Mechanisms of Hepatic Toxicity
I. TNF-induced liver injury*

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TUMOR NECROSIS FACTOR-α (TNF-α) is a cytokine produced mainly by activated macrophages and in smaller amounts by several other cell types. TNF-α exerts a variety of effects on different cell types and is implicated as an important mediator in various physiological and pathophysiological conditions. In addition, it has become clear that TNF-α is an important mediator of apoptosis (programmed cell death). To better understand the pleiotropic effects mediated by TNF-α, much effort has been made to characterize the intracellular pathways by which it impacts various biological processes such as cell proliferation, inflammation, and apoptosis.

TNF LIGAND AND RECEPTOR FAMILIES

TNF-α belongs to a family of nine ligands (TNF-α, lymphotoxin-α, TNF-β, Fas ligand, OX40L, CD40L, CD27L, CD30L, 4-1BB, and lymphotoxin-β) that activate structurally related receptor proteins known as the TNF receptor superfamily. Twelve transmembrane proteins consisting of two identical subunits have been identified as members of this family: TNF receptor 1 (TNF-R1, p55), TNF-R2 (p75), TNF-RP, Fas, OX-40, 4–1BB, CD40, CD30, CD27, pox virus PV-T2, PV-A53R gene products, and the p75 NGFR. TRID is a somewhat similar situation may exist for TNF-R1 and TNF-R2, in that soluble forms of the receptor are generated by proteolytic cleavage of the extracellular domain. The physiological role of soluble TNF receptors is not completely understood. However, some reports indicate that the interaction between TNF-α and the soluble receptors increases the half-life of TNF-α in the serum. Additionally, the soluble receptors block the interaction of TNF-α with the transmembrane receptors and thus act as antagonists of TNF-α (32).

INTRACELLULAR PATHWAYS ACTIVATED THROUGH TNF RECEPTORS

The intracellular regions of TNF-R1 and TNF-R2 lack catalytic kinase domains. Instead, receptor-associated proteins function as the transducers in TNF receptor-dependent signaling. The death domain is a conserved protein-protein interaction motif of ~80 amino acids that was first identified in the COOH-terminal regions of TNF-R1 and the Fas receptor. A number of death domain-containing receptor proteins have been subsequently identified, including reaper, RIP, DR3, DR4, and DR5. After ligand binding, the...
death domain of the Fas-associated death domain protein (FADD) interacts directly with Fas, while FADD binds TNF-R1 via the adapter protein TNF-R1-associated death domain protein (TRADD). FADD also contains a death effector domain, which mediates its interaction with caspase 8/FLICE/MACH, thus linking both receptors to the caspase cascade activated during apoptosis. A dominant-negative mutant of FADD that is unable to bind caspase 8 blocks both TNF-α- and Fas-induced apoptosis, indicating the critical role of this interaction for apoptosis signaling from either receptor protein (see Fig. 1) (4).

In addition to interacting with FADD, both TNF receptors bind TNF receptor-associated factors 1 and 2 (TRAF1 and TRAF2), which activate downstream signaling proteins, including the mitogen-activated protein kinase family member c-J un NH2-terminal kinase (JNK; also known as stress-activated protein kinase) and the transcription factor nuclear factor-κB (NF-κB). TRAF2 homodimers or TRAF1/TRAF2 heterodimers directly bind to a 78-amino acid region at the COOH-terminal domain of TNF-R2. In contrast, the NH2-terminal region of TRADD binds TRAF2, mediating the interaction between TRAF2 and TNF-R1 (10). The NH2-terminal ring finger domain of TRAF2 mediates the downstream signals that result in NF-κB activation. In particular, overexpression of a truncated form of TRAF2 lacking the NH2-terminal ring finger domain (TRAF2Δ) acts as a dominant-negative TRAF2 molecule, blocking NF-κB activation from either TNF receptor (10). However, recent studies (35) with TRAF2 (-/-) mice show that activation of NF-κB by TNF-α is only mildly impaired in these animals, indicating that NF-κB activation occurs via both TRAF2-dependent and -independent pathways, perhaps involving other TRAF family members or RIP. TRAF-interacting protein inhibits the activation of NF-κB by associating with the TNF-R2 complex through its interaction with TRAF2.

NF-κB is normally retained in the cytoplasm through interaction with its inhibitor IκB and is activated by a recently elucidated kinase cascade (13). The serine/threonine kinase NF-κB-inducing kinase directly binds to TRAF2 and in turn activates IκB kinase-α (IKK-α).

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**Fig. 1.** The 2 tumor necrosis factor receptors (TNF-R1 and TNF-R2) are depicted along with the intracellular pathways and the adaptor molecules involved in the different signaling cascades that diverge at the intracellular domain of TNF-R1 and TNF-R2. TRADD, TNF-R1-associated death domain protein. FADD, Fas-associated death domain protein. NF-κB, nuclear factor-κB. TRIP, TRAF-interacting protein. NIK, NF-κB-inducing kinase. IKK-α, IκB kinase-α. TRAF2, TNF-receptor-associated factor 2.
IKK-α phosphorylates IκBα at serines 32 and 36, resulting in ubiquitination and rapid degradation of IκB by the proteasome. The loss of IκB binding unmask nuclear localization signals on NF-κB, resulting in NF-κB translocation to the nucleus and transcriptional activation of its target genes. NF-κB activation results in cellular protection from apoptosis (33), since blocking NF-κB activation by chemical inhibitors or a dominant negative form of IκB significantly sensitizes cells to TNF-α-induced apoptosis. NF-κB (p65) (−/−) mice display massive hepatic apoptosis during development, resulting in embryological lethality; cell lines derived from these mice exhibit dramatically decreased viability after TNF-α treatment (5, 6).

TRAF2 also mediates the activation of the JNK pathway, as shown by JNK inhibition by TRAF2Δ and in TRAF2 (−/−) mice (19, 24). TNF-α-mediated induction of JNK involves activation of the small GTPase Rac1 and the signaling kinases MEKK and M KK4/ J NKK/SEK-1. JNK phosphorylates the activation domains of the transcription factors c-jun, ATF-2, and Elk-1. c-jun and ATF-2 heterodimers induce transcription of the c-jun gene, whereas Elk-1 transactivates c-fos. c-jun and c-Fos are both members of the activator protein-1 (AP-1) family of bZIP transcription factors. The role of JNK activation in apoptosis signaling is controversial. It has been reported that JNK activity is required for the induction of Fas- and ceramide-mediated apoptosis in fibroblasts and leukemia cells, as well as in growth factor-deprived sympathetic neurons. However, other results demonstrate that JNK activity is not required for TNF-α-mediated apoptosis in 293, HeLa, and MCF7 cells or Fas-mediated apoptosis in Jurkat cells (18, 19). Interestingly, lymphocytes derived from TRAF2 (−/−) mice were sensitized to TNF-α-mediated apoptosis despite intact activation of NF-κB, suggesting that JNK activity may play a protective role in this context (35).

**ROLE OF TNF-α IN LIVER REGENERATION**

TNF-α plays a key role in the proliferative response of the regenerating liver. After partial hepatectomy, the remaining hepatocytes promptly leave G0 and enter the proliferative stages of the cell cycle. Proliferation continues until the liver is restored. The interaction between mitogens and cytokines in this in vivo model of cellular proliferation is under intense investigation. The hepatic levels of TNF-α are rapidly increased after partial hepatectomy. It has been found that bilary epithelial cells and venous endothelial cells (20), not Kupffer cells, the resident macrophages in the liver, are the source of TNF-α, based on in situ PCR and the failure of GdCl3, a Kupffer cell toxin, to block TNF-α induction and hepatocyte proliferation after partial hepatectomy. The first evidence for a critical role for TNF-α in liver regeneration was a demonstration that hepatocyte DNA synthesis is inhibited after partial hepatectomy in rats pretreated with a neutralizing TNF-α antibody (1). In these same animals, the induction of JNK and AP-1 was inhibited, suggesting that TNF-α, JNK, and AP-1 may contribute to hepatic proliferation (6). In primary cultures of adult rat hepatocytes, incubation with TNF-α promotes the proliferative actions of hepatocyte mitogens, supporting the in vivo evidence that TNF-α potentiates hepatocyte proliferation (6).

The requirement for TNF-α as well as the cytokine interleukin-6 (IL-6) in normal hepatic regeneration after partial hepatectomy was further established by the use of IL-6 and TNF-R1 null mice. Partial hepatectomy in the IL-6 (−/−) mice resulted in liver necrosis and failure, with G1 cell cycle phase abnormalities, including decreased activation of AP-1 and the absence of activation of Stat3, a transcription factor induced by IL-6. Partial hepatectomy in TNF-R1 (−/−) mice resulted in decreased DNA synthesis, delayed restoration of liver mass, and increased mortality, with an absence of Stat3 and NF-κB activation (34). In both knockout mice, normal hepatic regeneration was restored by the administration of exogenous IL-6. These experiments suggest that TNF-α binds to the TNF-R1 of Kupffer cells or other nonparenchymal cells, inducing secretion of IL-6. The IL-6 then binds to its receptor on hepatocytes, leading to the activation of Stat3 (see Fig. 2).

To assess the role of NF-κB induction in liver regeneration after partial hepatectomy, NF-κB was specifically and selectively blocked by adenoviral-mediated delivery of an IκB superrepressor that is resistant to phosphorylation and degradation and thus inhibits NF-κB activation. Blocking NF-κB increased apoptosis and decreased the mitotic index after partial hepatectomy, resulting in liver failure (12). Thus the induction of NF-κB by TNF-α during liver regeneration appears to be required both to prevent apoptosis and to allow normal cell cycle progression (Fig. 2).

**ROLE OF TNF-α IN MODELS OF ACUTE LIVER INJURY**

The role of TNF-α in liver injury has been studied in several animal models. By using neutralizing anti-TNF-α antibodies or knockout mice for TNF-α, TNF-R1, or TNF-R2, it has become evident that TNF-α triggers apoptosis and/or necrosis of hepatocytes in vivo. In different animal models of liver injury, TNF-α plays a central or an additive role in the pathogenesis of acute liver injury. Here, we review the endotoxin/GalN model, the bacterial cell wall component lipopolysaccharide (LPS) is used to initiate the inflammatory response. Because rodents are less sensitive to LPS exposure than humans, LPS is combined with the amino sugar GalN to sensitize the animals. GalN is metabolized in the liver and results in selective depletion of uridine nucleotides, which specifically inhibits the transcription of hepatocytes. The essential role of TNF-α in the LPS/GalN model has
been shown by comparison of wild-type C3H mice and the endotoxin-resistant strain C3H/FeJ. An increase in TNF-α levels in the wild-type C3H mice precedes liver failure, whereas in the resistant C3H/FeJ strain, the increase in TNF-α levels and the induction of liver injury are abolished. More specifically, TNF-R1 is required for the induction of liver damage (26).

During LPS/GalN-induced liver injury, TNF-α induces the transcription of several proinflammatory genes, including chemokines, nitric oxide synthase, and adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and P-selectin. These changes are essential to trigger the extravasation of neutrophils into the liver parenchyma, which produces cytotoxic liver cell damage. In this scenario, a stepwise cascade has been described that consists of three events: 1) sequestration of neutrophils in the liver vasculature, 2) transendothelial migration, and 3) adherence-dependent cytotoxicity against hepatocytes. The initial sequestration of neutrophils in the liver sinusoids seems to be a predominantly passive trapping process induced by a variety of proinflammatory mediators. However, the transendothelial migration process is controlled by adhesion molecules that are expressed on hepatocytes and nonparenchymal cells. The initial step in transmigration is mediated by VCAM-1, which is expressed on sinusoidal, endothelial, and Kupffer cells, but not hepatocytes. ICAM-1 seems to be involved at a later step, when it becomes expressed on hepatocytes. The β-integrins, such as very late activating antigen-4, are the adhesion molecules on neutrophils that interact with VCAM-1 and ICAM-1 during transmigration. During the third step, hepatocyte apoptosis occurs initially, followed by hepatocyte necrosis. Interestingly, hepatocyte apoptosis is required for transmigration of neutrophils and subsequent necrosis (14). Thus early apoptosis of hepatocytes in the LPS/GalN model amplifies the extent of necrotic liver injury.

GalN/TNF-α MODEL

The administration of GalN and TNF-α triggers apoptosis of hepatocytes in vivo and in vitro. TNF-R1 knockout mice are resistant to TNF-α/GalN treatment, demonstrating the essential role of TNF-R1 in this apoptosis model (17). This suggests that the transcriptional block induced by GalN directly inhibits synthesis of antiapoptotic proteins induced by the TRAF2 pathway. In these mice, pretreatment with either IL-1 or nitric oxide prevents TNF-α/GalN-mediated apoptosis in a transcription-dependent manner (3). IL-1 via TRAF6 may thus activate protective genes, parallel to TNF-α via TRAF2/NF-κB. Interestingly, TNF-R2 knockout mice are more susceptible than wild-type mice to TNF-α/GalN treatment. Because transcriptional events cannot explain this observation, other mechanisms have to be invoked (G. Tiegs, personal communication). One explanation might be that in the absence of TNF-R2 more TNF-α is available to bind TNF-R1 and induce apoptosis. An alternative explanation could be the activation of additional protective pathways that do not require de novo gene transcription.

ConA MODEL

ConA is a lectin with high affinity for the hepatic sinus (29). Accumulation of ConA in the hepatic sinus results in an increased influx of circulating lymphocytes into the hepatic sinus and subsequent local proliferation via blastoid formation. The assembly of immune-activated cells in the liver results in an increase of several cytokines that have an essential effect on the degree of liver damage in the ConA model (7, 22, 29). Selective immunosuppression by cyclosporin A or FK506 completely prevents liver injury after ConA injection, demonstrating the important role of T cell activation in this model (22).

Interestingly, the activated lymphocytes start to infiltrate the liver tissue 8 h after ConA injection, after liver
damage has already begun. In contrast, maximal levels of most cytokines were reached before infiltration of lymphocytes occurred, showing that the early increase in cytokine levels is pivotal in triggering liver cell damage (22, 31). TNF-α and IFN-γ have been shown to directly contribute to liver cell damage, since anti-TNF-α and anti-IFN-γ antibodies protect against liver cell damage in this model (7). Additionally, it has been demonstrated that IFN-γ and TNF-α (-/-) mice are protected from ConA-induced liver cell damage, further supporting the role of these two cytokines for pathogenesis in the ConA model (15, 28). IL-6 and IL-10 inhibit liver cell damage by reducing the serum levels of IFN-γ and TNF-α, demonstrating the protective effect of certain cytokines in the ConA model (22).

Until now, a stepwise process of liver damage similar to the endotoxin/LPS model had not been defined for the ConA model. Adhesion molecules such as ICAM-1 or VCAM-1 seem to play a minor role, since mice pretreated with antibodies against either adhesion molecule as well as ICAM-1 knockout mice still undergo liver cell injury (Plümpe and Trautwein, unpublished results; Tiegs, unpublished results). However, ICAM-1 expression is clearly upregulated on hepatocytes, which correlates with the strong and immediate activation of NF-κB after ConA injection (Ref. 30; J. Plümpe, B. Fregien, and C. Trautwein, unpublished results). It seems possible that other NF-κB-dependent target genes might trigger mechanisms that contribute to liver injury in this model.

TNF-α IN HUMAN LIVER DISEASES

TNF-α was originally identified by its capacity to induce hemorrhagic tumors in mice. Attempts to use TNF-α for systemic anti-cancer chemotherapy have failed due to the appearance of severe side effects before therapeutic doses could be reached. One of the side effects of TNF-α treatment was an elevation in serum levels of transaminases and bilirubin levels, indicating a direct cytotoxic role of TNF-α in human hepatocytes. Subsequent studies have shown TNF-α may be involved in viral hepatitis, alcoholic liver disease, and fulminant hepatic failure. TNF-α serum levels are clearly elevated in patients with fulminant hepatitis (23). In addition, it was found that serum TNF-α levels were significantly higher in patients who died than in patients who survived (2). Studies on the expression of TNF-R1 and TNF-R2 during fulminant hepatic failure are still lacking.

A role for TNF-α in the pathogenesis of chronic hepatitis B and C viral infection has been suggested. Both viruses induce TNF-α expression in human liver and human hepatoma cell lines (8). Patients with chronic hepatitis B have elevated plasma TNF-α levels, and their peripheral blood mononuclear cells show enhanced TNF-α production in vitro. In addition, chronic hepatitis B-infected patients undergoing interferon-α treatment, a massive increase in spontaneous TNF-α production by blood mononuclear cells was observed at the time of successful antigen seroconversion (5), suggesting that the increased TNF-α levels may be involved in hepatitis B virus clearance. Furthermore, the serum levels of soluble TNF-R1 and TNF-R2 are significantly elevated in chronic hepatitis B infection. The serum levels of soluble TNF-R2 correlate closely with the extent of inflammation and hepatocyte death in the liver. During interferon therapy, the response and the increase in transaminases are associated with an increase in soluble TNF-R2 serum levels. For hepatitis C patients, interferon treatment clears the virus and reduces TNF-α levels to normal in responsive patients (16). Interestingly, pretreatment levels of TNF-α were higher in unresponsive compared with responsive patients (16). Hepatitis C proteins interact with the TNF receptor, although whether this interaction promotes or prevents apoptosis is not clear (27).

TNF-α serum levels are increased in patients with alcoholic hepatitis, and the levels correlate inversely with patient survival. TNF-α concentrations were significantly higher in patients who did not survive an episode of acute alcoholic hepatitis (2). Monocytes isolated from patients with alcoholic hepatitis spontaneously produced higher amounts of TNF-α compared with healthy controls. Monocytes derived from patients with alcoholic hepatitis also produced significantly more TNF-α in response to LPS than normal monocytes.

Several hypotheses have been developed to explain increased TNF-α levels in patients with chronic ethanol exposure. Chronic ethanol feeding increases the permeability of the gut to bacterial products such as LPS, potentially inducing TNF-α production in macrophages (21). In addition, recent studies investigated the promoter polymorphism in patients with alcoholic steatohepatitis. These experiments indicated that patients with alcoholic steatohepatitis had a mutation in the TNF-α promoter that increases its activity (9). Thus genetic factors may be involved in the increased TNF-α production in patients with alcoholic hepatitis.

REFERENCES


