HHS Public Access

Author manuscript

Gastroenterology. Author manuscript; available in PMC 2015 May 26.

Published in final edited form as:

Gastroenterology. 2015 March ; 148(3): 501–505. doi:10.1053/j.gastro.2015.01.012.

Impaired Autophagy Triggers Chronic Pancreatitis: Lessons From Pancreas-Specific *Atg5* Knockout Mice

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A few years ago, autophagy would require a detailed background introduction, but more and more, this fundamental cell biology mechanism of lysosome-driven degradation and recycling of cellular organelles, long-lived proteins, and lipids is becoming a common knowledge. Its main pathway, macroautophagy (herein referred to as autophagy), sequesters material destined for degradation into autophagosomes, which then fuse with lysosomes, forming the autolysosomes where cargo is degraded. The degradation products are recycled for the cell's energy and biogenesis needs. Autophagosome formation is a complex process governed by sequential formation of protein complexes controlled by evolutionarily conserved *ATG* (autophagy-related) genes; ATG5, ATG7, and ATG8/LC3 are among the key ATG proteins. Autophagy is a major quality control mechanism and is critical for cell survival in starvation and other stress conditions.

Numerous reviews (eg, references[1–4]) discuss diverse roles of autophagy in health and disease, which have been studied more extensively in some disease states (neurodegenerative diseases, cancer) and organs (heart, and more recently, liver[5]) than in others. The essential function of autophagy as a homeostatic mechanism is demonstrated by the embryonic lethality of mice with general deletion of major Atg proteins (eg, Atg5 or Atg7) and pathologic changes in specific organs targeted by conditional autophagy-gene knockouts.[2,6] Further, impaired autophagy is increasingly implicated in the pathogenesis of various diseases.[1–3,5–8] Autophagy impairment can be caused by defects in autophagosome formation, their fusion with lysosomes, and lysosomal degradative function. Impaired autophagy often manifests itself in accumulation of large cytoplasmic vacuoles containing undegraded or partially degraded material. Accumulation of such vacuoles in acinar cells is a long-noted feature of both human and experimental pancreatitis; however, the investigation of the status and roles of autophagy in normal and diseased exocrine pancreas has started only recently (reviewed in references[7,8]). In particular, it was shown[9] that autophagic flux is impaired in various experimental models of acute pancreatitis, mediating not only vacuolization but also increased intra-acinar trypsin activity, a signature response of pancreatitis. However, genetic approaches to examine the role of autophagy in pancreatitis

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Conflicts of interest

The authors disclose no conflicts.

have not been actively pursued[10,11]; in fact, the study by Diakopolous et al[12] in this issue of *Gastroenterology* represents the first detailed analysis of the effects of genetic ablation of a key autophagy mediator in pancreas.

The authors crossed "floxed" *Atg5*$^{F/F}$ mice and *Ptf1a-Cre* mice expressing Cre recombinase under control of the transcription factor Ptf1a that directs pancreas organogenesis, [13] to generate mice (termed *A5*) in which Atg5 is specifically deleted in acinar and islet cells at a distinct developmental age. Loss of Atg5 triggered the development of chronic pancreatitis (CP), with fibrosis, macrophage-type inflammation, acinar-to-ductal metaplasia, apoptosis, and pancreatic atrophy that progressed from 4 to 36 weeks of age. Such progression is also characteristic of human CP. Serum lipase was increased at the early (4 weeks) but not late (18 weeks) time point, reminiscent of human CP; intrapancreatic trypsin and cathepsin B activities were also increased at 4 weeks. A block in normal autophagy was manifest by a decrease in the autophagosomal marker LC3-II, a dramatic increase in p62/SQSTM1 (sequestosome 1), a protein degraded through autophagy, and the appearance of large ("improperly formed"[12]) autophagic vacuoles containing undegraded material. Dilated endoplasmic reticulum (ER) was also prominent in acinar cells early on.

Surprisingly, the development of CP, with all pathologic responses, was much less pronounced in female than in male *A5* mice. Because the early pancreas damage (at 4 weeks) was morphologically similar between males and females, the authors postulate that pancreas regeneration in *A5* mice is sex dependent, but another possibility is that female pancreas is more resistant to the effects of impaired autophagy. Whatever the mechanism, these findings present a mechanistic correlate to the known greater prevalence of CP in male human population.[14]

The *Ptf1a-Cre*–driven recombination occurs during pancreas development, and the defective autophagy impacted both acinar and islet cells in *A5* mice. Similarly, both exocrine and endocrine pancreas destruction was found[11] in *Pdx-Cre;Atg7* and *Pdx-Cre;Atg5* conditional knockouts (in which *Cre* expression is driven by Pdx1, another pancreatic progenitor transcription factor[13]), although the tissue damage was not studied in detail. These findings prompted a question about the primary role of the exocrine vs. endocrine compartment in pancreas injury. Diakopoulos et al[12] present several lines of evidence indicating that β-cell dysfunction (strictly speaking, diabetes) is not the primary driver of CP in *A5* mice. Thus, although insulin treatment of male *A5* mice reduced serum glucose levels, it did not rescue acinar cells from damage. Furthermore, inducing loss of insulin by streptozotocin treatment did not cause pathologic alterations in acinar tissue of *Ela-Cre*^{ER}*;Atg5* mice (expressing inducible *Cre*^{ER} under *ElastaseI* promoter) in which Atg5 is specifically deleted in adult acinar cells.

The focus of the Diakopoulos et al study[12] is on cellular and molecular pathways perturbed by impaired autophagy in *A5* pancreas. Transcriptomic analysis revealed marked enrichment of gene sets associated with pathways related to inflammation (ie, innate and adaptive immunity, cytokines), fibrosis, cell stress, and apoptosis. Autophagy is emerging as a major regulator of cellular metabolism,[15] and the metabolomic analysis showed disturbances of multiple pathways in *A5* pancreas. Mitochondrial dysfunction was manifest by abnormal

ultrastructure, lower activities of respiratory complexes, and dramatic decreases in CREB, a key transcription factor driving mitochondrial biogenesis. Based on these analyses, the authors stress the importance of reactive oxygen species (ROS) accumulation and reduced availability of anabolic substrates caused by glutamate deficiency (although limited information is presented on the latter). They further focus on the p62/Nrf2/Nqo1/p53 pathway. Nrf2, a key transcription factor regulating antioxidant gene expression, is activated by ROS and p62; Nqo1, a target of Nrf2, stabilizes p53 protein, a major driver of apoptosis. The expression of Nrf2- and p53-regulated genes was enhanced in *A5* pancreas. To elucidate the role of these pathways, Diakopoulos et al[12] generated *A5;p62* and *A5;p53* pancreas-specific double knockouts by crossing corresponding "floxed" mouse strains. Pancreas damage was reduced in each of these double knockouts (compared with *A5* alone), with less inflammation, fibrosis, acinar cell apoptosis, and compensatory proliferation. The results show conclusively that both p62 and p53 mediate the effects of impaired autophagy in *A5* pancreas.

To validate the detrimental effects of ROS accumulation and metabolic disturbances, male *A5* mice were given a diet containing palm oil, a rich source of antioxidants and fatty acids; a separate cohort received the antioxidant *N*-acetylcysteine. These treatments not only reduced ROS levels and cardiolipin oxidationin *A5* pancreas (as might be expected), they improved pancreatic morphology, preserved acinar tissue, and alleviated apoptosis. However, there was no improvement of endocrine dysfunction. On a molecular level, palm oil diet counteracted the p62 increase and CREB decrease in *A5* pancreas. (Of note, palm oil was shown[16] to improve CP in an experimental model.) Finally, some features of the *A5* pancreatic phenotype were displayed in human tissue from CP patients, including dilated ER, damaged mitochondria, and accumulation of p62 (previously reported[17]), Nqo1, and p53. However, exon sequencing of *ATG5* in 267 patients with hereditary or idiopathic CP (159 female and 108 male) did not find any genetic alterations associated with CP.

The work by Diakopoulos et al[12] is bursting with data that should stimulate broadly future basic research. These include issues related to the mechanisms of observed effects, for example, deficiencies in glutamate-dependent metabolism (does glutamate level decrease in *A5* pancreas?); or the decreased mRNA expression of p53, which is usually regulated at the protein level. Another aspect is the effects of Nrf2 activation known to be "context dependent" (promoting apoptosis, inflammation, and fibrosis in Atg5-deficient liver,[18] but suppressing oxidative stress and apoptosis in cardiomyocytes[19]).

More important, there is much to be learned about the pathways that can link defective autophagy with pancreatitis pathologies, namely, inflammation, necrosis, and fibrosis. Some of these pathways, both established and putative, are illustrated in Figure 1 based on the findings from[12] as well as other studies.[7–9,17,20–24] In particular, 1 mechanism whereby impaired pancreatic autophagy can promote inflammation is through activation of the p62–TRAF6–nuclear factor-κB pathway, leading to cytokine/chemokine release.[8,25] Another likely mechanism[8,21] involves inflammasome activation caused by ROS increase and the release of DAMPs (danger/damage-associated pattern molecules) from damaged mitochondria and necrotic cells. Of note, deficient autophagic clearance hinders the elimination of both inflammasomes and damaged mitochondria.[1–3,5] p62 accumulation also

can promote pancreatic damage by activating ER stress, as shown in the CP model induced by IKKα deficiency.[17] Recent evidence[22] indicates an important role for maladaptive ("terminal"[12]) ER stress in the pathogenesis of pancreatitis, but the underlying mechanisms remain largely unknown. Both ER stress and p53 up-regulation promote apoptosis and thus pancreatic atrophy. The *A5;p62* and *A5;p53* double knockouts generated in Diakopoulos et al's study[12] should help to clarify these issues. Pathways linking impaired epithelial autophagy and fibrosis, in general, are only starting to be unraveled,[5,26] but the vicious cycle of inflammation and necrosis is a major driver of pancreatic fibrosis, providing, in particular, cytokines and other factors that mediate the activation and proliferation of stellate cells.[24] The mechanisms whereby impaired autophagy can induce pancreatic necrosis (Figure 1) include mitochondrial dysfunction that leads to decreased cellular ATP,[23] and intra-acinar trypsinogen activation.[8,9,20]

As stated, pancreas-specific Atg5 knockouts driven by the developmental regulators Ptf1a[12] or Pdx1[11] both caused pancreas damage. In contrast, acinar tissue morphology remained normal in *Ela-Cre^{ER};Atg5* mice[12] in which Atg5 deletion was triggered by tamoxifen treatment specifically in adult acinar cells (expressing inducible *Cre^{ER}*). This is in agreement with the results of an earlier study[10] that used noninducible *Ela-Cre* to generate *Ela-Cre;Atg5* mice in which Atg5 is deleted in acinar cells starting from late embryo. One possible reason for the limited pancreatic damage in *Ela-Cre;Atg5* mice is the known suboptimal efficiency of the *Ela-Cre* driver.[27] However, the previous study[10] reported an improvement in acute cerulein pancreatitis in *Ela-Cre;Atg5* mice; this issue needs to be revisited.

The study by Diakopoulos et al[12] provides strong evidence that autophagy is critical to pancreas homeostasis and its impairment leads to pancreatitis. This conclusion is in accord with recent findings in other genetic models[11,17] and experimental pancreatitis models.[7–9,28] Further, the study uncovers cellular and molecular mechanisms mediating the effects of impaired autophagy in pancreas. One may speculate that autophagy is of particular importance to the pancreatic acinar cell, which relies on coordinated function of various organelles (ER, zymogen granules, endolysosomes, mitochondria) to maintain the high level of protein synthesis and secretion. Not only is there a profound autophagy impairment in various experimental models of pancreatitis (induced by administration of cerulein, choline-deficient/ethionine supplemented diet or L-arginine,[9] or by a combination of ethanol diet and lipopolysaccharide[28]), but, remarkably, alterations in many disparate pathways, which in one way or another cause impaired autophagy, all result in pancreas damage. Besides Atg5[11,12] and Atg7[11] knockouts, the examples (discussed in references[7,8,17]) include genetic ablation of Spink3, IKKα, LAMP-2, and inactivation of the mannose-6-phospate pathway of hydrolase delivery to the lysosome. The fact that screening of CP patients[12] has not (so far) found changes in *ATG5* does not rule out a causative role for genetic alterations in autophagy regulating proteins in human disease. Although the search has only begun, it has revealed that p62 accumulation, a marker of dysfunctional autophagy, is prominent in human CP.[12,17] Because multiple routes lead to defective autophagy, mutations in other proteins affecting ATG protein levels or ability to regulate autophagy, could promote pancreatitis.

The novel genetic model of CP,[12] using deletion of a key autophagy mediator, opens new venues for both elucidating the pathogenic mechanism of pancreatitis and developing/testing therapeutic approaches. One question, which would need to be clinically tested, is whether palm oil[12,16] may be effective in patients with CP (although, some say, it tastes terrible).

Acknowledgments

The authors thank Fred Gorelick for critical reading of this editorial.

Funding

Our research on autophagy in pancreatitis is supported, fully or in part, by NIH grants DK059936, AA019730, and DK098108.

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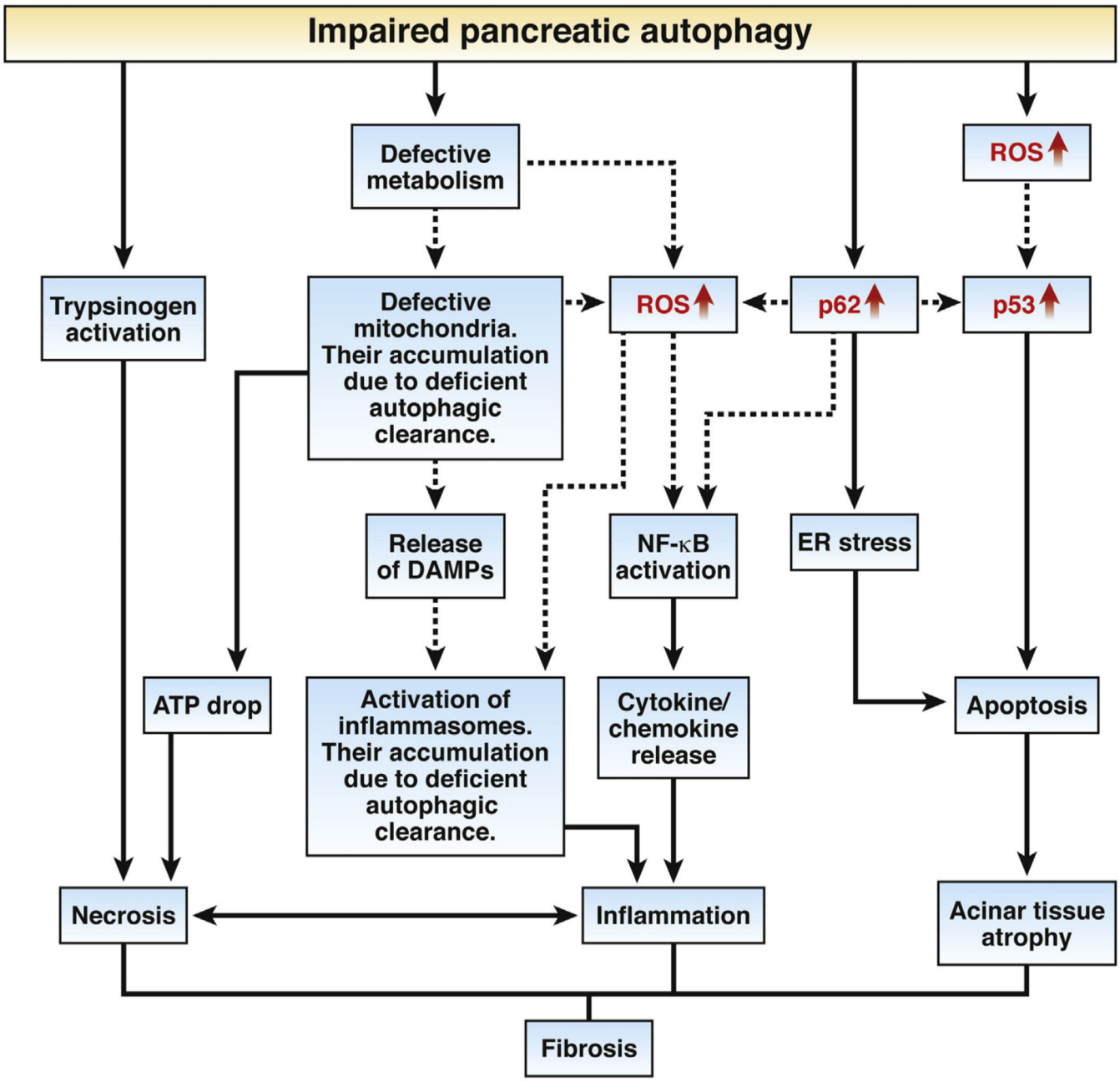

Figure 1.

Pathways linking impaired autophagy to pancreatitis. Solid lines indicate pathways operating in pancreas, based on the results of Diakopoulos et al[12] and other studies.[7–9,17,20–24,28] Dashed lines indicate pathways that are likely to be involved but not yet proven in the pancreas. Key molecular signals identified in Diakopoulos et al[12] are shown in red.