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Murine Hepatocyte Apoptosis Induced In Vitro and In Vivo by TNF-α Requires Transcriptional Arrest

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Freshly isolated mouse hepatocytes were essentially insensitive to TNF-α cytotoxicity. However, TNF-α induced a concentration-dependent cell death in hepatocytes that had been pretreated with the transcriptional inhibitors actinomycin D (ActD), D-galactosamine, or α-amanitin. Unlike RNA synthesis inhibition, a translational block in the presence of cycloheximide (CHX) or puromycin did not sensitize hepatocytes to TNF. On the contrary, these agents prevented hepatocytotoxicity induced by ActD/TNF. Pretreatment with peroxides or glutathione depletors had no significant influence on TNF cytotoxicity. In vivo treatment of mice with ActD/TNF caused hepatic failure, which was significantly reduced by co-treatment with CHX. These findings demonstrate that protein synthesis is required for this mechanism of cell death. To test whether TNF may trigger an endogenous suicide program in hepatocytes, we examined whether DNA fragmentation preceded cell death. In the culture system, hepatocellular DNA fragmentation in the presence of ActD/TNF was observed several hours before lactate dehydrogenase release and was inhibited by CHX. Similar results were obtained in vivo. Chromatin condensation and the formation of apoptotic bodies were observed in livers from mice treated with ActD/TNF and significant DNA fragmentation was detected as early as 4 h after challenge. At this time, organ total glutathione content and plasma transaminase levels were not significantly different from those of untreated controls. The findings of this study demonstrate that direct hepatotoxicity of TNF-α is associated with an apoptotic mechanism that becomes manifest under the metabolic condition of arrested transcription and functional translation.

TNF-α is a cytokine with a vast spectrum of physiologic and pathophysiologic functions, which are characterized initially by its tumor-necrotizing and cachexia-inducing properties. At high systemic concentrations, the cytokine may cause hyperthermia and leukocyte alterations followed by irreversible pathophysiologic events such as circulatory and multiorgan failure, which may eventually end in lethal shock. Concordant with these findings, passive immunization of animals with anti-TNF Ig completely prevented lethality or organ damage in experimental models of septic shock and related systemic inflammatory response syndromes (reviewed in Refs. 1 and 2).

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In vitro TNF has been shown to be cytotoxic to a large variety of transformed and nontransformed cell lines (3–6). Although numerous intracellular responses after receptor activation with native or recombinant TNF or agonist Abs have been described, the individual contributions and significance of the various signal transduction mechanisms are not fully understood in detail. It seems that sensitivity to TNF cytotoxicity is not simply correlated with the number of receptors (7), but rather depends on cellular characteristics not as yet identified. An essential determinant of the action of TNF on different cell types is the balance of detrimental vs protective mechanisms, which are both induced by the cytokine. The induction of mitochondrial manganese superoxide dismutase (8), heat shock proteins (9), and the antiprotease plasminogen activator inhibitor type 2 (10) are among the protective effects evoked by TNF. Consequently, blocking the synthesis of such protective proteins by inhibition of translation or transcription sensitizes many, but not all (11, 12), cell types to TNF toxicity. In such in vitro systems both necrosis and apoptosis

have been described as the mode of cell death (13, 14), the latter often being associated with DNA fragmentation (15).

In vivo mice sensitized with transcriptional inhibitors, such as GalN[2] (16), ActD (17), or α-amanitin (18), have been used for more than two decades for the study of organ failure and as models for endotoxin shock in rodents. Sensitization of mice was observed not only to LPS, but also to TNF, one of the terminal mediators of the cascade of endotoxic reactions (19, 20). The increased susceptibility of rodents to TNF-induced lethality after pretreatment with inhibitors of RNA synthesis is largely caused by a relatively selective liver failure (21, 22). The biochemical basis of this hepatotrophic toxicity is the fact that GalN is metabolized along the exclusively hepatic galactose pathway until the entire cellular uridine nucleotide pool is trapped in the form of GalN adducts, thereby leading to a liver-specific transcriptional arrest (reviewed in Ref. 23). The exact consequences of this hepatic predisposition for TNF-induced liver failure, however, await elucidation.

This study was designed to determine the direct toxic effects of TNF on primary mouse hepatocytes after different pretreatment regimens. Furthermore, we examined whether TNF induced programmed cell death in liver cells and in which metabolic situation DNA fragmentation occurs in these cells. The ultimate aim of this investigation was to verify the findings in vivo. Our data suggest that when transcription is impaired, the cytokine TNF initiates an internal suicide program in mouse liver parenchymal cells, which is dependent on functioning protein synthesis and independent of other nonparenchymal cell types.

Materials and Methods

Reagents

rmuTNF-α and rhuTNF-α were generously provided by Dr. Adolf, Boehringer Institute (Vienna, Austria), recombinant murine granulocyte/macrophage-CSF and recombinant human granulocyte-CSF were a kind gift from Dr. Seiler, Behring Werke (Marburg, Germany), rmuIFN-γ was purchased from Boehringer Mannheim (Mannheim, Germany), and rmuIL-1β was from R&D Systems (Minneapolis, MN). Anti-muTNF antiserum and an IgG fraction thereof was kindly provided by S. Jilg from our laboratory. Cell culture medium was from Biochrom (Berlin, Germany). GalN was purchased from Roth (Karlsruhe, Germany). Arachidonic acid metabolites were from Paesel (Frankfurt, Germany). Collagenase, MTT, ActD, CHX, LPS, and all substances that are not otherwise specified were purchased from Sigma Chemical Co. (Deisenhofen, Germany).

Animals

Male BALB/c mice (25 g, from the internal animal breeding house, University of Konstanz, Konstanz, Germany) were maintained under controlled conditions (22°C, 55% humidity, 12 h day/night rhythm) and fed a standard laboratory chow (Altromin 1313). C57Bl6/ScCr mice were kindly provided by Drs. M. Freudenberg and C. Galanos (Max-Planck-Institute for Immunobiology, Freiburg, Germany). For cell preparations, mice were fed ad libitum, whereas they were starved overnight for the in vivo experiments. Substances were injected either into the tail vein (TNF) in a volume of 300 μl saline containing 1% BSA or i.p. (ActD, CHX) in a volume of 200 μl saline. All animals received humane care in adherence to the National Institutes of Health guidelines as well as to legal requirements in Germany.

Animal experiments

To determine liver damage, animals were challenged at 8 am with TNF (3.3 μg/kg) and liver damage was assessed by measuring plasma enzymes 8 h after TNF injection, according to the method of Bergmeyer (24). Blood was sampled in heparin-coated syringes by cardiac puncture immediately after killing of the animals by cervical dislocation. ActD or CHX was given 2 or 20 min, respectively, before TNF injection.

To determine glutathione content and DNA fragmentation, mice were anesthetized after 4 h with pentobarbital (100 mg/kg). Livers were perfused for 10 s with cold buffer (50 mM phosphate, 120 mM NaCl, and 10 mM EDTA, pH 7.4) before one lobe was excised and frozen in liquid nitrogen for glutathione determination, according to the enzymatic cycling method of Tietze (25), after homogenization in 3% metaphosphoric acid. The remaining parts of the excised liver were treated with three strokes of an Elvenhejm-type homogenizer. The 20% homogenate (in perfusion buffer) was centrifuged at 13,000 × *g* for 20 min. Either the supernatant was further diluted 270-fold and directly used in an ELISA designed to detect DNA fragmentation or DNA was precipitated from 500 μl supernatant by the addition of 1 ml ethanol (−20°C) plus 50 μl sodium chloride (5 M) and stored at −20°C for further analysis on an agarose gel.

Cell cultures

Hepatocytes were isolated by using the two step collagenase perfusion method of Seglen (26) as modified by Klaunig et al. (27), with a viability exceeding 80% according to the trypan blue exclusion method. Cells were plated in 200 μl RPMI 1640 medium containing 10% newborn calf serum in 24-well plates at 8×10^4 hepatocytes/well. They were allowed to adhere to culture plates for 5 h before the medium was replaced with RPMI 1640 medium without serum. Experiments were conducted for the times indicated in an incubator run at 5% CO_2, 40% O_2, and 55% N_2. Hepatocyte cultures contained 5% Kupffer cells, as determined by latex phagocytosis and esterase staining.

HepG2 cells were maintained in RPMI 1640 supplemented with 10% FCS. For experiments, they were plated out at a density of 8×10^4 cells/well and left to adhere overnight. For toxicity determinations, medium was replaced with RPMI 1640 and cells were incubated under the same conditions as were freshly prepared hepatocytes.

Cytotoxicity assays

LDH was determined (24) in culture supernatants (S) and in the remaining cell monolayer (C) after lysis with Triton X-100. The percentage of LDH release was calculated from the ratio of S/(S+C). Alternatively, the capacity to produce formazan from MTT was measured essentially according to the method of Mosmann (28). Briefly, cells were incubated with 0.4 mg/ml MTT for 30 min before medium removal. Reduced MTT was measured spectrophotometrically in an ELISA reader at 560/690 nm after lysis of cells (containing formazan crystals) with isopropanol/formic acid, 95/5.

Protein and RNA synthesis

To determine protein or RNA synthesis, the label (1.85×10^5 [^{3}H]leucine or 8.4×10^4 Bq [^{3}H]uridine) was added in a volume of 10 μl to hepatocytes in 24-well plates. After 2 h, the medium was removed and cells were washed three times with ice-cold 10% TCA and dried with −20°C methanol. Next, they were lysed for 8 to 12 h at 37°C with 300 μl 0.5 M NaOH 1 mM EDTA/0.1% Triton. An aliquot of 250 μl was used to detect acid-precipitable radioactivity by β-scintillation counting. Ten microliters of the remaining lysis buffer was diluted 30-fold and used for protein determination (29).

DNA fragmentation

Cells were grown in 6-well plates at a density of 4×10^5 cells/well in 1 ml RPMI 1640. After 13 h of incubation with different stimuli they were

[2] Abbreviations used in this paper: GalN, D-galactosamine; ActD, actinomycin D; CHX, cycloheximide; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-3,5-diphenytetrazolium bromide; SDH, plasma sorbitol dehydrogenase; rhu, recombinant human; rmu, recombinant murine; IC_{50}, concentration needed for 50% inhibition.

lysed directly within the microtiter plate with 0.1% Triton X-100 and DNA fragmentation was determined according to McConkey et al. (30). Results are expressed as the ratio of the amount of unfragmented (pelleted at 27,000 × *g*) and fragmented DNA (supernatant), which were measured using the diphenylamine method (31).

Alternatively, DNA fragmentation was measured by quantitation of cytosolic oligonucleosome-bound DNA by using an ELISA kit (Boehringer Mannheim), according to the manufacturer's instructions. Briefly, the cytosolic fraction (13,000 × *g* supernatant) of approximately 330 cultured hepatocytes or the cytosol from liver homogenates was used as Ag source in a sandwich ELISA with a primary anti-histone Ab coated to the microtiter plate and a secondary anti-DNA Ab coupled to peroxidase. From the absorbance values, the percentage of fragmentation in comparison to controls was calculated according to the following formula:

$$\% \text{ of control} = (100\%) \times \frac{(\text{absorbance}_{\text{stimulated cells}} - \text{absorbance}_{\text{blank}})}{(\text{absorbance}_{\text{control cells}} - \text{absorbance}_{\text{blank}})} \quad (1)$$

Semi quantitative determination of DNA fragmentation (32) was performed by analyzing the pattern of low m.w. DNA that was stained with ethidium bromide after extraction by phenol/chloroform, precipitation in ethanol, and subsequent electrophoresis on 1% agarose gels.

Microscopy

Fluorescence microscopy was performed on an Axiovert 35, Carl Zeiss (Oberkochen, Germany) using hepatocytes grown on collagen-coated coverslips fixed for 10 min in 3.7% formaldehyde and stained for 15 s with 8 μg/ml bisbenzimide H33258 (Riedel-de Haen, Seelze, Germany). Kupffer cells were detected either by esterase staining with a kit from Technikon (Bad Vilbel, Germany) or by phagocytosis of fluorescein-labeled latex (ϕ 0.75 μm) from Polyscience Inc. (Eppelheim, Germany). Livers were fixed for histologic examination with Carnoy's solution and imbedded in paraplast. The 5 μm sections were stained with hematoxylin and eosin and photographed at 1008-fold magnification.

Statistics

Data are expressed as the means ± SD with $n = 3$. Unless otherwise indicated, individual data points in the diagrams that display no error bars represent SD smaller than the size of the symbol. Statistical significances were determined with use of the unpaired Student's *t*-test, if applicable, or with the Welsh test if variances were nonhomogeneous (F test <0.05). All data from cell culture experiments are on the basis of at least three individual cell preparations.

Results

Synergistic toxicity of TNF and transcriptional, but not translational, inhibitors to primary hepatocyte cultures

First, we examined the in vitro toxicity of TNF in cultures of freshly isolated adherent murine hepatocytes at concentrations ranging from 1 ng/ml (i.e., 20 pM) to 50 μg/ml (i.e., 1 μM). Even in cells exposed to high cytokine concentrations over a period of 20 h, viability was never reduced by more than 5% compared with incubations lacking TNF. These experiments demonstrate that mouse liver cells are extremely resistant to direct toxic effects of the cytokine. However, when incubations were performed in the presence of the transcriptional inhibitor ActD, the hepatocytes were susceptible to TNF-induced toxicity at concentrations of this mediator greater than 10 ng/ml (≅200 pM) (Fig. 1*A*). Thus, ActD was able to sensitize hepatocytes analogously to many different tumor cell lines.

To determine whether this sensitization was a general characteristic of transcriptional inhibitors, we incubated

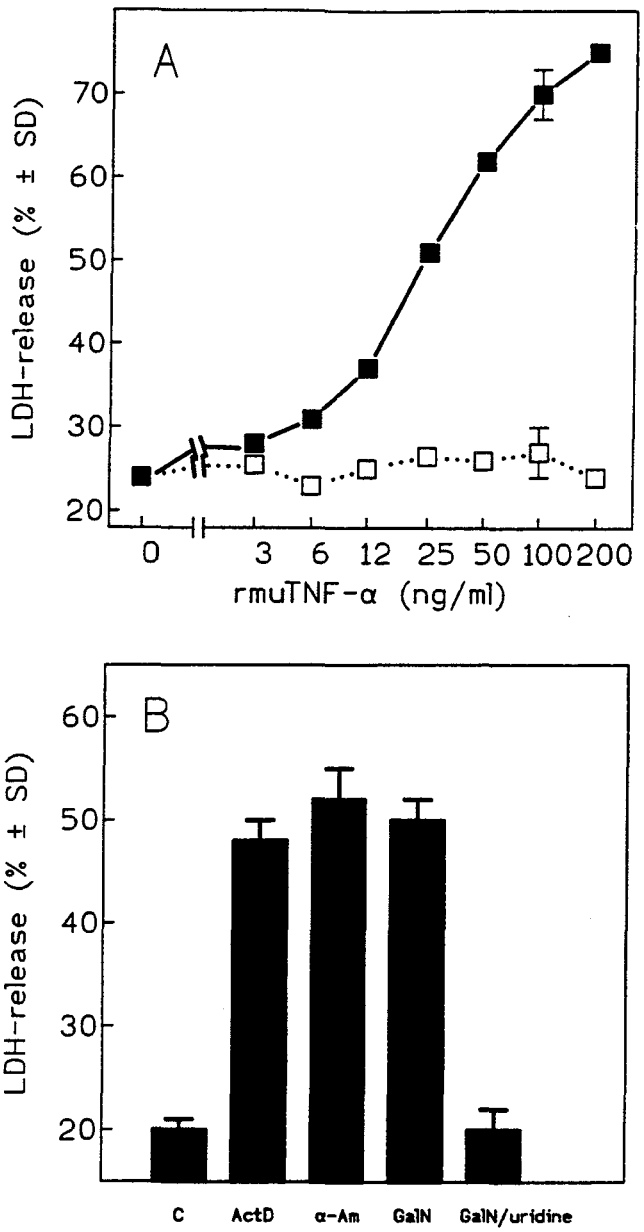


FIGURE 1. Sensitization of freshly isolated hepatocytes or cells cultivated for 24 h to varying concentrations of rmuTNF-α in the presence of transcriptional inhibitors. *A*: cells were incubated with 333 nM ActD/TNF for 20 h either 5 (filled squares) or 24 h (open squares) after isolation. *B*: cells were preincubated for 30 min with control medium (C), medium containing 333 nM ActD, 25 μg/ml α-amanitin (α-Am), 5 mM GalN, or 5 mM GalN/10 mM uridine (GalN/uridine) before TNF (20 ng/ml) was added. Toxicity was determined after 20 h by LDH release. Enzyme release in the presence of TNF alone was not significantly different from control incubations.

hepatocytes with GalN or α-amanitin, i.e., inhibitors having a different mechanism of action than ActD. All inhibitors of RNA synthesis had a strong sensitizing effect to TNF in our cell culture system without being toxic when

Table I. *Correlation between inhibition of RNA synthesis and sensitization to TNF in primary mouse liver cell cultures*

	Inhibition of Transcription[a] (%)			TNF Induced Toxicity[b] (%)	
ActD	0.5 h	7 h	18 h	11 ng/ml TNF	33 ng/ml TNF
15 nM	41 ± 3	42 ± 2	57 ± 5	0	3 ± 2.7
45 nM	54 ± 2	59 ± 2	66 ± 4	3 ± 1.4	3.2 ± 0.8
111 nM	76 ± 3	85 ± 3	96 ± 1	42 ± 1.3	58 ± 3.9
333 nM	81 ± 0.4	92 ± 1	100	57 ± 2.2	79 ± 1.9

[a] After the time points indicated, 8.4×10^4 Bq [^{3}H]uridine was added to the cultures. The amount of acid-precipitable incorporated radioactivity was determined after 2 h. Results are given as percentage of untreated controls.
[b] ActD was added 30 min before TNF. After 20 h, toxicity was determined from the ratio of formazan produced by TNF-treated cells and untreated controls. ActD alone had no significant cytotoxic effect.

incubated alone (Fig. 1*B*). These experiments suggest that sensitization of primary hepatocyte cultures to TNF is a general characteristic of transcriptional inhibitors. Restoration of transcriptional capacity by uridine repletion in GalN-treated hepatocytes resulted in a loss of sensitization to TNF, whereas uridine had no effect on the TNF-induced toxicity in ActD-sensitized hepatocytes (data not shown). These results further corroborate the interpretation that sensitization of hepatocytes to TNF is caused by a compromised transcriptional capacity.

Experiments in which the time point of addition of ActD was varied showed that this transcriptional inhibitor required incubation with hepatocytes at least 15 min before the addition of TNF for an optimal sensitization. Incubation of cells with ActD 15 to 240 min after TNF resulted in a fading sensitization, which was no longer significant after 4 h (data not shown). This observation may indicate that TNF rapidly desensitized cells to their own toxicity unless they were pretreated with an RNA synthesis inhibitor. An apparently different form of desensitization was observed when cells that had been precultured for 24 h before the experiments were used instead of fresh hepatocytes. Under such conditions, they became completely refractory to the toxicity induced by ActD/TNF (Fig. 1*A*).

To assure the correlation of transcriptional depression and sensitization to TNF, we measured RNA synthesis in hepatocytes after incubation with ActD, GalN (5 mM), or α-amanitin (25 μg/ml). The latter two substances caused a 60% inhibition of RNA synthesis compared with that of untreated control cells as early as 30 min after addition to hepatocytes. This inhibition further increased to approximately 75% compared with that of untreated controls after 18 h of incubation. The effects of ActD were examined in greater detail (Table I). Inhibition of RNA synthesis was dose and time dependent. The critical threshold concentration to initiate hepatocytotoxicity of TNF was 50 to 100 nM and caused an inhibition of transcription of $\geq$55%. In the presence of 333 nM ActD, inhibition of protein synthesis was negligible during the first 2 h and increased to 40% (data not shown) after 7 h of incubation. Thus, all three substances caused a sensitization of hepatocytes when RNA synthesis inhibition was greater than 60% of that of controls. No such correlation was found between inhibition of translation and sensitization for TNF toxicity. TNF alone (100 ng/ml) influenced the rate of neither transcription nor translation over 20 h.

Specificity of the toxic effect of TNF on hepatocytes

Next, we examined whether alternative sensitizers may substitute for transcriptional inhibitors. We found that neither translational inhibitors, such as puromycin ($\leq$5 μM) and CHX ($\leq$300 μM), nor glutathione-depleting agents, such as maleic acid diethyl ether (500 μM), buthionine-sulfoximine (250 μM), and phorone (500 μM), were able to sensitize the cells to TNF, although their effectiveness with respect to protein synthesis inhibition or glutathione depletion was confirmed in our culture system (data not shown). Moreover, inducers of oxidative stress, such as hydrogen peroxide ($\leq$5 mM), did not synergize with TNF. Therefore, we conclude that synergistic toxicity between TNF and RNA synthesis inhibitors represents a specific phenomenon.

When we examined the effects of other immune modulators such as rmuIL-1, rmuIL-2, rmuIFN-γ, recombinant murine granulocyte/macrophage-CSF, recombinant human granulocyte-CSF, leukotrienes B_4, C_4, and D_4, platelet-activating factor, and the stable thromboxane agonist U46619, no significant toxicity to ActD-sensitized hepatocytes was observed. We also checked whether TNF is responsible for the hepatocytotoxicity of a mixture of macrophage secretory products, which were obtained by stimulation of the mouse macrophage cell line RAW264.7 with 10 μg/ml LPS. Neutralization of TNF with an antiserum specific for muTNF-α completely prevented the toxicity of this macrophage-derived cytokine mixture to ActD-sensitized liver cells (data not shown).

Next we addressed the question of the role of nonparenchymal liver cells (which were present as contaminants in hepatocyte cultures) on TNF toxicity during a transcriptional block. When Kupffer cells were stimulated by the addition of LPS (0.1 to 10 μg/ml) to the culture medium during the incubations neither a significant change of basal LDH release nor of the toxicity of TNF (11 to 100 ng/ml) in ActD-sensitized hepatocytes was observed (data not shown). To determine whether LPS contaminations caused by the isolation procedure had possibly prestimulated the control cells, hepatocytes were prepared from C57BL/ScCr mice, which are known to have macrophages that are insensitive to LPS. Cell preparations obtained from this mouse strain reacted similarly to those prepared from normal C57BL or BALB/c mice (data not shown). In addition, the hepatoma cell line HepG2, which is resistant to TNF alone (33), was used for similar experiments. In this human cell system, which is free of any accessory cells, a

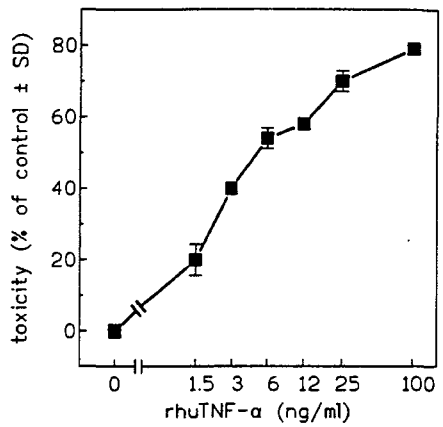


FIGURE 2. Toxicity of rhuTNF-α in ActD-sensitized HepG2 cells. HepG2 cultures (human hepatoma cells) were pretreated for 30 min with 333 nM ActD before rhuTNF was added. Cytotoxicity was determined 20 h after the addition of TNF by measuring MTT reduction.

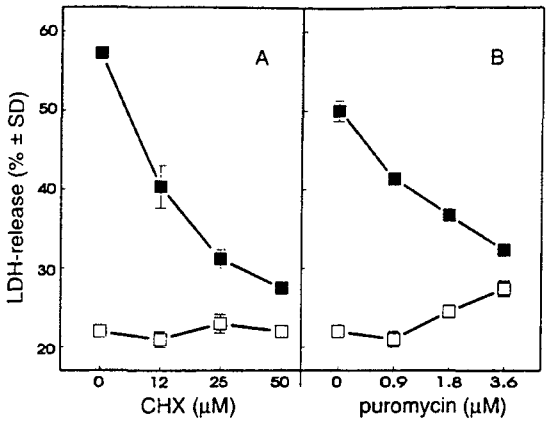


FIGURE 3. Protection of hepatocytes sensitized by ActD (333 nM) against TNF (20 ng/ml) toxicity by translational inhibitors. Cells were incubated with ActD alone (open squares) or ActD/TNF (filled squares) for 20 h in the presence of varying concentrations of protein synthesis inhibitors. CHX (*A*) or puromycin (*B*) was added 30 min before TNF and simultaneously with ActD.

similar synergistic effect between ActD and TNF was observed, as it was seen in freshly prepared primary cell cultures from mouse liver (Fig. 2). These data suggest that nonparenchymal liver cells are unlikely to contribute to the toxicity of TNF to hepatocytes pretreated with transcriptional inhibitors. They further demonstrate that TNF toxicity under these conditions is not confined to cells from rodents and can be studied without the requirement of isolating primary cells.

TNF-induced toxicity in ActD-sensitized hepatocytes or ActD-sensitized mouse livers requires intact protein synthesis

The following experiments addressed the question of whether protein synthesis played a role in the induction of cell death caused by ActD/TNF. Although 200 μM CHX inhibited transcription by more than 50% after an incubation time of 3 h, preincubation of hepatocytes with CHX (10 to 300 μM) for 4 h did not sensitize them to TNF. However, 25 μM of this inhibitor was sufficient to block protein synthesis completely and immediately (data not shown). These findings suggested that a simultaneous inhibition of translation and transcription (as in the case of CHX) might prevent the sensitization to TNF, which was seen when RNA synthesis only was inhibited (as in the case of ActD). This assumption was checked by testing the influence of a variety of different inhibitors of protein synthesis on our system. Puromycin (IC_{50} = 1.8 μM) or CHX (IC_{50} = 18 μM) protected hepatocytes in a concentration-dependent from ActD/TNF toxicity (Fig. 3). Analogous results were obtained for ricin D (IC_{50} = 4 μg/ml; data not shown). The findings demonstrate that the common property of these compounds, inhibition of protein synthesis, is the principle that blocks sensitization of the cells against TNF, which we observed under transcriptional arrest.

To investigate the relevance of this effect in vivo, we challenged mice with combinations of ActD, CHX, and TNF. None of these substances alone had any effect on the release of liver enzymes into the plasma, i.e., on the membrane integrity of hepatocytes. CHX and ActD both sensitized mice strongly to TNF-induced mortality. When plasma transaminases or plasma SDH were determined as parameters for hepatocellular injury, CHX proved to protect against hepatotoxicity induced by ActD/TNF (Table II). Thus, TNF-induced cell death in hepatocytes sensitized by transcriptional arrest requires functioning protein synthesis in vivo and in vitro.

Table II. *Differential influences of ActD or CHX on TNF-inducible liver injury in mice in vivo*

Treatment[a]	ALT (U/L)	AST (U/L)	SDH (U/L)
Saline	30 ± 10	82 ± 84	15 ± 12
TNF	161 ± 163	112 ± 159	26 ± 43
TNF + ActD	11,280 ± 5,331[b]	4,528 ± 5,331[b]	5,540 ± 2,373[b]
TNF + ActD + CHX	613 ± 287[c]	438 ± 125[c]	445 ± 185[c]
TNF + CHX	494 ± 561	240 ± 263	96 ± 105

[a] Dosages and application routes: TNF (3.3 μg/kg) was given i.v. ActD (800 μg/kg) and CHX (40 mg/kg) were given i.p. 2 or 20 min before TNF, respectively. Plasma enzymes were determined 8 h after the challenge.
[b] $p < 0.05$ vs control.
[c] $p < 0.05$ vs TNF + ActD; data, the means ± SD, $n = 6$.

TNF induces apoptosis and DNA fragmentation in ActD-sensitized mouse livers and primary hepatocytes

The requirement of intact protein synthesis for the manifestation of TNF-induced hepatocytotoxicity suggests that the induction of an internal suicide program could possibly be responsible for the cell death observed in this model. Because such suicidal processes are commonly associated morphologically with apoptotic changes and biochemically with DNA fragmentation, we examined these two

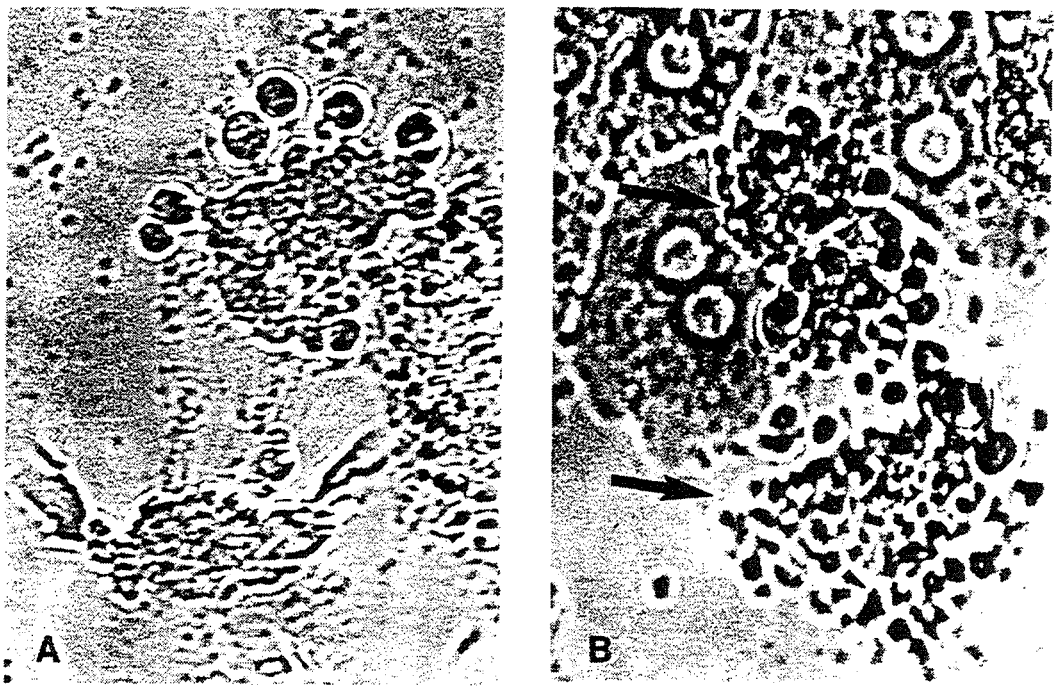


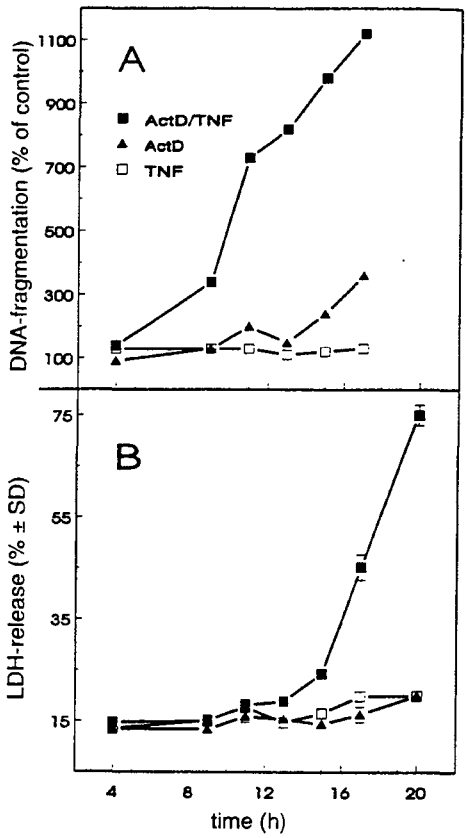
FIGURE 4. Morphological changes of hepatocytes treated with ActD (333 nM)/TNF (100 ng/ml). *A*: hepatocyte during an initial phase of cell death (13 h after challenge) showing a row of clearly detached blebs. *B*: two hepatocytes (indicated by arrows) that are completely disintegrated after 20 h of treatment with ActD/TNF. No such alterations were observed after treatment with ActD or TNF alone. To the left and right of the upper disintegrated cell two undamaged surviving binucleated hepatocytes are shown.

issues. By using light microscopic examinations, approximately 30 to 60% of the TNF-treated hepatocytes were found to be disintegrated into large blebs with diameters of 5 to 10 μm (Fig. 4). In parallel, a strong nuclear condensation and chromatin margination was observed in hepatocytes treated with ActD/TNF and stained with the fluorescent DNA stain bisbenzimide H33258. Control cells displayed neither of the above mentioned characteristics. Chromatin fragmentation into oligonucleosomes was determined by an ELISA specific for cytosolic histone-bound DNA or by agarose gel electrophoresis. ActD/TNF-induced DNA fragmentation started before LDH release was detected as a sign of toxicity (Fig. 5). From this time course of events it is concluded that the process observed is a cause rather than a consequence of cell death. Moreover, this TNF-inducible DNA fragmentation was dependent on protein synthesis, as concluded from the protective effect of CHX (50 μM), which reduced ActD/TNF-induced DNA fragmentation during a 13-h incubation by 54 ± 1%, as measured by the ELISA. Quantitative determination of the percentage of fragmented DNA confirmed the results obtained in the ELISA, i.e., after 13-h incubation ActD/TNF induced a DNA fragmentation of 57 ± 2% vs 26 ± 2% in controls.

Finally, we addressed the question of whether such a cytokine-induced apoptotic cell death may be involved in fulminant liver failure, which developed in ActD-sensitized mice challenged with TNF. Actually, chromatin fragmentation was observed clearly at a time point before any other signs of toxicity. Liver glutathione content and plasma transaminase levels remained unaltered when there was already a significant rise of DNA fragmentation, as determined by measurement of cytosolic oligonucleosomes (Table III). Further corroboration of this finding comes from the experiment shown in Figure 6. There we demonstrate that a classical oligonucleosome DNA ladder was obtained when low m.w. DNA from liver homogenates was analyzed on an agarose gel. In addition, morphologic changes typical of apoptosis, such as the appearance of intracellular apoptotic bodies and hyperchromatic changes of the nuclear lining, were frequently seen in liver sections from ActD/TNF-treated mice. Thus, the entire set of data provides independent evidence for the activation of an internal suicide program by TNF in ActD-sensitized hepatocytes in vivo and in vitro.

Discussion

This study was designed to examine under which metabolic condition and according to which mechanism the cytokine TNF induces hepatocyte damage. Hepatocytes express TNF receptors like most other cells (34), but they are damaged by TNF neither in the isolated perfused liver (35) nor in primary cultures (36, 37). This is in contrast to the in vivo effects of the cytokine, because TNF has been reported to cause hepatotoxicity in humans (reviewed in Ref. 38) and animals (39, 40). Two alternative assumptions that are not mutually exclusive may explain this apparent discrepancy. The first is



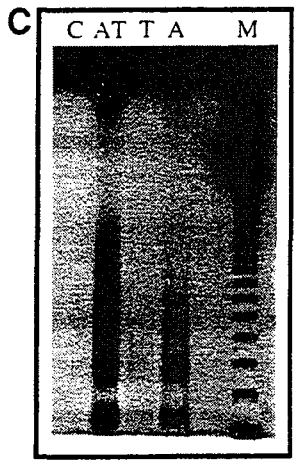


FIGURE 5. Time course of DNA fragmentation and LDH release in primary hepatocyte cultures challenged with ActD/TNF. Hepatocytes were incubated for 20 h with control medium, 100 ng/ml TNF with 333 nM ActD, or combinations of both substances. LDH release or DNA fragmentation were determined from parallel incubations at the times indicated. *A*: DNA fragmentation was determined by quantifying the amount of oligonucleosome-bound DNA in the 20,000 *g* supernatant of cell lysates. Data represent the means from three determinations. SD was <10%. *B*: LDH release in control cells was not significantly different from that in ActD-treated cells. Data represent the means ± SD from triplicate determinations. *C*: hepatocytes were incubated for 13 h with 100 ng/ml TNF (T), 333 nM ActD (A), saline (C), or ActD/TNF (AT). DNA was prepared from 13,000 *g* supernatants of the hepatocytes after lysis with 0.5% Triton X-100/10 mM EDTA and analyzed on a 1% agarose gel.

Table III. *Plasma enzymes, intrahepatic glutathione content, and DNA fragmentation in livers of mice treated with ActD/TNF in vivo*

Treatment[a]	ALT (U/L)[b]	Glutathione (nmol/mg protein)	DNA Fragmentation[c]
Control	41 ± 28	26.6 ± 2.5	100 ± 36
TNF	39 ± 34	22.7 ± 2.3	95 ± 8
ActD	25 ± 15	16.6 ± 0.4	157 ± 17
ActD/TNF	25 ± 4	20.5 ± 1.5	363 ± 97[d]

[a] TNF (3.3 μg/kg) was given i.v. ActD (800 μg/kg) was given i.p. 2 min before TNF. After 4 h, animals were killed, blood was withdrawn by cardiac puncture, and livers were processed for biochemical determinations.
[b] Analogous results were obtained when AST and SDH were measured.
[c] Percentage of control determined by ELISA quantitating cytosolic oligonucleosome-bound DNA.
[d] $p < 0.05$ vs ActD, data are means ± SD, $n = 3$.

that endothelial cells or neutrophils, which are both known to be activated by TNF (41), may be required to mediate the hepatic damage induced by this cytokine. However, liver damage caused by TNF occurred in mice even when leukocyte infiltration was inhibited (42). Another study reports on hepatocyte damage preceding leukocyte infiltration when induced by stimulation of the *fas*/Apo-1 Ag (43), a structural relative of the TNF receptor (44) that mediates apoptosis analogously to TNF (45), but clearly by an independent mechanism (46).

Alternatively, conditions that arise only under certain pathophysiologic situations may predispose hepatocytes to direct TNF toxicity in vivo. The results presented in this study favor the latter hypothesis as outlined in Figure 7, which is intended to illustrate the reasoning of the following discussion.

Any cellular resistance against TNF toxicity, be it caused by the induction of protective proteins, such as mitochondrial superoxide dismutase (8) or heat shock proteins (9), or by a lack of suicidal pathways inducible by the cytokine, requires an intact metabolism of the cell. The known sensitization of cell lines to TNF by the DNA intercalating molecule ActD can be explained by inhibition of transcription of message coding for protective proteins. In our system, inhibition of transcription by mechanistically different substances, such as the uridine depletor GalN or the RNA polymerase inhibitor α-amanitin, or by ActD resulted in an extreme sensitization of these cells to TNF. Because all other metabolic inhibitors (especially CHX and puromycin) and toxins tested did not sensitize freshly isolated murine liver cells to TNF, the cytotoxicity of the cytokine in hepatocyte cultures becomes manifest only under the metabolic condition of transcriptional arrest. Such a transcriptional arrest was a necessary, but not sufficient, condition, because ActD incubation did not facilitate TNF toxicity in cells precultivated for 24 h or in cells co-incubated with CHX.

The dependence of cell death on protein synthesis in our system suggests that an activation-dependent mechanism is involved. ActD/TNF-induced hepatotoxicity was prevented by CHX in vitro and also in vivo. Note that CHX

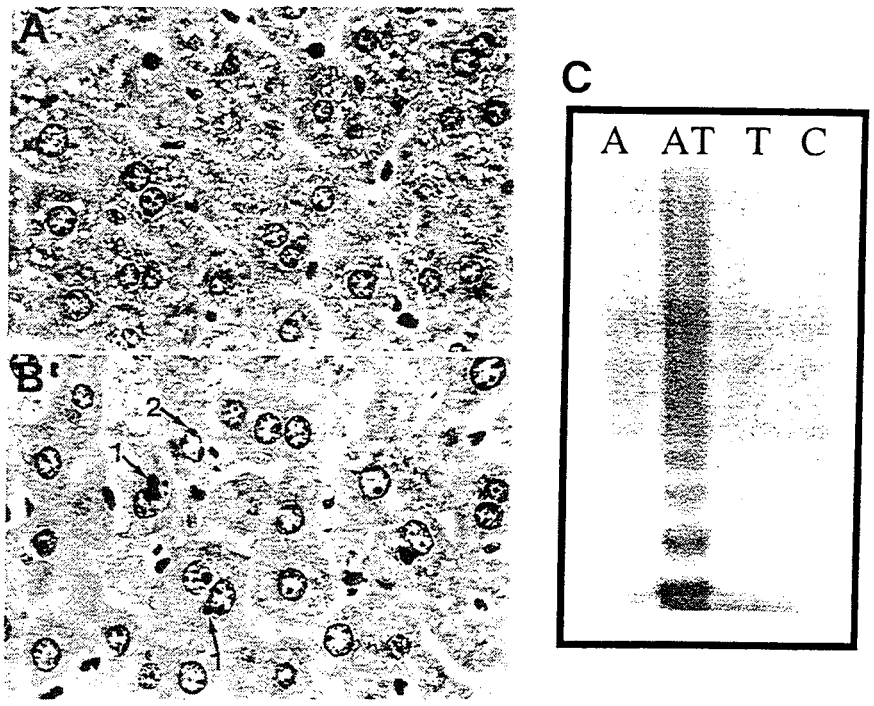


FIGURE 6. Apoptosis and DNA fragmentation in livers from mice treated with 800 μg/kg ActD and 3.3 μg/kg TNF for 4 h. *A*: hematoxylin and eosin-stained liver section from a mouse treated with ActD alone. Minor diffuse cloudy swelling of the hepatocellular cytoplasm is evident. *B*: Hematoxylin and eosin-stained liver section from a mouse treated with ActD/TNF. Numerous intrahepatocellular chromatin-containing apoptotic bodies (arrow 1) and hyperchromatic nuclear membranes (arrow 2) can be detected. *C*: DNA was prepared from 13,000 *g* supernatants of liver homogenates and stained with ethidium bromide after analysis on a 1% agarose gel. C, saline control; T, TNF only; A, ActD only; AT, ActD/TNF.

inhibited cell death in completely different models of toxicity (30, 47–49) and that pretreatment with CHX protected mice against lethal liver damage induced by GalN/LPS (50), whereas simultaneous administration of CHX and LPS caused synergistic lethality as a result of renal coagulopathy (51). Nevertheless, the seemingly paradoxic protection of CHX against ActD/TNF in our system and its inability to sensitize hepatocytes, although it inhibits RNA synthesis, requires a mechanistic explanation (Fig. 7). On one hand, TNF may initiate the induction of proteins protective against TNF effects that are inhibited by ActD. On the other hand, it may induce the translation of proteins from preformed RNA, which are required for cell killing. This would lead to a mutual neutralization of protective and toxic properties of TNF on hepatocytes if present alone, whereas only the protective properties would be abrogated by co-incubation with ActD. If so, co-incubation of TNF with CHX or CHX/ActD should restore the balance and leave cells unaffected. Although entirely speculative, these assumptions seem to reconcile the observations made in mouse hepatocyte cultures; however, we are well aware that mechanisms may be different in other cellular systems in which CHX sensitizes cells to TNF-induced cytotoxicity (1).

In previous work, investigators have noticed that TNF impaired mitochondrial functions of rat hepatocytes and caused a minor increase of enzyme leakage (52). TNF-induced damage was also observed in primary murine hepatocyte cultures at TNF concentrations of 1 to 10 μg/ml (53). This cytotoxicity was associated with oxidative stress and enhanced by IFN-γ. In our system, however, rmuIFN-γ (≤5000 U/ml = 1 μg/ml) had no significant effect. Moreover, in view of a

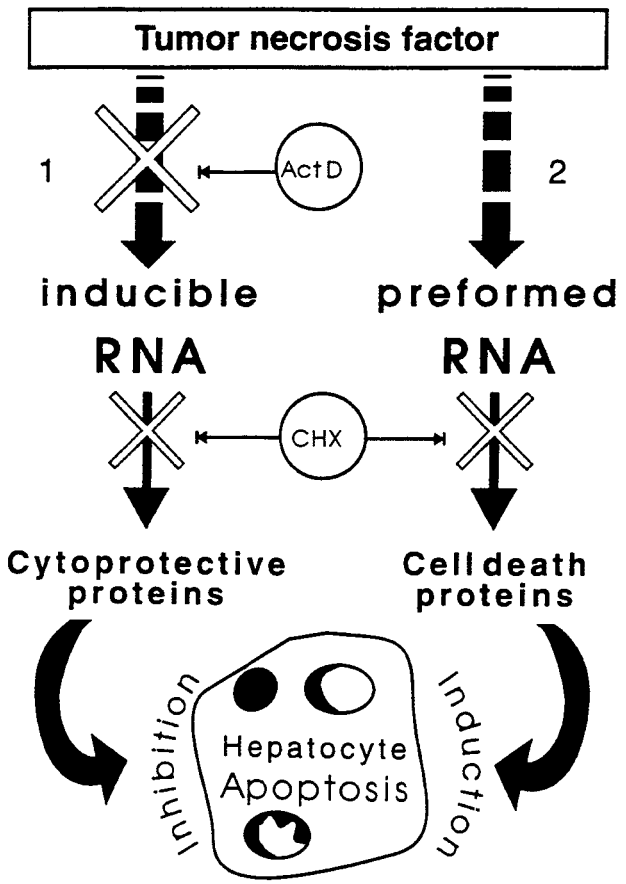


FIGURE 7. Two pathway hypothesis explaining the differential actions of ActD and CHX in TNF-challenged hepatocyte cultures.

lack of influence of either glutathione depletion or augmentation on TNF toxicity there was no evidence to assume the involvement of oxidative events in causing cell death.

The potentiation of TNF toxicity by transcriptional inhibition might be of prime relevance for the role of the cytokine in the host defense against intracellular pathogens such as bacteria (54) and viruses (reviewed in Refs. 55 and 56). For example, viral infection is known to induce TNF production, inhibit transcription of cellular proteins (57), and predispose cells to TNF-induced apoptosis (58). Moreover, TNF was implicated in the selective apoptotic killing of hepatocytes expressing the hepatitis B virus large-envelope polypeptide in a transgenic mouse model (59). Thus, analogous to ActD-sensitized hepatocytes, infected cells may also undergo suicide signaled by TNF. Such an apoptotic mechanism seems to be different from the one associated with liver size regression as described by Oberhammer et al. (60). Under the conditions chosen by these authors no DNA fragmentation was observed.

TNF-induced apoptotic death of hepatocytes may also be of major importance under conditions causing acute liver failure in septic shock. In various experimental models for sepsis, plasma concentrations of free TNF-α of up to 10 to 50 ng/ml are found (22). Nitric oxide, which is also known to be released during such conditions (61), has been reported to inhibit protein synthesis in hepatocytes (62). Therefore, it seems feasible that under further sensitization to TNF caused by hypoxia or ischemia, as is also observed in conditions of shock and experimental sepsis (63–65), the cytokine may deliver the terminal fatal signal to hepatocytes sensitized in multiple ways.

The interesting question in the context of liver pathophysiology was whether an in vivo correlate to the in vitro system would allow such speculations. The answer is clearly positive because our experiments in ActD/TNF-treated mice show proof of the principle. Indeed, a relatively selective hepatocyte damage (characterized by a ratio of plasma alanine aminotransferase to plasma aspartate aminotransferase >1.3) was found 8 h after challenge of mice with ActD/TNF; this liver failure was clearly preceded by DNA fragmentation and the appearance of apoptotic bodies. Thus, TNF may cause organ failure directly under various pathophysiologic conditions via a suicide program and might, therefore, act as a tissue apoptosis factor. In fact, hepatic failure and tissue destruction as a result of endogenously produced TNF has recently been demonstrated to be mediated by the 55-kDa TNF receptor (66), which is structurally related to the *fas*/Apo-1 Ag. The latter molecule has been reported to induce apoptosis and massive tissue destruction in murine liver (43). However, *fas* signals apoptosis distinctly from TNF (46) and *fas*-induced liver failure does not require transcriptional arrest. Therefore, two independent mechanisms for eliciting programmed cell death are likely to exist in parenchymal liver cells under specific metabolic situations.

A typical condition for TNF-induced liver damage includes the experimental situation generated by treatment of animals with GalN, which leads to a several thousandfold sensitization to the lethal effects of LPS or bacteria of both Gram types. Galanos et al. (67) have performed a series of elegant studies in mice that did not allow measurement of biochemical effects of the amino sugar. From these in vivo experiments they concluded that hypersensitivity in this model of LPS toxicity is a result of transcriptional sensitization to endogenously produced TNF. That this is actually the case is clearly demonstrated herein on the basis of biochemical and histologic measurements on the target cell level and might provide a molecular explanation for this long known experimental experience.

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