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