Concanavalin A-Induced Liver Cell Damage: Activation of Intracellular Pathways Triggered by Tumor Necrosis Factor in Mice

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Background & Aims: Concanavalin A (con A) induces tumor necrosis factor (TNF)-dependent hepatocyte apoptosis resembling immune-mediated fulminant hepatic failure in humans. Intracellular pathways originating at the TNF receptor are either linked to apoptosis, nuclear factor (NF)- κ B translocation, or Jun kinase (JNK) activation. The aim of this study was to study TNF-dependent pathways after con A injection in vivo.

Methods: Con A, con A plus anti-TNF, and control buffer were injected into BALB/c mice. Immunofluorescence, Western blot, Northern blot, gel shift, Erk, and JNK activity and DNA fragmentation experiments were performed at different time points after injection. **Results:** DNA fragmentation in hepatocytes was increased 4–24 hours after con A injection. JNK was activated maximally (>20-fold) directly after con A injection, whereas binding and nuclear translocation of NF- κ B was maximal after 4 hours. All pathways were blocked by anti-TNF. JNK activation was specific because related ERK 1 + 2 were not activated after con A. High nuclear expression of c-Jun was already evident 1 hour after con A injection; however, in contrast to JNK, anti-TNF treatment did not block c-Jun nuclear expression and DNA binding. **Conclusions:** In the con A model, activation of TNF-dependent pathways is associated with apoptosis of hepatocytes. Their modulation in vivo may have implications to develop new therapeutic strategies to prevent apoptosis.

Fulminant hepatic failure due to viral infection, intoxication, or other causes is a life-threatening event. Immune-mediated mechanisms that ultimately lead to apoptosis of hepatocytes are frequently involved in this process.¹ Therefore, animal models that mimic the condition of immune-dependent hepatocyte cell death are important to understand the mechanisms leading to liver failure and to develop new therapeutic approaches that may also be important in humans.

A new animal model of immune-mediated liver injury was developed² in which concanavalin A (con A) injection into mice leads to dose-dependent apoptosis of hepatocytes.^{3,4} Con A has high affinity toward the hepatic sinus, leading to T-cell activation in the liver.^{3,5} A subset of T lymphocytes, the CD4-positive cells, are activated as anti-CD4 antibodies block activation, and consequently, liver cell death.² In addition to the activated T lymphocytes, polymorphonuclear cells are found in the infiltrated liver, possibly recruited by CD4-positive cells.⁵

The assembly of immune-activated cells in the liver results in increased levels of several cytokines involved in cell to cell communication and inflammation. High levels of the cytokines interleukin (IL)-2, tumor necrosis factor (TNF)- α , IL-6, granulocyte-macrophage colony-stimulating factor, and IL-1 were found after con A injection, showing a time-dependent release into the plasma.^{2,3,5} Because high circulating TNF levels were found early after con A injection, blocking experiments with anti-TNF antibodies were performed. Interestingly, anti-TNF completely blocked liver cell injury, indicating that TNF plays a major role in mediating apoptosis of hepatocytes in this model.^{3,5}

Activation of several cell surface receptors can mediate apoptosis. The TNF receptor 1 belongs to the TNF/nerve growth factor receptor family and has a so-called death domain in the cytoplasmic part of the receptor.^{6,7} Binding of TNF to TNF receptor 1 activates diverse intracellular pathways. Besides its capacity to induce apoptosis,

Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; JNK, Jun kinase; NF, nuclear factor; SDS, sodium dodecyl sulfate; TNF, tumor necrosis factor; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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other pathways that lead to nuclear factor (NF)- κ B and Jun kinase (JNK) activation have been characterized recently in more detail.⁸⁻¹¹ These experiments showed that the signal cascades that either activate NF- κ B, JNK, or apoptosis directly diverge at the inner cell membrane. TRADD, which binds to the cytoplasmic domain of TNF receptor 1, is important for the activation of all of these pathways.^{8,11} On TNF receptor activation, three proteins, FADD/MORT1, TRAF2, and RIP, are capable of binding TRADD (TNF receptor 1-associated death domain protein). TRAF2 and RIP are important in activating downstream events leading to nuclear translocation of NF- κ B and JNK activation, whereas FADD (Fas-associated death domain protein)/MORT1 mediates essential signals for the induction of apoptosis involving interleukin-converting enzyme proteases.⁸⁻¹² The TNF receptor 2, which also binds TNF- α , can only activate TRAF2-dependent signals but not FADD-dependent apoptosis.¹³

So far the activation of TNF-dependent pathways has been mainly investigated by *in vitro* studies.⁸⁻¹⁰ *In vivo* results showing the importance of these pathways in animal models are lacking. Because apoptosis of hepatocytes in the con A model is mediated by TNF- α ,^{3,5} we were interested in studying whether these pathways are also involved in this process *in vivo*. Therefore, these cascades were investigated after con A injection and after pretreatment with anti-TNF antibodies. JNK, NF- κ B, and apoptosis become activated during con A-induced apoptosis in a TNF-dependent fashion. Differences in the time courses of these pathways show that NF- κ B and JNK are not necessary to maintain apoptosis. Because the balance between the different pathways that diverge at the TNF receptor dissect between apoptotic and antiapoptotic mechanisms, this *in vivo* model has implications for developing strategies that prevent apoptosis *in vivo*.

Materials and Methods

Animals, Con A Injection, and Preparation of Liver Nuclear Extracts

Pathogen-free male BALB/c mice were obtained from the Animal Research Institute of the Medizinische Hochschule Hannover. All experiments were started between 8 AM and 10 AM and were performed in agreement with the German legal requirements. Animals were anesthetized by an intraperitoneal injection of a combination of xylazine and ketamine hydrochloride as described previously.¹⁴ At least 4 animals were treated in parallel for each time point.

Con A (20 mg/kg) was injected intravenously. Anti-TNF immunoglobulin (Ig) was administered 15 minutes before con A injection when indicated.^{2,3} In control-treated animals, only the carrier solution, phosphate-buffered saline, was injected. At

the indicated time points, a small subxiphoid incision was made, blood samples were taken, and the liver was removed. The livers from animals treated in parallel were pooled. A part of the liver was frozen for Northern blot analysis, immunofluorescence, and DNA fragmentation assays. The remaining liver was used to prepare liver nuclear extracts.

For preparation of nuclear extracts, the pooled livers were rinsed in ice-cold phosphate-buffered saline and liver nuclear proteins were prepared as described previously.¹⁴ All steps were performed at 4°C. Nuclear proteins were aliquoted and immediately frozen in liquid nitrogen.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis with liver nuclear proteins were performed as described.¹⁵ Antibodies for NF- κ B and c-Jun proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Gel Retardation Assays

Gel retardation assays with liver nuclear extracts were performed with consensus oligonucleotides for c-Jun (5'-CGC-TTG-ATG-ACT-CAG-CCG-GAA-3') or NF- κ B site (5'-TAG-TTG-AGG-GGA-CTT-TCC-CAG-GCA-3') as described previously.¹⁶ Supershift experiments were performed with either an antibody directed against c-Jun or NF- κ B (Santa Cruz Biotechnology).

Northern Blot Analysis

Northern blot analysis was performed according to standard procedures, as described previously.¹⁴ c-Jun and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) complementary DNA probes were labeled with [α -³²P]adenosine triphosphate (ATP) according to Rediprime (Amersham, Braunschweig, Germany). The hybridization procedure was performed as described previously.¹⁴

Immunofluorescence

For immunofluorescence experiments, cryosections (4–5 μ m thick) were performed and immediately fixed in ice-cold acetone for 5 minutes, air-dried, and either stored at –80°C or used immediately. Immunofluorescence staining was performed essentially as described previously.¹⁷ Anti-NF- κ B (Santa Cruz Biotechnology) and anti-cytochrome P450 2D6 were both incubated as primary antibodies. Anti-rabbit IgG Cy3-conjugated antibodies (Sigma Chemical Co., St. Louis, MO) and anti-human fluorescein isothiocyanate (FITC)-conjugated antibodies (Dianova, Hamburg, Germany) were used as secondary antibodies. Sections were analyzed through either a 615-nm filter (Cy3 staining) or a 525-nm filter (FITC staining) with an Olympus fluorescence microscope (Hamburg, Germany).

In Vitro Kinase Assays

JNK activity was assessed by an in vitro kinase assay as described previously¹⁸ using recombinant glutathione *S*-transferase (GST)-c-Jun protein (1–79). The proteins were fractionated using 12.5% SDS-PAGE and visualized and quantitated using Fujix Bas 1000 Bioimaging Analyser (Raytest, Straubenhardt, Germany). Coomassie staining was used to show equal protein loading.

ERK activity was assessed similarly using an in vitro immune complex kinase assay with recombinant GST-Elk protein as a substrate. Immunoprecipitation was performed on whole-cell extracts using an anti-ERK-2 antibody (Santa Cruz, C14; cross-reactive with ERK-1). The proteins were fractionated using 12.5% SDS-PAGE and visualized and quantitated using PhosphorImager analysis. Coomassie staining was used to demonstrate equal protein loading.

DNA Fragmentation

For determination of DNA fragmentation by enzyme-linked immunosorbent assay (ELISA), one liver lobe (20%) was treated in TE buffer with five strokes of a homogenizer (pestle B). The 20% homogenate was centrifuged at 13,000*g* for 20 minutes. The supernatant was further diluted 200-fold and directly used to determine DNA fragmentation by the commercially available ELISA system cell death detection (Boehringer Mannheim) according to the manufacturer's instructions, designed to quantify cytosolic oligonucleosome-bound DNA (histone ELISA). The level of DNA fragmentation found before treatment was set to 1, and changes were shown as fold activation.

Immunohistochemical detection of apoptosis was performed using the in situ cell death detection kit (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling [TUNEL] Test; Boehringer Mannheim). Fixation of the cells was performed with acetone instead of paraformaldehyde. All other steps were performed according to manufacturer's instructions. The fluorescence-labeled DNA fragments were analyzed using an immunofluorescence microscope (Olympus IMT2-BH2; Tokyo).

Quantification

Quantification of results was performed with a Fujix Bas 1000 Bioimaging Analyser (Raytest) or by a densitometer as described before.¹⁴

Results

Con A Injection Induces Time-Dependent Apoptosis of Liver Cells

To test the time course of T cell-mediated apoptosis after con A injection, DNA fragmentation was measured in the liver at different time points after injection in comparison to the pretreatment status. By histone ELISA, DNA fragmentation started as early as 4 hours after con A injection (Figure 1A). The increase after

Figure 1. DNA fragmentation after con A injection. DNA fragmentation of liver cells was either monitored by (A) histone ELISA or (B–E) TUNEL-Test as described in Materials and Methods. Apoptotic hepatocytes by TUNEL-Test are shown (B) before injection, (C) 4 hours after con A injection, (D) 4 hours after con A injection plus anti-TNF pretreatment, and (E) after 4 hours when only the carrier solution was injected. Histone ELISA was performed at all different time points. The value before treatment of the animals was set to 1, and all other values were calculated accordingly and expressed as fold activation.

4 hours was >40% of background level. Maximal increase occurred after 8 hours, exceeding the pretreatment level more than sixfold. High cytosolic DNA fragmentation was monitored for up to 24 hours after treatment; the amount of DNA fragmentation was reduced after 48 hours; and pretreatment levels were reached again after 72 hours.

The time course found by TUNEL staining showed a slight variation compared with the histone ELISA (Figure 1B–E). The amount of positive nuclei was already high after 4 hours (Figure 1C). The number of positive cells

decreased after 24 hours. Treatment of con A with anti-TNF antibodies reduced apoptosis (Figures 1A and D).

Translocation, Nuclear Expression, and DNA Binding of NF- κ B

Binding of TNF to TNF receptor 1 may induce apoptosis and NF- κ B activation.⁸ In the con A model, DNA fragmentation was first detected 4–8 hours after con A injection. We therefore studied *in vivo* nuclear translocation of NF- κ B p65 by immunofluorescence (Figure 2A, C, E, and G). Already 1 hour after con A injection, NF- κ B was found in the nucleus of hepatocytes. An additional increase was observed for up to 4 hours, and NF- κ B expression remained high for up to 12 hours, after which nuclear staining of NF- κ B p65 decreased (data not shown). At time points later than 48 hours after con A injection, only a weak signal for NF- κ B p65 was found in the nucleus of hepatocytes. The nuclear translocation of NF- κ B p65 was completely blocked 4 hours after con A and anti-TNF administration (Figure 2E).

To assess that the nuclear translocation of NF- κ B really occurred in hepatocytes, double staining was performed with an anti-cytochrome P450 2D6 antibody (Figure 2B, D, F, and H). Cells positive for NF- κ B in the nucleus also had a cytoplasmic staining for cytochrome P450 2D6 (Figure 2D). These experiments showed that NF- κ B activation occurred in hepatocytes.

The increase in the nuclear translocation of NF- κ B p65 was quantitated by Western blot experiments of nuclear extracts. A greater than 10-fold increase in nuclear accumulation of NF- κ B p65 was evident 4 hours after con A injection (Figure 3A). Pretreatment levels were reached again 96 hours after injection. When the animals were pretreated with anti-TNF antibodies before con A injection, only a slight increase was detectable after 2 hours, and no change was found when only the carrier solution was used (Figure 3B and C). A weaker, but very similar, time course was found when the nuclear expression of p50 was tested, showing nuclear translocation restricted to con A injection (Figure 3, lower panels).

Higher nuclear NF- κ B expression correlated with its DNA binding. In gel shift experiments, an increase in complex formation was observed for up to 4 hours, and high-affinity binding lasted from 4 to 12 hours after con A injection (Figure 4A). This observation was restricted to T-cell activation after con A injection. Anti-TNF pretreatment nearly completely blocked complex formation (Figure 4B), and no specific band appeared after injection of the buffer control (Figure 4C). The complex was completely supershifted with an anti-p65 antibody,

whereas anti-p50 only supershifted the lower of the two bands (Figure 4D). These results indicated that the upper band most likely consists of a p65 homodimer and the lower band of a p65/p50 heterodimer.

Figure 2. Enhanced nuclear translocation of NF- κ B after con A injection. Site of NF- κ B p65 in liver tissue. Double immunofluorescence staining was performed with antibodies directed against NF- κ B (anti-rabbit) and cytochrome P450 2D6 (anti-human). Cy3-conjugated anti-rabbit and FITC-conjugated anti-human antibodies were used as secondary antibodies. NF- κ B staining was detected with a 615-nm filter (*left panels*) and cytochrome P450 2D6 with a 525-nm filter (*right panels*). Liver sections are shown (A and B) before treatment, (C and D) 4 hours after con A injection, (E and F) 4 hours after con A injection plus anti-TNF treatment, and (G and H) 24 hours after con A injection.

Figure 3. Higher nuclear expression of NF-κB p50 and p65 after con A-induced T-cell activation in liver cells. Western blot analysis was performed with liver nuclear extracts and anti-p65 (*upper panel*) and anti-p50 (*lower panel*) antibodies. Animals were either treated with (A) con A, (B) con A plus anti-TNF, or (C) the carrier solution alone. Nuclear extracts were used either before treatment (*lane 1*) or at different time points after injection as indicated. The positions of NF-κB p65 and p50 are indicated. MW, molecular weight.

Activation of JNK, but Not ERK Kinase, Is Associated With Apoptosis of Hepatocytes After T-Cell Activation

Cell culture experiments provided evidence that apoptosis induced by Fas and TNF is associated with JNK activation.¹⁹⁻²¹ In the con A model, several cyto-

Figure 4. DNA binding of NF-κB is enhanced after con A injection in liver nuclei. Liver nuclear extracts (1 μg) were incubated with a ³²P-labeled oligonucleotide representing an NF-κB consensus sequence. Liver nuclear extracts from mice were either treated with (A) con A, (B) con A plus anti-TNF, or (C) the carrier solution alone. Nuclear extracts were used either before injection (*lane 1*) or at different time points after treatment. The positions of the bound and free DNA are marked. (D) Supershift experiments. Liver nuclear extracts were used before treatment (*lane 1*) or at 4 hours after con A injection (*lanes 2-8*). Increasing amounts of anti-p50 (*lanes 3-5*) or anti-p65 (*lanes 6-8*) were added to the incubation mix.

kines are elevated, which are involved in different signaling pathways.^{3,5} Thus, *in vivo* after T-cell stimulation, the situation is more complex. We studied two MAPK pathways: activation of JNK and ERK.²² *In vitro* gel kinase assays were applied to measure the activity of JNK and ERK after induction of T cell-mediated apoptosis in hepatocytes.

One hour after con A-induced T-cell activation, JNK activity exceeded the pretreatment level more than 20-fold (Figure 5A). JNK activity remained high for up

to 4 hours. At later time points, when DNA fragmentation started, JNK activity clearly decreased, and pretreatment levels were found again 24 hours after con A injection. Activation of JNK was nearly completely blocked by pretreatment with anti-TNF antibodies. The maximal increase (fourfold) was found 4 hours after con A and anti-TNF treatment. No significant change was evident when the buffer control was administered.

At the early time points after con A injection, after 2 hours, the increase in JNK was only twofold compared with the pretreatment level and was not significantly higher than in animals in which only the carrier solution was injected. Twelve hours after T-cell stimulation by con A and anti-TNF treatment, JNK activity peaked and was fourfold higher than the pretreatment levels. After this time point JNK activity returned to nearly normal.

The cascade that leads to the activation of ERK 1 + 2 resembles the pathway involved in JNK activation but is stimulated by different factors. In contrast to JNK, ERK activity was only slightly increased after con A-induced T-cell activation (Figure 5B). The increase in ERK activity was only 2–3-fold and lasted for up to 12 hours. Even after injection of the carrier solution, ERK activity increased nearly 2-fold after 2 hours. The increase in ERK 1 + 2 activity, therefore seemed not to be specifically linked to con A-induced liver damage in contrast to the results obtained for JNK.

Figure 5. JNK, not ERK, activation is associated with apoptosis. (A) Recombinant GST-Jun protein was incubated with 25 μ g of liver nuclear extracts from mice treated with the substrates (control, con A, and con A + anti-TNF) at various time points after injection. Phosphorylation of GST-Jun by extract-derived JNK was measured by incorporation of [γ -³²P]ATP as determined by SDS-PAGE and PhosphorImager analysis. Extracts from the murine fibroblast cell line 3T3 were used as positive control (+); the same extracts incubated with GST alone were used as a negative control (-). (B) ERK-1 and ERK-2 proteins were immunoprecipitated from 25 μ g of the same liver nuclear extracts using a cross-reactive anti-ERK-2 polyclonal antibody. *In vitro* kinase reactions were performed on the immune complexes using recombinant GST-Elk protein as a substrate and [γ -³²P]ATP as a phosphodonator. Reactions were subjected to SDS-PAGE and PhosphorImager analysis. Extracts from 3T3 cells were used as a positive control when incubated with GST-ELK (+).

Con A Injection Activates c-Jun Expression and DNA Binding Via a JNK-Independent Pathway

Activation of JNK and consecutive phosphorylation of serine 63 and 73 in the transactivation domain of c-Jun is associated with increased transcription of c-Jun-dependent genes.²³ Additionally, c-Jun controls its own transcription by binding to a site in its own promoter.²⁴ We therefore investigated whether JNK activation is associated with higher c-Jun messenger RNA (mRNA) levels by using Northern blot analysis. A slight increase in c-Jun mRNA expression was found already after 1 hour (Figure 6A). A constant, more than 10-fold, increase was observed for up to 8 hours, after which c-Jun mRNA levels decreased. Pretreatment levels were reached after 48 hours. After pretreatment with anti-TNF antibodies, only a slight increase of c-Jun mRNA was evident (Figure 6B). The levels were back to normal 24 hours after injection, and no change was found when the carrier solution was injected (Figure 6C).

Because hepatic c-Jun mRNA showed a distinct increase after con A injection, we studied how its increase is reflected on the protein level. Western blot analysis of liver nuclear extracts was performed with an anti-c-Jun

antibody (Figure 7A). The level of nuclear c-Jun protein expression was significantly higher already after 1 hour. Nuclear c-Jun expression increased slightly until 8 hours and was high for up to 24 hours after con A injection. c-Jun expression decreased after 24 hours, and pretreatment levels were reached after 96 hours (Figure 7A). Unexpectedly, nuclear c-Jun expression was high already 2 hours after con A and anti-TNF injection and was elevated for up to 12 hours (Figure 7B). At later time

points, c-Jun expression was low again. No changes were found in the control-treated animals (Figure 7C).

To further confirm the differences in c-Jun mRNA and protein expression, gel shift experiments were performed. In agreement with Western blot analysis, complex formation was clearly enhanced 1 hour after con A injection (Figure 8A). Complex formation increased and was maximal after 8 hours. No specific complex formation was observed after 96 hours. Also, after pretreatment with anti-TNF, an increase in binding was observed after 2 hours and maximal binding was evident at 4 and 12 hours (Figure 8B). Complex formation was comparable to that in the animals only treated with con A within the

Figure 6. Higher c-Jun mRNA expression after con A injection. Northern blot analysis was performed at different time points after (A) con A injection and (B) con A plus anti-TNF treatment. (C) Animals treated with the buffer control. Specific signals were detected with a ^{32}P -labeled probe for c-Jun or GAPDH as indicated.

Figure 7. Nuclear c-Jun protein expression is increased after con A and con A plus anti-TNF treatment. Nuclear proteins derived at different time points from mice after (A) con A injection and (B) con A plus anti-TNF treatment and from (C) control-treated animals were separated on a 10% SDS gel, and Western blot analysis using an anti-c-Jun antibody was performed. The position of c-Jun is indicated.

Figure 8. Higher DNA binding of c-Jun after con A and con A plus anti-TNF treatment. Liver nuclear extracts (1 μ g) were incubated with a 32 P-labeled oligonucleotide representing the AP1 consensus sequence. Liver nuclear extracts from mice were either treated with (A) con A, (B) con A plus anti-TNF, or (C) control buffer. Nuclear extracts were used either before injection (lane 1) or at different time points after treatment. The positions of the bound and free DNA are marked. (D) Supershift experiments. Liver nuclear extracts were used before treatment (lane 1) or at 8 hours after con A injection (lanes 2–6). Increasing amounts of anti-c-Jun (lanes 3–6) were added to the incubation mix.

first 12 hours. No complex formation was observed in the animals in which the carrier solution was injected (Figure 8C). The specificity of the complex that appeared after con A injection was confirmed by competition studies with nuclear extracts of animals treated with con A for 8 hours (data not shown). Supershift experiments were also

performed with an anti-c-Jun antibody (Figure 8D), showing that the complex consisted mainly of c-Jun. Only a lower band was not supershifted with the anti-c-Jun antibody, indicating that an additional protein was present in the complex.

Discussion

Con A injection in mice leads to apoptosis of hepatocytes. Earlier results showed that con A activates CD4-positive cells infiltrating into hepatic tissue. Activation of T cells and mononuclear cells results in the production and release of various cytokines, e.g., TNF- α and interferon gamma (IFN- γ).^{2,5,25} Anti-TNF antibodies are able to block liver injury. Therefore, it was suggested that TNF- α may directly or indirectly be involved in inducing apoptosis in this model. Recent experiments showed that both TNF receptors are essential for inducing liver cell damage in the con A model because TNF receptors 1 and 2 knockout mice are protected. Evidence shows that TNF receptor 2 might be essential in the communication between lymphocytes and might be involved in the induction of IFN- γ .²⁶ The role of TNF receptor 1 seems more directly involved in inducing apoptosis of hepatocytes in the con A model. The aim of this study was to investigate intracellular pathways that originate at the TNF receptor of hepatocytes after con A injection in vivo.

Recent experiments by Hsu et al. suggested that different signaling cascades are activated through the cytoplasmic death domain of the TNF receptor 1. These pathways lead to either nuclear translocation of NF- κ B, activation of JNK, or induction of apoptosis.^{8,11,12} Our results show that also in vivo, after con A-mediated T-cell activation, all three pathways became activated. Furthermore, anti-TNF treatment blocked activation of NF- κ B translocation, JNK activation, and apoptosis. These results were obtained using whole liver tissue. Immunofluorescence studies indicated that most of the activation may occur in hepatocytes. However, some of the activation might have occurred in the nonparenchymal cells.

The TNF receptor mediates JNK activation via a signal transduction pathway consisting of the sequential activation of distinct protein kinases.²⁷ Recently, the activation of JNK was linked to apoptosis.^{19,20} Mutant forms of the kinases involved in this pathway acting as dominant negative repressors of JNK activation prevented the induction of apoptosis in this in vitro system.²⁸ However, there is no experimental proof that this regulation is also crucial in more complex systems. In contrast, recent experiments provided strong evidence

that the pathways leading to JNK activation and apoptosis can be dissected from each other. These experiments also indicated that JNK is not a direct regulator of apoptosis because blocking of the JNK pathway did not alter induction of apoptosis.¹¹

Maximal activation of JNK after con A injection was found very early, after 1 hour, following con A-induced T-cell activation. In contrast, the beginning of DNA fragmentation can first be observed after 4 hours and is maximal after 8 hours. The time course of JNK activation *in vivo* does not argue against an involvement of JNK in triggering apoptotic processes. At later time points, however, when DNA fragmentation is most prominent *in vivo*, JNK activity is low again. These results thus show that JNK is at least not required to maintain DNA fragmentation in hepatocytes.

Several cytokines become secreted into the plasma after con A injection, which in turn lead to the induction of different intracellular signal transduction cascades in hepatocytes. As an example for one of these cascades, ERK 1 + 2 was studied. The pathway leading to ERK 1 + 2 activation is closely related to the pathway controlling JNK activation.²⁰ However, activation of ERK 1 + 2 seems, in contrast to the results obtained for JNK, not related to the events found after con A-mediated liver damage.

After con A injection, high levels of the transcription factor c-Jun, which is the direct substrate of JNK, have been found at time points when DNA fragmentation has not yet been started. c-Jun might play a role in inducing the apoptotic processes observed at later time points. Earlier experiments also showed that a dominant negative form of c-Jun (TAM 77) is able to block apoptosis in U 937 cells.²⁹ However, blockage of the TNF action by using an anti-TNF antibody shows that the close link between c-Jun and induction of apoptosis is not as evident in con A-induced apoptosis. Despite anti-TNF treatment, c-Jun is highly expressed in the nucleus and shows high DNA binding, although no apoptosis occurs. Expression of c-Jun therefore seems unrelated to apoptosis in hepatocytes.

The signals that regulate c-Jun expression after con A injection and anti-TNF pretreatment are unknown. However, protein expression of c-Jun after con A injection seems to be mainly controlled on a posttranscriptional level, because an increase in c-Jun mRNA level was found after the increase in c-Jun protein expression. A pathway activated by one of the other cytokines may likely have c-Jun as a target, possibly leading to either its high nuclear translocation or protein stabilization.

After con A injection also nuclear translocation of NF- κ B is controlled in a TNF-dependent manner. In

contrast to JNK activation, which was already most prominent after 1 hour, nuclear translocation of NF- κ B increased until 4–8 hours after con A injection. Earlier studies showed that NF- κ B is able to prevent apoptosis in different cell types^{11,30–33} and may have a similar function in hepatocytes. Therefore, differences in the activation kinetics of TNF-dependent signal transduction cascades might be responsible for the decision toward apoptosis or NF- κ B nuclear translocation. Maximal activation of DNA fragmentation and nuclear NF- κ B translocation was found at nearly the same time points. Therefore, the intracellular cascades that induce apoptosis are activated before that time point, and NF- κ B with its prolonged kinetic is unable to prevent apoptosis and liver injury.

Besides its possible involvement in the prevention of apoptosis, earlier studies showed that NF- κ B is associated with cell cycle progression in hepatocytes during liver regeneration.³⁴ Knockout mice lacking the Rel A component of NF- κ B have a defect in normal liver development.³⁵ NF- κ B thus seems crucial for events that induce cell proliferation and thereby repair of the liver. After con A-induced T cell-mediated apoptosis and an increase in aminotransferase levels, the liver enters the cell cycle after 24 hours and normal liver function is reconstituted (T. Rakemann and C. Trautwein, unpublished results). TNF-dependent signals may have two different functions after con A-induced T-cell activation. While inducing apoptosis via FADD-dependent mechanisms, the receptor activates pathways via TRAF2 and RIP, which are important to reconstitute the organ and to establish normal liver function.

The mechanisms that control whether apoptosis or NF- κ B activation is the more prominent signal at TNF receptor 1 are not yet defined. Additional signals may be crucial for influencing the balance in a certain direction. Because several cytokines are secreted early during this process, they could synergistically trigger the pathways that initiate apoptosis. Recent studies of Küsters et al.²⁵ indicate that, in addition to TNF, IFN- γ is also critical for controlling apoptosis after con A injection. They showed that anti-IFN- γ treatment prevented hepatocytes from apoptosis after con A-induced T-cell activation. Lower TNF- α levels were found after anti-IFN- γ pretreatment. However, addition of TNF- α , to substitute its decrease after anti-IFN treatment, is not sufficient to induce apoptosis. There is no clear evidence that IFN- γ induces apoptosis after binding to its receptor. It is more likely that IFN- γ modulates the pathways that are activated after TNF-TNF receptor 1 binding. IFN- γ blocks cell proliferation via Stat1.³⁶ Therefore, high IFN- γ levels directly counteract the known effects of NF- κ B on cell proliferation and possibly also other

undefined target genes. These results indicate that further studies can directly address the question how Stat1 may interfere with cell cycle control and thus trigger apoptosis.

Fas also induces apoptosis after binding to its ligand.^{37,38} Both Fas and TNF receptor 1 are expressed on hepatocytes^{1,4} and use the same intracellular molecule, FADD/MORT1, which links their activation to apoptosis.³⁹ Additional studies using this animal model should help to develop new therapeutic options to prevent FADD-dependent apoptosis of hepatocytes, which seems also crucial during immune-mediated fulminant hepatic failure in humans.

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