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APOPTOSIS IN ALCOHOLIC AND NONALCOHOLIC STEATOHEPATITIS

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1. ABSTRACT

Alcohol liver disease (ALD) as well as nonalcoholic fatty liver disease (NAFLD) are two of the most common forms of chronic liver disease worldwide and may progress to cirrhosis and end stage liver disease. ALD and NAFLD seem to share many pathophysiologic mechanisms with the accumulation of lipids in the liver being the first step in the development of both conditions. While mitochondrial dysfunction and production of reactive oxygen species seem to play an important role in the progression from simple steatosis to steatohepatitis in both diseases, the pathogenesis of ALD and NAFLD as it relates to tissue injury remains poorly understood. Insights into these mechanisms are of significant clinical importance because current therapies for both conditions are limited and future therapies will be predicated by an understanding of their pathogenesis. In this review we focused on the current evidence for a central role of hepatocellular apoptosis, a specific form of cell death, in the pathogenesis of ALD and NAFLD as well as the current knowledge regarding the subcellular and molecular mechanisms involve in triggering hepatocyte apoptosis in these diseases.

2. INTRODUCTION

2.1. Alcohol liver disease (ALD) and nonalcoholic fatty liver disease (NAFLD)

Alcohol liver disease (ALD) as well as nonalcoholic fatty liver disease (NAFLD) are two of the most common forms of chronic liver disease worldwide (1; 2). Both conditions may progress to end stage liver disease (3; 4). ALD and NAFLD are indistinguishable by histopathology which may range from simple accumulation of fat in the liver (steatosis), to steatosis plus inflammation,

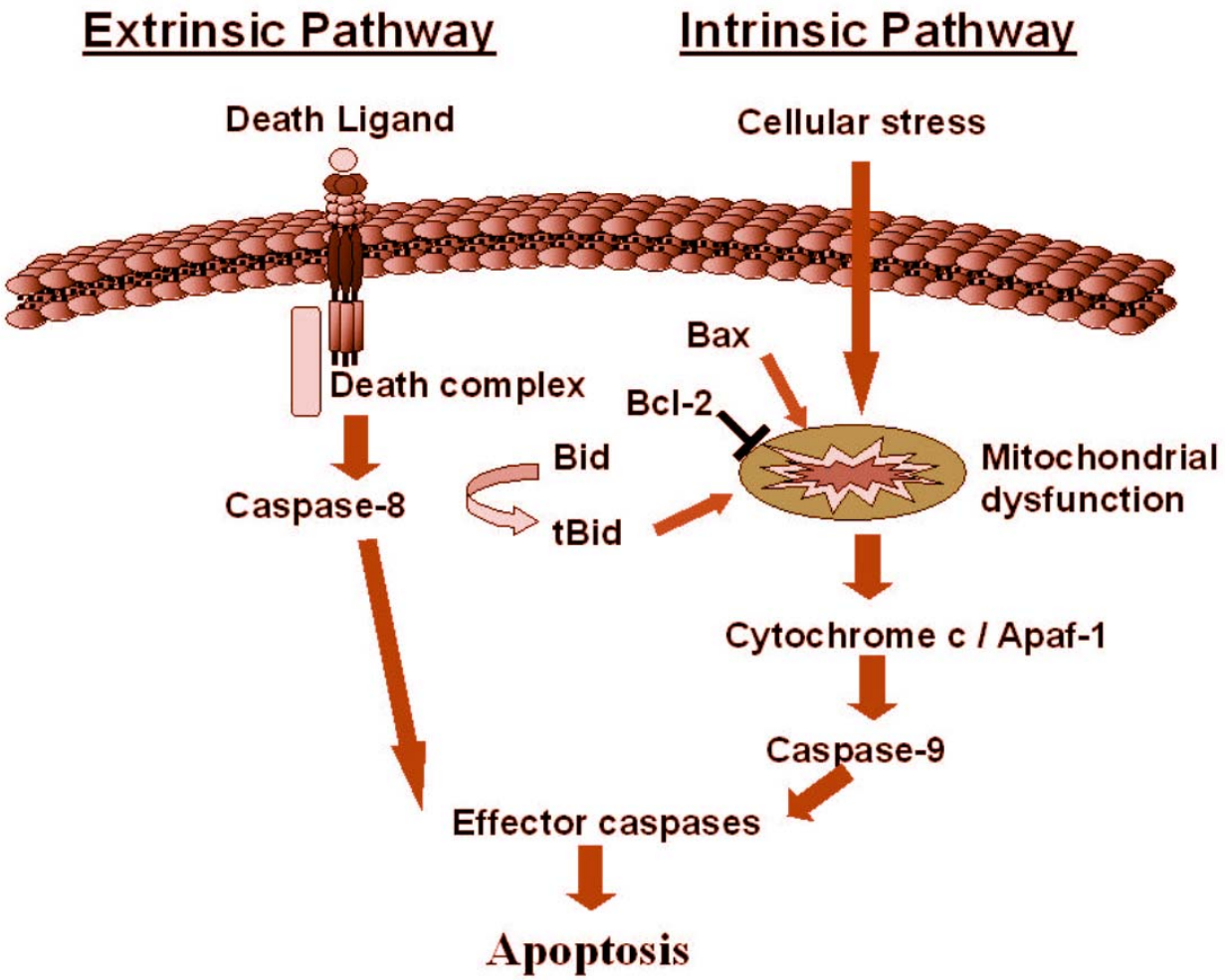

Figure 1. Apoptosis pathways. Two fundamental pathways may execute apoptosis. In the first one death ligands activate their receptors, resulting in the formation of a death complex, which then activates caspase-8. The second pathway, cellular stress results in mitochondrial dysfunction with release of cytochrome c into the cytoplasm, which then forms an activation complex with apoptotic-protein activation factor-1 (Apaf-1) and caspase 9, the apoptosome. This complex activates the downstream effector caspases 3, 6 and 7 which execute the final apoptotic changes. Cytochrome c release may be prevented by anti-apoptotic members of the Bcl-2 family and promoted by pro-apoptotic members of this family, such as Bax.

mallory bodies, ballooned hepatocytes, to advanced fibrosis and cirrhosis (5). ALD and NAFLD also seem to share many pathophysiologic mechanisms with the accumulation of lipids in the liver being the first step in the development of both conditions (6). While mitochondrial dysfunction and production of reactive oxygen species seem to play an important role in the progression from simple steatosis to steatohepatitis in both diseases (7; 8), the pathogenesis of ALD and NAFLD as it relates to tissue injury remains incompletely understood.

2.2. Mechanisms of apoptosis

Apoptosis is a form of cell death characterized by organized nuclear and cellular fragmentation. During apoptosis cells are fragmented into small membrane-bound apoptotic which are then removed by phagocytosis. Apoptosis may be executed by two fundamental pathways (9; 10) (Figure 1): one is mediated by death receptors on the cellular surface, the so call extrinsic pathway; and the other is organelle based, the so call intrinsic pathway. The extrinsic pathway is initiated by death receptors including CD95 (APO-1/Fas), TNF-R1 and TRAIL-Rs. When engaged by their natural ligands, these receptors trigger intracellular cascades that active death-inducing proteolytic enzymes, especially caspases. In the intrinsic pathway, apoptosis can be initiated by several intracellular organelles. Indeed, lysosomal permeabilization, endoplasmic reticulum stress, nuclear DNA damage, and mitochondrial dysfunction can all trigger apoptosis (11; 12; 13). In liver cells, mitochondrial dysfunction plays a critical role by amplifying the apoptotic signal and integrating both pathways into a final common one (14; 15). Mitochondrial dysfunction results in release of several pro-apoptotic proteins into the cytosol including cytochrome c, which then forms an activation complex with apoptotic-protein activation factor-1 (Apaf-1) and caspase 9, the apoptosome.

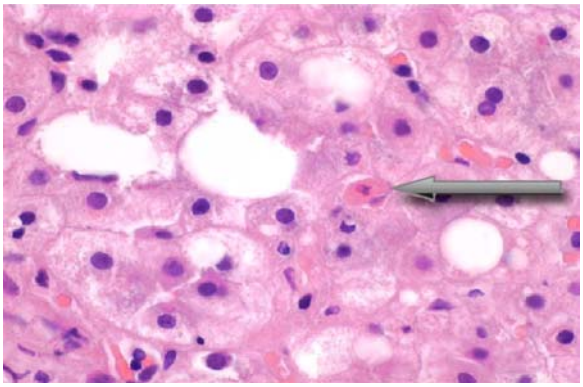

Figure 2. Example of hepatocyte apoptosis detected on an H&E stain liver specimen from a patient with NASH.

This complex activates the downstream effector caspases 3, 6 and 7 which execute the final apoptotic changes (11). The mitochondrial events are regulated by the Bcl-2 family of proteins which include anti-apoptotic (Bcl-2, Bcl-x_L) and pro-apoptotic (Bax, Bak) members (16).

2.3. Apoptosis and the liver

Hepatocellular apoptosis is emerging as an important, if not critical, mechanism contributing to the progression of human liver diseases (14; 17; 18; 19; 20; 21). The ensuing responses of cell repair, inflammation, regeneration, and fibrosis may all be triggered by apoptosis (14; 17). Of these processes, hepatic fibrosis has the potential to be the most deleterious, as progressive fibrosis may result in cirrhosis and its fear complications of portal hypertension, liver failure, hepatocellular carcinoma. Both experimental and human studies suggest a link between hepatocyte apoptosis and liver fibrogenesis (22). For instance, attenuation of hepatocyte apoptosis also reduces fibrogenesis in animal models of cholestasis (23; 24) . Engulfment of apoptotic bodies by hepatic stellate cells stimulates the fibrogenic activity of these cells and may be one mechanism by which hepatocyte apoptosis promotes fibrosis (25). In humans with chronic HCV infection as well as NAFLD the magnitude of hepatocyte apoptosis correlates with the severity of fibrosis (26; 27). Although, the presence of apoptotic bodies referred to as Councilman or pyknotic bodies has long been recognized in histopathologic specimens of patients with ALD and NAFLD (Figure 2), it was not until recent years that apoptosis was recognized as a potential significant mechanism contributing to inflammation and fibrosis . In this review we describe the current evidence for the role of apoptosis in the pathogenesis of ALD and NAFLD as well as the current knowledge regarding the subcellular and molecular mechanisms involve in triggering hepatocyte apoptosis in these diseases.

3. HEPATOCYTE APOPTOSIS IN ALD

Despite years of investigative efforts, the pathogenesis of ALD remains poorly understood (1; 3; 28). It is known that chronic alcohol consumption may lead to steatosis, which in a subgroup of patients may progress to steatohepatitis and cirrhosis (29; 30). While alterations in lipid metabolism including increase fatty acid synthesis and inhibition of mitochondrial β-oxidation secondary to an alter redox state results in fatty liver, the mechanisms responsible for disease progression are less clear. A large body of evidence suggest that hepatocyte apoptosis may be a key mechanism of alcohol-induced liver injury (31; 32; 33). Several studies have now demonstrated that hepatocyte apoptosis is a significant pathologic featured of human ALD. Using the TUNEL assay, two early studies reported the presence of hepatocyte apoptosis in liver biopsy specimens of ALD patients (34; 35). These observations were further confirmed and expanded in three subsequent studies. By using the TUNEL assay and immunohistochemical detection of caspase activation, Natori *et al* (36) demonstrated that in patients with ALD the magnitude of hepatocyte apoptosis correlated with the severity of disease as hepatocyte apoptosis was more pronounced in patients with high bilirubin and AST levels as well as patients with grade 4 steatohepatitis. Ziol *et al* (37) reported that the apoptotic index in liver specimens from ALD patients correlated with Maddrey's discriminant function, the presence of ascites and serum bilirubin. Finally, in the Ribeiro study (38), apoptotic cell death correlated with histological disease severity, including degree of inflammation and stage of fibrosis. Experimental studies have shown that alcohol exposure results in an increase in cell death by apoptosis both in-vivo and in-vitro. In animal models of ALD, apoptosis extending from acinar zone 3 into zone 2 has been demonstrated in both mice and rats following ethanol feeding (33; 39). In-vitro exposure of human and rat hepatocytes to ethanol results in a dose-dependent induction of apoptosis (40).

3.1. The intrinsic pathway of apoptosis in ALD

Several mechanisms have been postulated to play a role in alcohol induced hepatocyte apoptosis including oxidative stress induced mitochondrial dysfunction and death receptor mediated apoptosis. Alcohol-induced liver injury has been linked to an increase in reactive oxygen species (ROS) production including superoxide anion (O_2-) and the hydroxyl radical (OH-) (7). Potential sources of ROS include Kupffer cells and hepatocytes cytochrome P450 2E1 (CYP2E1) induction by ethanol (7). Excessive production of ROS may result in oxidative damage to lipids, proteins, and DNA (41). Polyunsaturated fatty acids (PUFA), which are found predominantly in cellular membranes, are especially vulnerable to attack by ROS because of the high concentration of allylic hydrogens in their structure (41). Mitochondrial membranes have a substantial concentration of phospholipids containing PUFA which makes the mitochondria particularly vulnerable to ROS toxicity. Mitochondrial dysfunction may result in further production of ROS that may induce apoptosis directly by mechanisms incompletely understood or by induction of Fas-ligand (42; 43; 44). Also ROS may promote the onset of the mitochondrial permeability transition (MPT) by inducing the opening of the permeability transition pore and / or more recent evidence suggest by inducing translocation of Bax from cytosol to mitochondria (45). These events result in mitochondrial permeabilization and release of cytocrhome c triggering the

apoptotic cascade describe earlier in the text. When the insult is more severe and involves most of the mitochondria, ATP becomes profoundly depleted and may result in a switch from apoptosis to oncotic necrosis (46). Consistent with a role for oxidative stress in hepatocyte apoptosis in ALD, Kurose *et al* (43) demonstrated that glutathione depletion in a rat model of ALD resulted in an increase in the apoptosis rate and that antioxidant therapy may reduce apoptosis in the same model (43). Chen *et al* showed enhanced lipid peroxidation and apoptosis in a human cell line that overexpresses CYP2E1 after exposure to arachidonic acid (47).

3.2. The extrinsic pathway of apoptosis in ALD

Death receptor induced apoptosis have also been associated with ethanol-induced hepatocyte apoptosis, especially the Fas/Fas-ligand and TNF receptor 1/TNF-α systems. Taieb and co-worker reported elevated levels of soluble Fas and Fas-ligand in the plasma of patients with ALD (48). While others have also found raised TNF-α plasma levels and this have been shown to be a strong predictor of mortality in alcohol-dependent subjects (49; 50). In the Natori study (36), Fas receptor was strongly expressed in the hepatocytes of patients with ALD, while Ribeiro *et al* (38) found increased expression of both Fas and TNF-R1. As previously mentioned, oxidative stress has been associated with increase in Fas-ligand expression. In patients with ALD, Galle *et al* (51) reported an increased in Fas-ligand mRNA. Because hepatocytes also expressed Fas receptor, in alcoholic liver damage, death of hepatocytes might occur by paracrine or autocrine mechanisms mediated by the hepatocytes themselves.

Experimental studies have also provided strong evidence for a role of death receptor mediated apoptosis in the pathogenesis of ALD. Indeed, limuro and colleagues (52) showed that treatment with antibodies to TNF-α attenuated liver injury in a continuous intragastric feeding rat model of ALD. Whereas, a subsequent study by Yin *et al* using a modification of the continuous alcohol enteral feeding used in the limuro study demonstrated that TNR-R1 knock out mice are protected against alcohol-induced steatosis and liver injury (53). Pastorino *et al* demonstrated that TNF-α toxicity in the setting of ALD may be in part mediated by induction of MPT in a p38 MAPK dependent fashion (54; 55). A recent study by Rodriguez and coworkers (56) showed that exposure of human and rat liver cells to pharmacologic concentrations of ethanol increases expression of TNF-R1 levels. These findings suggest a direct role for ethanol in modulation of TNF-R1 expression in hepatocytes. However the subcellular mechanisms by which ethanol may induce TNF-R1 generation remains unknown.

4. HEPATOCYTE APOPTOSIS IN NAFLD

Obesity, a serious public health problem, and its associated metabolic syndrome are the main risk factor for the development of NAFLD, now the most common cause of chronic liver disease in the United States and many other countries (2; 8). NAFLD is a clinicopathological syndrome with a wide spectrum of liver damage ranging form simple bland steatosis, to steatohepatitis, to cirrhosis (57). Nonalcoholic steatohepatitis (NASH) represents a more severe form of NAFLD and is broadly defined by the presence on liver biopsy of steatosis plus inflammation and variable degrees of scarring, in the absence of significant alcohol consumption (58). The pathogenesis of NASH remains poorly defined. Current concepts suggests that the development of NASH is a "two hit" process (59; 60). The first hit is the development of hepatic steatosis. Insulin resistance which is almost universally present in patients with NAFLD is thought to play a pivotal role in the accumulation of lipids in the liver (61; 62). Insulin resistance and subsequent hyperinsulinemia may result in alterations of the hepatic pathways of uptake, synthesis, degradation, and secretion of free fatty acids (FFA) (61; 62). Although, the relative importance of each of these pathways has not been completely elucidated, the net result is that of hepatocyte accumulation of fatty acids which renders the liver more susceptible to a "second hit" which then triggers an inflammatory response and progressive liver damage. The nature of the "second hit" remains poorly understood but is of significant biomedical importance as identification of these processes may help to identify novel therapeutic targets to treat this disease.

Emerging data suggest hepatocyte apoptosis may be a key component of the "second hit" involved in the progression of NAFLD to NASH (63). By using the TUNEL assay and immunohistochemical detection of active caspase 3 in a large a well characterized NAFLD patient population, we were able to demonstrate (27) that hepatocyte apoptosis is a prominent pathologic feature of human NAFLD. Moreover, we showed a positive correlation between hepatocyte apoptosis and AST/ALT ratio as well as degree of inflammation and stage of fibrosis (27). In a subsequent study, by using a similar approach Ribeiro *et al* (38) confirmed our findings. In-vivo animal models of NAFLD as well as in-vitro cell models of hepatocyte steatosis are now also providing evidence that fatty acid accumulation results in an increase in cell death by apoptosis. In the methionine choline deficient (MCD) animal model of NAFLD, hepatocyte apoptosis is detected early on after initiation of the diet (Feldstein *et al*, unpublished data). We have shown an increase sensitivity to Fas mediated hepatocyte apoptosis in a dietary model of NAFLD induced by feeding mice with a high carbohydrate diet, which recapitulates many of the cardinal features of human NAFLD including obesity, insulin resistance, hyperleptinemia, elevated serum FFA and hepatic steatosis (64). Studies using a genetic model of NAFLD, the ob/ob mice, that are deficient in leptin, have shown a significant increase expression of different specific mediators of both the intrinsic and extrinsic pathway of apoptosis without a significant increase in hepatocyte apoptosis per se (65). Interestingly, this model is characterized by a lack of fibrosis development (66). In-vitro exposure of human hepatocytes to long chain saturated FFA results in a dose-dependent induction of apoptosis (64).

4.1. The intrinsic pathway of apoptosis in NAFLD

When FFA over accumulate in non-adipose tissue such as the liver they may enter nonoxidative deleterious

pathways triggering cell injury and apoptosis. Several mechanisms have been postulated to play a role in FFA induced apoptosis. These include generation of ROS, a lysosomal pathway, and death receptor mediated pathway. In liver specimens from patients with NASH, Seki *et al* (67) reported an increase in by-products of lipid peroxidation. Increased production of ROS in the presence of excess FFA has been demonstrated in several animal models of NAFLD (41). In a similar fashion as in ALD increase production of ROS may result in apoptosis by inducing mitochondrial dysfunction and/or by induction of Fas-ligand and activation of the extrinsic pathway. Yet, up to date, very limited information is available regarding the possible role of oxidative stress in triggering hepatocyte apoptosis in this condition.

By using an *in vitro* cell model of hepatocyte steatosis we have recently demonstrated that FFA induce cytosol-to-lysosome redistribution of Bax, a pro-apoptotic member of the Bcl-2 protein family, which upon certain stimuli translocates to membranes inducing channel formation (68). This results in activation of the lysosomal pathway of apoptosis by inducing lysosomal permeabilization and release of cathepsin B (ctsb), a lysosomal protease and specific mediator of apoptosis by acting on mitochondria to induce mitochondrial dysfunction. In the same study, we demonstrated that genetic or pharmacological inactivation of ctsb protected against diet induced hepatic steatosis and liver injury (68).

4.2. The extrinsic pathway of apoptosis in NAFLD

Death receptor induced apoptosis may also play a role in disease progression from NAFLD to NASH. We have recently reported that Fas protein expression is increase in liver specimens from NASH patients (27). By performing semiquantitative analyses we showed that Fas immunostaining was significantly higher in NASH patients as compared to patients with simple steatosis and normal controls. In a subsequent study we were able to demonstrate that exposure of human liver cells to FFA results in upregulation of Fas expression and increase sensitivity to Fas mediated apoptosis (64). The precise mechanisms by which FFA promotes Fas generation as fatty acids, triglycerides or via generation of ROS, will require further studies. Crespo and co-workers reported an increase in both TNF-α and TNFR1 mRNA levels in the liver of NASH patients as compared to an obese group of similar age without NASH (69). Moreover, this increase of TNF-α mRNA was higher In patients with more advance stage of fibrosis (69). While Ribeiro *et al* (38) reported an increase protein expression of TNFR1 in liver specimens from NASH patients. More recently, Hui and colleagues reported an increase in serum levels of TNF- α in NASH patients (70). Experimental studies have also provided strong evidence for a role of TNF receptor 1/TNF-α systems in the pathogenesis of NAFLD. Indeed, Li and colleagues (71) showed that treatment with antibodies to TNF-α attenuated liver injury in a mice model of NAFLD. Whereas, we demonstrated that TNR-R1 knock out mice are protected against diet-induced steatosis and liver injury (68).

5. PERSPECTIVES

Accumulating evidence now suggests that apoptosis may play a significant role in the pathogenesis of both ALD and NAFLD. As outlined above, many of the mechanisms leading to hepatocyte apoptosis may be shared by the two conditions. However, still limited information is available regarding the specific triggers of apoptosis, as well as the subcellular and molecular mechanisms involve.

A better understanding of the key molecular players responsible for triggering apoptosis in ALD and NAFLD may not only improve our understanding of the pathophysiology of these diseases but have significant therapeutic implications and provide new targets for developing of novel pharmacologic agents.

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