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Obesity, autophagy and the pathogenesis of liver and pancreatic cancers

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Abstract

Liver and pancreatic cancers are both highly lethal diseases with limited to no therapeutic options for patients. Recent studies suggest that deregulated autophagy plays a role in the pathogenesis of these diseases by perturbing cellular homeostasis and laying the foundation for disease development. While accumulation of p62 upon impaired autophagy has been implicated in hepatocellular carcinoma, it's role in pancreatic adenocarcinoma remains less clear. This review will focus on recent studies illustrating the role of autophagy in liver and pancreatic cancers. The relationships between autophagy, nuclear factor-κB signaling and obesity in hepatocellular carcinoma will be discussed, as well as the dual role of autophagy in pancreatic adenocarcinoma.

Keywords

autophagy; hepatocellular carcinoma; p62; nuclear factor-κB; pancreatic ductal adenocarcinoma

Introduction

Autophagy, or "self-eating," was discovered as a proteolytic process activated upon starvation to recycle cellular building blocks for new protein synthesis and energy production[1]. Autophagy is controlled by a series of ubiquitin-like protein modification reactions which promote formation and subsequent elongation of the phagophore or isolation membrane that enwraps cytoplasmic constituents and organelles leading to an enclosed double membrane structure called the autophagosome[1]. Eventually, the autophagosome fuses with the lysosome and its contents are degraded by various lysosomal acid hydrolases. Mechanistically, this process is controlled by conjugation of the ubiquitin-like protein LC3 (ATG8) with phosphatidylethanolamine (PE) through an enzymatic cascade catalyzed by ATG7, ATG3 and ATG12-ATG5[1]. PE-conjugated LC3, termed $LC3_{II}$, is present on both the inner and outer isolation membranes, serving as a recognition site for LC3-binding chaperones, such as p62, that deliver their cargo to autophagosomes[2]. In addition to its role in cell survival during periods of starvation, autophagy has emerged as a major quality control mechanism required for maintenance of cellular homeostasis[3]. Constitutive autophagy prevents accumulation of misfolded and unfolded proteins, which would otherwise form inclusion bodies, and damaged organelles, including non-functional mitochondria that leak electrons and produce reactive oxygen species (ROS). It is

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autophagy's role in degrading damaged cellular components that when deregulated promotes the pathogenesis of a number of diseases, including hepatocellular carcinoma and pancreatic adenocarcinoma as discussed below.

Autophagy & Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC), the most common form of liver cancer, is the third leading cause of cancer-related deaths worldwide, and only second to pancreatic cancer as the most aggressive and incurable[4]. HCC frequently develops in patients with chronic liver disease (CLD) caused by hepatitis B or C infections, chronic alcohol consumption, hemochromatosis, exposure to liver toxins and obesity[5,6]. These conditions induce hepatocyte death, thereby eliciting a cyclical inflammatory response that further triggers cell death and subsequent compensatory proliferation, laying the foundation for eventual development of liver fibrosis, cirrhosis and/or HCC. As treatment options for HCC patients are limited at best, surgical resection with or without subsequent liver transplantation is currently the most effective way to combat disease, yet for most patients the cancer is too advanced upon diagnosis to qualify for this procedure. Therefore, a better mechanistic understanding of the molecular pathogenesis of HCC is imperative to generate novel therapeutic solutions that can regress disease progression.

Suppressed autophagy has been linked to a number of cancers as the essential autophagy gene, *beclin 1*, is monoallelically deleted in 40–75% of cases of human breast, ovarian, and prostate cancers[7]. This observation prompted mouse genetic studies which showed that mice heterozygous for *beclin 1* develop spontaneous cancers in the liver, as well as lung and lymphoid tissues, indicating that *beclin 1* is a haplo-insufficient tumor suppressor gene[8]; yet, because *beclin 1* is known to regulate the endocytic pathway in addition to autophagy[7], the effect of defective autophagy on tumorigenesis remained elusive. More definitive evidence of suppressed autophagy playing a causal role in HCC was recently shown in mice with liver-specific deletion of the autophagy gene, *Atg7* (*Atg7*$^{\Delta hep}$)[9]. These mice, as well as mice mosaically deleted for *Atg5*[10], which both contain mutations that prevent autophagosome-formation, display severe hepatomegaly accompanied by hepatocyte hypertrophy and chronic liver injury[9]. Hepatocytes from these mice exhibit deformed mitochondria, accumulation of ubiquitin- and p62-containing aggregates, increased oxidative stress and genomic instability[9,10]. Eventually, these mice develop spontaneous hepatocellular adenomas[10,11], most likely as a result of chronic liver damage and oxidative stress, yet the exact mechanism remains to be fully elucidated. Interestingly, these tumors lacked a malignant morphology, suggesting that defective autophagy may lead to deregulated proliferation but needs to be coupled with other genetic changes that result in dedifferentiation and full acquisition of the transformed phenotype.

Remarkably, other than organelle accumulation, most of the pathologies observed in livers of autophagy-deficient mice, including tumor development, are suppressed by whole body ablation of p62, a multifunctional protein that directs ubiquitinated protein aggregates to autophagosomes for degradation and which accumulates in autophagy-deficient hepatocytes[12]. Curiously, p62 accumulation was also observed in a variety of human chronic liver diseases, including alcoholic hepatitis, NASH (non-alcoholic steatohepatitis) and HCC[13,14]. In fact, p62 together with polyubiquitinated proteins to which it binds[2] is a major component of Mallory bodies, which are found in many CLDs, and hyaline bodies which are found in HCC[13,14]. Accumulation of p62 is a sign of impaired autophagy[2] and therefore these findings strongly suggest that chronic liver diseases and HCC development are either caused by or strongly associated with an autophagy defect.

Accumulation of p62 also results in stabilization of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2)[15], which regulates expression of detoxifying enzymes that protect the cell from oxidative and electrophilic stresses[16]. Nrf2 constitutively interacts with the E3 ubiquitin ligase adaptor protein, Keap1 (kelch-like ECH-associated protein 1), targeting it for proteasomal degradation. Upon exposure to ROS and electrophiles, Keap1 is inactivated, resulting in stabilization and nuclear localization of Nrf2. p62 competitively binds the Nrf2-binding site of Keap1 such that accumulation of p62, as in autophagy-deficient hepatocytes, enables stabilization of Nrf2 and transcription of its target genes[15]. Recent reports have suggested that persistent activation of Nrf2 promotes pancreatic[17] and liver tumor[11] development, most likely by reducing oxidative stress that would otherwise induce cell death and thereby enabling proliferation of cells that contain genomic aberrations. In support of this notion, p62- and Keap1-positive aggregates were detected in over a quarter of human HCCs with most tumors highly expressing Nrf2 target genes[11]. Therefore, while p62 causes oxidative stress and ROS accumulation[18], it also induces expression of antioxidant genes through Nrf2 stabilization[15], further complicating the protumorigenic nature of this multifunctional protein.

NF-κB Signaling & Hepatocellular Carcinoma

While multiple signaling pathways have been implicated in regulating autophagy, it has been shown *in vivo* that hepatocytes depleted of IKKβ (IκB kinase β), one of two catalytic subunits of the IKK kinase complex responsible for NF-κB (nuclear factor κB) activation, are defective in starvation-induced autophagy[19]. NF-κB, in response to pro-inflammatory stimuli, regulates cell survival, immunity, and inflammation[20], and mouse models of liver carcinogenesis have revealed that NF-κB signaling plays an important role in liver injury and inflammation. In fact, we found that liver-specific depletion of IKKβ (*Ikkβ* $^{\Delta hep}$) increases the susceptibility of mice to chemically-induced HCC upon exposure to the chemical carcinogen, diethylnitrosamine (DEN)[20,21]. DEN is metabolically activated in zone 3 hepatocytes[20], forming DNA adducts, and when administered to mice at 2 weeks of age, or to adult mice in conjunction with a tumor promoter, induces hepatocyte death, compensatory proliferation and eventual HCC development[21], closely resembling human HCC with poor prognosis[22]. DEN-injected *Ikkβ* $^{\Delta}_{hep}$ mice exhibit increased ROS production, which results in persistent activation of Jun kinases (JNK)[21] and the STAT3-activating kinase, JAK2[23], as a result of oxidation and inactivation of their respective phosphates[23,24]. Dietary administration of the antioxidant butylated hydroxyanisole (BHA) to *Ikkβ* $^{\Delta hep}$ mice prevents sustained JNK and STAT3 activation and suppresses DEN-induced carcinogenesis[21,23]. Moreover, crossing *Ikkβ* $^{\Delta hep}$ mice to *Jnk1*$^{-/-}$ mice suppresses hepatocyte death, compensatory proliferation and HCC development[21]. Liver-specific *Stat3* knockout mice also display resistance to DEN-induced hepatocarcinogenesis[23]. These results illustrate the significance of NF-κB-dependent transcription of antioxidant genes, such as superoxide dismutase 2 (SOD2) and ferritin heavy chain (FHC), in preventing ROS accumulation and negatively controlling JNK and STAT3 activation. Interestingly, depleting IKKβ in both hepatocytes and resident liver macrophages (Kupffer cells) reduces hepatocarcinogenesis as a result of decreased secretion of growth factors and inflammatory cytokines from Kupffer cells and consequently, reduced compensatory proliferation of hepatocytes[21]. The importance of NF-κB signaling in preventing hepatocarcinogenesis has also been demonstrated by others using mice with hepatocyte-specific depletion of the regulatory subunit of the IKK kinase complex, IKKγ/NEMO (*Ikkγ* $^{\Delta hep}$)[25], and the IKK complex activating kinase, TAK1 (*Tak1*$^{\Delta hep}$)[26]. Both mouse models are completely defective in NF-κB signaling, unlike *Ikkβ* $^{\Delta hep}$ mice, which retain residual IKK activity likely due to compensation from the remaining catalytic subunit, IKKα[20]. Correspondingly, *Ikkγ* $^{\Delta hep}$ and *Tak1*$^{\Delta hep}$ mice exhibit spontaneous liver injury, hepatosteatosis, fibrosis and HCC development without exposure to a chemical carcinogen[25, 26].

We have also found that NF-κB signaling is important for obesity-promoted chemically-induced HCC development[27]. Epidemiological studies have shown that obesity increases risk of cancer-related deaths for a number of cancers with the strongest enhancement seen in HCC[28]. Fatty liver disease, or hepatosteatosis, is commonly caused by excessive dietary fat intake and is quite prevalent in the United States, affecting up to 24% of the population[29]. Hepatosteatosis can progress into a chronic inflammatory response (steatohepatitis) as a result of cellular damage caused by lipid overload, increasing the likelihood of progression to HCC[6, 28]. Moreover, our laboratory recently reported that obesity, through enhanced expression of the pro-inflammatory cytokines TNF and interleukin 6 (IL-6) and activation of the transcription factor STAT3, promotes hepatosteatosis followed by chronic liver inflammation and tumorigenesis in mice[27]. Mice exposed to DEN at two weeks of age and then maintained on a high-fat diet (HFD) following six weeks of age develop more HCC in comparison to DEN-injected mice kept on a low-fat diet. Remarkably, ablation of either type I TNF receptor (TNFR1) or IL-6 reduced JNK and STAT3 activation, decreased hepatosteatosis, steatohepatitis and HCC development[27]. Interestingly, autophagy has been shown to regulate lipid metabolism in the liver and $Atg7^{\Delta}{}_{hep}$ mice display hepatosteatosis[30], suggesting that defective autophagy may contribute to the increase in hepatosteatosis and enhanced chemically-induced HCC development observed in obese mice.

As both $Ikk\beta^{\Delta hep}$ and obese mice exhibit defective autophagy[19, 31], we are currently investigating whether defective autophagy contributes to the increased susceptibility of these mice to HCC. Despite its key role in liver pathophysiology, the role of autophagy and its contribution to chemically-induced liver damage has not been investigated. In agreement with previous reports that autophagy is suppressed in $Ikk\beta^{\Delta hep}$ mice[19], we observed accumulation of p62 in these mice without any stimulus. Exposure to DEN further enhanced p62 accumulation in $Ikk\beta^{\Delta hep}$ mice, suggesting that DEN and other toxic chemicals may cause or exacerbate an autophagy defect. Remarkably, concurrent deletion of *Stat3* in hepatocytes prevents p62 accumulation and restores p62 levels to those of wild type (WT) mice, indicating that STAT3 negatively regulates autophagy. As STAT3 activation is imperative for hepatocarcinogenesis in both $Ikk\beta^{\Delta hep}$ and obese mice, we are curious to determine whether STAT3's role in autophagy and p62 accumulation is what drives tumorigenicity in these models. As both STAT3 activation and defective autophagy correlate with poor prognosis in human HCC[23, 32, 33], our results imply that these clinical observations are linked and that targeting STAT3 in patients may be an effective approach to stimulating autophagy and promoting HCC regression and/or prevention.

Dual roles of autophagy in pancreatic cancer

In addition to hepatocellular carcinoma in the liver, dysfunctional autophagy has also been linked to pancreatitis, in which an autophagic morphology is detected in the pancreas[34]. Pancreatitis, together with old age, smoking, alcohol abuse and obesity, is a risk factor for pancreatic ductal adenocarcinoma (PDAC), the major type of pancreatic cancer and one of the deadliest malignant diseases with median survival of less than 6 months after conventional therapy and 5-year overall survival rate of less than 5%[35]. Resistance to conventional therapy and its aggressive nature make PDAC an incurable cancer. Curiously, all PDAC risk factors can lead to impaired autophagy[1]. In PDAC, autophagy is a double-edged sword: during the tumor initiation stage autophagy functions as a tumor suppressor, while in advanced PDAC cells autophagy provides a survival function and promotes tumor growth under stress, including hypoxia, nutrient deprivation and chemotherapy[36].

Autophagy serves as a barrier to limit tumor initiation of PDAC

Autophagy is responsible for lysosomal clearance of protein aggregates and damaged organelles, especially damaged mitochondria that produce reactive oxygen species (ROS)[7]. Impaired autophagy can occur due to perturbation of any step of the autophagy process, leading to accumulation of protein aggregates and damaged organelles (including mitochondria), thereby causing ER stress and ROS accumulation[7]. ROS can cause DNA damage, genomic instability and tumorigenesis[37]. In addition, inhibition of autophagy increases necrosis and inflammation, which is partially responsible for the tumorigenesis by providing a protumorigenic inflammatory microenvironment.

Almost all of human PDACs carry gain-of-function mutations in *K-Ras* gene, suggesting that K-Ras mutation may represent an initiating event[38]. It is generally believed that PDAC initiated from metaplastic conversion of normal cells to noninvasive ductal precursors, which form pancreatic intraepithelial neoplasia (PanIN) lesions[35]. Mouse models have revealed that mutant K-Ras can reprogram pancreatic acinar cells to PanIN/PDAC lineage via a process termed acinar to ductal metaplasia (ADM). There is ample evidence that DNA damage response gene expression and genomic instability occur in PanIN lesions[39]. Furthermore, oncogenic Ras can stimulate autophagy[40], and autophagy plays a special role in survival of Ras transformed cells[40]. Oncogenic K-Ras induces proliferative arrest or premature senescence and that transformation of K-ras expressing cells requires cooperating oncogenes and inactivation of tumor suppressors, such as p16INK4a or Trp53[41,42]. It was demonstrated that autophagy is induced during oncogene-induced senescence (OIS)[43], and induction of the autophagy protein ULK3 was sufficient to stimulate autophagy and premature senescence[43]. Autophagy is an important component required for efficient establishment of the senescent phenotype[43,44] and its impairment facilitates escape from Ras-induced senescence, thereby contributing to increased tumorigenesis. Senescence is an early barrier to oncogenesis whose abrogation due to an autophagy defect may increase cancer incidence. As a result, two questions need to be addressed: how autophagy facilitates K-Ras-induced senescence and whether autophagy defects stimulate pancreatic tumorigenesis via a bypass of K-Ras-induced senescence. Related to this, it was reported that Ras induces autophagy via upregulation of the BH3-protein Noxa and the autophagy regulator Beclin 1, and this may limit the tumorigenic potential of K-Ras transformed cells[45]. Silencing of Noxa or Beclin 1 reduced Ras-induced autophagy and increased the tumorigenic potential and clonogenic survival of transformed cells[45].

One consequence of defective autophagy is accumulation of p62 aggregates[12]. Accumulation of p62 was observed in human pancreatitis and PDAC samples (unpublished data). p62, a multidomain signaling adaptor protein, acts as a signaling hub to recruit and oligomerize important signaling molecules to control cell survival and apoptosis[2]. p62 directly interacts with LC3, localized on autophagosomes[2]. p62 recruits polyubiquitinated, misfolded, aggregated proteins and dysfunctional organelles for clearance by autophagy, and p62 itself is cleared through autophagy[2]. p62 accumulation has been observed in autophagy-deficient mice[12], and it has been proposed that autophagy suppresses tumorigenesis via elimination of p62[18]. In addition to enhanced ER stress, oxidative stress and DNA damage caused by p62 accumulation, p62, as a signaling molecule, has been shown to be essential for NF-κB activation[2,46]. Ras-induced transformation was impaired in immortalized embryonic fibroblast from $p62^{-/-}$ mice due to reduced NF-κB activity[46]. Loss of p62 reduces formation of Ras-induced lung adenocarcinoma[46]. In HCC, persistent activation of transcription factor Nrf2 due to excess p62 accumulation contributes to HCC development in autophagy-deficient mice[11]. Similarly, K-Ras-induced expression of Nrf2 promotes ROS detoxification and tumorigenesis in pancreatic cancer[17]. Whether or not p62 regulates the

turnover of Nrf2 in PDAC or PanIN lesions remains unclear. Curiously, however, low grade PanINs from Nrf2-deficient mice are proliferative and demonstrate more senescence[17].

Defective autophagy is also involved in pancreatitis[34], which contributes to PDAC through inhibition of K-Ras-induced senescence[42]. Pancreatitis is an important risk factor for PDAC. It has been demonstrated that autophagy is impaired in acute pancreatitis[34], which mediates both acinar cell vacuolation and trypsinogen activation[34]. It was indicated that autophagic flux is reduced in pancreatitis due to deficient lysosomal degradation caused by impaired cathepsin processing[34]. Caerulein-induced pancreatitis promotes oncogenic transformation of adult pancreatic acinar cells by oncogenic K-Ras[42,47]. Perhaps this tumor promoting effect of caerulein is related to its effect on autophagy[42,47].

Autophagy supports cancer cell survival in advanced PDAC

Whereas autophagy suppresses tumor initiation, established cancer cells may depend on autophagy for survival. In established tumors, autophagy is up-regulated in hypoxic regions, where cancer cells use the catabolic function of autophagy to tolerate stress[36]. Basal autophagy is low in lower-grade PanIN1 and PanIN2, which may allow the accumulation of ROS and genomic instability, promoting tumor initiation. However, basal autophagy is elevated in high-grade PanIN3 and PDAC[48]. Particularly, in pancreatic cancer cell lines with K-Ras mutations, basal autophagy is elevated due to K-Ras-induced autophagy[48]. Compared to other human cancer cell lines, PDAC cell lines exhibit elevated basal autophagy, which makes them more sensitive to autophagy inhibition[48]. Furthermore, PDAC requires autophagy to maintain energy homeostasis and tumor growth[48]. However, how autophagy is involved in energy generation and biosynthesis remains unclear. It has been indicated that autophagy is required to maintain the pool of functional mitochondria necessary to support growth of Ras-driven tumors[49]. Upon matrix detachment, increased numbers of $Atg5^{-/-}$ cells continue to proliferate relative to $Atg5^{+/+}$ controls. However, unlike nontransformed autophagy-deficient cells, loss of *Atg5* impairs rather than enhances the ability of $H\text{-}Ras^{V12}$ or $K\text{-}Ras^{V12}$ transformed embryonic fibroblasts (MEFs) to proliferate during extracellular matrix (ECM) detachment[49]. Increased glycolysis, a main strategy to generate energy in cancer cells, in autophagy-competent cells facilitates Ras-driven adhesion-independent transformation and proliferation in Ras-transformed MEFs[50]. Genetic (RNAi-mediated silencing of ATG genes) or pharmacologic inhibition of autophagy leads to ROS accumulation, DNA damage and metabolic defects, resulting in tumor regression and prolonged survival in PDAC xenograft and genetic models[48]. Transformation by oncogenic K-Ras in PDAC may cause addiction to autophagy to maintain energy balance for tumor growth. Elevation of basal autophagy possibly serves as an adaptation to prevent accumulation of ROS generated by Ras-induced transformation, reduce oxidative stress and provide key intermediates to sustain cell metabolism and maintain tumor growth. Autophagy is required for tumorigenic growth of pancreatic cancer, and drugs that inactivate the autophagy process may have a unique clinical utility for treating pancreatic cancer. Chloroquine (CQ) and its derivatives, which block lysosomal acidification and autophagosome degradation, inhibiting autophagy, and have been used safely in human patients for other purposes, are now being tested in treatment of pancreatic cancer. As pancreatic cancer is sensitive to autophagy inhibition but resistant to other conventional therapies, this may prove to be an effective approach to regressing disease.

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