Mechanisms of Liver Injury.

I. TNF-α-induced liver injury: role of IKK, JNK, and ROS pathways

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TNF-induced liver injury

TNF-induced liver injury is mediated by the production of inflammatory mediators and cell death, and plays a major role in the pathophysiology of septic shock and the wasting syndrome. In the liver, TNF-α is involved in the pathophysiology of viral hepatitis, alcoholic liver disease, nonalcoholic fatty liver disease, and ischemia-reperfusion (I/R) injury. TNF-α plays a dichotomous role in the liver, where it not only acts as a mediator of cell death but also induces hepatocyte proliferation and liver regeneration.

TNF-α is produced mainly by macrophages but also by a broad variety of other cell types including lymphoid cells, mast cells, endothelial cells, fibroblasts, and neuronal cells (43). TNF-α is primarily produced as a type II transmembrane protein but may be released in soluble trimeric form via proteolytic cleavage by the metalloprotease TNF-converting enzyme (TACE) (43). In response to LPS and other bacterial products, large amounts of TNF-α are generated. TNF-α exerts its biological functions via interactions with two cognate membrane receptors, TNF-R1 and TNF-R2. Although TNF-α may act as a potent activator of both proinflammatory and proapoptotic pathways, these signaling pathways interact in a complex network at several levels, and activation of one pathway often depends on the inactivation of another pathway, suggesting that cells are capable of directing the TNF-α-induced signal toward the appropriate response. The ability of TNF-α to bind to two different receptors, which transmit distinct intracellular signals with different affinities, adds yet another level of control over TNF-α-induced cellular responses. Whereas TNF-R1 is efficiently activated by soluble TNF-α, TNF-R2 activation requires the binding of membrane-bound TNF-α (reviewed in Ref. 43). After TNF-α binding, TNF receptors undergo a conformational change allowing them to recruit adapter molecules that then initiate the activation of intracellular signaling pathways. The intracellular region of TNF-R1 contains a conserved protein-protein interaction motif of ~80 amino acids termed the “death domain,” which interacts with the adapter molecule TNF receptor-associated protein with death domain (TRADD), which contains a similar death domain. TNF-R1-bound TRADD then serves as an assembly platform for binding of TNF-α receptor-associated factor (TRAF2), receptor-interacting kinase (RIP), and the adapter molecule Fas-associated death domain (FADD) (43). In contrast, TNF-R2 does not contain a death domain and directly interacts with TRAF2. Activation of TNF-R1 may lead to the activation of NF-κB, JNK, and p38 through RIP1 and TRAF2, whereas activation of caspases and apoptosis is mediated through FADD (43). Although death receptors of the TNF receptor family such as Fas and TNF-related apoptosis-inducing ligand (TRAIL) efficiently form a “death-inducing signaling complex” (DISC) in which caspase-8 activation is initiated, such a complex has not been detected in TNF receptor signaling. Most likely, TNF-R1 only induces a weak and transient formation of this complex because of the TRAF2-mediated recruitment of inhibitor of apoptosis (IAP) molecules, which interfere with the activation of caspase-8 (43). This concept is further supported by the finding that TNF-induced death signals generally require additional mitochondrial signals, whereas Fas is capable of inducing apoptosis independently of this mitochondrial pathway in many cell types. If antiapoptotic signals such as TRAF2 and NF-κB are blocked, TNF-induced caspase-8 activation leads to the activation of proapoptotic members of the Bcl-2 homology (BH) domain proteins, mitochondrial depolarization, cytochrome c release, and the activation of executioner caspases (4, 43). Although TNF-R2 exclusively activates proinflammatory pathways and does not induce apoptosis, the cross talk between TNF-R1- and TNF-R2-induced signals may modulate TNF-R1-induced effects including apoptosis. TNF-R2 may enhance TNF-R1-induced cell death by several mechanisms such as 1) lowering the availability of TRAF2 after its binding to TNF-R2, 2) inducing the degradation of TRAF2 through a cIAP1-mediated mechanism, 3) TNF-R2-mediated prolonged JNK activation, and 4) TNF-R2 induced TNF-α secretion (13). However, the role of TNF-R2 in liver pathophysiology remains largely unknown.

TNF-INDUCED CELL DEATH IN HEPATOCYTES

Role of IKK/NF-κB in protection from TNF-induced cell death. NF-κB transcription factors are master coordinators of immune and inflammatory responses and play a major role in

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the protection from cell death (11). NF-κB consists of homo- and heterodimers composed of the NF-κB members including p50, p52, p65, and c-Rel. The most prominent form of NF-κB is the p50-p65 heterodimer, which strongly induces transcription of NF-κB-responsive genes (11). p50-p50 homodimers, on the other hand, downregulate NF-κB-dependent gene transcription because of the lack of a transactivation domain in the p50 subunit. NF-κB dimers and NF-κB homodimers are held in an inactive state in the cytoplasm by their association with IκB proteins. NF-κB is activated after IκB is phosphorylated and subsequently degraded by the proteasome, enabling the liberated NF-κB dimers to enter the nucleus and initiate transcription of genes with κB sites (11). Phosphorylation of IκB is mediated by a high-molecular-weight IκK complex that consists of the regulatory subunit IκK-γ and two catalytic subunits termed IκK-α and IκK-β. Although both catalytic IκK subunits are capable of inducing IκB phosphorylation, IκK-β is a more efficient IκB kinase than IκK-α, and IκK-β mediates the majority of IκB phosphorylation in vivo (11). In contrast, IκK-α seems to play a predominant role in the phosphorylation of the NF-κB subunit p100 in the lymphotxin pathway (33). NF-κB induces the expression of several antiapoptotic proteins including cIAPs, c-FLIP, A1, A20, TRAF2, and Bcl-xl, which block the activity of either death receptors or the mitochondrial death pathway (43). Recently, it was shown that the attenuation of TNF-induced JNK activation represents another important mechanism by which NF-κB protects from TNF-induced cell death (9, 37). NF-κB regulates several factors that have been implicated in the repression of JNK activity, among them the transcription factors XIAP and GADD45 as well as the antioxidant enzyme SOD2. Transcription of the mitochondrial form of SOD (SOD2), an antioxidant enzyme that can prevent TNF-induced cell death, is controlled by NF-κB, providing a link between NF-κB activation and suppression of ROS. Whereas Gadd45−/− and Xiap−/− fibroblasts do not show any change in the kinetics of JNK activation (1, 18), antioxidants such as butylated hydroxianisole (BHA) are capable of blocking the prolonged JNK activation after TNF-α (17). Thus TNF-induced ROS production seems to be the crucial factor that induces prolonged JNK activation in the absence of NF-κB activation (see Fig. 1). This notion is further supported by the findings that RelA- and IκK-γ-deficient fibroblasts show an increased production of ROS after TNF-α treatment but not after IL-1 (17).

The crucial cytoprotective role of NF-κB is seen during embryonic development, in which NF-κB protects the liver against TNF-induced apoptosis (11). Mice that are deficient in the p65 NF-κB subunit, the noncatalytic subunit IκK-γ, or the catalytic subunit IκK-β lack this protection and die around embryonic days 12.5–13.5 because of increased hepatocyte apoptosis. In contrast, mice lacking the Iκκ-α subunit survive until birth, suggesting that Iκκ-α is not required for protection from TNF-induced apoptosis in the liver. Despite these apparently clear-cut roles for IκK-α and IκK-β, it is unclear whether IκK-α and IκK-β fulfill similar functions in adult hepatocytes. Using dominant negative IκK-α and IκK-β, we (28) have shown that IκK-β mediates the majority of TNF- and IL-1-induced NF-κB activation in rat hepatocytes in vitro and that dominant negative IκK-β sensitizes hepatocytes to TNF-mediated apoptosis. IκK-β seems not only to be involved in the regulation of IκB phosphorylation but also to be the predominant kinase to phosphorylate NF-κB subunit p65 in its transactivation domains in hepatocytes and other cell types (28, 31). However, there is evidence that hepatic Iκκ-α may substitute for hepatic IκK-β to a higher degree than described in other organs because a hepatocyte-specific deletion of IκK-β does not induce extensive hepatocyte apoptosis after TNF-α administration in vivo (22, 23). Several other kinases including glycogen synthase kinase (GSK)-3β and Tank-binding kinase 1/NF-κB-activating kinase (TBK1/NAK) regulate

Fig. 1. Role of IKK, JNK, and ROS in TNF-α-induced cell death and proliferation. Soluble TNF-α (sTNF) binds to TNF-R1, whereas membrane TNF-α (mTNF) predominantly binds to TNF-R2. After binding, both TNF receptors recruit the adapter molecules TRADD, TRAF2, and RIP to activate the IKK and JNK pathways. IKK phosphorylates IκB and p65 resulting in IκB degradation and NF-κB activation. Prolonged activation of JNK requires TNF-induced reactive oxygen species (ROS) production because ROS contribute to JNK activity by oxidizing and inactivating several members of the MAP kinase phosphatases (MKPs). Prolonged JNK activation shifts the balance toward cell death by inducing phosphorylation of the E3 ligase Itch and subsequent ubiquitination and degradation of the NF-κB-regulated caspase-8 inhibitor c-Flip. NF-κB activation prevents prolonged JNK activation and cell death by inducing transcription of the antioxidant MnSOD. Shorter activation of JNK induces proliferation through its target c-Jun.
TNF-induced NF-κB activation in hepatocytes, and animals lacking these kinases die during embryonic development because of TNF-induced hepatocyte apoptosis (3, 15, 30).

**ROS-JNK pathway in TNF-induced hepatic apoptosis and necrosis.** TNF-α strongly activates JNK, which phosphorylates its targets c-Jun, ATF-2, and JunD. These transcription factors are members of the AP-1 family and induce the transcription of AP-1-dependent genes, many of which are involved in the regulation of inflammation, proliferation, and cell death. JNK activation is initiated after TRAF2 and RIP bind to TRADD, leading to the phosphorylation of JNK kinase kinases such as MEK kinase 1 (MEKK1) and apoptosis signaling kinase 1 (ASK1). TRAF2 induces the activation of JNK at least in part through a ROS-dependent pathway that activates ASK1. JNK is phosphorylated by MKK4 and MKK7 on different residues, but only MKK7 is efficiently activated by TNF-α, and disruption of MKK7, but not of MKK4, blocks TNF-induced JNK activation (38). In addition, TNF-induced ROS oxidize and inactivate MAPK phosphatases (MKP) (17), which dephosphorylate JNK, leading to a prolonged activation of JNK (see Fig. 1). The proapoptotic role of JNK in TNF-induced cell death has emerged in recent years because of the availability of specific JNK inhibitors and the understanding that the duration of JNK activation is critical for its proapoptotic effects. Prolonged TNF-induced JNK activation requires the absence of NF-κB activity (9, 37). Recent data suggest that ROS are crucial mediators in this process and that NF-κB upregulates antioxidative defenses and thus prevents ROS generation and prolonged JNK activation (27). The antioxidant BHA suppresses TNF-induced cell death (12, 27, 42), and this effect is mediated by blocking the TNF-induced prolonged activation of JNK (17). In hepatocytes, TNF induces prolonged JNK activation after NF-κB inhibition (20, 32) and prolonged JNK activation contributes to TNF-induced apoptosis (32). Although JNK induces AP-1-dependent transcription by phosphorylating and transactivating its classic targets c-Jun, ATF2, and JunD, the mechanism by which JNK enhances TNF-induced apoptosis seems to be independent of these factors. In hepatocytes, the proapoptotic effects of JNK do not require transcription and c-Jun and involve targets upstream of the mitochondria (32). The requirement for a prolonged JNK activation suggests that 1) a threshold of JNK activity is required that can only be achieved by prolonged activation, 2) the prolonged activation of JNK reveals targets that are only available at later time points after initial TNF-α binding, or 3) the JNK target requires the activity of other pathways, which may be only available at later timepoints, to induce apoptosis. A recent report has shown that TNF-α-mediated JNK activation accelerates turnover of the NF-κB-induced antiapoptotic protein c-FLIP and thus sensitizes to TNFα-induced cell death (5a). JNK phosphorylates and activates the E3 ubiquitin ligase Itch, which specifically ubiquitinates c-FLIP and induces its proteasomal degradation. Accordingly, Itch-deficient mice are resistant to TNFα-induced acute liver failure, and cells from these mice do not display inducible c-FLIP ubiquitination and degradation (5a). Additionally, it has been suggested that JNK induces the cleavage of Bid to a specific fragment termed “jBid,” which then acts on the mitochondria to release Smac, a cofactor that is required to inhibit the TRAF2-IAP-mediated block of caspase-8 activation (8). Additionally, it has been suggested that JNK has targets in the mitochondria and that mitochondrial JNK activation in response to ROS causes cytochrome c release and cell death (2). JNK exists in two isoforms in the liver, JNK1 and JNK2. Although it was initially reported that JNK2 has a 25-fold higher affinity for c-Jun, it has become evident that this high affinity is present only in unstimulated cells and that the binding of JNK2 to its target c-Jun primarily regulates c-Jun stability (26). When cells are stimulated with agonists that induce JNK activity, JNK1, on the other hand, mediates the majority of c-Jun phosphorylation (26). Thus JNK1 and JNK2 play distinct roles in the response to TNF and other agonists. Indeed, it has been shown that JNK1 is required to induce proliferation (26). In addition, JNK1 but not JNK2 is required for the proapoptotic effects of TNF-α in fibroblasts (21). Experiments addressing the role of JNK1 vs. JNK2 in TNF-induced apoptosis have not been performed in primary hepatocytes. However, both JNK1 and JNK2 deficiency block concanavalin A (ConA)-induced liver injury in vivo (23).

**Determinants of TNF-induced apoptosis and necrosis in hepatocytes.** Hepatocytes often show morphological features of apoptosis and necrosis, for which the term “necrapoptosis” has been coined (16). TNF induces apoptosis as well as necrosis in hepatocytes in vitro and in vivo. There seem to be several cellular determinants that shift the balance from one to the other pathway. Apoptosis requires ATP, and a switch from apoptosis to necrosis occurs when cells are devoid of ATP. The cleavage of poly(ADP-ribose) polymerase (PARP)-1 in early phases of apoptosis is an important event in proapoptotic signaling, because the activation of PARP after cellular insults consumes large amounts of NAD⁺ and, in efforts to resynthesize NAD⁺, may cause massive ATP depletion that in turn switches the cellular response to necrosis. Fibroblasts from PARP-1-deficient mice are protected against ATP depletion and necrotic, but not apoptotic, cell death (14). The adapter molecule RIP seems to be another important determinant of the form of cell death that is induced after TNF-α. Under apoptotic conditions caspase-mediated cleavage of RIP may block the necrotic pathway, and the complete absence of RIP increases cellular resistance to H₂O₂-mediated necrosis (34). It seems that RIP promotes necrosis through a JNK-dependent pathway (34).

**ROLE OF IKK/NF-κB, JNK, AND ROS IN IN VIVO MODELS OF HEPATIC INJURY**

**Role of ROS-JNK pathway in hepatic I/R injury.** Hepatic ischemia followed by reperfusion (I/R) is a major clinical problem during transplantation, liver resection, and circulatory shock, producing apoptosis and necrosis. TNF-α, but not Fas, is a crucial mediator in hepatic reperfusion injury (25). Inhibition of TNF-α signaling by TNF antiserum or genetic inactivation of TNF-R1 ameliorates hepatic reperfusion injury and prolongs survival (7, 25). Several intracellular signaling pathways are induced after I/R, including NF-κB and JNK. We (19) have shown that blocking hepatic ROS production by overexpression of SOD1 almost completely prevented hepatic JNK activation and injury, suggesting that ROS are major contributors of JNK activation and injury in I/R. In a follow-up study, we showed that inhibition of JNK by three novel pharmacological inhibitors improved 7-day survival from ROS from 20–40% to 60–100% (40). JNK inhibition strongly reduced
Bid degradation, mitochondrial cytochrome c release, caspase-3 activation, and lipid peroxidation, suggesting that JNK acts upstream of the mitochondria in I/R (40). In a model of orthotopic liver transplantation, JNK inhibitors showed a similar efficacy in decreasing pericentral hepatocyte necrosis and nonparenchymal cell death (41). Thus the ROS-JNK pathway represents a promising new target for the treatment of hepatic I/R injury (see Fig. 2).

Role of IKK/NF-κB in hepatic I/R injury. Whereas NF-κB has a protective role in TNF-induced hepatocyte apoptosis, it promotes cell death in hepatic I/R. Inhibition of NF-κB by an adenoviral IkB superrepressor reduces TNF-α levels and protects from hepatic I/R (36), suggesting that NF-κB acts upstream of TNF-α in the pathology of hepatic I/R, whereas it is downstream of TNF-α in hepatic injury after TNF-α challenge. TNF-α, in concert with other cytokines, mediates the recruitment of neutrophils to the liver that induce inflammation and cell death. It has been suggested that NF-κB activation in response to oxidative stress is not mediated by the classic IKK-proteasome dependent pathway but instead depends on tyrosine phosphorylation of IkB-α by Src family members (10). Accordingly, there is no strong activation of IKK after hepatic I/R (39). However, a recent study reported that mice carrying a hepatocyte-specific deletion of IKK-β are resistant to hepatic I/R injury (22). Moreover, specific pharmacological inhibitors of IKK-β were highly effective in preventing hepatic I/R injury (22). Although this result seems to contradict previous studies, it is possible that the presence of IKK-β is required for NF-κB activation despite the lack of strong IKK activation. In intestinal reperfusion injury, IKK is activated after reperfusion, but, in contrast to the liver, IKK-β deletion worsens intestinal I/R injury (6). Thus IKK-β is a potential target for the treatment of hepatic I/R injury (see Fig. 2).

Role of IKK/NF-κB in TNF- and ConA-induced liver injury. TNF-α does not induce liver injury in normal hepatocytes in vivo because of the strong activation of cytoprotective pathways such as NF-κB. However, hepatic TNF-α toxicity is induced when hepatic gene transcription is blocked by coadministration of galactosamine, which mimics hepatic conditions occurring in several disease states. Administration of ConA induces a T cell-mediated hepatitis with a high degree of hepatocellular death without the requirement to block NF-κB or transcription. In the ConA model, infiltrating T lymphocytes express high amounts of membrane-bound TNF-α, which achieves a stronger and prolonged activation of JNK because of the simultaneous activation of TNF-R1 and TNF-R2 (23). Recently, two mouse models with a hepatocyte-specific deletion of IKK-β were created (22, 23). Surprisingly, the administration of soluble TNF-α or LPS was not sufficient to induce considerable amounts of hepatic apoptosis in these models (22, 23). In contrast, mice with a hepatocyte-specific IKK-γ deletion are exquisitely sensitive to hepatocyte apoptosis induced by soluble TNF-α (22). The ability of TNF-α to induce apoptosis appeared to correlate with NF-κB activity in these mouse models: IKK-γ-deficient hepatocytes displayed little NF-κB activation and high amounts of cell death, whereas mice with IKK-β-deficient hepatocytes displayed moderate to strong hepatic NF-κB activation and little or no cell death (22, 23). Whereas the study by Maeda et al. (23) found that IKK-β−/− mice are more susceptible to ConA-induced liver failure, the study by Luedde et al. (22) did not find an increased susceptibility to ConA. Maeda et al. (23) argues that IKK-β is required to protect hepatocytes from membrane-bound TNF-α, which is predominantly induced by ConA and achieves a stronger activation of IKK through the concurrent activation of TNF-R1 and TNF-R2. However, the absence of liver injury at 10 mg/kg ConA in wild-type mice in the study by Maeda et al. (23) raises doubt and suggests that hepatic IKK-β is not required to protect from either soluble or membrane-bound TNF-α as suggested by Luedde et al. (22). Thus it seems that IKK-β deletion does not completely prevent TNF-induced NF-κB activation and that IKK-α can indeed substitute IKK-β to some degree in adult hepatocytes.

Role of JNK and ROS in TNF- and ConA-induced liver injury. In the liver, prolonged activation of JNK mediates TNF-induced cell death both in vitro and in vivo. After ConA administration, JNK activity correlates with hepatic damage (35). The crucial role of JNK was demonstrated by Maeda...
et al. (23), who showed that mice lacking either JNK1 or JNK2 are highly resistant to ConA- and LPS plus galactosamine-induced liver failure and show considerably lower amounts of apoptotic and necrotic cell death in the liver. Similarly, the antioxidant BHA blocks the prolonged JNK activation in mice treated with ConA and protects them from ConA-induced liver failure (17). Thus antioxidants and JNK inhibitor appear to be useful drugs for the treatment of TNF-dependent hepatitis (see Fig. 3).

Role of IKK, JNK, and ROS in liver regeneration and hepatocarcinogenesis. Whereas TNF induces hepatocyte death on the one hand, it also promotes hepatocyte proliferation on the other hand and thus contributes to the restoration of liver mass after massive liver injury. We (29) have recently shown that JNK is a crucial downstream mediator of TNF in liver regeneration after partial hepatectomy and that blocking JNK inhibits hepatocyte proliferation and liver regeneration. In contrast, hepatocytes do not require activation of NF-κB pathway for proliferation (5). However, NF-κB indirectly regulates hepatocyte proliferation by controlling the transcription of mediators in Kupffer cells that drive hepatocyte proliferation. This differential role of IKK/NF-κB in hepatocytes and Kupffer cells was recently demonstrated in the diethylnitrosamine model of hepatocarcinogenesis: a hepatocyte-specific deletion of IKK-β increased ROS production, JNK activation, and hepatocyte death (24). This increase in hepatocyte death stimulated Kupffer cells to release proinflammatory and proliferative mediators that enhance compensatory proliferation of surviving hepatocytes and hepatocarcinogenesis (24). The opposite results, i.e., decreased hepatocyte proliferation and hepatocarcinogenesis, were obtained when IKK-β was deleted in Kupffer cells because of the diminished secretion of factors that are crucial in driving hepatocyte proliferation (24). These results clearly show that IKK-β exerts specific functions in each hepatic cell population. In hepatocytes, IKK-β protects...
from TNF-induced apoptosis. In Kupffer cells, IKK-β induces the transcription of proinflammatory and proliferative mediators. These results are not only interesting in the clinical context of hepatocarcinogenesis but also for many other hepatic diseases that involve hepatocyte apoptosis and Kupffer cell activation, such as hepatitis and fibrosis. Future therapies may specifically target IKK-β in Kupffer cells to prevent hepatocellular damage and increased proliferation in precancerous lesions (see Fig. 4). However, it remains to be clarified whether complete IKK-β inhibition in Kupffer cells or JNK blockade in hepatocytes may have deleterious effects in an already injured liver by blocking compensatory hepatocyte proliferation and liver regeneration.

**SUMMARY**

In recent years, it has become evident not only that the IKK, JNK, and ROS pathways are crucial in the regulation of TNF-induced cell death, inflammation, and proliferation but that these signaling pathways are highly intertwined at several levels. The functions of IKK, JNK, and ROS in the liver have been extensively characterized, and their importance in a number of hepatic diseases has been clearly demonstrated by using specific inhibitors or knockout mouse models: the production of ROS is responsible for the prolonged activation of JNK, which in turn is associated with hepatic apoptosis. Inhibition of ROS production and JNK activation has proven to efficacious in liver injury after ConA administration and hepatic I/R. IKK-β protects hepatocytes from TNF-induced cell death and regulates the transcription of proinflammatory and proliferative mediators in Kupffer cells. Inhibition of IKK-β in Kupffer cells, but not hepatocytes, reduces hepatocarcinogenesis. Moreover, inhibition of IKK-β has been shown to prevent inflammation and cell death after hepatic I/R. These recent advances in basic science provide a basis to evaluate the potential therapeutic use of specific IKK and JNK inhibitors and antioxidants for the treatment of fulminant hepatitis, hepatic I/R injury, and hepatocellular carcinoma in clinical settings.

**REFERENCES**


