytes to TNF- α toxicity through distinct cell death pathways that diverge below the level of FADD. ActD-induced hepatocyte sensitization to TNF- α cytotoxicity occurs through a FADD-dependent, caspase-independent pathway of apoptosis.

Prominent among the varied physiological effects of the cytokine tumor necrosis factor- α (TNF- α)¹ is its ability to act as a cytotoxin and induce apoptotic or necrotic cell death (1). Al-

though TNF- α cytotoxicity has been widely investigated in the context of its potential as an antineoplastic agent, recent studies have demonstrated that TNF- α may also induce death in cells in normal tissue undergoing injury or inflammation. TNF- α toxicity is particularly important to the pathophysiology of liver disease, and TNF- α has been implicated as a mediator of hepatocyte death following injury from toxins, ischemia/reperfusion, and hepatitis virus (for a review, see Ref. 2). In toxin-induced liver injury, endogenously produced TNF- α induces a significant proportion of the subsequent liver cell death as evidenced by the ability of TNF- α neutralization to dramatically reduce liver injury from toxins such as carbon tetrachloride (3), actinomycin D (ActD) (4), and ethanol (5). Hepatocytes are normally resistant to TNF- α cytotoxicity (6, 7); therefore, these toxins sensitize hepatocytes to cell death from TNF- α by an as yet unknown mechanism. *In vitro* investigations into the mechanisms of TNF- α cytotoxicity in nonhepatic cells have demonstrated that binding of TNF- α to tumor necrosis factor receptor 1 (TNFR-1) results in receptor trimerization and the recruitment of a series of intracellular proteins (1). Initially, TNFR-associated death domain protein binds to the TNFR-1. TNFR-associated death domain protein then recruits TNFR-associated factor 2, Fas-associated protein with death domain (FADD), and receptor-interacting protein (1, 8). Binding of TNFR-associated death domain protein and FADD to the TNFR-1 leads to the recruitment, oligomerization, and activation of caspase-8 (8, 9). Activated caspase-8 subsequently initiates a proteolytic cascade involving other caspase family members, ultimately leading to apoptosis (10, 11). Activation of these downstream caspases may be amplified by factors released from mitochondria such as cytochrome *c* (12, 13). Alternative caspase-8-independent mechanisms by which TNF- α receptor binding initiates downstream caspase activation may also exist. Investigations have demonstrated a FADD-independent pathway of TNF- α -induced caspase activation involving RAIDD (14). Despite their differences, these pathways all ultimately transduce the TNF- α death signal through the activation of caspases.

The resistance of nontransformed cells to TNF- α -induced cytotoxicity is thought to depend on the ability of TNF- α signaling to up-regulate a protective cellular gene(s). This conclusion is based on the finding that inhibition of RNA synthesis by ActD or of protein synthesis by cycloheximide sensitizes nonhepatic cells (15) and hepatocytes (4, 7) to TNF- α -induced cell death. Recent investigations have demonstrated that cellular activation of the transcription factor NF- κ B is critical for the induction of resistance to TNF- α toxicity (16–19). Blocking NF- κ B activation in cultured hepatocytes (12, 20) or in the liver *in vivo* (21), converts the hepatocellular TNF- α response from

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¹ The abbreviations and trivial names used are: TNF- α , tumor necrosis factor- α ; RALA, RALA255-10G; ActD, actinomycin D; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TNFR-1, tumor necrosis factor receptor 1; FADD, Fas-associated protein with death domain; IDN-1529, N-[(indole-2-carboxyl)alaninyl]-3-amino-4-oxo-5-fluoropentanoic acid; IDN-1965, N-[(1,3-dimethylindole-2-carboxyl)-valinyl]-3-amino-4-oxo-5-fluoropentanoic acid; PAGE, polyacrylamide gel electrophoresis; FACs, fluorescence-activated cell sorting.

one of proliferation to one of apoptosis. These results have suggested that toxins such as ActD or carbon tetrachloride may sensitize hepatocytes to TNF- α toxicity by blocking up-regulation of an NF- κ B-dependent protective gene. However, because of differences in the amount of cell death caused by these two forms of TNF- α toxicity (7, 20), we hypothesized that ActD and NF- κ B inhibition sensitize hepatocytes to TNF- α -induced death by different mechanisms. By contrasting the involvement of caspases, FADD, and cytochrome *c* in these two forms of sensitization to TNF- α -induced cytotoxicity, the present studies demonstrate that two distinct TNF- α cell death pathways exist in hepatocytes.

EXPERIMENTAL PROCEDURES

Materials—All reagents were from Sigma unless otherwise indicated.

Cells and Culture Conditions—The rat hepatocyte cell line RALA255-10G (22) was cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 4% fetal bovine serum (HyClone, Logan, UT), 2 mM glutamine, and antibiotics (Life Technologies, Inc.). These cells are conditionally transformed with a temperature-sensitive T antigen. At the permissive temperature of 33 °C, they express T antigen, remain undifferentiated, and proliferate. Culture of the cells at the restrictive temperature of 37 °C suppresses T antigen expression, markedly slows growth, and allows differentiated hepatocyte gene expression (22). For these experiments, the cells were cultured at 33 °C until confluent, trypsinized, and replated at 0.65×10^6 cells/dish on 35-mm plastic dishes (Falcon; Becton Dickinson, Lincoln Park, NJ). After 24 h, the medium was changed to Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum, glutamine, antibiotics, and 1 μ M dexamethasone, and the cells were placed at 37 °C.

After 3 days of culture at 37 °C, cells receiving adenovirus were infected with 2×10^9 particles of the appropriate virus/dish ($\sim 1 \times 10^3$ particles/cell or 5–10 plaque-forming units/cell). In the case of double virus infections, dishes received 2×10^9 particles of each virus simultaneously. Additional Ad5LacZ control virus was added when necessary to make the total viral load equal in each dish. Three hours later, infected and uninfected cells received fresh serum-free medium containing dexamethasone. Medium was supplemented with dexamethasone to optimize hepatocyte differentiation as described previously (22). Twenty hours later, some cells were pretreated with ActD (15 ng/ml) for 30 min. Untreated, infected, and ActD-treated cells then received rat recombinant TNF- α (R & D Systems, Minneapolis, MN; ED₅₀ of 10–20 pg/ml as measured by cytotoxicity in the L-929 cell line) at a concentration of 10 ng/ml.

To inhibit caspase activity, cells were pretreated for 1 h before the addition of TNF- α with the following caspase inhibitors dissolved in dimethyl sulfoxide: 50 μ M N-[(indole-2-carboxyl)alaninyl]-3-amino-4-oxo-5-fluoropentanoic acid (IDN-1529) or N-[(1,3-dimethylindole-2-carboxyl)-valinyl]-3-amino-4-oxo-5-fluoropentanoic acid (IDN-1965) (IDUN Pharmaceuticals, La Jolla, CA), 100 μ M Ac-Tyr-Val-Ala-Asp-chloromethylketone, Ac-Asp-Glu-Val-Ala-Asp-aldehyde, and Val-Ala-Asp-fluoromethylketone (BACHEM, Torrance, CA). IDN-1529 has broad anti-caspase activity, inhibiting caspase-1, -3, -6, and -8, and IDN-1965 is a pancaspase inhibitor with some selectivity toward caspase-6 and -8.²

Adenovirus Construction and Infection—The recombinant, replication-deficient adenovirus Ad5I κ B was used as described previously to inhibit NF- κ B activation (20). This adenovirus contains an I κ B construct in which serines 32 and 36 are mutated to alanines, driven by the cytomegalovirus promoter-enhancer. This mutant I κ B cannot be phosphorylated and therefore irreversibly binds NF- κ B, preventing its activation. In addition, the previously described viruses Ad5LacZ, which contains the *Escherichia coli* β -galactosidase gene; a CrmA-expressing adenovirus (12); and NFD-4, a dominant negative FADD adenovirus (12), were employed. All viruses were grown in 293 cells and purified by banding twice on CsCl gradients as described previously (20). Numbers of viral particles were determined by optical densitometry, and recombinant virus was then stored in 25% (v/v) glycerol at -20 °C.

MTT Assay and Cell Counts—The amount of cell death was quantified from determinations of cell number with the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay (23), as de-

scribed previously (20). The percentage of cell survival was calculated by taking the optical density of cells given a particular treatment, dividing that number by the optical density for the untreated, control cells, and then multiplying by 100.

Alternatively, cell death was determined by doing manual counts of the numbers of trypan blue-excluding cells following trypsinization. The percentage of cell survival was calculated by taking the number of trypan blue-excluding cells following treatment, dividing by the number of untreated, control cells, and multiplying by 100.

Microscopic Determination of Apoptosis—Phase-contrast and fluorescent microscopy were conducted as described previously (24). The relative number of apoptotic cells was determined by fluorescent costaining with acridine orange and ethidium bromide as previously employed (20). The percentage of cells with apoptotic morphology (nuclear and cytoplasmic condensation, nuclear fragmentation, membrane blebbing, and apoptotic body formation) was determined by examining >400 cells/dish. Necrosis was determined by positive ethidium bromide staining. Fluorescent micrographs were taken on an Olympus IX microscope with $\times 40$ long working distance infinity corrected optics.

Protein Isolation and Western Blot Analysis—To isolate protein for caspase immunoblots, cells were scraped in medium and centrifuged. The cell pellet was resuspended in lysis buffer containing 10 mM HEPES (pH 7.4), 42 mM MgCl₂, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM dithiothreitol, and 2 μ g/ml pepstatin A, leupeptin, and aprotinin. The solution was then mixed at 4 °C for 30 min. After centrifugation, the supernatant was collected, and the protein concentration was determined by the Bio-Rad protein assay (Bio-Rad).

Fifty micrograms of protein were heated in 1 \times SDS gel loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) at 100 °C for 2 min. The samples were subjected to 10 or 12% SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Schleicher & Schuell) in transfer buffer containing 39 mM glycine, 48 mM Tris, pH 8.3, 0.037% SDS, and 15% methanol. Membranes were stained with Ponceau Red to ensure equivalent amounts of protein loading and electrophoretic transfer among samples. Blocking of the membranes was performed using a solution of 5% nonfat milk, 10 mM Tris, pH 8.0, 0.15 M NaCl, and 0.05% Tween (TBS-T) for 1 h. Rabbit anti-caspase-2 polyclonal IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-caspase-3 polyclonal IgG, rabbit anti-caspase-7 polyclonal IgG, or rabbit anti-caspase-8 polyclonal IgG (IDUN Pharmaceuticals), were used as primary antibodies at 1:1000, 1:2000, 1:1000, and 1:2000 dilutions, respectively, in 5% milk-TBS-T for 2 h. A goat anti-rabbit IgG conjugated with horseradish peroxidase (Life Technologies, Inc.) was used as a secondary antibody at a 1:10,000 dilution in 5% milk-TBS-T blocking solution for 1 h. Proteins were visualized by chemiluminescence (Supersignal Ultra; Pierce).

For Western immunoblots of poly(ADP-ribose) polymerase (PARP), centrifuged cells were suspended in lysis buffer composed of 20 mM Tris, pH 7.5, 1% SDS, 2 mM EDTA, 2 mM EGTA, 6 mM β -mercaptoethanol, and the protease inhibitors described above. After a 10-min incubation on ice, cell suspensions were sonicated. Fifty micrograms of protein were subjected to 8% SDS-PAGE as described above. Membranes were exposed to rabbit anti-PARP polyclonal antibody (Santa Cruz Biotechnology) at a 1:4000 dilution followed by goat anti-rabbit secondary antibody at a 1:20,000 dilution.

Quantification of DNA Hypoploidy by FACS—Identification of apoptotic cells by detection of DNA loss after controlled extraction of low molecular weight DNA was performed as described previously (25). At various time points, cells were trypsinized, washed in Hanks' buffered saline solution, and pelleted. The cell pellets were resuspended and fixed in 70% ethanol and stored at -20 °C for up to 1 week. After washing twice in Hanks' buffered saline solution, 1-ml Hanks' buffered saline solution cell suspensions were incubated with 0.5 ml of phosphate-citric acid buffer (0.2 M Na₂HPO₄, 0.1 M citric acid, pH 7.8) for 5 min to extract low molecular weight DNA from apoptotic cells. Subsequently, the cells were centrifuged, and the pellet was resuspended in 0.5 ml of Hanks' buffered saline solution containing 20 μ g/ μ l propidium iodide and RNase (10 μ g/ml). Following a 30-min incubation at room temperature, cells were analyzed on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) at an excitation of 488 nm. DNA fluorescence pulse processing was used to discriminate between single cells and aggregates of cells (doublet discrimination) by evaluating the FL2-width versus FL2-area scatter plot. Light scatter gating was used to eliminate smaller debris from analysis. DNA content was displayed on a 4-decade logarithmic scale. An analysis gate was set to limit the measurement of hypoploidy to an area of 10-fold loss of DNA content (25).

² J. Wu, personal communication.

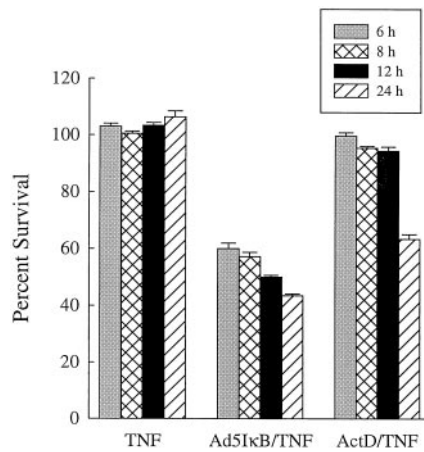


FIG. 1. Inhibition of NF- κ B activation or RNA synthesis sensitized RALA hepatocytes to TNF- α -induced cell death. Cells were treated with TNF- α alone (TNF), infected with Ad5I κ B and treated with TNF- α (Ad5I κ B/TNF), or treated with ActD and TNF- α (ActD/TNF) as described under "Experimental Procedures." The percentage of survival was determined at 6, 8, 12, and 24 h relative to untreated controls by MTT assay. Data are from three independent experiments, each with duplicate dishes for every condition.

Caspase-3-like Enzyme Activity Assay—Caspase-3-like enzyme activity was assayed in cells using a caspase-3 activity kit (BIOMOL, Plymouth Meeting, PA) according to the manufacturer's instructions.

Mitochondrial Protein Isolation and Western Blots—Mitochondrial fractions were prepared from RALA hepatocytes by differential centrifugation in 250 mM sucrose buffer as described previously (26). Fifty micrograms of mitochondrial protein were subjected to 15% SDS-PAGE as described above. A mouse anti-cytochrome *c* monoclonal IgG (Pharmingen, San Diego, CA) and a mouse anti-cytochrome oxidase subunit IV monoclonal IgG (Molecular Probes, Inc., Eugene, OR) were used at 1:1000 dilutions together with a goat anti-mouse IgG conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY).

Statistical Analysis—All numerical results are reported as mean \pm S.E. and represent data from a minimum of three independent experiments.

RESULTS

Sensitization to TNF- α Cytotoxicity Occurs More Rapidly following NF- κ B Inactivation than RNA Synthesis Inhibition—If NF- κ B inactivation and RNA synthesis inhibition both sensitize hepatocytes to TNF- α toxicity by blocking TNF- α -inducible expression of a common protective gene(s), then the timing of cell death should be similar after either form of sensitization. The time course of TNF- α -induced cell death was determined by MTT assay following inhibition of NF- κ B activation by infection with the mutant-I κ B-expressing adenovirus Ad5I κ B or inhibition of RNA synthesis by ActD. While TNF- α alone was nontoxic to RALA hepatocytes, TNF- α treatment of Ad5I κ B-infected cells resulted in rapid hepatocyte death with a 44% decrease in cell number within only 6 h (Fig. 1), as previously established (20). By 24 h after TNF- α administration, cell death further increased only slightly (Fig. 1). Infection of hepatocytes with a control virus, Ad5LacZ, that expresses the β -galactosidase gene, failed to sensitize the cells to TNF- α toxicity (data not shown). In contrast, TNF- α administration to hepatocytes pretreated with ActD caused no cell death within 6 h (Fig. 1). By 24 h, ActD/TNF- α treatment did result in 37% cell death (Fig. 1). Pretreatment of cells with ActD at earlier time points failed to alter the timing or amount of cell death following TNF- α administration (data not shown).

To ensure that the MTT assay accurately represented the numbers of cells surviving the various treatments, cell survival was also determined by counts of trypan blue-excluding cells. The amount of cell death as determined by this method (Table

TABLE I
Cell survival following Ad5I κ B/TNF- α or ActD/TNF- α treatment without caspase inhibitor (\emptyset) or in the presence of IDN-1529 (+1529)

Survival was determined by counts of trypan blue-excluding cells and is expressed as a percentage of untreated control cells. Cells were pretreated with the caspase inhibitor IDN-1529 1 h before the addition of TNF- α . Results are from three independent experiments performed in duplicate.

Time	Ad5I κ B/TNF- α		ActD/TNF- α	
	\emptyset	+1529	\emptyset	+1529
<i>h</i>		%		%
6	56.3 \pm 1.6	86.8 \pm 4.7	100.0 \pm 4.7	ND
8	50.8 \pm 4.1	ND ^a	101.0 \pm 5.0	ND
12	51.0 \pm 3.5	ND	90.8 \pm 2.9	ND
24	40.2 \pm 2.7	66.3 \pm 3.5	62.0 \pm 3.4	58.8 \pm 1.4

^a ND, not done.

I) was virtually identical to the MTT data (Fig. 1). These results demonstrate that the time course of cell death after TNF- α treatment differs markedly depending on the form of sensitization, suggesting that NF- κ B inactivation and RNA synthesis inhibition may sensitize RALA hepatocytes to TNF- α cytotoxicity by distinct mechanisms.

NF- κ B Inactivation and ActD Both Sensitize RALA Hepatocytes to Death by Apoptosis—TNF- α -induced cell death is predominantly apoptotic but may also occur by necrosis. We have previously established that TNF- α treatment following NF- κ B inactivation leads to hepatocyte apoptosis, with a marked increase in the number of apoptotic cells occurring within 1–6 h (20). To determine whether ActD/TNF- α -induced cell death also occurred by apoptosis, cells were examined by fluorescent microscopy after costaining with acridine orange and ethidium bromide. In ActD/TNF- α -treated cells, no increase in the number of apoptotic cells occurred within 6 h after TNF- α treatment (Fig. 2), consistent with the failure to detect any cell death at this time point by MTT assay and cell counts. The percentage of apoptotic cells did increase 2- and 4-fold by 8 and 12 h, respectively, after ActD/TNF- α treatment (Fig. 2). ActD sensitization did not induce necrosis, because the percentage of necrotic cells as indicated by ethidium bromide positivity was less than 1% in control and treated cells at the 6-, 8-, 12-, and 24-h time points. Although sensitization by ActD led to a delayed cell death relative to NF- κ B inactivation, both forms of sensitization caused TNF- α -induced death through apoptosis.

ActD/TNF- α -induced Apoptosis Is Associated with Caspase Activation, PARP Cleavage, and DNA Hypoploidy—Most forms of apoptosis are mediated by the proteolytic actions of caspases (10). Previous studies have demonstrated that TNF- α treatment of RALA hepatocytes sensitized by inhibition of NF- κ B activation resulted in caspase activation and PARP degradation (20). Caspase activation during ActD/TNF- α -induced apoptosis was assessed by Western immunoblot analysis of protein isolates from ActD/TNF- α -treated hepatocytes. Levels of procaspase-2, -3, -7, and -8 were unchanged at 6 h following ActD/TNF- α treatment, indicating that no caspase activation had occurred by this time (Fig. 3). Significant caspase activation was also not detected at 8 and 12 h after ActD/TNF- α treatment (data not shown). By 24 h, caspase activation had occurred as demonstrated by the loss of procaspase-2, -7, and -8, and the appearance of the processed subunits of caspase-3, -7, and -8 (Fig. 3). Decreases in procaspase levels 24 h after ActD/TNF- α treatment were much less than the dramatic decreases that occurred in Ad5I κ B/TNF- α -treated cells at 6 h (Fig. 3). No caspase activation occurred at 6, 8, 12, or 24 h in cells treated with TNF- α alone (Fig. 3 and data not shown).

The immunoblot evidence of caspase activation in ActD/TNF- α -treated cells at 24 h was substantiated by measure-

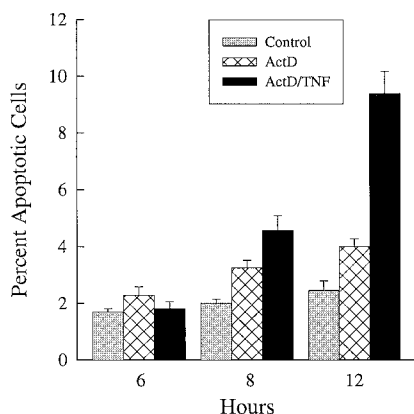


FIG. 2. Time course of induction of apoptosis by TNF- α /ActD treatment. RALA hepatocytes were untreated (*Control*) or treated with ActD alone (*ActD*), and ActD combined with TNF- α (*ActD/TNF*). The percentage of apoptotic cells was determined at 6, 8, and 12 h by fluorescent staining with acridine orange and ethidium bromide as described under "Experimental Procedures." Results are from three independent experiments each with duplicate dishes for every data point.

ments of caspase-3-like activity. At 8 h, no increase in activity could be detected in ActD/TNF- α -treated cells (Fig. 4). However, at 24 h, a 2.7-fold increase in caspase-3-like-activity was present in ActD/TNF- α -treated cells as compared with untreated control cells (Fig. 4).

To further ensure that the processed caspases were functionally active, ActD/TNF- α treated cells were examined for PARP cleavage. No PARP cleavage could be detected at 6, 8, or 12 h (data not shown), but PARP cleavage did occur within 24 h after ActD/TNF- α treatment (Fig. 5). The appearance of PARP cleavage therefore coincided with the timing of procaspase processing by immunoblot analysis and the detection of caspase-3-like activity.

DNA hypodiploidy assays of hepatocytes stimulated by TNF- α after sensitization with ActD were performed as an additional biochemical marker of the presence and timing of caspase activation and apoptosis. No significant increase in the proportion of cells with reduced DNA content occurred at 8 h after ActD/TNF- α administration (Fig. 6). By 24 h, a 4-fold increase in the number of cells with DNA hypodiploidy had occurred with ActD/TNF- α treatment (Fig. 6). Caspase activation, PARP cleavage, and DNA fragmentation all occurred after a similar delay in ActD/TNF- α -treated cells.

Caspase Inhibition Prevents TNF- α -induced Apoptosis following NF- κ B Inactivation but Not after ActD Treatment—To determine whether activated caspases mediate TNF- α toxicity in hepatocytes sensitized by either inhibition of NF- κ B activation or RNA synthesis, the ability of CrmA and chemical caspase inhibitors to prevent cell death was evaluated by MTT assay. The viral caspase inhibitor CrmA was expressed in RALA hepatocytes by an adenoviral vector. CrmA expression reduced the amount of apoptosis in Ad5I κ B/TNF- α -treated cells by 52% at 6 h (Fig. 7). The chemical pancaspase inhibitors IDN-1529 and IDN-1965 almost completely inhibited cell death in Ad5I κ B/TNF- α -treated hepatocytes (Fig. 7), similar to results previously published with lower doses of these inhibitors (20). In contrast, apoptosis caused by ActD/TNF- α treatment was not blocked by either CrmA expression or the addition of the chemical caspase inhibitors IDN-1529 and IDN-1965 (Fig. 7). Counts of trypan blue-excluding cells confirmed that IDN-1529 inhibited Ad5I κ B/TNF- α -induced apoptosis but did not block cell death from ActD/TNF- α (Table I). The additional caspase inhibitors Ac-Tyr-Val-Ala-Asp-chloromethylketone (100 μ M), Ac-Asp-Glu-Val-Asp-aldehyde (100 μ M), and Val-Ala-

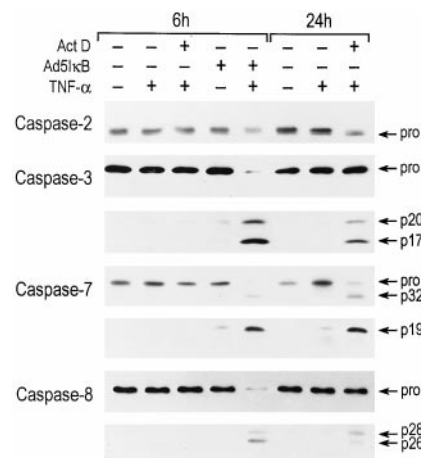


FIG. 3. Ad5I κ B/TNF- α and ActD/TNF- α treatments induced caspase activation. Aliquots of total cell lysates were subjected to SDS-PAGE, and immunoblotting was performed using anti-caspase-2, -3, -7, and -8 antibodies as described under "Experimental Procedures." Proteins were isolated from untreated cells, cells treated with TNF- α or ActD/TNF- α , and Ad5I κ B-infected cells with or without TNF- α at 6 and 24 h as indicated. Levels of procaspase-2, -3, -7, and -8 (indicated by *pro*) along with the processed caspase-3, -7, and -8 subunits are shown.

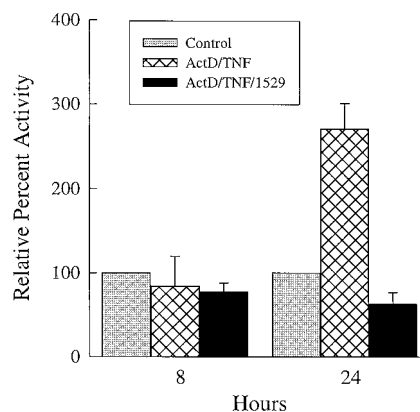


FIG. 4. Caspase-3-like enzyme activity was increased by 24 h after ActD/TNF- α treatment. Cells were untreated (*Control*) or treated with ActD/TNF- α alone (*ActD/TNF*) or together with IDN-1529 (*ActD/TNF/1529*). Caspase-3-like enzyme activity was determined at 8 and 24 h after TNF- α treatment. The changes in activity are shown relative to control cell activity normalized to 100%. Results are from three independent experiments with duplicate dishes for each point.

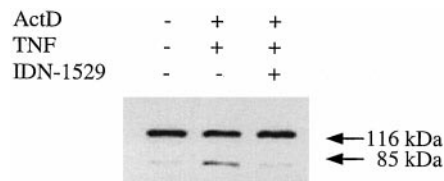


FIG. 5. TNF- α induced PARP cleavage in ActD/TNF- α -treated cells at 24 h. RALA hepatocytes were treated with ActD/TNF- α alone or together with the caspase inhibitor IDN-1529 for 24 h. Aliquots of total cell lysates were subjected to SDS-PAGE, and immunoblotting was performed with an anti-PARP antibody. The intact 116-kDa PARP and its 85-kDa cleavage product are indicated.

Asp-fluoromethylketone (100 μ M) also failed to prevent ActD/TNF- α -induced apoptosis (data not shown). Cell death that occurred in ActD/TNF- α -treated hepatocytes in the presence of caspase inhibitors was apoptotic as indicated by the presence of morphological features characteristic of apoptosis on fluorescent microscopy (Fig. 8). The percentage of apoptotic cells after 12 h of treatment with ActD/TNF- α ($7.6 \pm 0.4\%$) was unaltered

FIG. 6. ActD/TNF- α treatment induced caspase-dependent DNA degradation at 24 h. Apoptosis was quantitated by flow cytometric analysis of propidium iodide-stained cells as described under "Experimental Procedures." **A**, DNA histograms demonstrating the distribution of DNA content in untreated cells (*Control*) and cells treated with ActD/TNF- α alone (*ActD/TNF*) and together with IDN-1529 (*ActD/TNF/1529*) for 8 and 24 h. The percentage of cells that contained sub-G₁ amounts of DNA in this representative experiment are indicated. **B**, percentage of sub-G₁ cells in control and in ActD/TNF- α - and ActD/TNF- α /IDN-1529-treated cultures after 8 and 24 h. Results are from three independent experiments with duplicate dishes for each data point.

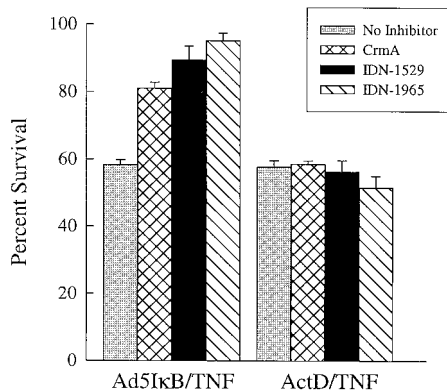
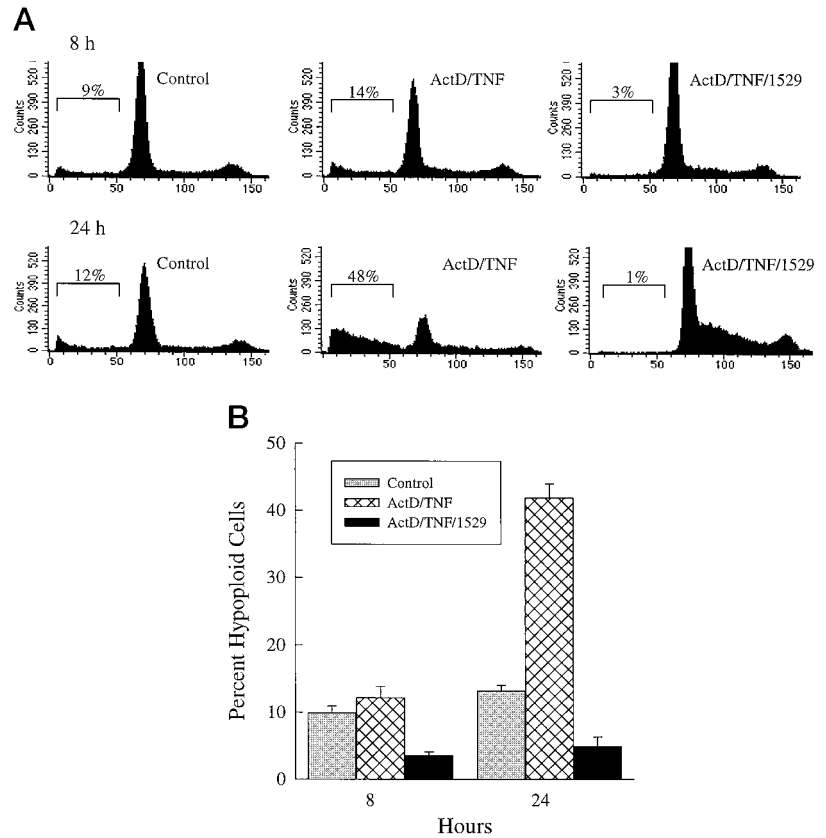


FIG. 7. Caspase inhibition prevented cell death from treatment with Ad5I κ B/TNF- α but not ActD/TNF- α . Cells were infected with an adenovirus expressing CrmA or pretreated with the caspase inhibitors IDN-1529 and IDN-1965. Percentage of survival at 6 h (Ad5I κ B/TNF- α) or 24 h (ActD/TNF- α) was calculated relative to untreated controls by MTT assay. Data are from duplicate dishes in each of three independent experiments.

by the addition of IDN-1529 ($7.2 \pm 0.6\%$). The percentage of necrotic cells at 12 h ($1.2 \pm 0.2\%$) was also unchanged by IDN-1529 ($1.0 \pm 0.1\%$). Caspase inhibition, therefore, did not convert ActD/TNF- α -induced cell death from apoptosis to necrosis.

To ensure that the failure of caspase inhibitors to block ActD/TNF- α -induced apoptosis was not due to pharmacological inactivity peculiar to this cell treatment, the effectiveness of caspase inhibition was assessed in both models of sensitization. Treatment of hepatocytes with IDN-1529 effectively blocked the increases in caspase-3-like enzyme activity that occurred after Ad5I κ B/TNF- α or ActD/TNF- α treatment. In ActD/TNF- α -treated cells, IDN-1529 prevented the rise in caspase-3-like activity and even lowered activity 23 and 38% below levels in

untreated control cells at 8 and 24 h, respectively (Fig. 4). Caspase inhibition also prevented PARP cleavage at 24 h (Fig. 5). Finally, the addition of this caspase inhibitor to ActD/TNF- α -treated cultures completely abrogated internucleosomal DNA cleavage as determined by FACS. Caspase inhibition reduced the percentage of cells displaying a sub-G₁ DNA content to levels below that seen in control cells (Fig. 6). Therefore, the failure of caspase inhibition to prevent TNF- α -induced apoptotic death in ActD-sensitized hepatocytes was not secondary to insufficient inhibition of caspase activity.

TNF- α Cytotoxicity following Inhibition of either NF- κ B Activation or RNA Synthesis Is FADD-dependent—Critical to the initiation of the TNF- α death response is the binding of FADD to the TNF- α receptor binding complex (1, 8), although FADD-independent pathways have been reported (14). The ability of ActD/TNF- α to trigger apoptosis independent of caspase activation suggested that this form of apoptosis may occur independently of FADD as well. To determine whether FADD is integral to the death pathways activated in Ad5I κ B- and ActD-sensitized hepatocytes following TNF- α stimulation, FADD function was inhibited by an adenovirus expressing a dominant negative FADD. Cell death in hepatocytes sensitized to TNF- α toxicity by Ad5I κ B and ActD was inhibited 81 and 47%, respectively, by FADD dominant negative expression. These findings indicate that ActD/TNF- α -induced apoptosis was at least partially dependent on FADD, despite the fact that the resultant cell death occurred by a caspase-independent mechanism.

Ad5I κ B/TNF- α but Not ActD/TNF- α Treatment Causes Mitochondrial Cytochrome *c* Release—Despite the common involvement of FADD in both forms of sensitization to TNF- α cytotoxicity, the time differential in caspase activation between the two models suggested that other events upstream of caspase activation must differ in the two types of sensitization. Mitochondrial release of cytochrome *c* is a common mechanism of caspase activation during apoptosis (27) and has been re-

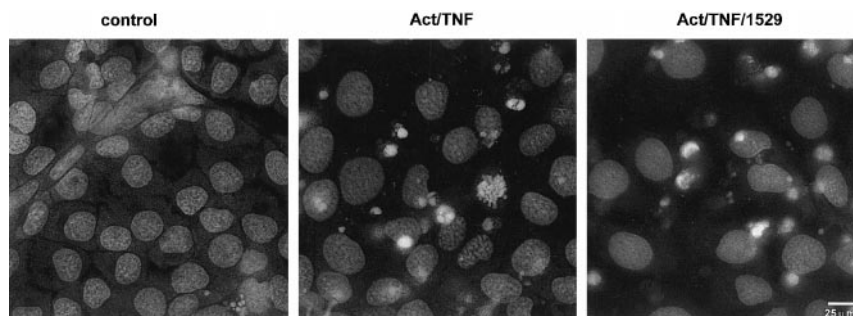


FIG. 8. ActD/TNF- α -induced cell death occurred by apoptosis despite the presence of a caspase inhibitor. Fluorescent micrographs are from acridine orange-stained untreated cells (*control*), and cells treated for 24 h with ActD/TNF- α alone (*Act/TNF*) or together with pretreatment with IDN-1529 (*Act/TNF/1529*). In the absence or presence of caspase inhibitor, ActD/TNF- α -treated cells underwent apoptosis as indicated by numerous shrunken cells with condensed and fragmented chromatin.

ported to occur in Ad5I κ B/TNF- α -treated primary hepatocytes (12). Mitochondrial loss of cytochrome *c* occurred in Ad5I κ B/TNF- α -treated RALA hepatocytes at 6 h (Fig. 9). In contrast, no cytochrome *c* release resulted from ActD/TNF- α treatment at 6, 8, or even by 24 h (Fig. 9). To ensure that equal amounts of mitochondrial protein had been isolated from each sample, levels of cytochrome oxidase, a mitochondrial protein not released during apoptosis (28), were determined by Western blot analysis and shown to be equivalent in all protein isolates (Fig. 9). This selective induction of mitochondrial cytochrome *c* release during Ad5I κ B/TNF- α -induced but not ActD/TNF- α -induced apoptosis further demonstrates that these two TNF- α death pathways diverge below the level of FADD.

DISCUSSION

TNF- α has multiple biological effects on hepatocytes that include either the stimulation of cellular proliferation or the initiation of cell death. In rat liver, TNF- α induces both responses under different physiological circumstances, stimulating hepatocyte proliferation after partial hepatectomy (29, 30) and inducing cell death during hepatotoxic injury (3). Our previous investigations have demonstrated that hepatocyte NF- κ B activation is essential to preventing TNF- α cytotoxicity (20), similar to findings in some nonhepatic cells (16–19). Despite the complex and diverse signaling cascades initiated by TNF- α , inhibition of activation of the single transcription factor NF- κ B is sufficient to convert the TNF- α response from proliferation to cell death both in cultured RALA hepatocytes (20) and in hepatocytes *in vivo* following partial hepatectomy (21). These results suggest that NF- κ B is the transcriptional regulator of a cellular gene(s) that when up-regulated by TNF- α blocks the cellular death response to TNF- α and allows hepatocellular proliferation to occur.

The concept that NF- κ B up-regulates a protective cellular gene is consistent with findings that inhibition of RNA or protein synthesis sensitizes resistant cells including hepatocytes to cytotoxicity from TNF- α (4, 7, 15). Previous studies in RALA hepatocytes demonstrated that ActD did not prevent TNF- α -induced activation of NF- κ B as measured by DNA binding activity, but ActD did partially inhibit NF- κ B-dependent gene expression (20). NF- κ B inactivation or RNA/protein synthesis inhibition may therefore act at different levels to ultimately block expression of the same NF- κ B-dependent cellular gene. To examine this question, TNF- α -induced cell death in RALA hepatocytes was compared following NF- κ B inactivation or ActD treatment. The present results indicate that the two forms of TNF- α -induced cell death differ significantly in several respects. The first difference is in the timing of cell death. NF- κ B inactivation resulted in immediate cell death with increased numbers of apoptotic cells within only 1 h of TNF- α treatment (20) and the majority of cell loss occurring in 6 h. In

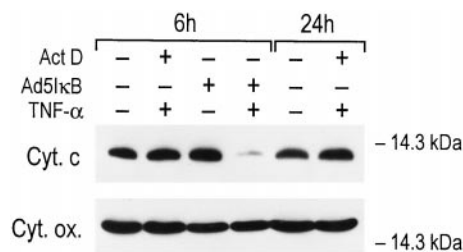


FIG. 9. Mitochondrial cytochrome *c* release occurred with Ad5I κ B/TNF- α but not ActD/TNF- α treatment. Mitochondrial fractions were prepared from untreated cells, cells treated with ActD/TNF- α , and Ad5I κ B-infected cells with or without TNF- α as indicated at 6 and 24 h. Aliquots of mitochondrial lysates were subjected to SDS-PAGE and immunoblotting performed with anti-cytochrome *c* (Cyt. *c*) and anti-cytochrome oxidase (Cyt. *ox.*) antibodies.

contrast, ActD/TNF- α treatment led to a delayed induction of cell death. No death occurred within 6 h after treatment, and a small increase in the number of apoptotic cells was detected only after 8 h of treatment. This disparate timing of cell death in the two models suggests that the two forms of sensitization activate distinct cell death pathways.

Despite the time differential, both forms of TNF- α toxicity resulted in apoptotic cell death. Consistent with prior findings that RALA hepatocyte death from Ad5I κ B/TNF- α occurred by apoptosis (20), ActD/TNF- α -induced cell death was also apoptotic as determined by fluorescent microscopic studies, PARP cleavage, and FACS analysis. All of these measures of apoptosis confirmed MTT and cell count data indicating that cell death did not commence until 8 h after ActD/TNF- α treatment. An additional hallmark of apoptosis in ActD/TNF- α -treated cells was the presence of caspase activation. Western immunoblotting and caspase-3-like enzyme activity assays demonstrated that caspase activation occurred following TNF- α stimulation in cells sensitized by either Ad5I κ B infection or ActD treatment. In keeping with the divergent time courses of apoptosis in the two models, caspase activation following ActD sensitization occurred much later than that seen following inhibition of NF- κ B. Caspase activation was not evident until 24 h, in contrast to the rapid caspase activation previously detected within 2 h after Ad5I κ B/TNF- α treatment (20). Although both models of TNF- α sensitization resulted in caspase activation at times appropriate to the occurrence of apoptosis, viral and chemical caspase inhibitors only blocked death in Ad5I κ B/TNF- α -treated cells. The failure of caspase inhibitors to prevent ActD/TNF- α -induced apoptosis was not because of insufficient caspase inhibition, because the chemical inhibitor IDN-1529 markedly suppressed caspase-3-like activity in these cells and completely abrogated the degradation of cellular DNA. These data are consistent with reports of a caspase-

activated DNAase responsible for DNA fragmentation in apoptosis (31) and the fact that DNA fragmentation is a late phenomenon not essential for the occurrence of apoptotic cell death (32).

RALA hepatocytes still exhibited morphological features of apoptosis on fluorescent microscopy despite effective caspase inhibition. The dissociation between the caspase-dependent apoptotic parameters of PARP and internucleosomal DNA cleavage and the caspase-independent morphological changes demonstrated in the present study are consistent with recent reports identifying the ability of a caspase-independent factor to induce an apoptotic nuclear morphology (33, 34). Although caspase activation occurred with ActD/TNF- α treatment and was associated with caspase-dependent biochemical hallmarks of apoptosis, caspase activation did not mediate apoptosis from ActD/TNF- α treatment. Results in ActD/TNF- α -treated cells are in marked contrast to those in Ad5I κ B/TNF- α -treated cells in which caspase activation was critical to the induction of apoptosis. These data demonstrate that NF- κ B inactivation and ActD sensitize RALA hepatocytes to TNF- α cytotoxicity by distinct mechanisms. In the absence of NF- κ B, a critical inhibitor of the TNFR-1-triggered caspase cascade may not be up-regulated, leading to a rapid caspase activation that commits the cell to apoptosis. ActD sensitized hepatocytes to TNF- α -induced death by a slower, and as yet undetermined mechanism in which caspase activation was not critical for the commitment to cell death. To our knowledge, these findings represent the first report of TNF- α -induced apoptosis occurring by a caspase-independent mechanism and add to the other recent examples of caspase-independent apoptosis (32, 35).

These results differ from a recent report by Li *et al.* (36), who demonstrated that in primary rat hepatocyte cultures ActD/TNF- α caused a rapid, caspase-dependent apoptosis associated with cytochrome *c* release. A potential explanation for these divergent findings is that Li *et al.* employed a much higher ActD concentration (200 ng/ml), which by itself causes a significant amount of hepatocyte apoptosis. Unlike our lower ActD dose, their concentration may have more effectively inhibited NF- κ B-dependent gene transcription, making their model equivalent to our Ad5I κ B/TNF- α treatment. In addition, chemotherapeutic drugs that interfere with macromolecular synthesis induce apoptosis through the Fas death pathway (37, 38), and high dose ActD may also activate this caspase-dependent pathway in hepatocytes. Alternatively, primary hepatocyte cultures exist in a nonproliferative, proapoptotic state that may alter cell death responses as compared with RALA hepatocytes or hepatocytes *in vivo*.

To determine the level at which the two TNF- α death pathways diverge in RALA hepatocytes, the function of the TNFR-1-binding protein FADD was blocked. Expression of a dominant negative FADD significantly reduced cell death following either Ad5I κ B/TNF- α or ActD/TNF- α treatment, indicating that these pathways diverge below the level of FADD. These data demonstrate that in RALA hepatocytes FADD can transduce the TNF- α death signal via a caspase-independent pathway, in addition to the pathway involving caspase-8 activation previously described in nonhepatic cells (9). This finding, together with the recent demonstration of FADD-dependent, caspase-independent induction of necrosis in Jurkat cells by Fas ligand (39), points to the ability of FADD to initiate cell death through caspase-independent mechanisms. Subsequent to the engagement of FADD, the two pathways of TNF- α -induced cell death in hepatocytes diverge, with mitochondrial cytochrome *c* release occurring with Ad5I κ B/TNF- α - but not ActD/TNF- α -induced apoptosis. Mitochondrial release of cytochrome *c* is not essential for many forms of death receptor-

induced apoptosis but has been proposed to serve as an accelerator of this process (40). Receptor-mediated apoptosis has been demonstrated to result from cytochrome *c*-independent caspase activation through the direct actions of autoactivated caspase-8 on caspase-3 and -7 (40). In RALA hepatocytes, cytochrome *c* release may be required for the induction of a rapid, caspase-dependent apoptotic pathway. In the absence of cytochrome *c* release, the engagement of FADD triggers an alternative, caspase-independent death pathway. In nonhepatic cells, FADD mediates activation of acid sphingomyelinase, leading to ceramide generation (41). We have previously demonstrated that RALA hepatocytes sensitized to ceramide toxicity by ActD undergo caspase-independent apoptosis (42). It is therefore possible that FADD-dependent ceramide signaling induces cell death in ActD/TNF- α -treated RALA hepatocytes. Cell death may occur from mitochondrial damage with resultant toxic generation of reactive oxygen species and depletion of ATP. Further studies must now identify both the mechanism of this caspase-independent death and the factors that regulate whether sensitized hepatocytes enter a rapid, caspase-dependent or slower, caspase-independent pathway of TNF- α -induced apoptosis.

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