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Apoptosis and Cytokines in Nonalcoholic Steatohepatitis

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is one of the commonest causes of chronic liver disease in the United States, and represents several overlapping clinicopathological states, ranging from simple steatosis to nonalcoholic steatohepatitis (NASH). Although dysregulated lipid accumulation occurs across the spectrum of NAFLD, features of liver cell injury such as hepatocyte ballooning, cytoskeletal changes (Mallory-Denk bodies) and hepatocyte apoptosis occur predominantly in NASH, and distinguish NASH from simple steatosis. Indeed, NASH is a more serious form of liver damage, as cirrhosis and / or hepatocellular carcinoma are potential outcomes of NASH, but rarely occurs in individuals with simple steatosis. Hepatic injury and apoptosis that occur in adults is often dysregulated and is accompanied by the accumulation of immune cells, which produce cytokines and growth factors that drives chronic inflammation and may result in fibrosis. The purpose of this review is to summarize the process of apoptosis and roles of putative cytokines in progressive NAFLD.

Keywords

apoptosis; cytokines; inflammation; fibrosis; nonalcoholic steatohepatitis

Introduction

Nonalcoholic fatty liver disease (NAFLD) is now the leading cause of chronic liver disease in the United States (1). It is closely associated with the metabolic syndrome, which is a constellation of insulin resistance, central obesity, hypertension and dyslipidemia (2). Histologically, NAFLD may range from simple steatosis to steatohepatitis and cirrhosis (3, 4). Individuals with simple steatosis rarely develop significant disease, whereas, nearly 20% of those with nonalcoholic steatohepatitis (NASH) progress to end-stage liver disease (5,6). Evidence that cirrhosis and hepatocellular carcinoma are more likely to develop in individuals

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with NASH rather than simple steatosis, suggests that NASH is a more serious form of liver injury (5,7,8).

The ‘two-hit’ hypothesis is a widely accepted paradigm to explain the progression of NAFLD, from simple steatosis (fatty liver) to NASH (8). The first hit involves dysregulated hepatic lipid accumulation (steatosis). Second hit(s) include oxidative, metabolic and *cytokine* stresses that overwhelm hepatocyte survival mechanisms, leading to hepatocyte cell death (*apoptosis*). Indeed, NASH differs from simple steatosis, mainly, in the degree of hepatocyte injury and apoptosis (9,10). We have previously proposed that hepatocyte apoptosis is the critical ‘third-hit’ that drives the progression from NASH to cirrhosis (11). Hepatocyte apoptosis triggers regenerative mechanisms to replace dead hepatocytes (12). However, aberrant responses may occur in some individuals, resulting in the activation of hepatic stellate cells (HSC) to myofibroblasts and the hepatic recruitment of pro-inflammatory, pro-fibrogenic immune cells.

In this review, we will discuss the role of apoptosis and impact of putative cytokines in the progression of NAFLD.

Apoptosis

Programmed cell death or apoptosis, is a vital component of normal cellular turnover, and development. It is an ATP-dependent process, characterized by cell shrinkage, chromatin condensation (pyknosis), membrane blebbing and budding (13,14). When appropriately regulated, the process of apoptosis and/or clearance of apoptotic bodies is limited to specific cells, and is not associated with an inflammatory reaction (15-17). In contrast, apoptosis occurring in adult tissues in response to noxious insults is typically dysregulated, prolonged (18), and inflammatory in nature. Adding to the insult, it may ultimately promote fibrosis (19-21).

Apoptosis is mediated by either the extrinsic (death receptor) pathway or intrinsic (mitochondrial) organelle-based pathway (22). Both pathways converge on a similar execution pathway, which is initiated by the cleavage of caspase-3 (14,23). Activation of caspases occurs through the cleavage of aspartate residues and requires caspase activity. This proteolytic cascade amplifies the apoptotic signaling pathway and leads to rapid cell death.

In the liver, apoptosis is typically triggered by ligation of surface death receptors (24), including Fas (CD95), tumor-necrosis factor (TNF) receptor 1, and tumor necrosis factor-related apoptosis-inducing ligand receptors 1 and 2 (TRAIL-R1 and -R2) (24,25). Expression of Fas/CD95 is enhanced in patients with viral hepatitis, alcoholic hepatitis, chronic biliary disease and acute liver failure (26). The binding of ligand to its cognate receptor results in the recruitment of cytoplasmic adaptor molecules, Fas-associated protein with death domain (FADD) and TNFRSF1A-associated via death domain (TRADD), and the subsequent activation of caspase-8 (27-29). Caspase-8, in turn, activates caspase-3, committing the cell to the final, common pathway of apoptosis (14). This pathway was demonstrated when mice that were administered anti-Fas antibodies went on to develop massive hepatocyte apoptosis and die from fulminant hepatic failure (30).

Apoptosis and Inflammation

The link between apoptosis and inflammation was demonstrated in skin and peritoneal experiments as mice injected subcutaneously with anti-Fas antibody developed a robust local inflammatory infiltrate (31), and inoculation of Fas-L expressing tumor cells into the murine peritoneal cavity resulted in an interleukin (IL) - 1 β -mediated neutrophilic infiltration (32).

Relevant to the liver, inflammation is the critical stage in the progression from steatosis to steatohepatitis (33). The number of inflammatory cells is minimal in simple steatosis, but is significantly up-regulated in individuals with steatohepatitis (34,35). This increase in inflammatory infiltrate is mirrored by the degree and extent of hepatocyte apoptosis (9,36). Supporting this, recent studies have shown that hepatocyte apoptosis may directly or indirectly promote inflammation (37-40). Infection with *Listeria monocytogenes* triggered hepatocyte apoptosis and release of neutrophil chemoattractants (41). Subsequent work demonstrated that MIP2 and IL8 regulate hepatic neutrophil infiltration (42). The use of cathepsin B knock-out mice and pharmacological inhibitors by Canbay et al. demonstrated that apoptosis induced by bile-duct ligation is associated with the production of pro-inflammatory chemokines, CXCL1 and MIP2 (43). Similar observations were noted with experiments using Fas-L agonists (39, 44). The inflammatory infiltrate was composed predominantly of neutrophils; immune recruitment was mediated largely by CXCL1. When investigators inhibited apoptosis using the caspase inhibitor, zDEVD-fmk, they noted a corresponding reduction in CXCL1 and MIP2 production, as well as in the severity of hepatic inflammation.

Ligation of TNF-R1/CD120a triggers nuclear factor κ B (NF- κ B) activation, up-regulation of pro-inflammatory cytokines and adhesion molecules (25). In the galactosamine/endotoxin shock model, TNF- α mediated, caspase-3 activation, triggered parenchymal cell apoptosis and neutrophil transmigration (38,45), while supplementation with the caspase-inhibitor abrogated cellular apoptosis, neutrophil transmigration and neutrophil-related injury. These studies lend support to the concept that cellular apoptosis is a signal for inflammatory cell recruitment (38).

Tissue inflammation may similarly ensue during the clearance of apoptotic bodies (15). Apoptotic bodies are typically rapidly phagocytosed by neighboring cells with the process being non-inflammatory, a hallmark of physiological apoptosis (16). In contrast, the engulfment of apoptotic bodies by monocytes or kupffer cells (liver resident macrophages) during liver injury is associated with the up-regulation of the death ligands, CD95, TRAIL and TNF- α (46-48). Complementing this, the depletion of kupffer cells by gadolinium-chloride is associated with impaired apoptotic body engulfment, reduced expression of the chemoattractant, MIP2 and amelioration of liver injury (49).

Experimental evidence for this 'apoptosis – inflammation' axis is supported by findings of hepatocyte apoptosis in patients with alcoholic steatohepatitis (50) and NASH (9,51,52). In both, hepatocyte apoptosis correlates strongly with clinical and histological disease severity. Additionally, the co-localization of apoptotic hepatocytes with polymorphonuclear cells suggests an apoptosis-dependent immune cell recruitment (53).

Apoptosis and Fibrosis

The nexus between apoptosis and fibrosis was initially explored by Canbay and co-workers (20). Mice treated with bile-duct ligation (BDL) to induce chronic liver injury and fibrosis expressed increased amounts of α -SMA, TGF- β , collagen α 1(I) and TIMP1, compared to sham-operated and Fas-deficient (lpr) mice (54). Phagocytosis of apoptotic bodies by macrophages stimulated the production of TGF- β , a key pro-fibrogenic cytokine (47,49), whereas treatment with gadolinium chloride to induce macrophage depletion reduced amounts of TGF- β , collagen α 1 (I) and α -SMA after BDL. In a similar fashion, engulfment of apoptotic bodies by primary or immortalized HSC was shown to trigger their own activation and promote fibrosis (48). Both processes could be inhibited pharmacologically with antagonists to PI3-K (LY294002) and p38 MAPK (SB203580). Phagocytosis of apoptotic bodies by HSC was confirmed by recent *in vivo* data, in 3 different models of liver fibrosis (19). The engulfment

of apoptotic bodies triggered NADPH-oxidase activation and superoxide production. In turn, these reactive oxygen species stimulated further apoptosis and enhanced fibrosis (55,56).

Further evidence for a link between apoptosis and fibrosis is derived from caspase inhibition studies in animals. The administration of the pan-caspase inhibitor, IDN-6556, to rodents subjected to BDL resulted in the attenuation of hepatocyte apoptosis, injury, inflammation and fibrosis (57). Similar observations were recorded in animals treated with an antagonist to Cathepsin B (R-3032) (43).

Apoptosis in NAFLD

Hepatic steatosis occurs as a result of abnormal lipid handling by the liver (58-61) which sensitizes the liver to injury and inflammation (8). Obese *ob/ob* mice harbor a homozygous mutation in the leptin gene, and are unable to synthesize leptin (62). They develop spontaneous hepatic steatosis, and when injected with anti-Fas antibody, exhibit massive liver injury (63). Similarly, mice fed the carbohydrate diet for 8 weeks develop macrovesicular steatosis and up-regulate expression of the death receptor, Fas (64). Treatment with Jo2 (anti-Fas antibody) enhanced hepatocyte apoptosis, hepatic injury, chemokine production (CXCL1 and MIP2), and infiltration of neutrophils. HepG2 cells cultured in the presence of free fatty acids also developed cellular steatosis, up-regulated Fas expression and were vulnerable to the Fas-L.

NASH, a more advanced lesion than simple steatosis, is characterized by increased hepatocyte injury and apoptosis (9). The same is true in alcoholic steatohepatitis (ASH) (50,52,65). Livers obtained from individuals with ASH and NASH show enhanced caspase-3 and -7 activation, as well as Fas and TNF-R1 expression. Using immunohistochemical approaches, Ribeiro and co-workers noted that individuals with NASH up-regulated NF- κ B expression, a transcription factor that promotes the expression of pro-inflammatory cytokines, death receptors and death ligands such as TNF- α (66,67). When compared to normal individuals, those with NASH had higher serum levels of TNF- α (68-70). However, studies using the TNFR1 knock-out mice indicated that TNF- α was not always critical for the development of NASH (71-73). Rather, other molecules signaling through the TNF-R superfamily could be involved. For example, livers from patients with excessive alcohol intake show greater induction of TRAIL. When exposed to free fatty acids, hepatocyte-derived cell lines up-regulate TRAIL-receptors (74).

Mice fed the methionine-choline deficient (MCD) diet are commonly used in the study of NASH as they exhibit histologic similarities to human disease (75-77). 8-weeks of MCD treatment result in increased hepatocyte apoptosis by TUNEL-staining and active-caspase-3 assays (Witek, RP et al. *Manuscript in submission*), with the onset of apoptosis commensurate to the development of steatohepatitis (75). In the latter study, the investigators noted a sustained up-regulation of hepatic p53 tumor suppressor gene. p53 activation was directly associated with Bcl-XL suppression, Bid cleavage, caspase-3 activation and p21 induction. Interestingly, p53 is also known to regulate TRAIL-R expression, and its expression is enhanced in patients with NASH (78) and in obese *ob/ob* mice (79).

Oxidative stress is one of the second hits believed to mediate the progression to NASH (8, 33). When the amount of ROS overwhelms buffering capacity, DNA mutations, peroxidation of membranes and generation of additional free radicals can occur (80). At low levels, ROS may activate NF- κ B to induce synthesis of pro-inflammatory cytokines and death receptor expression (81,82). In a recent study, rats fed the Lieber-DeCarli high-fat diet (71% of energy from fat) for 6 weeks expressed increased rates of hepatocyte apoptosis that mirrored necro-inflammatory changes and oxidative stress (83). The authors noted higher phosphorylated JNK and Bax (pro-apoptotic protein) compared to controls. JNK activation has been shown to regulate cellular apoptosis (83-85), possibly through the regulation of the Bcl-2 family. In addition, JNK1 has been shown to promote the development of murine NASH (77).

The identification of apoptosis as a critical mediator of inflammation and fibrosis in liver disease is important as it allows the design of future drug therapy and development of noninvasive biomarkers (Figure 1). In this respect, we observed a significant reduction in hepatic fibrosis when genetically obese, diabetic *db/db* mice were treated with a pan-caspase inhibitor (Witek, RP et al. *Manuscript in submission*), while Feldstein and colleagues measured serum cytokeratin-18 fragments (a caspase-3-cleavage product) in human subjects and demonstrated a strong correlation with histological severity (10).

Cytokines in Steatohepatitis

In the recent decades, investigators have defined the critical roles of pro-inflammatory cytokines in the pathogenesis of ASH(50,86). It was noted that patients with severe ASH exhibited high serum levels of TNF- α (87-89), which correlated with clinical severity. Similar cytokine changes were observed in animal models of alcoholic injury (90,91). Given that NASH and ASH share common histopathologic features, it is conceivable that similar immunopathogenic mechanisms may be involved in the development of NASH (86).

Tumor-necrosis factor (TNF) - α

TNF- α impairs insulin action *in vitro* and *in vivo* (92-95) and individuals with insulin resistance show higher serum levels of TNF- α . Administration of TNF- α to individuals also results in impaired insulin sensitivity (96). The mechanisms responsible for TNF- α effects appear to be related to the sustained activation of inflammatory kinases, such as Jun-N-terminal kinase (JNK) and inhibitor of K-kinase β (IKK β) (97). JNK activation inhibits the phosphorylation of insulin receptor substrate (IRS)-1 (98,99) while IKK β activity leads to the activation of NF- κ B and the induction of additional pro-inflammatory cytokines (100). Conversely, neutralization of TNF- α improved hepatic insulin resistance in *ob/ob* mice through reductions in JNK and IKK β activities (101,102). Similarly, probiotic therapy reduced injury and inflammation in *ob/ob* mice, likely via the down-regulation of JNK and IKK β . TNF- α also modulates the expression of sterol regulatory element binding proteins (SREBP), transcription factors involved in regulating enzymes of lipid synthesis (103). Levels of SREBP-1c are elevated in *ob/ob* mice (104). Exogenous TNF- α promotes the expression of SREBP-1c (105) while neutralizing antibodies to TNF- α decreases expression of SREBP-1c.

TNF- α expression is up-regulated in obesity (106) and serum TNF- α levels are increased in patients with NASH (68). Gene expression in adipose tissue and liver are similarly enhanced in NASH, and correlated with the stage of disease (107). More recently, TNF- α polymorphisms have also been noted in individuals with NAFLD compared to the control population (108, 109). Indeed, treatment with metformin and pentoxifylline, drugs which antagonize TNF- α , improve NASH (110,111). Similar changes in serum and tissue TNF- α levels are observed in animal models of obesity (112) and NASH (113). Moreover, mice genetically deficient in TNF-R1 are resistant to NASH by the MCD and high-carbohydrate diets (71,73). Specifically, TNF-R deficient mice exhibit reduced kupffer cell activation and fibrogenesis, suggesting a role of TNF- α in modulating HSC activation (102,114). More recent work by Yamaguchi et al, however, highlighted the possibility that TNF- α alone may be insufficient in the development of fibrosis, as treatment of obese and diabetic *db/db* mice with diacylglycerol acyltransferase 1 (DGAT1) antisense oligonucleotides resulted in worse fibrosis despite reductions in the amount of steatosis and TNF- α levels (115).

The effects of TNF- α may lie, in part, with its biological relationship with adiponectin, an adipose-tissue derived protein. *ob/ob* mice have low levels of adiponectin compared with TNF- α (116) and the injection of adiponectin to *ob/ob* mice reverses NASH and TNF- α levels. Similar changes were observed in KK-Ay mice, another model of NAFLD (117,118). Individuals with NASH have lower levels of plasma adiponectin compared with controls

(119,120). Importantly, circulating adiponectin levels may inversely correlate with hepatic inflammation (107,121), while, weight reduction has been shown to increase the ratio of adiponectin to TNF- α and improve NASH (122,123).

Leptin and Th1 / Th2 cytokines in NASH

Leptin is a highly conserved cytokine-like hormone secreted not only by the adipose tissue, but also activated T cells (124). Leptin binds to the leptin receptor (Ob-R) that stimulates the Janus-kinase signal transduction and activator of transcription (JAK-STAT) signaling pathways (125).

Leptin receptors are found on immune cells and leptin has been shown to modulate T cell responses and viability (126,127). Obese *ob/ob* mice are genetically deficient in leptin (128) and spontaneously develop features of the metabolic syndrome and hepatic steatosis. They also develop thymic atrophy and exhibit changes in neurohumoral factors (129) that lead to the selective reduction in hepatic NKT cells (130). Restoration of norepinephrine levels in *ob/ob* mice reduced NKT cell apoptosis and increased NKT cell numbers (131). NKT cells are critical modulators of the innate and adaptive immune response, and produce both pro-inflammatory (Th1) cytokine (IFN- γ) and anti-inflammatory, pro-fibrogenic (Th2) cytokines (IL4, IL13) (132). Livers from *ob/ob* mice show significant reductions in IL4 compared with IFN- γ (Th1 polarization) (130). This may explain their relative resistance to fibrosis despite persistent chronic liver injury. The pro-Th1 milieu would also account for their sensitivity to endotoxin-mediated (lipopolysaccharide) hepatotoxicity (113), one of the putative second hits in the progression of NAFLD. When *ob/ob* mice are corrected for leptin deficiency, they reduce weight, develop less hepatic inflammation but develop fibrosis (133-135), exhibiting features seen in individuals with progressive NASH. In addition to NKT cell numbers, restoration of leptin levels could promote fibrogenesis through increases in TGF- β secretion by macrophages (135,136). Similarly, *ob/ob* mice supplemented with norepinephrine develop less injury and lower amounts of pro-inflammatory cytokines, but express increased TGF- β expression, HSC activation and fibrosis (137). Collectively, the current data suggests that the balance of Th1 and Th2 cytokines in the microenvironment may determine disease outcome.

As hepatic NKT cells are a predominant source of Th2 cytokines, IL4 and IL13, depletion of NKT numbers would imply a dearth of pro-fibrogenic factors. NKT cells accumulate in chronic viral hepatitis (138-140), primary biliary cirrhosis (141,142) and Wilson's disease (143). Indeed, hepatic and circulating NKT cells from individuals with chronic viral hepatitis show enhanced IL4 and IL13 production (138). IL13 has been shown to activate hepatic stellate cells via IL13-Ra2 (144) and activate macrophages via the alternative pathway (145). In the TNBS model of chronic colitis, IL13 signaling has been found to initiate a cascade of pro-fibrogenic events that involve TGF- β activation and myofibroblast production of collagen (146); conversely, antagonism of IL13 signaling ameliorated murine schistosomiasis hepatic fibrosis (147). Recent work in our laboratory have shown that wild-type mice with intact leptin signaling possess greater number of NKT cells and exhibit greater fibrosis when treated with the MCD diet for 8 weeks, and α GalCer-activated NKT cells promote hepatic stellate cell activation in vitro (*unpublished*). Explanted livers from patients with NASH cirrhosis also contain up to 4-fold more NKT cells than normal human livers (*unpublished*). Further studies will be needed to determine if NKT-associated cytokines such as IL4 and IL13 regulate NASH progression. The identification of such cytokines could potentially provide novel targets for NASH therapy (Table 1).

Summary

NASH develops in a subgroup of individuals with NAFLD, and differs from simple steatosis with regard to the degree of hepatocyte injury and apoptosis. Hepatocyte apoptosis results in

the release of factors that promote the recruitment of inflammatory cells and trigger the deposition of type 1 collagen by hepatic myofibroblasts. Studies have shown that the degree of hepatocyte apoptosis may be assessed by serum measurements of cytokeratin-18 fragments (a caspase-3-cleavage product) in human subjects, and the use of caspase inhibitors may ameliorate the amount of fibrosis in vivo. NASH is also characterized by high levels of pro-inflammatory cytokines such as TNF- α , which promotes hepatic insulin resistance and drives the progression from simple steatosis to NASH. TNF- α may activate downstream kinases that induce further cytokine production in a feed-forward loop, while attenuating the expression and activity of adiponectin. In aggregate, the balance of Th1 (IFN γ) and Th2 (IL4, IL13) cytokines in the microenvironment may play a critical role in shaping disease outcomes.

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Figure 1. Hepatocyte Apoptosis during liver injury leads to Inflammation and Fibrosis
 During liver injury, ligation of surface death receptors by FasL, TNF α or TRAIL (extrinsic apoptosis pathway), promotes hepatocyte apoptosis, through recruitment of cytoplasmic adaptor molecules and activation of caspase-8. Caspases amplify the apoptotic signaling cascade, and commits the hepatocyte to the final execution (apoptosis) pathway (activated caspase-3). The organelle-based (intrinsic apoptosis pathway), non-receptor mediated apoptosis-pathway involves various stimuli (growth factors, cytokines, free radicals, hypoxia, viral infections and toxins) that disrupt the mitochondrial permeability transition pore, and loss of the mitochondrial transmembrane potential. These changes lead to the release of pro-apoptotic proteins, which initiate the mitochondrial-apoptosis pathway. Clearance of apoptotic

bodies occurs through phagocytosis by resident macrophages (Kupffer cells) and hepatic stellate cells. Engulfment of apoptotic bodies is associated with increased ROS production and activation of transcription factors, NF- κ B. In turn, this leads to enhanced production of pro-inflammatory cytokines (TNF α) and chemokines (MIP2, IL8). TNF α may further drive the extrinsic apoptosis pathway through a feed-forward, paracrine loop. Phagocytosis of apoptotic bodies also induces the production of the pro-fibrogenic cytokines, TGF β and IL13, via the PI3K and MAPK pathways.

Table 1**Summary of cytokines in NAFLD**

	Th1 Pro- inflammatory	Th2 Pro- Fibrogenic	Major source(s) of cytokines and other known function(s)
IL4	–	+	S: Th2 lymphocytes, NKT F: Promotes Th2 differentiation
IL10	–	– / +	S: Monocytes, Th2 lymphocytes, (Foxp3) Treg, NKT F: Down-regulates Th1 cytokine expression Suppresses antigen-presentation
IL13	–	+	S: Th2 lymphocytes, NKT F: Induces secretion of TGF- β Alternative macrophage activation Allergic inflammation, IgE secretion
IFN γ	+	–	S: Th1 lymphocytes, NK and NKT F: Promotes Th1 and suppresses Th2 differentiation Classical macrophage activation Anti-viral and Anti-tumor activity
TNF α	+	–	S: macrophage, lymphoid and other tissues F: inflammatory, apoptotic activity, cell survival, proliferation and differentiation via NF- κ B, MAPK and caspase activity
Leptin	+	+	S: adipose tissue, liver, brain, muscle, T cells F: regulate energy balance T cell survival and response (Th1 vs Th2 cytokines)
TGF β	–	+	S: (Foxp3) Treg, immune cells, stellate and epithelial cells F: apoptosis, cell cycle regulation, angiogenesis Suppress lymphocyte activation (immunosuppression)

+: pro; -: anti; S: source; F: function