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Autophagy impairment in pancreatic acinar cells causes zymogen granule accumulation and pancreatitis

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ABSTRACT

Basal autophagy degrades many kinds of proteins and organelles to maintain quality and plays important roles in cellular homeostasis. However, the impact of basal autophagy on zymogen granules in pancreatic acinar cells is unknown. In the present study, we examined the influence of autophagy impairment in acinar cells on zymogen granules and homeostasis of the pancreas, using mice with pancreas-specific autophagy impairment (*Pdx1-Cre*$^{+/-}$ *Atg7*$^{fl/fl}$ mice). The number of zymogen granules in acinar cells from these mice did not differ from that in acinar cells from their wild-type littermates at 3 weeks of age. However, the number of zymogen granules in acinar cells drastically increased at 4 weeks of age in mice with pancreas-specific autophagy impairment. In addition to the increased number of zymogen granules, serum lipase was elevated, and the pancreas became oedematous at 4 weeks of age, suggesting pancreatitis. After 5 weeks of age, acinar cell death was accelerated, and several histological features of chronic pancreatitis were observed, including glandular atrophy and pseudotubular complexes with fibrotic changes. In conclusion, the impairment of pancreas-specific basal autophagy caused spontaneous zymogen granule accumulation in acinar cells and pancreatitis, which eventually led to chronic pancreatitis.

1. Introduction

Macroautophagy (hereafter, autophagy) serves as a degradation system for proteins and organelles in the cytoplasm [1,2]. During the early stage of autophagy, autophagosomes enclose some proteins and organelles by a lipid bilayer and isolate them from the cytoplasmic fraction of the cell. These autophagosomes then fuse with lysosomes, leading to the degradation of proteins and organelles within the autophagosomes [1,2]. Proteins and organelles that are degraded by autophagy can be used as an energy source, and autophagy is induced by starvation [1,2]. Degraded proteins and organelles can also be used for the synthesis of new proteins or organelles. Therefore, the basal level of autophagy plays important roles in maintaining proteins and organelles.

In pancreatic acinar cells, autophagosomes are observed under physiological conditions [3]. Recently, the suppression of autophagy in acinar cells was reported to induce chronic pancreatitis in pancreas-specific Atg5-knockout (KO) mice [4], pancreas-specific Atg7-KO mice [5] or LAMP-2-KO mice [6]. Diakopoulos et al. reported that autophagy impairment in acinar cells causes mitochondrial dysfunction and p62 up-regulation, leading to an increase in reactive oxygen species, which causes acinar cell death [4]. These reports support the idea that basal autophagy is essential for maintaining acinar cells, which is similar to other cells, including neural cells [7] and hepatocytes [8]. One of the characteristics of acinar cells is the presence of zymogen granules [9–11]. Pancreatic zymogen granules are defined as cytoplasmic vesicles that contain inactive precursors of enzymes (zymogens), such as trypsinogen, proelastase and prolipase [9–11]. Trypsinogen is the precursor of trypsin and is activated by enteropeptidase, which is secreted by the duodenal mucosa [9–11]. Since the activation of zymogen granules in acinar cells induces self-digestion of the pancreas, which leads to pancreatitis, it is important for unreleased-zymogen granules to remain inactivated to avoid causing pancreatitis [9–11].

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Regarding the relationship between autophagy and pancreatitis, Gukovsky, I et al. reported that autophagy was impaired in pancreatitis, as evidenced by the accumulation of abnormally large vacuoles, which were considered to be autolysosomes that contained poorly degraded material [12,13]. Decreased autophagic function was also reported in an ethanol/lipopolysaccharide model of pancreatitis [14]. However, the significance of autophagy for zymogen activation and the pathophysiology of pancreatitis has not yet been sufficiently clarified. In addition, the roles of basal autophagy in the control of zymogen granules and homeostasis of the pancreas under physiological conditions remain unclear.

In the present study, we addressed these issues by using pancreas-specific KO mice for Atg7 (*Pdx1-Cre*$^{+/-}$ *Atg7*$^{fl/fl}$ mice), which codes an essential protein for executing autophagy [8]. We revealed that pancreas-specific Atg7 KO spontaneously increased the number of zymogen granules in acinar cells and caused pancreatitis in mice at 4 weeks of age. Following the pancreatitis, these mice exhibited continuous acinar cell death and histological findings consistent with chronic pancreatitis. These results revealed that basal autophagy is essential for the control of zymogens and homeostasis of the pancreas.

2. Materials & methods

2.1. Mice

Mice that contain an *Atg7* gene with 2 loxP sequences [8] were provided by the RIKEN BioResource Research Center (BRC) through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT) in Japan. Pdx-Cre transgenic mice were obtained from the National Cancer Institute (NCI) Mouse Repository (Frederick, MD). We generated pancreas-specific Atg7-KO mice by crossing the two strains. All animal experiments were performed in accordance with protocols approved by the institutional animal committee. All mice were treated humanely.

2.2. Western blotting

Samples of pancreas tissues were lysed with lysis buffer, and the protein concentrations were adjusted as described previously [15]. The proteins were electrophoretically separated, followed by blotting. We used the following antibodies as primary antibodies: an anti-Atg7 antibody from Cell Signaling Technology (Danvers, MA), an anti-LC3 antibody from MBL Life Science (Nagoya, Japan), an anti-β-actin antibody from Cell Signaling Technology and an anti-trypsinogen antibody from Abcam (Cambridge, UK).

2.3. Electron microscopy

Pancreases were fixed by cardiac perfusion using a 2.5% glutaraldehyde solution buffered at pH 7.4 with 0.1 M Millonig's phosphate and postfixed with a 1% osmium tetroxide solution. After dehydration in graded concentrations of ethanol, the samples were embedded in Nissin EM Quetol 812 epoxy resin. Ultrathin sections with a thickness of 80 nm were cut with an ultramicrotome. The sections were stained with uranyl acetate and lead citrate and observed with a Hitachi H-7650 electron microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) at 80 kV.

2.4. Primary culture

To evaluate the secretory capacity of pancreatic acinar cells, exocrine pancreatic cells were isolated and incubated as described below [16–20]. According to the method of Geron, E et al. [20], the basic medium for the experiment contained 140 mM NaCl, 4.7 mM KCl, 1.16 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM glucose, 10 mM HEPES (pH 7.4 with NaOH), 0.1 mg/ml soybean trypsin inhibitor (Sigma Aldrich, T9003), and 1% bovine serum albumin (Merck Millipore, 810033). The dissociation medium also contained 75 U/ml purified collagenase (Worthington, LS005273). Pancreas tissues were divided into fragments with scissors, shaken in dissociation medium for 10 min (120 cycles/min) at 37 °C, pipetted up and down through polypropylene pipettes (first with a 2 mm orifice and then with a 0.9 mm orifice), filtered through 160 μm nylon mesh filters (Merck Millipore, NY6H 090 00) and washed three times with basic medium. The tissues were incubated with or without 50 pM or 100 pM cholecystokinin (CCK) (Sigma Aldrich, C9026) or 1 μM carbachol (Sigma Aldrich, PHR1511). Samples were incubated and culture supernatants were removed to measure the amylase levels in the medium at each time point. Amylase levels were measured using Phadebas® Amylase test tablets (Shino-Test Corporation, Tokyo, Japan). Just before incubation, some samples were lysed with the culture medium, and the amylase levels of the whole samples were measured. The amylase secretion ratio after X-hour culture was calculated by the following formula: ((amylase level of medium after X-hour culture) - (amylase level of medium before culture))/((amylase level of whole sample before culture) - (amylase level of medium before culture)).

2.5. Histologic examination

Formalin-fixed, paraffin-embedded (FFPE) tissues from the mouse pancreas were sectioned (4 μm) and stained with haematoxylin and eosin (HE). To assess fibrosis, the pancreas sections were stained with Sirius Red.

2.6. Assessment of chronic pancreatitis

Chronic pancreatitis was evaluated according to the histological grading scale for chronic pancreatitis [21,22]. Briefly, areas of abnormal architecture were scored from 0 to 4, and the presence of glandular atrophy, pseudotubular complexes and fibrosis within abnormal areas were each scored from 0 to 3. The total of these scores (0–13) was used to evaluate chronic pancreatitis.

2.7. TUNEL staining

To detect apoptotic cells, the pancreas sections were subjected to terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labelling (TUNEL) staining, as previously described [23].

2.8. Biochemistry

Mouse serum was used to measure amylase and lipase levels with commercially available assays (Nagahama Life Science Laboratory, Nagahama, Japan).

2.9. Statistical analysis

Bar graphs present the standard mean ± standard deviation (SD). Comparisons of histology scores between two groups were analysed by the Mann-Whitney *U* test. Unpaired t-tests were used for other comparisons between two groups. $P < 0.05$ was regarded as significant.

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