Recent Advances in Alcoholic Liver Disease

IV. Dysregulated cytokine metabolism in alcoholic liver disease

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Submitted 27 April 2004; accepted in final form 11 May 2004

ALCOHOLIC LIVER DISEASE (ALD) remains a leading cause of death from liver disease in the United States. The exact number of patients in the United States with some form of ALD is unclear and probably underestimated, but it likely exceeds 2 million persons. In studies from the Veterans Administration, patients with cirrhosis and superimposed alcoholic hepatitis (AH) had 65% mortality over a 4-yr period, with most of those deaths occurring in the first few months (2). Thus the prognosis for this disease is more ominous than for many common types of cancer such as breast, prostate, and colon. Defining mechanisms for liver cell death in ALD will provide new insights into the development of this process and should provide potential targets for therapeutic intervention. New therapies are sorely needed for ALD because there currently is no FDA-approved treatment.

The goal of this concise article is to review the critical role of dysregulated cytokine metabolism as one such mechanism in the development of ALD. The cytokine of major focus in this article and of major focus in our laboratory is TNF-α. Cytokines are low molecular weight mediators of cellular communication produced by multiple cell types in the liver. Kupffer cells are prominent producers of proinflammatory cytokines, such as IL-1 and TNF-α, as well as certain anti-inflammatory cytokines, such as IL-10. Hepatocytes are increasingly a recognized source of cytokine production and not just a target of cytokine toxicity in liver injury. Hepatocytes are important producers of chemokines such as IL-8, which regulates the inflammatory response. Sinusoidal endothelial cells express adhesion molecules that modulate white blood cell adhesion and transmigration. Stellate cells are activated and produce collagen in response to the profibrotic cytokine transforming growth factor-β (TGF-β). This article will review the role of cytokines in liver injury, hepatic fibrosis, liver regeneration, and therapies that modulate cytokine metabolism.

DYSREGULATED CYTOKINE METABOLISM IN ALD

Overview. Abnormal cytokine metabolism is a major feature of ALD. We were the first to report dysregulated TNF-α metabolism in AH with the observation that cultured monocytes, which produce the overwhelming majority of systemic circulating TNF-α and are a surrogate marker for Kupffer cells, from AH patients spontaneously produced TNF-α and produced significantly more TNF-α in response to an endotoxin (LPS) stimulus (18). Increased serum TNF-α concentrations in AH were then reported by several groups, and values correlated with disease severity and mortality (17). A TNF-α promoter polymorphism was linked with susceptibility to alcoholic steatohepatitis, suggesting that a subset of people who drink alcohol may be genetically predisposed to the development of ALD (8). Elevated serum concentrations of TNF-α-inducible cytokines/chemokines, such as IL-6, IL-8, and IL-18, were reported in patients with AH and/or cirrhosis, and levels correlated with markers of the acute-phase response, liver function, and clinical outcome (reviewed in Ref. 17).

Concomitant with these human studies, complementary studies in rats, mice, and tissue culture evaluated the role of cytokines (especially TNF-α) in experimental models of liver disease (reviewed in Ref. 17). More than a decade ago, it was shown that rats chronically fed alcohol were more sensitive to hepatotoxic effects of injected LPS. Subsequently, we (11) showed that rats chronically fed alcohol had much higher LPS-stimulated plasma levels of TNF-α than control rats, and liver injury could be attenuated by giving a prostaglandin analog that downregulated TNF-α production. Studies (14, 19) using the intragastric alcohol-feeding model demonstrated that the development of liver injury coincided with an increase in TNF-α mRNA in the liver and in isolated Kupffer cells. These rats were fed ethanol intragastrically, had high blood LPS levels, and increased expression of cytochrome P450 2E1, which could cause generation of reactive oxygen intermediates (ROI). Indeed, markers for oxidative stress and lipid peroxidation were noted in these animals.

Not only are there increased levels of proinflammatory cytotoxic cytokines in ALD, but there is diminished monocyte and Kupffer cell production of protective anti-inflammatory cytokines such as IL-10. The importance of this observation in humans with ALD has been extended by using IL-10 knockout mice.

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mice that are much more susceptible to ethanol hepatotoxicity and exhibit increased levels of proinflammatory cytokines such as TNF-α. IL-10 not only plays an important physiological role in modulating TNF-α, but it also has antiinflammatory effects. Thus a deficiency in this cytokine could play an etiologic role in the development/progression of ALD (reviewed in Ref. 17).

Several strategies were devised to decrease cytokine production/activity in an attempt to block or attenuate liver injury. Examples include giving probiotics or antibiotics to modulate gut flora and LPS, gadolinium chloride to destroy Kupffer cells, and antioxidants, such as the glutathione prodrug Pro-cysteine, to inhibit cytokine production. All of these strategies were successful in attenuating alcohol-induced liver injury in rats (reviewed in Ref. 17). Perhaps the most compelling data relating TNF-α to alcohol-induced liver injury came from the late Ron Thurman’s laboratory (12), which successfully used anti-TNF-α antibody to prevent liver injury in alcohol-fed rats. Similarly, mice lacking the TNF-α type I receptor also did not develop alcoholic liver injury (24).

Two major stimuli for cytokine production in ALD are thought to be ROI and gut-derived LPS (17). In ALD, there is increased intestinal permeability and a high frequency of endotoxemia (reviewed in Ref. 17). LPS activates the redox-sensitive transcription factor, NF-κB, in Kupffer cells, resulting in the production of certain cytokines such as TNF-α (Fig. 1) (17). TNF-α can increase gut permeability, induce oxidative stress, and perpetuate this cycle (reviewed in Ref. 9). Generation of ROI through the metabolism of alcohol also can activate NF-κB (21). Recently, other unique mechanisms of relevance to ALD have been shown to play a role in stimulation of cytokine production. Elevated apoptosis is increasingly recognized as an important mechanism for cell death in ALD. Studies from Gores’ laboratory (3) have shown that as hepatocytes die of apoptosis, they can be taken up by Kupffer cells and stimulate TNF-α production. It was recently reported (13) that when hepatocytes die of proteasome inhibition-mediated apoptosis, the dying hepatocytes release IL-8 and IL-18 to cause sustained inflammation (Fig. 2). Several recent studies document the role of impaired proteasome function in the liver injury and development of alcoholic hyaline in ALD. Thus in ALD, hepatocyte apoptosis can be a maladaptive inflammatory process, instead of the predicted “sterile” programmed death.

This dysregulation of cytokine metabolism in ALD is associated with an activated functional/phenotypic change in monocytes/macrophages/Kupffer cells. One of the most widely used markers of monocyte activation in humans is the serum or urinary neopterin level. Neopterin is a pyrazino-pyrimidine compound produced in large amounts by macrophages after stimulation with TNF-γ. Neopterin levels are consistently elevated in ALD. Increased levels of cell adhesion molecules, such as leukocyte function-associated antigen-3, are expressed on monocytes in patients with ALD and also reflect monocyte activation. Mechanisms involved in this activation include increased LPS binding protein, increased levels of CD14, enhanced signaling through Toll-like receptors, direct effects of alcohol, oxidative stress, and others. It is important to note

Fig. 1. Selected mechanism(s) for enhanced TNF-α production and “sensitization” of hepatocytes to TNF-α-induced apoptosis in alcoholic liver disease. Sam, S-adenosylmethionine; mito, mitochondria; ROI, reduced oxygen intermediates; TNF, TNF-α.

Fig. 2. HepG2 Cells were either untreated (UT), treated with TNF-α alone (T), treated with the proteasome inhibitor MG 132 alone (M), or treated with TNF-α plus MG 132. IL-8 levels were determined by ELISA. Proteasome inhibition by itself induced IL-8 production, and this was enhanced with a combination of proteasome inhibition plus stimulation by TNF-α.
that this activation takes place over a prolonged time period. Thus acute alcohol administration has a inhibitory effect on proinflammatory cytokine production. When experimental animals are given an acute dose of alcohol, they show evidence of depressed serum concentrations of TNF-α after LPS stimulation. Similarly, short-term incubation of monocytes with alcohol in vitro results in suppressed LPS-stimulated proinflammatory cytokine production. On the other hand, when animals are chronically fed alcohol for at least 4–6 wk, they have increased LPS stimulated TNF-α production (11). Similarly, when a monocyte cell line was incubated with varied concentrations of ethanol for 1–7 days, it produced variable amounts of TNF-α depending on the duration of exposure to alcohol. Acute alcohol exposure modestly inhibited TNF-α production. However, by day 6, ethanol treatment significantly upregulated TNF-α production in association with increased generation of ROI (25).

The increased TNF-α production could be attenuated by antioxidants such as N-acetylcysteine. Thus chronic alcohol exposure causes monocytes/Kupffer cells to become activated or primed to overexpress certain cytokines in response to a stimulus such as LPS.

TNF-α signaling. Because TNF-α appears to be a critical cytokine in the development of ALD and many of its systemic manifestations, it is important to understand the TNF-α signaling pathway (9). TNF-α signals through two distinct cell surface receptors (TNF-R1 and TNF-R2). Multiple experimental approaches have revealed that TNF-R1 initiates the majority of TNF-α biological activities (Fig. 3). The initial step in TNF-α signaling involves the binding of the TNF-R1 trimer to the extracellular domain of TNF-R1 and the release of the inhibitory protein, silencer of death domains, from TNF-R1’s intracellular domain (ICD). The resulting aggregated TNF-R1 ICD is recognized by the adaptor protein, TNF-receptor-associated death domain, which recruits additional adaptor proteins receptor-interacting protein (RIP), TNF-R2, and Fas-associated death domain (FADD). These latter proteins recruit key enzymes to TNF-R1 that are responsible for initiating signaling events. FADD also contains a death effector domain, which then recruits procaspase-8. Activation of procaspase-8 through self-cleavage leads to a series of downstream events, including the caspase cascade, resulting in activation of caspase-3, or the mitochondrial pathway in which Bid is truncated and activated by caspase-8 and activated Bid translocates to mitochondria and induces cytochrome c release. Cytochrome c activates Apaf-1, in the presence of dATP, which, in turn, activates procaspase-9. Activated caspase-9 can then cleave downstream effector caspases including caspase-3 (4). In addition, substantial evidence has demonstrated that induction of mitochondrial permeability transition (MPT) is required for TNF-α induced cytochrome c release and apoptosis in hepatocytes. TNF-α-induced opening of MPT pore results in reduced mitochondrial transmembrane potential and has been shown to constitute a critical event of the cell death process.

TNF-α also simulates JNK activation. JNK is part of the evolutionarily conserved MAPK family and represents one subgroup of MAPKs implicated in cell-death pathways activated primarily by cytokines and exposure to environmental stress (6, 16). Once activated, JNK proteins can move from the cytoplasm of the cell into the nucleus. A major target of the JNK signaling pathway is the activation of the activator protein-1 transcription factor that is mediated, in part, by the phosphorylation of c-Jun and related molecules. Numerous studies have implicated both proapoptotic and antiapoptotic functions for the JNK pathway. These inconsistencies are due to the fact that the effect of activation of the JNK pathway on apoptosis depends on cell type, the nature of the death stimulus, the duration of its activation and probably, most importantly, the activity of other signaling pathways (6, 16). Recent data suggest that sustained hepatocyte JNK activation leads to apoptosis/hepatic injury.

In addition to activating the death pathway, TNF-α also activates the NF-κB survival pathway. TNF-α-induced activation of NF-κB relies on phosphorylation-dependent ubiquitination and degradation of IκB proteins. NF-κB is normally retained in the cytoplasm through interaction with its inhibitor, IκB. IKK-α phosphorylates IκBα at Ser22 and Ser26, resulting in ubiquitination and rapid degradation of IκB by the proteosome. The loss of IκB binding unmasks 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 (22), because blocking NF-κB activation by chemical inhibitors or a dominant negative form of IκB significantly sensitized cells to TNF-α-induced apoptosis. NF-κB p65−/− mice display massive hepatic apoptosis during development, resulting in embryonic lethality. Cell lines derived from these mice exhibit dramatically decreased viability after TNF-α treatment.

An interesting feature of the TNF-α signaling network is the existence of extensive cross talk between apoptosis and survival signaling pathways that emanate from TNF-R1. An enhanced apoptosis signal or decreased NF-κB activity will result in cellular susceptibility to TNF-α-induced apoptosis, whereas enhanced activation of NF-κB protects against apoptosis.

Hepatocyte sensitization to TNF-α killing. Importantly, hepatocytes are normally resistant to the above-noted TNF-α-induced killing/apoptosis. Thus in ALD there is not only increased TNF-α production, but alcohol must somehow render hepatocytes to be susceptible to TNF-α killing. There are

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**Fig. 3. Simplified TNF-α signaling pathways.** FADD, Fas-associated death domain; DD, death domain; TNF-R1, TNF-α receptor 1.
several known mechanisms by which hepatocytes can be rendered “sensitive” to TNF-α killing (Fig. 1). HepG2 cells can be sensitized to TNF-α killing by inhibitors of RNA or protein synthesis (e.g., actinomycin D and cycloheximide) (15). Pretreatment of HepG2 cells with a low dose of TNF-α prevented subsequent TNF-α cell killing, even when cells were sensitized as previously described (10). This is a form of in vitro tolerance to TNF-α killing, and it probably represents a mechanism whereby the host attempts to protect itself against ongoing cell injury. Several laboratories (20, 23) independently demonstrated that alcohol sensitizes primary hepatocytes and HepG2 cells to TNF-α-mediated hepatotoxicity. Studies by Hoek’s laboratory (20) demonstrated that both primary hepatocytes from rats chronically fed alcohol and HepG2 cells, which express CYP2E1, have increased TNF-α cytotoxicity associated with MPT pore opening. Multiple elegant studies from Kaplowitz’s group (5) document that decreasing mitochondrial GSH in chronic alcohol-fed rats sensitizes liver to TNF-α hepatotoxicity. This group has shown that decreasing mitochondrial GSH in vitro sensitizes to TNF-α-induced hepatocytes killing (5). Moreover, it has been reported that slightly increasing the temperature of the incubation medium increases cytotoxicity (10). This may have direct relevance to patients with AH who very often have fever as one of their complications. We recently showed (21a) that enhancement of intracellular 5-adenosylhomocysteine (SAH), a metabolite of 5-adenosylmethionine (SAM), and a potent inhibitor of hepatic transmethylation reactions, sensitized hepatocytes to TNF-α killing. This has potential relevance to ALD in which elevated levels of homocysteine and SAH occur. Moreover, we (13) have shown that proteasome inhibition sensitizes to TNF-α hepatotoxicity. Multiple groups have documented impaired function of the 26S proteasome in animal models of ALD. Thus there are many ways of sensitizing hepatocytes to TNF-α killing that have direct relevance to ALD.

CYTOKINES (TGF-β) AND FIBROSIS

The discovery that cytokines, particularly TGF-β1, control liver fibrogenesis has opened an important area of research and potential therapeutic intervention. TGF-β1 is a critical factor promoting stellate cell transformation into fibroblasts, with subsequent shift of balance in the extracellular matrix degradation synthesis toward synthesis. The expression of this cytokine is upregulated in AH, both in laboratory animals and in patients with alcohol-induced liver fibrosis. Liver fibrosis is regarded as an imbalance between synthesis and degradation of the extracellular matrix components, thereby implicating the regulation of matrix metalloproteinases (MMP) in liver fibrogenesis. It is currently known that TNF-α upregulates the expression of MMP and, to a lesser extent, their natural inhibitors, tissue inhibitors of MMP (TIMP) in the stellate cells, whereas TGF-β1 upregulates only TIMPs. Thus inhibition of MMPs leads to a subsequent shift of the balance toward fibrosis. Of particular interest is the fact that TNF-α is classically recognized as a causal factor in the early stages of ALD, whereas TGF-β1 intervenes in the later stages. In agreement with this scenario, clinical data show that during early stages of ALD in humans, MMP activation is markedly increased. However, as the ALD progresses toward fibrosis, a decrease in the activity of these enzymes takes place. Besides TNF-α and TGF-β1, liver fibrogenesis is also controlled by other cytokines such as connective tissue growth factor, which is induced by TGF-β, IL-10, IL-6, and others.

CYTOKINES AND LIVER REGENERATION

Diehl’s laboratory (1) first suggested a role for cytokines in liver regeneration in ALD with the observation that anti-TNF-α antibody inhibited liver regeneration after partial hepatectomy. Alcohol consumption impairs liver regeneration, likely, in part, through alcohol effects on cytokine expression and secretion. Among cytokines implicated in the liver regenerative response are IL-6, TNF-α, cytokine growth factor (HGF), TGF-α, and EGF (7), and alcohol affects these cytokines’ expression and secretion. TNF-RI knockout mice have an impaired liver regeneration after partial hepatectomy. The role of TNF-α in liver regeneration has raised the issue of the dual role of this cytokine: as deleterious (e.g., in ALD) and as beneficial (e.g., during regeneration) for the liver. Thus excess TNF-α can produce hepatotoxicity in a sensitized hepatocyte, whereas a low “basal” amount of TNF-α supports liver regeneration.

Recently, it has been recognized that IL-6 plays a central role in liver regeneration, especially after partial hepatectomy. Classically, the role of IL-6 in liver regeneration has been viewed as inducing acute-phase protein response of the liver and, therefore, maintaining body homeostasis. However, an expanded role of IL-6 in liver protection/regeneration has recently evolved. Experiments in IL-6 knockout mice demonstrated that IL-6: 1) induces hepatocyte proliferation through a mechanism independent on HGF and EGF, 2) induces antiapoptotic genes, which explains its protective effect against Jo2-induced apoptosis of the hepatocyte, and 3) downregulates proapoptotic genes such as caspase-3. The last two effects place IL-6 in the category of antiapoptotic cytokines. Therefore, the role of IL-6 in liver regeneration should be regarded as threefold: inducing acute-phase proteins, hepatocyte proliferation, and antiapoptosis.

The antiregenerative effects of alcohol are associated with dysregulated signaling mechanisms, such as those involving NF-κB, JNK, STAT3, phosphatidylinositol 3-kinase/Akt-kinase, and others. IL-6, on the other hand, is known to activate STAT3. In IL-6 knockout mice, alcohol-induced apoptosis is much greater than in the wild-type mice, and antiapoptotic proteins, such as Bcl-2 and Bcl-XL, are suppressed. In view of these and other experimental data, the increased levels of IL-6 in the circulation of ALD patients should probably be regarded as a defense mechanism against liver injury, rather than as an injurious factor.

THERAPY FOR ALD: CYTOKINE MODULATION

Despite the serious nature of this disease process, there is no FDA-approved therapy for any aspect of ALD. It is universally accepted that patients with ALD should stop drinking. They also should stop smoking and lose weight, if obese. Both smoking and obesity are associated with oxidative stress and may accelerate progression of ALD via oxidative stress (possibly through cytokine production).

Most patients with advanced ALD have malnutrition, and nutritional support is an important first-line therapy for patients with severe liver disease. Indeed, in a major randomized study
of enteral nutrition vs. steroids, patients with AH showed a similar initial outcome and fewer long-term infections in the group receiving enteral nutrition (2a). Thus traditional nutritional supplementation clearly improves nutritional status and, in some instances, hepatic function (and possibly morbidity and mortality in selected cases). Nutrition support may positively impact cytokine production, because enteral nutrition helps maintain gut integrity and decreases bacterial translocation. Nutrition support may also enhance nutrient antioxidants and potentially decrease cytokine production through decreased oxidative stress.

Steroids (prednisone) have been the most extensively studied therapy for AH. Meta-analyses support the use of prednisone in patients with moderate to severe AH who do not have contraindications such as infections, gastrointestinal bleeding, etc. Corticosteroids represent the first form of drug therapy in ALD patients whose mechanism of action is inhibition of proinflammatory cytokine production. With the recognition of the role of cytokines and cytokine receptors in a large number of diseases, multiple companies have developed agents aiming to: 1) neutralizing the circulating cytokines (using antibodies or soluble receptors), 2) neutralizing cytokine soluble or cell-bound receptors (using specific antibodies), 3) inhibiting cytokine synthesis (e.g., pentoxifylline and prostaglandin derivatives), 4) administering anti-inflammatory cytokines (e.g., IL-10), and 5) removal or neutralizing of inflammatory cytokine-producing cells. In patients with ALD, limited pilot studies using a TNF-α-soluble receptor and an anti-TNF-α antibody suggest that these compounds are generally safe and tolerable (18a). However, a multicenter study from France using both high-dose infliximab (anti-TNF-α antibody) and prednisone had to be discontinued due to increased infections (19a). Thus infection and impaired liver regeneration are two of the ongoing concerns with these types of drugs. The strategy employing inhibitors of cytokine synthesis or secretion, such as pentoxifylline, has generated promising results, especially in severe AH with early hepatorenal syndrome (2). The complementary and alternative medicine SAM may have efficacy in ALD via inhibition of TNF-α and stimulation of IL-10. In a large multicenter study from Spain, SAM was shown to improve survival and decreased need for liver transplantation in a group of patients with alcoholic cirrhosis (16a). The use of leukocyte apheresis with a good outcome has been reported in a case of severe acute AH (2). Administration of antibiotic agents are just beginning clinical trials. Cytokine/anticytokine therapy for ALD is in its infancy, but, on the basis of its success with other diseases, we predict rapid progress in this area.

GRANTS
This research was supported by the National Institute on Alcohol Abuse and Alcoholism Grants AA-010762 (to C. J. McClain), AA-014096 (to C. J. McClain), AA-013170 (to S. S. Barve), AA-000297 (to D. B. Hill), AA-01485 (to D. B. Hill), and AA-12314 (to I. Deacue); a Kentucky Science and Engineering Foundation Grant (to C. J. McClain); and the Department of Veterans Affairs Grant (to C. J. McClain).

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