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Autophagy and the Liver

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1. Introduction

Autophagy is a cellular process that involves lysosomal degradation and recycling of intracellular organelles and proteins to maintain energy homeostasis during times of cellular stress [1]. It also serves to remove damaged cellular components such as mitochondria and long-lived proteins. Autophagy is catabolic mechanism and although hepatic autophagy performs the standard functions of degrading damaged organelles/aggregated proteins and regulating cell death it also regulates lipid accumulation within the liver. Autophagy can be divided into three distinct sub-groups that are discussed below. This chapter focuses upon the role of autophagy in a variety of liver diseases including hepatocellular carcinoma (HCC) and viral hepatitis. The increased understanding of the cellular machinery regulating autophagy within the liver may foster the development of therapeutic strategies that will ultimately help treat liver disease.

As stated above autophagy involves lysosomal-dependent degradation of long-lived proteins and cellular organelles [2]. In rodent models of nutrient starvation autophagy was responsible for degradation of 35% of total liver protein within 24 hours [3] illustrating the role of autophagy in liver homeostasis and energy conservation. Conversely inhibition of autophagy in hepatocytes led to a 4-fold increase in liver mass because of failure to degrade a variety of cellular components [4]. Therefore autophagy plays an important role maintaining liver function under basal conditions but may also be manipulated by pathological processes to cause liver disease. The precise role of autophagy within the liver is detailed below. This chapter is not exhaustive with regard to the role of autophagy in liver disease but draws upon the current understanding of autophagy role in selected liver diseases.

2. Autophagic pathways

Three distinct types of autophagy have been described in eukaryotic cells; macroautophagy (referred to hereafter as autophagy), chaperone-mediated autophagy (CMA), and microau-

tophagy [5]. In autophagy, a portion of cytosol is engulfed by a double-membrane structure, termed an autophagosome, that fuses with a lysosome whose enzymes degrade the cellular constituents sequestered in the autophagosome [6]. The regulation of this process is highly complex and controlled by the co-ordinated actions of the evolutionarily conserved autophagy-related genes (Atgs). Over 32 Atg genes have been identified in yeast and humans [7, 8]. The source of the double membrane is controversial, but it might be derived from the endoplasmic reticulum (ER), mitochondria, or plasma membrane [9]. The double membrane of the autophagosome is formed and elongated by as yet unclear mechanisms, but a number of multi-protein complexes are known to mediate these processes [10]. CMA allows the direct lysosomal import of unfolded, soluble proteins that contain a particular pentapeptide motif. In the third form of autophagy, microautophagy, cytoplasmic material is directly engulfed into the lysosome at the surface of the lysosome by membrane rearrangement. Despite being three separate mechanisms each type of autophagy involves engulfment of a part of the cytosol and lysosomal dependent degradation within a double membrane. Each of these three autophagic processes is discussed below.

2.1. Autophagy (Macroautophagy)

32 Atg genes have been identified thus far that regulate autophagy of which 16 Atg genes are required for all types of autophagy [11]. The major cellular pathways regulating autophagy aside from the Atg proteins include the inhibitory mammalian target of rapamycin (mTOR) and class III phosphatidylinositol 3-kinase (PI3K). Thus the Atg proteins, PI3K and mTOR pathways all co-ordinate a highly complex cellular signaling pathways to regulate autophagy [8].

The formation of the autophagosome can be divided into several steps. The description that follows is a simplified pathway and in reality the process is likely to be much more complex. The autophagy process is induced by the ULK1 kinase complex [12] and later class III PI3K complex is involved in vesicle nucleation [13]. This is followed by membrane expansion that involves various Atg proteins including the Atg2-Atg18 complex, Atg12-Atg5-Atg16 conjugation system and Atg8-phosphatidylethanolamine (Atg8-PE) conjugation system [14]. The precise role of each molecular regulator is beyond the scope of this chapter although the process is discussed in detail below. The reader is referred to recent excellent reviews for more detailed reviews of the whole process [8, 10, 15].

In general, the Atg1-Atg13-Atg17 complex recruits and organizes other proteins for the developing autophagosome [16]. Activation of the Atg1-Atg13-Atg17 complex leads to organization of the Atg6/beclin-1-Vps34 complex on the lipid membrane [12]. Vps34 produces phosphatidylinositol 3-phosphate, which can recruit other proteins to the complex [17]. Vps34 is the target of the widely used pharmacologic inhibitor of autophagy 3-methyladenine (3-MA) [18, 19]. Importantly, beclin-1 is an important interface between the autophagic and cell death pathways, because the anti-apoptotic proteins Bcl-2 and Bcl-X_L bind beclin-1 to inhibit autophagy [20]. The regulation of this interaction is complex but includes its disruption by c-Jun N-terminal kinase 1-mediated phosphorylation of Bcl-2 [21].

Following the above, autophagosome formation and elongation involves 2 ubiquitin-like conjugation processes that generate membrane-bound protein complexes. In the first, Atg7

and Atg10 mediate the conjugation of Atg12 to Atg5 [22], which subsequently interact with Atg16 [23]. The Atg12–Atg5 complex associates with the membrane and then dissociates upon completion of the autophagosome. The second critical conjugation reaction involves Atg8 or microtubule-associated protein 1 light chain 3 (LC3). LC3 is constitutively cleaved by Atg4 to produce LC3-I. With a signal to induce autophagy, Atg7 and Atg3 mediate the conjugation of LC3-I to the membrane lipid PE to form LC3-II [24]. LC3-II associates with the autophagosomal membrane, where the lipidated protein can mediate membrane elongation and closure. LC3-II is degraded late in the autophagic pathway, after autophagosome fusion with a lysosome [25]. The formation of these autophagic vacuoles can be detected experimentally by labeling cells with the specific autophagic marker monodansylcadaverine (MDC).

Once formed, autophagosomes traffic along microtubules by a dynein-dependent mechanism to reach perinuclear lysosomes located near the microtubule-organising center. Another method to monitor the induction of autophagy is therefore to detect perinuclear, LC3-positive aggregates by immunofluorescence. Before they fuse with lysosomes, autophagosomes can fuse with early and late endosomes to form an amphisome. This process allows for a point of convergence between the pathways of autophagy and endocytosis [10]. Autophagosomes dock and fuse to form an autophagolysosome or autolysosome by a process that has not been well defined in mammalian cells. The term “autophagic vacuole” has been used for autophagosomes, amphisomes, and autolysosomes, which can be indistinguishable experimentally. In yeast, fusion is mediated by soluble N-ethylmaleimide-sensitive factor attachment protein receptors [26]. Whereas in mouse hepatocytes the soluble N-ethylmaleimide-sensitive factor attachment protein receptor protein vti1b mediates autophagosome-endosome fusion [27]. Other factors that regulate autophagosome fusion include the guanosine triphosphate binding protein Rab7 [28]. Autophagosome-lysosome fusion allows mixing of their contents and degradation of the cargo of the autophagosome by lysosomal acid hydrolases, which include proteases, nucleases, lipases, glycosidases, and phosphatases. The degraded products are then transported back to the cytosol for recycling. Constitutive levels of autophagy are required for cell survival and function in most organs, including the liver. Autophagy therefore has vital cellular functions even when not activated. The overall process of autophagy is summarised in Figure 1.

The formation of autophagy is commenced by ULK1 kinase complex. This process can be inhibited by mTOR. The process of membrane development involves beclin-1 a process that can be inhibited by Bcl2. Following this various Atg conjugation systems are involved in membrane elongation before the double membrane autophagic vacuoles fuse with the lysosome to form the autophagolysosome and degrade the autophagic vacuole cargo for recycling.

2.2. Chaperone Mediated Autophagy (CMA)

In CMA, soluble proteins with a specific pentapeptide motif are recognised by the chaperone protein Hsc70 for translocation to the lysosome, where binding to the lysosome-associated membrane protein type 2A (LAMP-2A) receptor leads to protein internalization and degradation [29]. Similar to autophagy, CMA is constitutively active and increases with cellular

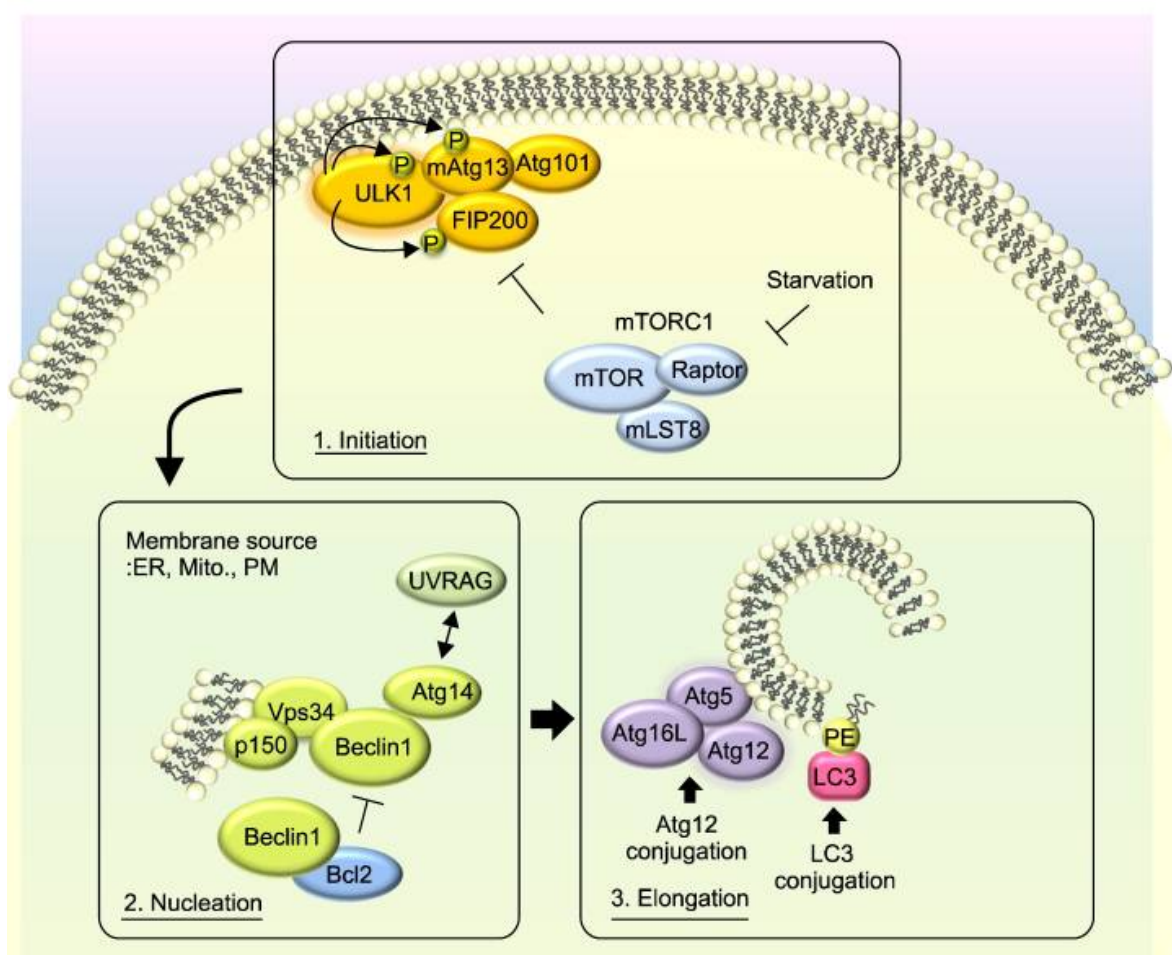


Figure 1. The Process of Autophagy.

stresses. CMA function in the liver has not been well studied [30], although CMA has been shown to mediate hepatocyte resistance to oxidant stress [31]. In addition few studies attempt to distinguish between CMA and autophagy. However, although autophagy and CMA are distinct pathways, they are likely to interact and a reduction in one pathway may lead to activation of the other. This interaction can complicate interpretation of the effects of inhibiting either one [32]. It is likely that both autophagy and CMA serve similar purposes within the cell.

2.3. Microautophagy

Microautophagy is a non-inducible, lysosomal internalisation of cellular constituents that occurs by invagination of the lysosomal membrane. Much of the data concerning microautophagy has focused upon the role of microautophagy of the mitochondria termed mitophagy [33]. Mitochondria are the central executioners for apoptosis and many times damaged mitochondria trigger cell death. If damaged mitochondria are limited to a fraction of mitochondria within the capacity of autophagic removal, cell death will be avoided. Nevertheless,

cells may eventually die if the number of damaged mitochondria is too overwhelming to be removed by autophagy. Thus, autophagy may increase the threshold for the death stimuli. Accumulating evidence demonstrates that pharmacologic or genetic inhibition of autophagy greatly enhanced cell death [34]. The trafficking of mitochondria to lysosomal structures has long been known to occur in liver, and the livers of mice with a conditional knockout of Atg7 develop massive hepatomegaly marked by the accumulation of deformed mitochondria [4]. The process of lysosomal removal of mitochondria by autophagy was first characterized in hepatocyte models by Lemasters *et al* [2]. Before removal of dysfunctional mitochondria, mitochondria undergo the mitochondrial permeability transition (MPT), in which inner mitochondrial membrane pores open, leading to adenosine triphosphate (ATP) depletion from the uncoupling of oxidative phosphorylation and outer membrane rupture with release of pro-apoptotic factors. The MPT induces mitophagy, because the MPT inhibitor cyclosporine blocks this process. Indeed after liver injury LC3-positive structures appear in hepatocytes in the area of injury [2]. These findings indicate that a mechanism exists for the selective targeting of MPT-damaged mitochondria for autophagic degradation. Recent studies in non-hepatic cells have implicated Atg32 [35], the Bcl-2 family member Nix [36], and the ubiquitin ligase Parkin [37] in this process, but further studies are required to determine whether these proteins mediate selective mitophagy in hepatocytes.

Mitochondrial damage that induces the MPT leads to death of hepatocytes [38], and these findings provide a mechanism by which insufficient or impaired microautophagy can promote hepatocyte cell death. These processes may become even more prominent during ischaemia and hypoxia that is often encountered during liver disease. Selective mitophagy might be a mechanism to protect against hepatocyte death, because removal of mitochondria that have undergone the MPT could prevent mitochondrial oxidative stress, ATP depletion, or the release of pro-apoptotic factors. The contribution of mitophagy to the response of the hepatocyte to various death stimuli needs to be more carefully examined.

Aside from mitophagy, microautophagy can also selectively occur in the endoplasmic reticulum [39] and peroxisomes [40]. These latter two processes will not be discussed further in this chapter.

3. Autophagy in liver diseases

As one of the most metabolically active organs, the liver plays a central role in regulating the overall organisms energy balance by controlling carbohydrate and lipid metabolism. The liver functions as a major buffering system to maintain the homeostasis of macro- and micronutrients to allow other tissues to function normally under physiological stress. Liver-targeted autophagy deficiency results in accumulation of protein aggregates, damaged mitochondria, steatosis and liver injury. These findings support a pro-survival and cyto-protective role of autophagy in maintaining protein, lipid and organelle quality control by eliminating damaged proteins and organelles as well as excessive lipid droplets in the liver during stress. In addition, accumulating evidence now indicates that autophagy is also involved in hepatocyte cell death,

steatohepatitis, hepatitis virus infection and HCC. The role of autophagy in a variety of liver diseases is discussed below.

3.1. Autophagy and liver Ischaemia Reperfusion Injury (IRI)

Liver IRI occurs during many clinical scenarios within the liver. It involves a period of oxygen and nutrient deprivation due to the lack of blood (ischaemia) followed by the re-introduction of blood to the liver (reperfusion). IRI is well known to have detrimental effects upon the liver and can induce considerable liver injury [41]. IRI is an obligatory part of liver transplantation but also occurs as part of the clinical scenario involved in haemorrhagic shock, abdominal trauma and liver resectional surgery. The ischaemia associated with these processes induces the formation of reactive oxygen species (ROS) that can mediate liver parenchymal injury [41, 42]. The main cellular target for the IRI is the hepatocyte. The restoration of blood flow although of undoubted benefit to the liver can perpetuate the accumulation of ROS and further accentuate liver damage [42, 43]. The precise role of autophagy within the context of oxidative stress within the liver remains controversial unlike the roles of apoptosis and necrosis that have firmly established as detrimental [43]. Recent evidence suggests that autophagy is primarily a cyto-protective mechanism during liver IRI *in vitro* at least [19].

A variety of experimental approaches have been used to assess the role of autophagy within the liver during IRI. *In vitro* studies have shown that hepatocytes up-regulate autophagy in a ROS-PI3K-Atg protein dependent manner to protect against primarily apoptotic cell death principally by inducing mitophagy [19]. It must be stressed however that these isolated studies have focused only upon autophagy within hepatocytes and the role of autophagy in cholangiocytes, endothelial cell and hepatic stellate cells remains to be established. These observations have also been extended to suggest that autophagy also limits necrotic cell death during ischaemia [44]. Various cellular pathways have been shown to be important in autophagy mediated hepato-protection during ischaemia including calcium-calmodulin dependent protein kinase IV [45], PI3K [19] and AMPK [46]. The emerging consensus *in vitro* appears to be that autophagy is a cyto-protective mechanism although this remains contentious [47].

In vivo autophagy appears to protect the liver against ischaemia and oxidative stress [47, 48] although studies report both increase and decrease in autophagy after liver ischaemia [49-51]. These observations are likely to reflect the method used to assess autophagy in liver tissue. For instance an increase in Atg protein levels does not necessarily equate to autophagy induction.

Very few studies have assessed the effect of autophagy in human livers. Domart *et al* study does provide some data as to the role of autophagy in patients undergoing liver surgery [52]. In this study the surgical technique of ischaemic preconditioning (IPC) was used to assess whether it was hepatoprotective. IPC involves short periods of total liver ischaemia followed by reperfusion with the premise being that this increases the resistance of the liver to oxidative stress. In this study, two liver biopsies were taken, one prior to ischemia required by liver resection and another after liver reperfusion. Although overall the study did not show any overall benefit from IPC a subgroup of patients who underwent IPC for 10 minutes followed by 10 minutes of reperfusion before the prolonged ischemia required by liver resection showed

a significant increase in liver cell autophagy [52]. This suggests that in this context, autophagy enhancement could allow for decreasing liver cell death.

Studies assessing autophagy during and after liver transplantation give contradictory results [44, 53, 54]. The explanation for such discrepancies may be the solution for cold preservation used. Indeed, a decrease in autophagy was observed in a study using a histidine–tryptophan–ketoglutarate cold-storage solution for 24 h cold preservation, while the contrary was reported when using the University of Wisconsin (UW) cold-storage solution. Importantly, UW cold storage solution does not contain amino acids. It is well demonstrated that amino acid depletion rapidly induces autophagy [55] and that anoxia decreases autophagy protein level. This induction of autophagy due to the absence of amino acids, may explain not only the apparent discrepancy between these studies but also the protection of the liver obtained with preservation solution such as the UW solution. Indeed, hypoxia/reoxygenation induces mitochondrial dysfunction [42]. Due to the decrease in autophagy proteins induced by anoxia/reoxygenation, autophagy fails to remove dysfunctional mitochondria, so that the mitochondria laden with ROS and calcium undergo the MPT, which in turn leads to uncoupling of oxidative phosphorylation, energetic failure, ATP depletion, and ultimately cell death. In case of associated nutrient depletion, autophagy is enhanced and facilitates autophagy of damaged mitochondria, leading to cell survival. This hypothesis is supported by the beneficial effect on liver tolerance to IRI of several strategies aiming at increasing autophagy in murine models [48, 49, 56]. It is striking to notice that many of the studies that suggest that inhibiting autophagy could ameliorate liver tolerance to ischemia used non-specific inhibitors of autophagy known to also have autophagy independent activities. This demands the development of specific autophagy inhibitors that will allow the dissection of autophagic pathways.

The role of autophagy in liver repair and regeneration will not be considered in this chapter but autophagy level decreases following partial hepatectomy suggesting a shift from the physiological steady state between anabolism and catabolism to the positive balance which is required for the compensatory growth of the liver after partial hepatectomy [57]. Finally no studies have yet specifically evaluated the autophagic pathway in liver sinusoidal endothelial cells or cholangiocytes. This is a limitation in understanding the effect of autophagy in liver IRI since these cells are also sensitive to ischemia and lesions to these cells are a key event in this context.

3.2. Autophagy and Alcoholic Liver Disease (ALD)

At the cellular level chronic alcohol abuse can result in mitochondrial damage, inhibition of insulin signaling, steatosis, apoptotic and necrotic cell death, all of which can be regulated by autophagy. Indeed, ethanol exposure is known to induces autophagy in primary cultured mouse hepatocytes [58] and in hepatoma cells expressing alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (Cyp2E1) [59]. Indeed ethanol treated hepatocytes demonstrate increased autophagosome number when compared to controls as assessed by electron microscopy and suppression of autophagy exacerbates alcohol-induced liver injury [58]. In ALD cell death may still occur in the presence of cyto-protective autophagy as in alcoholic patients have

other pathophysiological conditions such as diabetes, hyperinsulinemia, obesity and hepatitis C virus (HCV) that impair autophagy.

The majority of ethanol is metabolized in the liver and individuals who abuse alcohol by routinely drinking 50-60 g of ethanol per day are at risk for developing ALD [60]. ALD can ultimately progress to HCC. The pathogenesis of liver disease from alcohol abuse comes from the interaction of several factors, including the generation of oxidants and reactive metabolites from ethanol oxidation, which, in turn, causes other metabolic derangements [61]. Chronic alcohol consumption leads to liver steatosis and protein accumulation within the liver [62]. Furthermore chronic ethanol consumption slows down the catabolism of long-lived proteins in rodent livers [63] with reduction in the number of autophagosomes in hepatocytes [64]. This protein accumulation contributes to the formation of Mallory Denk (M-D) bodies in liver cells the hallmark of ALD [65]. Moreover recent evidence indicates that autophagy can degrade these insoluble complexes such as M-D bodies but clearance is hampered by an ethanol-elicited suppression of autophagy.

Several mechanisms have been suggested to contribute to ethanol-induced autophagy within the liver, including ROS production, ethanol metabolism and the suppression of mTOR although the precise mechanisms remain the subject of controversy. Oxidants such as ROS derived from ethanol metabolites such as acetaldehyde and malondialdehyde-acetaldehyde may impair autophagy [66]. Furthermore ethanol-induced ROS production can induce the expression of cytochrome Cyp2E1 and damage mitochondria [59]. ROS can also activate autophagy by the inactivation of Atg4B [67]. However, whether ethanol-induced ROS also inactivate Atg4B to promote autophagy in hepatocytes remains unknown. It is important that ROS may regulate the activity of many Atg proteins.

Chronic ethanol exposure may induce lysosomal fragility in an analogous manner to iron-induced oxidative stress [68]. Furthermore It is worth noting that lysosomes seem to exhibit differential sensitivity to ethanol levels in the serum [69] but even low levels of serum ethanol appear to be impair autophagy [70]. Ethanol-induced suppression of autophagy may result from alterations in hepatic amino acid pool sizes, especially those of leucine, which have been deemed regulatory amino acids and suppressors of autophagy. L-leucine is a potent autophagic suppressor reducing autophagy in chronic ethanol administration in rats when compared to control [71]. Thus, the association of an ethanol-induced reduction in autophagy with higher levels of intrahepatic leucine may partially explain autophagic suppression in the ethanol-fed state. Leucine accumulation could reflect a reduced ability of the liver to synthesize proteins, which indeed occurs in ethanol-fed animals [71].

Autophagic suppression by ethanol is well documented to disrupt protein trafficking in the liver. As discussed above autophagy requires the action of cytoskeletal elements to aid autophagosome formation and subsequent fusion with other vesicular bodies [72]. Disruption of vesicular movement within the hepatocyte by ethanol treatment occurs by mechanisms that are independent of the molecular motors, dyenin and kinesin, although there is evidence for alterations in the protein, dynamin [73]. Trafficking of exogenous proteins into the hepatocyte by endocytosis and the intracellular delivery of proteases to lysosomes are both inhibited by ethanol consumption. Furthermore, the anti-secretory properties of ethanol in the liver are well

documented. Studies with liver slices and cultured cells indicate that ethanol metabolism is required for disruption of these protein trafficking events [74]. *In vitro* investigations also revealed that acetaldehyde, the initial product of ethanol oxidation, inhibits the polymerization of tubulin to form microtubules, indicating that the reactive metabolite may impair protein trafficking by forming adducts with tubulin subunits, thereby blocking their polymerization into microtubules [75].

Acute and chronic ethanol treatment also suppress Akt function in the liver, through the upregulation of the PTEN (phosphatase and tensin homolog) phosphatase [76]. Suppression of mTOR leads to the activation of the downstream ULK1 complex to trigger autophagy. It remains to be seen whether alcohol can affect the ULK1 complex. In addition to mTOR, ethanol-induced autophagy also requires the activation of beclin 1/VPS34 PI3K complex because 3MA, a PI-3 kinase inhibitor, suppresses acute ethanol-induced autophagy. Furthermore, ethanol has been shown to suppress proteasome activity, induce ER stress and activate JNK in hepatocytes, and all of these mechanisms have been shown to induce autophagy in non-hepatocyte models. Whether proteasome inhibition, ER stress and JNK activation play a role in ethanol-induced autophagy in hepatocytes needs to be further studied.

In addition to the accumulation of potentially toxic proteins, ethanol also causes mitochondrial damage [77, 78]. It is crucial that such damaged mitochondria be removed by mitophagy. While there is no firm evidence of ethanol-elicited suppression of mitophagy, the detection of increased numbers of damaged mitochondria in livers of ethanol-fed animals provides circumstantial evidence of mitophagy inhibition. Autophagy protects against ethanol-induced toxicity in livers of mice. Reagents that modify autophagy might be developed as therapeutics for patients with ALD [58].

Finally it has been speculated that ethanol may inhibit autophagy because chronic ethanol consumption reduces AMPK activity in the liver. However the role of AMPK in autophagy is still controversial although AMPK agonists such as metformin significantly protect against ethanol induced liver injury.

3.3. Autophagy and viral hepatitis

Besides the physiological function of autophagy in maintaining cellular homeostasis detailed above, autophagy is a newly recognized facet of the innate and adaptive immune system. Hepatotrophic viruses such as hepatitis C virus (HCV) and hepatitis B virus (HBV) have developed strategies to subvert and manipulate autophagy for their own survival benefit [79]. The role of HCV and HBV will be considered separately below.

3.3.1. HCV

Several studies have assessed the autophagic pathway in hepatocytes infected with HCV both *in vitro* and in liver biopsies from chronic HCV patients [80]. Using various experimental approaches including LC3, Atg5 or Beclin-1 immunoblotting, electron microscopy or GFP-LC3 immunofluorescence, studies consistently demonstrated an accumulation of autophagic vacuoles in HCV-infected hepatocytes [81]. Importantly, other viruses such as vesicular

stomatitis virus and mutant herpes simplex virus 1 can be captured and eliminated by the autophagic pathway [82] but HCV has evolved to avoid and subvert autophagy using multiple strategies [83]. HCV appears to avoid its recognition by the cellular autophagic machinery [84]. This is based upon studies showing no or rare co-localization of HCV proteins with autophagic vacuoles.

HCV prevents the maturation of the autophagosome into an autolysosome [85]. For instance in HCV infected hepatocytes there is an increase in the number of autophagic vacuole without enhancement in autophagic protein degradation. Secondly, there is an absence of co-localization of lysosomes with autophagic vacuoles in HCV-infected cells in contrast to nutrient starved cells. Thirdly, there is a reduction in the number of autophagic vacuoles following HCV elimination and finally the absence of increase in the number of late autophagic vesicles in hepatocytes from chronic HCV patients as compared to controls, while a strong augmentation in the number of autophagic vesicles is observed. This may be related to a lack of fusion between autophagosome and lysosome.

HCV also utilizes functions or components of autophagy to enhance its intracellular replication [86]. Indeed, it has been recently shown that autophagy proteins are required for translation and/or delivery of incoming HCV RNA to the cell translation apparatus [87]. However, autophagy proteins are not needed for the translation of progeny HCV once replication is established since down-regulation of autophagy proteins 10 days after transduction had no effect on HCV replication. Therefore it is suggested that by remodelling endoplasmic reticulum membranes, the autophagy proteins or autophagic vesicles might provide an initial membranous support for translation of incoming RNA, prior to accumulation of viral proteins and the eventual establishment of virus-induced cellular modifications [88]. Alternatively, autophagy proteins might contribute directly or indirectly to the cytoplasmic transport of the incoming RNA to cellular factors or sites that are required for its translation. Importantly, autophagy proteins are required neither for HCV entry nor for HCV secretion. Altogether, these data explain the apparent contrast between the results of some *in vitro* studies reporting the implication of autophagy proteins in HCV replication and the absence of correlation between the number of autophagic vacuoles or the LC3-II level and the HCV load in chronic hepatitis C patients: autophagy proteins are required only for initial steps of HCV replication, but not once replication is established.

Notably, cytosolic RNA-sensing protein kinase PKR and eIF2- α phosphorylation regulate virus- and starvation-induced autophagy [89]. It is tempting to speculate that recognition of the incoming HCV RNA by RNA-sensing molecules induces autophagy and hence, favours its initial translation. Alternatively, constitutive basal autophagic vesicle formation might be required for this initial HCV RNA translation. The above observations suggest that autophagy proteins are pro-viral factors for HCV and can be manipulated to facilitate HCV infection of the liver.

3.3.2. HBV

HBV also induces autophagosomes in hepatocytes, as demonstrated both *in vitro* in several liver derived cell lines [90] and *in vivo* in the liver of transgenic mouse lines harboring HBV

DNA [91]. Importantly, this induction was also observed in the liver of an HBV-infected patients but not of a non-infected patients [92]. In contrast to HCV, HBV enhances the autophagic flux, as late autophagic vacuoles could be detected in mouse hepatocytes using electron microscopy and given the existence of an extensive co-localization of lysosome-associated membrane protein 1 (LAMP1) with GFP-LC3 puncta [93]. However, without being able to provide the reason for it, no significant increase in protein degradation was observed in HBV DNA-transfected cells.

An HBV-encoding protein, HBx, plays a crucial role in this HBV-induced autophagy. Indeed, transfection of Huh7.5 cells with an HBV unable to express HBx did not enhance autophagy [94]. Moreover, expression of HBx alone was sufficient to induce autophagy; similar results were obtained *in vivo* in transgenic mice [91]. This effect of HBx is due, at least partly, to its ability to bind to class III PI3K, a regulatory molecule that controls autophagy. Although conflicting, HBx may also up-regulate the transcription of beclin-1 thus sensitizes the cells to starvation-induced autophagy. Whether the role of HBx is confined to short nutrient starvation conditions or also exists in normal conditions remains controversial. If, in the same way as HCV, HBV subverts autophagy, the strategy applied is somewhat different. Autophagy enhances HBV replication mostly at the step of viral DNA replication, slightly at the step of RNA transcription, and not at other levels. How autophagy may enhance HBV DNA replication remains unresolved.

The question whether HBV could be engulfed in autophagic vacuoles is not fully elucidated despite the observation that HBV core/e antigens and surface antigens partially co-localized with autophagic vacuoles. Immuno-electron microscopy studies would be required to address this issue. However, as HBV seems to benefit from autophagy proteins and as the autophagic protein degradation rate is not increased, this hypothesis seems unlikely and autophagic vacuoles may rather serve as the sites for viral DNA replication and morphogenesis.

3.4. Autophagy and Hepatocellular Carcinoma (HCC)

The incidence of HCC is increasing worldwide primarily through the increase of cirrhosis secondary to viral hepatitis. The role of autophagy in the development of cancer has been comprehensively reviewed recently [95]. Autophagy is generally thought to be an anti-tumour mechanism in cells. The tumor suppressor role of autophagy is not yet clear but may involve limiting chromosomal instability, restricting oxidative stress and reducing intratumoral necrosis and local inflammation. The role of autophagy in the regulation of neoplasia has originated from observation where demonstrating mono-allelically deletion of Beclin-1 in 40–75% of cases of human breast, ovarian, and prostate cancer [96]. Moreover, the regulation of autophagy overlaps closely with signaling pathways that regulate tumorigenesis.

Studies assessing autophagy in HCC have clearly demonstrated *in vitro*, in mice and in patients that, in this context, autophagy is a tumor suppressor mechanism. This follows the general paradigm that autophagy is a cyto-protective mechanism. Murine models with heterozygous disruption of Beclin-1 have a high frequency of spontaneous HCC [97]. Moreover, crossing beclin-1 +/- mice, with mice, that transgenically express the HBV large-envelope polypeptide under the transcriptional control of the mouse albumin promoter,

resulted in the acceleration of the development of hepatitis B virus-induced small-cell dysplasia [98]. This maybe the prelude to the development of HCC. Expression of several autophagic genes such as Atg5, Atg7 and Beclin-1 and their corresponding autophagic activity is decreased in HCC cell lines compared to that in a normal hepatic cell line [99]. Similarly, Beclin-1 mRNA and protein levels are lower in HCC tissue samples than in adjacent non-tumor tissues from the same patients [100].

The most aggressive malignant HCC cell lines and HCC tissues with recurrent disease display much lower autophagic levels than less aggressive cell lines or tissues, especially when the anti-apoptotic B-cell leukemia/lymphoma (Bcl)-xL protein is over-expressed [101]. Interestingly, in a tissue microarray study consisting of 300 HCC patients who underwent curative resection, the expression of Beclin-1 was significantly correlated with disease-free survival and overall survival only in the Bcl-xL+ patients. Multivariate analyses revealed that Beclin-1 expression was an independent predictor for disease-free survival and overall survival in Bcl-xL+ patients [101]. In addition, there was a significant correlation between Beclin-1 expression and tumor differentiation in Bcl-xL+ but not in Bcl-xL- HCC patients. These data suggest that autophagy defect synergizes with altered apoptotic activity and facilitates tumor progression and poor prognosis of HCC. The role of other Atg proteins in the development and progression of HCC remains to be established but on the basis of the data with Beclin-1 it would appear that these proteins would also have anti-neoplastic effect.

The mechanisms responsible for this low autophagy protein level are not elucidated. However, a recent study has demonstrated that HAb18G/CD147, a transmembrane glycoprotein highly expressed in HCC, contributes to this decreased autophagic level in HCC through the class I phosphatidylinositol 3-kinase-Akt pathway upregulation [102]. Other oncoproteins such as the Bcl-2 family proteins may also be implicated in HCC, like in other cancers. Stimulation of hypoxia-inducible factors (HIFs) due to hypoxic stress within HCC may also contribute to autophagy modulation [103]. However many of these signaling pathways have not been conclusively shown to be involved in HCC progression. It does remain an attractive notion that these pathways are modulated by HCC leading to decrease levels of autophagy and increased susceptibility to HCC. Indeed pharmacological therapies that inhibit autophagy improves survival of patient undergoing liver transplantation for HCC when compared to non-HCC recipients suggesting the specificity of its beneficial impact for cancer patients [104]. In addition ROS accumulation, and DNA damage also facilitates the development and progression of HCC [105]. In studies carried out using tetrandrine, a calcium channel blocker, it regulated the expression of Atg7, which then promoted tetrandrine-induced autophagy [106]. In vitro and in vivo tetrandrine caused the accumulation of ROS and induced cell autophagy in a tumor xenograft model. Therefore, these findings suggest that tetrandrine is a potent autophagy agonist and may be a promising clinical chemotherapeutic agent [106]. It was further demonstrated that oroxylin A-triggered autophagy contributed to cell death using over-expression of Atg5 and Atg7 and inhibition of autophagy by siBeclin 1 and 3-methyladenine (3-MA) [99]. In vivo study, oroxylin A inhibited xenograft tumor growth and induced obvious autophagy in tumors. These findings define and support a novel function of autophagy in promoting death of HCC cells [99]. Furthermore miR-375 inhibits autophagy by

reducing expression of Atg7 and impairs viability of HCC cells under hypoxic conditions in culture and in mice. miRNAs that inhibit autophagy of cancer cells might be developed as therapeutics [107].

These data provide potential therapeutic targets to modulate the development of HCC. However until the precise regulatory role of autophagy in HCC is established these treatment cannot be used in the clinic.

3.5. Autophagy and other liver diseases

The role of autophagy in other liver disease has yet to be firmly established. However limited studies have been conducted in some liver diseases. In primary biliary cirrhosis (PBC) autophagy is specifically seen in the damaged small bile ducts along with cellular senescence. The inhibition of autophagy suppressed cellular senescence in cultured cells suggesting that autophagy may mediate the process of biliary epithelial senescence and be involved in the pathogenesis of bile duct lesions seen in PBC [108]. Furthermore the expression of LC3 was seen in coarse vesicles in the cytoplasm of bile ductular cells and significantly more frequently in PBC of both early and advanced stages when compared to control livers. Autophagy is frequently seen in bile ductular cells in ductular reactions (DRs) in PBC. Since cellular senescence of bile ductular cells is rather frequent in the advanced stage of PBC, autophagy may precede cellular senescence of bile ductular cells in DRs in PBC [109]. The aggregation of p62 is specifically increased in the damage bile ducts in PBC and may reflect dysfunctional autophagy, followed by cellular senescence in the pathogenesis of bile duct lesions in PBC [110]. Whether the same occurs in other biliary diseases such as primary sclerosing cholangitis is not known.

Recent reports have suggested a role for autophagy in alpha-1-antitrypsin (AT) deficiency where a mutant protein activates autophagy [111]. Autophagy is thought to be involved in the degradation of the mutant protein and hence defective autophagy may contribute to the hepatic fibrosis seen in AT [111].

Furthermore the role of autophagy in NAFLD, NASH and metabolic liver disease remains the subject of on-going research.

4. Conclusion

This chapter has outlined the extensive role played by autophagy within the liver and its role in various liver diseases. In general autophagy appears to be primarily a cyto-protective mechanism within the liver and especially hepatocytes. Autophagy induction appears to protect the liver from IRI and appears to negatively regulate neoplasia and reduce the effects of alcohol. However the autophagy cell machinery can be used by viral infections and biliary disease to aid disease propagation within the liver. It is important that aside from disease pathogenesis autophagy may have a role in liver repair and regeneration that has not been considered here.

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