Review

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Biomarkers of liver cell death

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Summary

Hepatocyte cell death during liver injury was classically viewed to occur by either programmed (apoptosis), or accidental, uncontrolled cell death (necrosis). Growing evidence from our increasing understanding of the biochemical and molecular mechanisms involved in cell demise has provided an expanding view of various modes of cell death that can be triggered during both acute and chronic liver damage such as necroptosis, pyroptosis, and autophagic cell death. The complexity of non-invasively assessing the predominant mode of cell death during a specific liver insult in either experimental *in vivo* models or in humans is highlighted by the fact that in many instances there is significant crosstalk and overlap between the different cell death pathways. Nevertheless, the realization that during cell demise triggered by a specific mode of cell death certain intracellular molecules such as proteins, newly generated protein fragments, or MicroRNAs are released from hepatocytes into the extracellular space and may appear in circulation have spurred a significant interest in the development of non-invasive markers to monitor liver cell death. This review focuses on some of the most promising markers, and their potential role in assessing the presence and severity of liver damage in humans.

Keywords: Biomarker; Cell death; Acute liver injury; Chronic liver injury.
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Abbreviations: NAFLD, Non-alcoholic fatty liver disease; NASH, Non-alcoholic steatohepatitis; APAP, acetaminophen; ALI, acute liver injury; ALF, acute liver failure; ALD, alcoholic liver disease; CK18, cytokeratin-18; FasL, Fas Ligand; MMP, matrix metalloproteinase; sFas, soluble Fas; sFasL, soluble FasL; TNF-α, tumor necrosis factor-alpha; TNFR, tumor necrosis factor receptor; sTNF-α, soluble TNF-α; sTNFR, soluble TNFR; DISC, death-inducing signaling complex; FADD, Fas-associated protein with death domain; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor; DR, death receptor; sTRAIL, soluble TRAIL; sDR, soluble DR; RAGE, receptor for advanced glycan endproducts; TLR, toll-like receptor; IRAK, interleukin-1 receptor-associated kinase; miRNA, microRNA; HCV, hepatitis C virus; HCB, hepatitis B virus; HBeAg, hepatitis B e antigen; MP, microparticle; ALT, alanine aminotransferase.

Evolving concepts of modes of liver cell death in acute and chronic liver disease

Growing evidence has demonstrated that several modalities of hepatocyte cell death occur in both acute and chronic liver diseases [1]. Indeed, excessive cell death has been identified as a central mechanism of liver damage in conditions such as acute and chronic viral hepatitis, alcoholic and non-alcoholic steatohepatitis (ASH and NASH), and drug-induced liver injury (DILI) [2–5]. Sustained hepatocyte cell death has also been implicated in the development of hepatic fibrosis [6,7]. The understanding and identification of key molecules involved in biochemical cascades leading to cell death in liver pathophysiology have offered new options for the development and testing of novel pharmacological and/or gene mediated therapies for patients with various liver diseases [8–10].

Each cell death pathway can, in principle, be distinguished on the basis of initiating events, intermediate changes, terminal cellular events, and effect on tissue [11,12]. In addition to the classical modes of cell death, such as apoptosis and necrosis (oncosis), several other forms of hepatic cell death have been described, including autophagic cell death, pyroptosis, and necroptosis [13–15]. Apoptosis, a highly organized and genetically controlled process, is the most investigated and best defined form of programmed cell death. Apoptosis is initiated by either membrane receptors (extrinsic pathway) or intracellular stimuli (intrinsic pathway). However, both pathways result in the activation of effector caspases 3 and 7, which execute the final apoptotic changes [16]. Controversy has existed over whether autophagy functions to initiate or prevent cell death [17]. Autophagy has been characterized as a type of cell death along with apoptosis and necrosis [18,19]. By contrast, many investigations have defined protective functions for autophagy [20–22]. Necroptosis is induced by the same death receptors that activate the extrinsic apoptotic pathway, namely TNF-R1, TNF-R2, and Fas [23]. Upon interaction of receptor protein kinases 1 and 3 (RIP1 and RIP3), and in the absence of activated caspase 8, cell death that morphologically resembles necrosis occurs [24]. Pyroptosis, a novel caspase 1 dependent form of programmed cell death, was characterized a decade ago by Cookson and Brannan [25]. Inflammasome dependent caspase 1 activation initiates (acting on more than 40 substrates) an inflammatory response, as proinflammatory cytokines pro-IL-1β and pro-IL-18 are made active via cleavage. Additionally, caspase 1 introduces the formation of discretely sized ion-permeable pores in the plasma membrane, which leads to water influx, cell swelling and finally cell lysis due

to increased osmotic pressure [26]. Necrosis, or oncosis, is an accidental form of cell death with the fatal consequence being cellular oxygen deprivation whereby the generation of reactive oxygen species (ROS) leads to mitochondrial dysfunction and a drop in ATP level below the threshold required to maintain cellular integrity [1,27]. Morphologically, oncosis is characterized by cellular swelling ('oncosis' in Greek), formation of membrane blebs lacking cellular organelles, and finally cell membrane rupture with the release of cellular contents [28,29] (Table 1).

Most forms of cell death have been extensively characterized *in vitro* in primary hepatocytes or several immortalized hepatocyte cell lines, but only a few have been well defined *in vivo* using various experimental animal models or patients with liver diseases. The complexity of studying cellular demise either *ex vivo* (explanted liver tissue from animal models or liver biopsy tissue from humans) or *in vivo* (model organism and/or humans) comes from the recognition that, in many instances, hepatic cell death represents a highly heterogeneous process. Moreover, frequent overlap and crosstalk between involved pathways may result in molecular transitions between different modalities. As such, the lines between programmed and non-programmed cell death can become blurred in tissues like the liver where dying cells and phagocytes are typically not in close contact. Therefore cells experiencing specific forms of programmed cell death during an acute or chronic insult could undergo secondary lysis *in situ* resulting in a mixed pattern of cell death. Despite these significant challenges, the prospect of developing mechanism-based, non-invasive biomarkers of cell death have gained significant attention. These markers may provide novel clues regarding the pathophysiology of disease in humans, may help stratify patients at risk and/or be used alongside current diagnostics to select patients that are likely to respond to specific therapies (e.g., caspase inhibitors). In the following sections, we will discuss some promising and well-studied blood biomarkers of cell death in various liver diseases. We will touch on their potential for use as non-invasive tools to monitor liver damage, their role and limitations in identifying cell death mode specificity and the future prospect of mechanism-based biomarkers for human liver disease.

Monitoring cell death *in vivo* – Non-invasive blood biomarkers

Soluble cytokeratin-18 (CK18) and fragmented CK18

The cellular content of the soluble fraction of cytokeratin-18 (CK18), the major intermediate filament protein in the liver, has been shown to be released into the extracellular space during cell death both *in vitro* and *in vivo*. Therefore, it has been hypothesized that blood measurements of soluble CK18 present a viable means for monitoring epithelial apoptotic cell death. Full length CK18 can be cleaved by caspase-6 and caspases-3 and -7, resulting in fragments of approximately 30 kDa and 45 kDa respectively [30]. The 30 kDa fragment can be detected using a specific antibody (M30), while a different antibody (M65) detects both full length and fragmented forms [31,32] (Fig. 1). It has been postulated that the M30:M65 ratio can effectively differentiate between apoptotic and necrotic cell death. This concept has recently come into question for a number of reasons: (1) It is now clear that apoptosis can occur independently of caspase activation, as many instances of caspase activation in non-lethal processes have been reported [33]; (2) A number of cell death modes, excluding necrosis, are associated with disruption of plasma membrane, which can result in the release of CK18 (e.g., pyroptosis); (3) In tissues from complex organisms, cells dying by apoptosis, or other forms of programmed cell death, can undergo secondary lysis and subsequently release CK18. Recently, Kramer and colleagues published an elegant study assessing the M30:M65 ratio *in vitro* in a tumor cell line and *in vivo* in sera from cancer patients after both were exposed to pro-apoptotic chemotherapy [34]. Their results show that more than 85% of CK18 released *in vitro* was comprised of caspase cleaved CK18, while the M30:M65 ratio *in vivo* was as low as 0.01. The authors concluded that treatment with a pro-apoptotic, caspase-activating drug resulted in a massive increase in circulating full-length CK18 (M65 positive).

Uncovering the importance of increased hepatocyte cell death, as a result of lipotoxic insults, in the development and progression of NASH led us to the following hypothesis: measuring circulating levels of soluble CK18 allows us to quantify hepatocyte cell death and therefore non-invasively diagnose NASH [35]. A recent meta-analysis showed that plasma CK18 levels exhibit a sensitivity of 78%, a specificity of 87%, and an area under the receiver operating curve (AUROC) of 0.82 (95% CI: 0.78–0.88) in the diagnosis of NASH among patients with NAFLD [36]. As shown among the 231 participants in the PIVENS (Pioglitazone *vs.* Vitamin E *vs.* Placebo for the Treatment of Non-diabetic Patients with Non-alcoholic Steatohepatitis) trial, every 100-U/L decline in serum CK-18 was significantly associated with overall histological improvement, the resolution of NASH, an improvement of at least 1 point in steatosis grade, hepatocellular ballooning, and an improvement in NAFLD activity score (NAS) [37]. The investigators measured CK18 levels at baseline and at 16, 48, and 96 months thereafter among 231 of the 247 patients enrolled in the PIVENS trial [37]. Moreover, CK18 was the only NASH biomarker included in the recent published AGA/AASLD/ACG guidance for diagnosis and management of NAFLD [38]. The recommendations were that "Although serum/plasma CK18 is a promising biomarker for identifying steatohepatitis, it is premature to recommend in routine clinical practice." (Strength – 1, Evidence – B).

Circulating levels of fragmented and full length CK18 have also been shown to be elevated in various other liver disorders. Bantel and coworkers have extensively studied hepatocyte cell death and the release of CK18 in patients with chronic hepatitis C (CHC) [39,40]. In sera of 59 patients with chronic hepatitis C, they found a marked increase in CK18 levels. More than 50% of the CHC patients with normal aminotransferase levels exhibited elevated serum CK18 levels and 30% of patients with normal aminotransferase levels but increased CK18 levels showed advanced stages of fibrosis [39]. The extent of liver steatosis quantified in liver biopsies was closely correlated with serum levels of CK18 in patients with CHC [40]. Measuring CK18 serum levels appeared to be a more sensitive method to detect early liver injury and fibrosis when compared to conventional surrogate markers. The usefulness of serum CK18 levels as a clinical marker for CHC patients was questioned by a large study in 267 patients with treatment-naïve CHC [41]. Jazwinski *et al.* found elevated CK18 levels in CHC patients when compared to controls and while the stage of fibrosis was associated with increasing serum CK18 levels there was no association between CK18 and the grade of steatosis [41]. In addition, Yilmaz *et al.* described higher CK18 levels in patients with NASH than in those with CHC infection

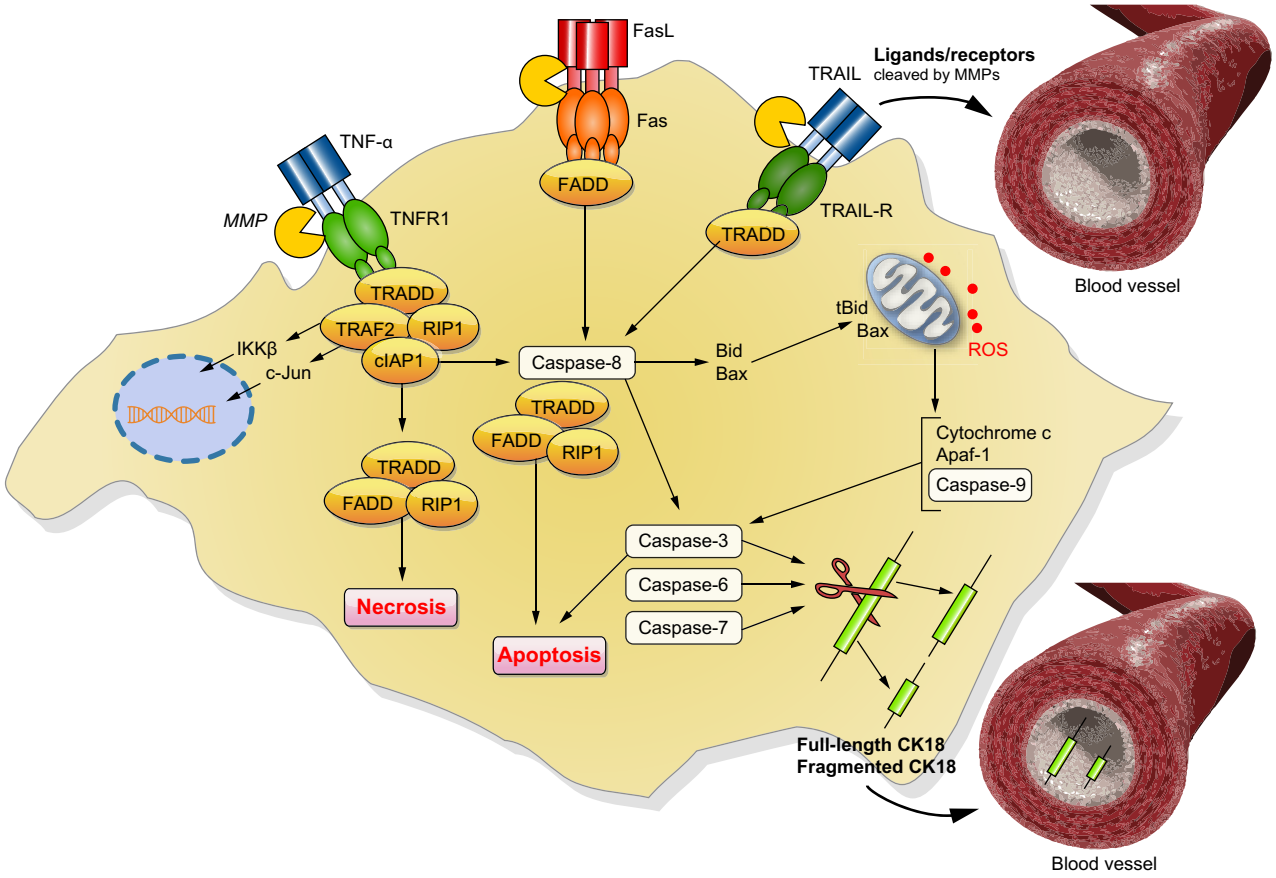

Fig. 1. Signaling pathways for death ligands/receptors and CK18. Full-length CK18 is digested by active caspase-3/6/7 and full-length and fragmented CK18 are released into the blood vessel. Death ligands bind death receptors followed by the activation of cell death pathways, leading to apoptosis or necrosis. The death complex is composed of FasL/Fas and FADD (Fas-associated protein with death domain) and triggers apoptosis through (I) activation of caspase-8 and caspase-3 or (II) activation of pro-apoptotic proteins, such as Bid and Bax, resulting in the assembly of the apoptosome complex (cytochrome c, Apaf-1, and caspase-9) and activation of caspase-3. The death complex is composed of TNF-α/TNFR1, TRADD (TNF-R associated death domain), TRAF2 (TNF receptor associated factor 2), RIP1 (receptor-interacting protein1), and cIAP1, and activates several pathways: (1) IKKβ, (2) c-Jun, (3) TRADD, FADD, and RIP1 complex followed by necrosis, or (4) activation of caspase-8, TRADD, FADD, and RIP1 complex triggers apoptosis. The death complex composed of TRAIL/TRAIL-R uses similar pathways to Fas/FasL and TNF-α/TNFR1. Death ligands and receptors can be digested by enzymes such as MMP (Matrix Metalloproteinase) and released into the blood vessel.

[42]. The authors concluded that NASH patients have an increased hepatocyte loss by apoptosis compared with CHC patients. While these studies support the association of CHC with increased circulating levels of CK18 fragments, the potential clinical role and potential utility of these biomarkers in patients with CHC remains uncertain. In particular, what is the advantage, or potentially added benefit to the current standard measurement of viral load determination for clinical decisions? Would these markers be used to assess the severity of steatosis, or fibrosis? Further, how would these measurements compare with the various serologic and imaging modalities in assessing liver fibrosis in CHC?

Serum CK18 fragments have also been assessed in the context of chronic hepatitis B. In a study including 115 patients with HBeAg-negative chronic hepatitis B, serum CK18 fragments correlated with serum transaminases, viraemia, and grading score, but not with fibrosis or steatosis severity [43]. The authors concluded that CK18 fragment levels may be a useful marker for differentiation between the inactive HBV carrier state and HBeAg-negative chronic hepatitis B, but not in estimating the severity of liver histological lesions among HBeAg-negative chronic hepatitis B patients.

CK18 aggregates are the main components of Mallory bodies, a hallmark in the diagnosis of alcoholic liver disease, which made CK18 seem to be a promising biomarker [44]. Indeed, serum levels of CK18 in heavy drinkers were higher than those of healthy controls, with serum CK18 levels also being higher in cases of alcoholic hepatitis when compared to cases of fatty liver [45]. Interestingly, urinary levels of full-length CK18 are increased in alcoholics [46]. A recent study by Lavallard and colleagues evaluated CK18 and CK18-fragments in serum samples of 143 heavy alcoholics. They confirmed the findings of previous studies by showing a strong correlation of CK18 and CK18 fragment with Mallory bodies, hepatocyte ballooning, and fibrosis [47]. Elevated levels of serum hepatocyte death and apoptotic markers were independent risk factors in predicting severe fibrosis. The authors concluded that CK18 and CK18-fragment serum levels could be useful to rapidly evaluate liver injuries and the efficacy of therapies in alcoholic liver disease [47].

The mechanisms of acute liver failure (ALF) in humans are complex, etiology-dependent, and are thought to be influenced by the balance between various cell death modes, mainly necrosis and apoptosis, as well as by cellular regeneration [48]. Therefore, measuring biomarkers that can help in the distinction

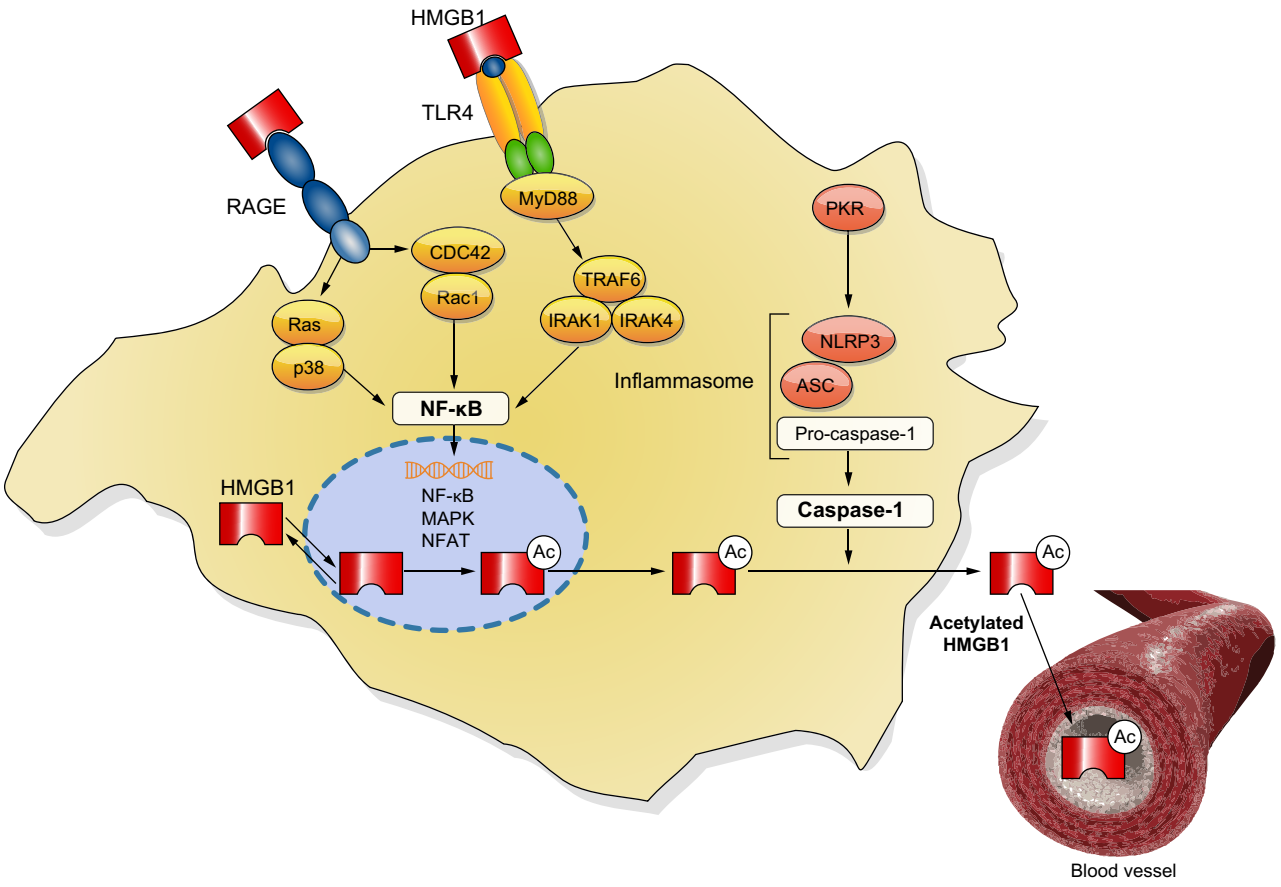

Fig. 2. The signaling pathway for HMGB1. HMGB1 binds to TLR (Toll-like receptor) 4 and recruits MyD88 followed by NF-κB activation via TRAF6 (TNF receptor associated factor), IRAK1 (interleukin-1 receptor-associated kinase 1), and IRAK4. HMGB1 also binds to RAGE (receptor for advanced glycan endproducts) and leads to NF-κB activation via CDC42/Rac1 or Ras/p38 activation. HMGB1 acetylation is mediated by NF-kB, MAPKM, and NFA in the nucleus and is released in the blood vessel correlated with inflammasome activation driven by PKR activation.

of cell death modes are attractive tools that may help in the understanding of mechanistic pathways of ALF and serve as potential prognosis indicators. In an early study in a group of 68 patients with ALF, Bechmann *et al.* found a strong correlation of non-spontaneous remission and total CK18 levels as measured by M65 determination [49]. They implemented a CK18 (M65)-based MELD (model of end-stage liver disease) score by replacing bilirubin with M65 in the MELD score calculation. They were able to demonstrate a high sensitivity and specificity in predicting survival of ALF patients. However, due to the low number of patients studied, and the lack of validation cohort, the accuracy of this new score for the different etiologies of ALF could not be assessed. The study however provided evidence supporting the concept that measuring different CK18 markers could help in the distinction between the different types of cell death involved in various forms of ALF. Subsequently, the largest study conducted to date by the Acute Liver Failure Study Group (ALFSG) included a total of 500 patients with ALF and demonstrated that M30 rather than M65 proved to be the most effective measurement to predict which patient would require liver transplantation or die. Based on these results the authors developed the ALFSG index which includes coma grade, INR, levels of bilirubin and phosphorus, and $\log_{10}$ M30 value, and subsequently validated these findings in a separate group of 250 patients with ALF [50].

In summary, CK18 and CK18-fragments are elevated in various forms of acute and chronic liver injury. CK-18-fragments have been established as the most reliable blood biomarker for predicting the presence of NASH in liver biopsy in both adult and children with NAFLD. Determination of CK-18 fragments as part of the ALFSG index better predicts outcomes in patients with ALF than both the King's College criteria (KCC) and MELD score. However, CK-18 measurements remain a research tool and are not currently available in the clinical setting. The role of these markers in diagnosis, or monitoring, various forms of liver disease remains unclear and future studies are still needed.

Death receptors and their ligands

Soluble Fas receptors and Fas ligand

Fas (CD95/APO-1) is a death receptor in the tumor necrosis factor receptor (TNFR) family and is expressed in a variety of tissue [51]. It is activated by the Fas Ligand (FasL/CD95L), which is a member of the tumor necrosis factor superfamily. Upon binding, the receptor undergoes trimerization and forms the intracellular death-inducing signaling complex (DISC) [52]. The DISC includes

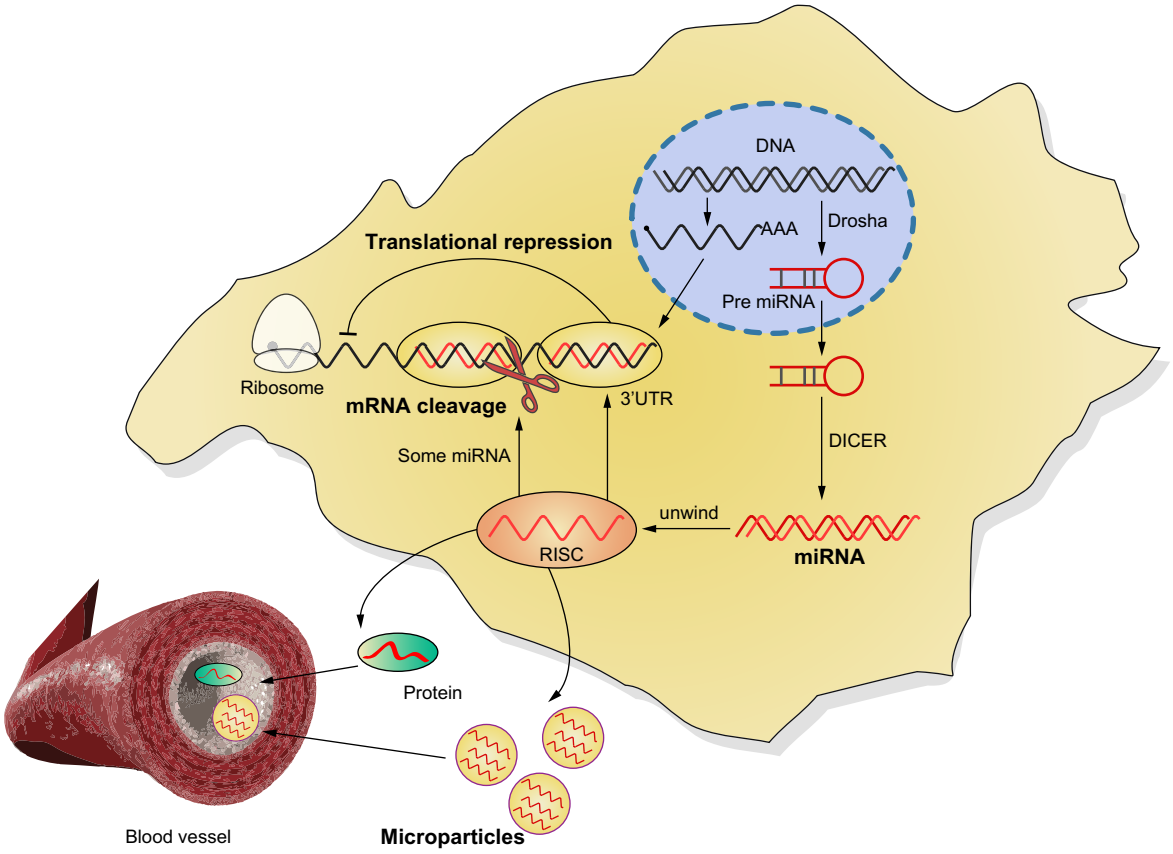

Fig. 3. miRNA mechanism. Pre-miRNA (precursor microRNA) is processed by Drosha, a nuclear RNase III enzyme, and is exported to the cytoplasm. There the pre-miRNA is manipulated to miRNA by Dicer. miRNA is unwound to single stranded RNA and loaded into the RISC complex. The RISC complex mainly binds to 3′UTR of target genes which can lead to translational repression. Some miRNA-RISC-complexes bind to the ORF (open reading frame) of a target sequence resulting in messenger RNA (mRNA) degradation. miRNAs might be encapsulated into microparticles and released into the bloodstream.

the Fas-associated protein with death domain (FADD) and triggers either a type I or type II signaling cascade. In type I cells, cleavage of the initator caspase-8 ultimately results in activation of apoptotic effector caspases (caspase-3, -6, and -7), whereas in type II cells the apoptosome complex is released from dysfunctional mitochondria [53] (Fig. 1). The Fas/FasL signaling pathway has been implicated in various liver pathologies including Wilson's disease, alcoholic liver disease (ALD), acute fulminant hepatitis, as well as chronic viral hepatitis [54–56]. Fas signaling has also been shown to function as a link between obesity-associated fatty liver and increased susceptibility to liver damage. We recently reported in a diet-induced obesity animal model, and in patients with non-alcoholic steatohepatitis, a significant increase in the abundance of hepatic Fas receptor, which led to increased liver sensitivity to the endogenous Fas ligand (FasL) [57]. Moreover, Zou and coworkers showed that chemically blocking Fas signaling with an inhibitory peptide (YLGA 12-mer) reverses liver damage in two established models of fatty liver [58].

Soluble Fas (sFas) is the soluble form of the membrane associated Fas and is generated by alternative mRNA splicing [59]. The soluble FasL (sFasL), however is converted from membrane-bound FasL by a matrix metalloproteinase (MMP)-like enzyme [60]. Both sFas and sFasL appear to intriguing non-invasive biomarkers to monitor cell death in the serum. Suzuki and colleagues found that serum sFasL levels are increased in the acute phase of hepatitis and their levels vary according to the cause of hepatitis [61]. In another study, Nakae and colleagues described that sFasL levels were significantly higher in patients with ALF when compared to patients with sepsis. However authors did not observe significant differences in sFas levels [62]. In patients with NASH, we recently demonstrated that serum levels of sFas and sFasL are significantly higher in patients with biopsy-proven NASH when compared to patients with simple steatosis [63]. We also generated a prediction model to diagnose NASH that includes CK18 fragments and soluble Fas with a sensitivity and specificity of 88% and 89%, respectively [63].

Soluble TNF receptors and TNF-alpha

Tumor necrosis factor-alpha (TNF-α) is a pleiotropic monocyte-derived cytokine that has been associated with cancer progression, severe inflammatory diseases, and liver injury [64]. In order to fulfill inflammatory reactions in the tissue, TNF-α binds to two distinct receptors: TNFR1 and TNFR2. While TNFR1 is expressed in almost all tissue, TNFR2 is expressed only on inflammatory cells. Once TNF-α ligates TNFR1 the TNF receptor-associated protein with death domain (TRADD)

Table 1. Different modes of liver cell death.

	Apoptosis	Necroptosis	Autophagic cell death	Pyroptosis	Necrosis/oncosis
Nucleus	Condensation DNA fragmentation	Normal	Condensation (late stage)	Condensation DNA fragmentation	Mild DNA damage
Cytoplasm/ membrane	Blebbing	Swelling/disruption	Autophagosomes, autolysosomes	Swelling/pores	Swelling/blebbing
Cellular components	Secretion	Release		Release	Release
Main pathway	Caspase-3/6/7	RIP1/RIP3	mTOR, Atg	Caspase-1	ROS

RIP1/3, receptor interacting protein 1/3; mTOR, mammalian target of rapamycin; Atg, autophagy-related; ROS, reactive oxygen species.

is recruited to the death domain of TNFR1. TRADD, as the central molecule in the TNF signaling cascade, can activate at least three different, and potentially contradictory, signaling cascades: (1) I-kappa B kinase (IKK) complex which induces pro-inflammatory and anti-apoptotic target genes; (2) c-Jun-kinase leading to an increase in pro-apoptotic and proliferation related genes; (3) death-inducing signaling complex (DISC) that induces apoptosis (Fig. 1).

It is known that inflammatory mediators such as cytokines and adipocyte derived cytokines, termed adipokines, play an important role in establishing NAFLD and the progression to more advanced stages of fatty liver diease [65,66]. The role of TNF-α as a proinflammatory mediator in insulin resistance, a key element in the development of NAFLD, was first described by Hotamisligil almost two decades ago [67]. Treatment with a neutralizing antibody improved insulin resistance in obese animals, which show TNF-α overexpression in the adipocytes. Notably, hepatic *TNF-α* and *TNFR1* mRNA levels were increased in patients with NASH when compared to patients with simple steatosis [68]. A study by Ribeiro *et al.* supported these findings and even found an increase in hepatic *TNFR1* mRNA levels in patients with ASH, further underlining this important principle in liver disease. In their study, hepatocyte apoptosis was also significantly increased in both NASH and ASH patients [69]. The TNF-α converting enzyme (TACE) cleaves receptor bound TNF-α and membrane incorporated TNFR1 or TNFR2, resulting in the release of soluble TNF-α (sTNF-α) and soluble TNFR1 or TNFR2 (sTNFR1 or sTNFR2) into the bloodstream [70,71]. Therefore, sTNF-α and sTNFR present interesting targets for non-invasive biomarkers to monitor cell death. An early report by McClain *et al.* described TNF-α metabolism dysregulation in ASH and reported that cultured monocytes from AH patients spontaneously produced TNF-α [72]. Numerous studies by other groups confirmed that increased serum TNF-α concentrations in patients with ASH were correlated with disease severity and mortality [73]. Felver *et al.* reported elevated plasma TNF-α concentrations in patients with severe ASH, either on admission or at discharge 30 days later, and found 82% mortality in ASH patients while 100% survival was seen in patients without elevated plasma TNF-α levels [74]. Bird *et al.* reported that patients with severe ASH who subsequently died had higher plasma TNF-α levels compared to those who survived, they also found that TNF-α levels significantly correlated with serum bilirubin and creatinine values [75]. Moreover, serum TNF-α levels were increased in children, adolescents and adults with NASH [76,77]. Plasma TNF-α levels also were significantly higher in patients with chronic hepatitis C, with liver cirrhosis, and hepatocellular carcinoma when compared to those with acute or mild chronic hepatitis C [78]. Additionally, a positive correlation of soluble TNFR1 and soluble TNFR2 and disease progression were described in patients with chronic hepatitis C [79].

Soluble TRAIL receptor and TRAIL

The TNF receptor family further incorporates the TNF-related apoptosis-inducing ligand receptor (TRAIL-R), TRAIL-RI, and TRAIL-RII, the last two known also as the death receptor 4 (DH4) and DH5 [80]. It has been hypothesized that TNF-related apoptosis-inducing ligand (TRAIL) can induce apoptosis because it activates caspases that are, to a wide extent, similar to those involved in Fas and TNF-α-induced cell death [81] (Fig. 1). TRAIL, as well as DH4 and DH5, are involved in a variety of diseases including vascular diseases, cancers, and infectious diseases [82,83]. All three TNF receptors have been shown to be upregulated in liver samples of patients with HCV-associated chronic liver injury and liver cirrhosis [84], as well as in patients with hepatitis B-mediated ALF [85]. Two studies by Malhi and Kahraman and colleagues showed enhanced DR5 expression in the livers of patients with NASH [86,87]. Furthermore, levels of serum sTRAIL have been shown to be significantly higher in patients with NAFLD [88], or hepatitis B virus (HBV) infection, when compared to healthy controls [89].

Based on these results, death receptors such as TNFR and TRAIL-R and their respective ligands are appealing as promising non-invasive biomarkers to monitor liver damage.

High mobility group box 1 (HMGB1)

The high-mobility group box-1 (HMGB1) protein, also known as high-mobility group 1 (HMG-1) and amphoterin, is a highly conserved, abundant, non-histone nuclear protein expressed in almost all eukaryotic cells [90]. Cellular effects of HMGB1 are induced via signal pathway receptors, such as receptor for advanced glycan end products (RAGE) and toll-like receptor 4 (TLR4). Both receptors activate NF-κB, whereas RAGE mediates this activation through Ras/p38 or CDC42/Rac1, TLR4 recruits MyD88 which then forms a complex involving TRAF6, interleukin-1 receptor-associated kinase 1 (IRAK1), and IRAK4 [91]. Within the nucleus, HMGB1 modulates and 'fluidizes' nucleosomes, bends DNA, and binds bent chromatin DNA, thus facilitating transcription of many genes [92] (Fig. 2). As a component of the innate immune system, the HMGB1 protein functions as a common signal that alerts the host to cell stress, unscheduled cell death, and to microbial invasion [93]. In response to inflammatory stimuli, HMGB1 can be secreted by numerous cells including

macrophages, natural killer cells, neutrophils and mature dendritic cells. Originally, it was thought that HMGB1 was released from only necrotic, but not apoptotic, cells [94]. Recent studies indicate that cells dying via apoptosis could also release HMGB1. In a number of human cancer cell lines HMGB1 is released following treatment with chemical inducers of cell death (staurosporine, etoposide, or camptothecin). This release can be diminished via application of the apoptosis-inhibitor Z-VAD-fmk [95]. Thus the release of HMGB1 from both apoptotic and necrotic cells can be observed [96]. The distribution and nuclear transport affecting the secretion of HMGB1 is controlled by several posttranslational modifications such as acetylation, cysteine oxidation, and phosphorylation [97,98]. HMGB1 is hyper-acetylated upon activation with LPS in monocytes and macrophages while unacetylated HMGB1 is thought to be released by necrotic or damaged cells [99,100]. Recently, Lamkanfi and coworkers described a dependency of HMGB1 secretion on NLRP3 inflammasome assembly and caspase-1 activation. They found that HMGB1 secretion from LPS-primed macrophages, or *Salmonella typhimurium* infected macrophages, required inflammasome components, such as apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC), caspase 1 and Nalp3, or caspase 1 and Ipaf respectively [101]. A subsequent study from Lu and coworkers showed that inflammasome activation in this context relies on a double-stranded RNA-dependent protein kinase (PKR, also known as EIF2AK2) [102].

The role of HMGB1 generation during acute liver failure has been explored in various experimental models. In a partial lobar liver warm ischemia mouse model Kamo *et al.* showed that ASC-mediated caspase-1/IL-1β signaling promotes HMGB1 to produce a TLR4-dependent inflammatory phenotype that leads to hepatocellular injury [103]. Another study addressed the role of intracellular HMGB1 in modulating the early activation of Kupffer cells (KCs) during Concavalin A (Con A) acute liver failure. The authors found that up-regulation of HMGB1 expression and the translocation of HMGB1 in KCs corresponded with early activation of KCs, while blockade of intracellular HMGB1 significantly inhibited production of pro-inflammatory cytokines [104].

Based on the importance of HMGB1 in the pathogenesis of liver damage during acute liver injury, as well as the concept that different forms of this molecule (hyper-acetylated *vs.* total) can serve to distinguish various modes of cell death Park and colleagues examined the role of HMGB1 as biomarker of liver cell death in acetaminophen (APAP) induced acute liver injury [105]. They demonstrated in a small pilot study that both total and hyper-acetylated HMGB1 levels were elevated in the sera of APAP overdose patients with liver injury compared to overdose patients without liver injury and healthy volunteers. Increased total and acetylated HMGB1 was associated with a worse prognosis (King's College Criteria) – patients died or required liver transplantation – when compared to those with spontaneous recovery. In a follow-up study by the same group, total HMGB1 levels in blood were assessed at the time of first presentation to the hospital in a total of 129 patients with acetaminophen-induced acute liver injury [106]. Total HMGB1 concentrations were found to be superior to serum ALT levels in identifying acute liver injury within 8 h of the overdose. Future studies by independent groups to validate these results, as well as to assess the utility of HMGB1 in non-APAP forms of acute liver injury, are needed. Unfortunately, to date, there are no specific antibodies to identify different functional isoforms of HMGB1, and mass spectrometry-based analysis presently remains the only option for identification. These methods have several limitations, including issues with reproducibility, operator dependence requiring skilled, trained analysts and a specialized proteomic laboratory, as well as low-throughput and high cost that currently limit their potential role in clinical medicine.

miRNAs

Small non-coding RNAs 21–25 nucleotides in size are known as microRNA (miRNA) and play an important role in the regulation of gene expression [107]. They are generated as a pre-miRNA and exported from the nucleus. In the cytoplasm, the pre-miRNAs are recognized by an endoribonuclease called Dicer, a member of the RNase III family, and cleaved into 21–25 base pair long miRNAs. Dicer also unwinds the cleaved portion(s) to a single strand and loads the miRNA into the RNA-induced silencing complex (RISC). The miRNA-RISC complex can interact with the 3′-end untranslated region (3′UTR) of the target gene's messenger-RNA resulting in the suppression of mRNA translation [107]. The miRNA-RISC complex can also bind directly to target sequences resulting in the degradation of mRNA [107] (Fig. 3). Since a single miRNA can have several target genes, it plays a significant role in gene regulation. Indeed, a number of miRNAs have been implicated in various disease processes, especially cancer, where miRNAs already serve as biomarkers [108]. miRNAs also offer an intriguing opportunity as therapeutic targets [109,110]. Several miRNAs are specifically expressed or enriched in the liver. The most abundant liver specific miRNA is miR-122, which is present in hepatocytes and can be released into circulation during hepatocyte damage [111,112]. Two independent studies by Wang *et al.* and Su *et al.* addressing acetaminophen (APAP) toxicity in experimental murine models identified miR-122 and miR-192 as potential biomarkers. They showed dose- and time-dependent changes in the blood levels of these miRNAs that paralleled histopathologic changes of liver damage [113]. Changes in plasma levels of these miRNAs could be detected early after APAP exposure and were more sensitive than ALT measurements in the assessment of liver injury [113,114]. Based on these results, Park and colleagues studied the plasma levels of these two miRNAs in a small cohort of patients with APAP-induced acute liver injury. Both miR-122 and miR-192 were found to be substantially elevated in patients with APAP-induced acute liver injury when compared to patients without liver disease [115]. Aforementioned miRNAs were also elevated when compared to a small group of patients with non-APAP acute liver injury [115]. In a follow-up study by the same group, measurement of miR-122, as well as other markers of cell death, significantly outperformed ALT, INR, and plasma acetaminophen concentration for the prediction of APAP-induced liver injury [116].

The potential of various miRNAs as biomarkers of disease severity in various chronic liver conditions have been recently explored [111]. Yamada and colleagues revealed that serum levels of miR-122, as well as miR-21, miR-34a, and miR-451 were increased in patients with NAFLD, when diagnosis was based on ultrasound determination of liver fat, and that miR-122 levels correlated with the severity of liver steatosis [117]. Furthermore, miR-103/107 levels were increased in liver biopsies from

Table 2. Soluble biomarkers in human liver disease.

Biomarker	Tested in acute liver injury	Tested in chronic liver disease	Pilot studies available	Validation studies available	Clinical correlation	Main cell death mode	[Ref.]
CK18 [full length CK18 (M65), fragmented CK18 (M30)]						Apoptosis/necrosis	
Fragmented CK18		NAFLD/NASH	Yes	Yes	Disease marker		[35] [36]
Full length CK18		NAFLD/NASH	Yes	No	Disease marker		[36]
Fragmented CK18		CHC	Yes	No	Disease progression		[39] [40]
Fragmented CK18		CHC	Yes	No	Advanced fibrosis		[41]
Fragmented CK18		CHC/NASH	Yes	No	Disease marker		[42]
Fragmented CK18		Hepatitis B	Yes	No	Disease distinguish		[43]
Full length/ fragmented CK18		ALD	Yes	No	Disease marker		[45] [46]
Full length/ fragmented CK18		ALD	Yes	No	Disease progression		[47]
Full length CK18	ALF		Yes	No	Disease prediction		[49]
Fragmented CK18	ALF		Yes	Yes	Disease prediction		[50]
Fas						Apoptosis	
sTNFR1		CHC	Yes	No	Disease progression		[79]
sTNFR2		NASH	Yes	No	Disease marker		[77]
sTNFR2		CHC	Yes	No	Disease progression		[79]
TRAIL						Apoptosis	
s-TRAIL		NAFLD	Yes	No	Disease marker		[88]
s-TRAIL		Chronic hepatitis B	Yes	No	Disease severity		[89]
HMGB1						Pyroptosis/necrosis	
Total/ hyperacetylated HMGB1	APAP		Yes	No	Worse prognosis		[105] [106]
miRNAs						Various	
miR-122	APAP/ ALF		Yes	No	Worse prognosis		[115]
miR-192	APAP/ ALF		Yes	No	Worse prognosis		[115]
miR-122		NAFLD	Yes	No	Disease severity		[117]
miR-122		CHC	Yes	No	Liver fibrosis/ inflammation		[112]
miR-155/125b/146a		CHC	Yes	No	Abundance		[120]
miR-29		Cirrhosis	Yes	No	Disease severity		[123]
miR-214-5b/221/222		Liver fibrosis	Yes	No	Disease severity		[125] [126]
Microparticles						Various	
MPs-CD11a(+)/ CD4(+)/CD235(+)		Cirrhosis	Yes	No	Abundance		[135]
MPs-CD14(+)		NASH	Yes	No	Disease severity		[136]
MPs-CD4(+)/ CD8(+)		CHC	Yes	No	Abundance		[136]

patients with ALD, NAFLD, and NASH, and those levels were closely associated with insulin resistance [118,119]. In addition, Bala and colleagues found that inflammation related miRNAs (miR-155, miR-125b, and miR-146a) were increased in the plasma of patients with CHC [120]. Another study by the same group using various experimental mouse models, demonstrated that circulating levels of these inflammation related miRNAs are different in response to the type of liver injury, such as APAP, ALD, and TLR9 and TLR4 ligand-induced liver damage [121]. The authors also explored mechanism of miRNA release and found that in ALD and in inflammatory liver injury, serum/plasma miR-122 and miR-155 were predominantly associated with the vesicles, whereas in DILI/APAP injury these miRNAs were present mainly in the protein-rich fraction. Consistent with these results we recently found that in murine models of NASH, microvesicles containing miR-122 are released by hepatocytes and can be measured in circulation (see below: "Microparticles").

Several studies showed that liver fibrosis caused by hepatic stellate cell (HSC) activation could be monitored by miRNAs. Lakner and coworkers recently showed that miR-19b is reduced in rodent and human liver fibrosis [122]. A similar study found reduced miR-29 family members in a carbon tetrachloride-induced mouse model of hepatic fibrogenesis (CCl_4), as well as in patients with advanced liver cirrhosis [123]. Using a bile duct-ligation fibrosis model in rats, Venugopal *et al.* described reduced levels of miR-150 and miR-194 [124]. Similarly, serum levels of miR-122 inversely correlated with severity of fibrosis in patients with CHC. The levels were decreased in patients with severe fibrosis, while patients with early stages of fibrosis and high inflammatory activity showed increased levels of miR-122 [112]. On the other hand, increased levels of miR-214-5p and miR-221/222 have been observed in rodent and human liver fibrosis [125,126]. Several key areas for future investigation include assessing the mechanisms and kinetics of liver microRNA release into blood and other potential biofluids reservoirs, as well as establishing the different compartments, such as extracellular vesicles, that comprise the predominant source of circulating miRNAs in various liver disease processes. Future prospective and longitudinal biomarker studies in APAP and non-APAP acute liver injury, as well as in various chronic liver diseases, will be required to determine whether miR-122 works in isolation, or in combination with other miRNAs, in order to create miRNA profiles that can provide added clinical utility and prognostic value to currently available tools.

Microparticles

Microparticles (MPs) are small membrane vesicles released in a highly regulated manner from dying or activated cells. MPs range between 100 and 1000 nm in size and are generated through cell membrane shedding. This process involves a sorting of membrane proteins into the shed MP and the inversion of phosphatidylserine from the inner to the outer membrane during cellular activation, or early apoptosis [127]. MPs can be classified as cell-to-cell communicators [128]. They carry specific signatures such as surface receptors, integral membrane, cytosolic and nuclear proteins, and RNAs including miRNAs from parental cells and deliver these signatures to other cells [129,130] (Fig. 3). Notably, MPs not only stay in the original tissue, but may also circulate in the bloodstream [131]. The first report of MP release into circulation during liver injury came from the AM Diehl group where they demonstrated an increase in blood and bile MPs using a bile duct ligation (BDL) rat model [132]. The Diehl group went on to show that these MPs contain biologically active Hedgehog (Hh) ligands and provided evidence suggesting that activation of Hh signals on hepatic sinusoid endothelial cells may be an important mechanism by which MPs contribute to tissue remodeling during chronic cholestatic liver injury. More recently, our group identified circulating MPs released in mice with diet-induced NASH as potential and novel biomarkers for the diagnosis of NASH [133]. We found that hepatocytes exposed to excess amounts of saturated fatty acids released membrane-bound microparticles that induced angiogenesis when administered to mice. Microparticles from the blood of mice with diet-induced steatohepatitis originated from the liver and triggered migration and tubular structure formation when applied to an endothelial cell line. The angiogenic effects of microparticles generated by hepatocytes exposed to saturated fatty acids, or of those from mice with diet-induced steatohepatitis, involved the uptake of the microparticles by endothelial cells, a process that required Vanin-1, an enzyme located on the surface of the microparticles. Thus, the pathological angiogenesis that can occur in steatohepatitis could be reduced by preventing endothelial cells from internalizing Vanin-1–positive microparticles from hepatocytes. The relevance of MP determination in human disease has been studied in recent pilot studies [134,132]. Rautou *et al.* reported elevated circulating levels of leuko-endothelial-derived CD31(+)/41(−) MPs, pan-leukocyte-derived CD11a(+) MPs, lymphocyte-derived CD4(+) MPs, and erythrocyte-derived CD235a(+) MPs in patients with liver cirrhosis [135]. Two recent studies by Kornek and Schuppan showed that MPs positive for various inflammatory cell markers such as CD4(+) or CD8(+) T cells, CD14(+) monocytes, and iNK cells can be detected in the plasma of patients with various liver conditions [136,137]. Notably, circulating MPs positive for monocyte-marker CD14, or iNK cell marker, were higher in a small group of patients with NASH when compared to those with hepatic steatosis, while patients with chronic hepatitis C infection had increased levels of MPs positive for CD4 and CD8 T cell markers [136]. Thus, extracellular vesicles, in particular MPs, are evolving as attractive biomarkers to diagnose and monitor liver injury in various acute and chronic liver conditions. MPs not only carry a specific signature of the cell of origin, but may also be important in the pathogenesis and progression of liver damage.

Future studies to better assess the profile of MPs present in blood, the mechanisms involved in their generation, and their potential contribution to the pathogenesis and progression of liver damage are warranted.

Conclusion

Hepatocyte cell death is a central mechanism involved in liver injury associated with both acute and chronic liver damage. A growing understanding of the signaling events involved in triggering cell death has allowed for the dissection of the different molecular pathways that result in hepatocyte cell death. The classical hepatocyte cell death model has been presented as having two mutually exclusive forms: programmed, or apoptotic cell death, *vs.* accidental, or necrotic cell death, based on morphological criteria. However, recent breakthroughs have identified a number of cell death modes that present with crosstalk and cooperation in the execution of cell death. Recent evidence indicates that during these processes certain intracellular and surface proteins are cleaved and/or released from hepatocytes, which has spurred a significant interest in the potential for development of non-invasive markers to monitor liver cell death. The determination of these markers in experimental models and patients with various forms of liver disease have provided important clues about the role of cell death in liver injury and suggested that biomarkers of cell death may be useful markers of liver damage (Table 2). In addition, a biomarker panel including detection of cell death and liver specificity will possibly increase accuracy of diagnosis. Future studies to better assess these markers in different types of acute and chronic liver injury, as well as to identify novel markers of hepatocyte cell death, are warranted and may provide crucial help in the diagnosis, monitoring and treatment of liver disease.

Key Points

- Non-invasive biomarkers of cell death are evolving as promising tools to diagnose and monitor liver damage in various acute and chronic liver diseases
- Various markers in blood have been proposed to allow the distinction between different modes of cell death in the liver contributing to the pathogenesis of liver damage. However, several limitations of current assays remain to be addressed
- Determination of soluble CK18 fragments is currently the most reliable single blood marker for diagnosis of NASH
- Determination of CK-18 fragments as part of the ALFSG index better predicts outcomes in patients with ALF than both the King's College criteria (KCC) and MELD score
- Death receptors such as TNFR and TRAIL-R and their respective ligands are appealing as promising non-invasive biomarkers to monitor liver damage
- Measurement of various functional isoforms of HMGB1 in blood may be used to assess the predominant types of cell death occurring during liver injury
- In pilot studies both acetylated and total HMGB1 are better predictors of outcomes in patients with APAP-ALI than serum liver enzymes, but independent, larger validation studies are needed
- Both microRNAs, and extracellular vesicles have various features that make them potentially ideal mechanism-based biomarkers with strong supporting experimental data. Currently, there are only a few very small pilot studies in humans and future studies are needed

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Conflict or interest

Dr. Feldstein and Dr. Eguchi report that their named as co-inventor on pending and issued patents filed by the Cleveland Clinic and University of California San Diego (UCSD) that refer to the use of biomarkers in fatty liver disorders.

Review criteria

A search for original articles and reviews published between 1998 and 2013 and focusing on biomarkers of liver cell death was performed in PubMed. The search terms used were "liver", "biomarker", "NAFLD", "NASH", "DILI", "hepatitis", "cytokeratin-18, CK18", "Fas", "TNF", "TRAIL", "cell death", "apoptosis", "autophagy", "necroptosis", "pyroptosis", "oncosis", "necrosis", "HMGB1", "miRNA", and "microparticles", alone and in combination. All articles identified were English-language, full-text papers. We also searched the reference lists of identified articles for further relevant papers.

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