Involvement of autophagy in trypsinogen activation within the pancreatic acinar cells

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Atg5 and Atg7 are required for autophagy-related 5 (ATG) genes that are required for autophagosome formation (Klionsky et al., 2003; Klionsky, 2005).

A
tophagy is mostly a nonselective bulk degrada-
tion system within cells. Recent reports indicate
that autophagy can act both as a protector and
killer of the cell depending on the stage of the disease or the
surrounding cellular environment (for review see Cuervo,
cytoplasmic vacuoles induced in pancreatic acinar cells
by experimental pancreatitis were autophagic in origin,
as demonstrated by microtubule-associated protein 1 light
chain 3 expression and electron microscopy experiments.

Introduction

There are at least three types of autophagy: (1) macroautophagy,
(2) chaperone-mediated autophagy, and (3) microautophagy
(Mizushima, 2005). Although autophagy is mostly a nonselective
bulk degradation system within cells, chaperone-mediated
autophagy is a selective degradation system in which cytoplasmic
proteins are refolded and transported into the lysosome
lumen across the membrane (for review see Cuervo, 2004).
In microautophagy, only a small portion of cytoplasm is taken
up directly by invagination of lysosome membrane into the
lumen. As macroautophagy is believed to be the primary means
for cytoplasm to lysosome delivery, it is most commonly
referred to simply as autophagy. Genetic studies on yeast have
identified >20 autophagy-related (ATG) genes that are required for
autophagosome formation (Klionsky et al., 2003; Klionsky, 2005).

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Abbreviations used in this paper: Atg, autophagy related; CCK, cholecystokinin; H&E, hematoxylin and eosin; LC3, light chain 3; TAP, trypsinogen activation peptide.
The online version of this article contains supplemental material.

To analyze the role of macroautophagy in acute pancreatitis,
we produced conditional knockout mice lacking the
autophagy-related 5 gene in acinar cells. Acute pancreatitis
was not observed, except for very mild edema in a re-
stricted area, in conditional knockout mice. Unexpectedly,
trypsinogen activation was greatly reduced in the absence
of autophagy. These results suggest that autophagy exerts
devastating effects in pancreatic acinar cells by activation
of trypsinogen to trypsin in the early stage of acute pancre-
atitis through delivering trypsinogen to the lysosome.

Autophagy-defective yeast mutants are not able to survive during
nitrogen starvation (Tsukada and Ohsumi, 1993). Similarly, most
of the Atg5−/− and Atg7−/− mice died within 1 d after birth
(Kuma et al., 2004; Komatsu et al., 2005). Thus, autophagy is
thought to be important for the cellular response to starvation
and the normal turnover of cytoplasmic constituents.

Acute pancreatitis has long been considered to be an auto-
digestive disorder in which inappropriate activation of tryp-
sinogen to trypsin within pancreatic acinar cells leads to the
development of pancreatitis (Hirota et al., 2006). However, the
mechanisms responsible for intracellular activation of trypsin
have not been elucidated with certainty. There are two major hy-
potheses: the colocalization hypothesis (van Acker et al., 2006)
and the autoactivation hypothesis (Leach et al., 1991). According
to the former hypothesis, digestive enzymes become colocalized
with lysosomal hydrolases, such as cathepsin B, and activate
trypsinogen in cytoplasmic vacuoles of acinar cells (Steer and
Meldolesi, 1987). The latter hypothesis suggests that trypsinogen
is autoactivated under low pH conditions in the presence of serine
protease (Leach et al., 1991). However, the mode of trypsinogen
delivery to the lysosomes or cellular compartments has been the subject of investigation. There are three possible mechanisms for delivery of trypsinogen to the cellular compartment where activation occurs. One is fusion of zymogen granules with lysosomes (crinophagy; Koike et al., 1982). The second is perturbation of normal intracellular trafficking of zymogen granules and lysosomal hydrolases. The third is endocytic vacuole formation through uptake of secreted digestive enzymes by acinar cells via endocytosis, transportation to endosomes, and fusion of endosomes with lysosomes (Sherwood et al., 2007). One important clue to distinguish between these possibilities is the appearance of cytoplasmic vacuoles within pancreatic acinar cells (Watanabe et al., 1984). This is an early feature of acute pancreatitis. EM and immunohistochemical studies suggested that many vacuoles observed in both experimental and human acute pancreatitis were autophagic in origin (Helin et al., 1980; Adler et al., 1985). Our previous study suggested that autophagy was induced in the acinar cells of mice with experimental pancreatitis induced by cerulein (cholecystokinin [CCK] analogue; Ohmuraya et al., 2005). Collectively, these results indicate that vacuoles are autophagic in origin and that autophagy is somehow involved in the development of pancreatitis.

In this study, we report that cytoplasmic vacuoles induced in experimental acute pancreatitis are autophagic in origin and that absence of autophagy in the Atg5 conditional knockout mouse results in greatly reduced acute pancreatitis caused by loss of trypsinogen activation in pancreatic acinar cells. Our results suggest that trypsinogen is delivered to the endosome or lysosome through autophagosome/autolysosome formation.

**Results and discussion**

We first analyzed the origin of vacuoles in cerulein-induced experimental pancreatitis. No changes in pancreatic morphology

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**Figure 1.** Autophagy induction in pancreatic acinar cells of cerulein-induced acute pancreatitis. Overnight-starved mice were treated by saline (A) or cerulein 6 (B), 9 (C), or 12 (D) times, and pancreatic sections were analyzed by H&E staining. Insets show higher magnifications of areas indicated in A–D. (E) Serum amylase activity in mice with cerulein-induced pancreatitis. Data are shown as mean ± SEM (error bars). *, P < 0.05. Bars, 50 μm.
autophagy, we performed EM examination, which revealed vacuoles containing zymogen granules (Fig. 2 A, asterisks) in acinar cells after nine cerulein injections (Fig. 2 A). Some vacuoles contained both zymogen granules and membrane-bound organelles (Fig. 2 A, arrows), suggesting nonselective uptake. EM images after 12 cerulein injections were characterized by an increase in vacuoles containing zymogen granules and organelles and by the appearance of large vacuoles with homogeneous material that seemed to be autolysosomes (Fig. 2 B). There are two forms of microtubule-associated protein 1 light chain 3 (LC3), a mammalian homologue of yeast Atg8. LC3-I is localized in the cytoplasm and is converted into LC3-II, which is associated with the formation of autophagosomes. The cytosolic LC3-I protein (16 kD) was converted into LC3-II (14 kD), and the amount of LC3-II was correlated with the extent of autophagosome formation. GFP-LC3 mice were treated with cerulein and analyzed by fluorescence microscopy. Bars: (A and B) 2 μm; (D) 10 μm.

were observed at any time in any sample obtained from saline-injected mice (Fig. 1 A). In contrast, acute pancreatitis was observed in cerulein-injected mice. The severity of acute pancreatitis increased with the number of cerulein injections. With six and nine cerulein injections, mild edema and acinar cell degeneration were observed (Fig. 1, B and C). With 12 cerulein injections, the pancreas showed severe acinar cell degeneration with significant edema and inflammatory cell infiltration in the interstitium (Fig. 1 D). In accordance with histological changes, we observed a significant increase in serum amylase activity (Fig. 1 E). The observed increase was proportional to the cerulein dose and the severity of acute pancreatitis. To examine the induction of autophagy, we performed EM examination, which revealed vacuoles containing zymogen granules (Fig. 2 A, asterisks) in acinar cells after nine cerulein injections (Fig. 2 A). Some vacuoles contained both zymogen granules and membrane-bound organelles (Fig. 2 A, arrows), suggesting nonselective uptake. EM images after 12 cerulein injections were characterized by an increase in vacuoles containing zymogen granules and organelles and by the appearance of large vacuoles with homogeneous material that seemed to be autolysosomes (Fig. 2 B). There are two forms of microtubule-associated protein 1 light chain 3 (LC3), a mammalian homologue of yeast Atg8. LC3-I is localized in the cytoplasm and is converted into LC3-II, which is associated with the
autophagosome membrane in a phosphatidylethanolamine-conjugated form (Ichimura et al., 2000; Kabeya et al., 2000). The amount of LC3-II is thus correlated with the extent of autophagosome formation. In control mice, LC3-II was increased slightly by starvation but was virtually unchanged during saline treatment (Fig. 2 C). In mice treated with cerulein, a dose-dependent increase in LC3-II level was observed. Autophagy can also be monitored by detection of GFP fluorescence in GFP-LC3 mice (Mizushima et al., 2004). After 24-h fasting, some dots that represent autophagosomes were detected in the cytoplasm in saline-injected mice (Fig. 2 D). After cerulein injection, many dots were observed, and the number of GFP-LC3 dots from mice receiving 12 cerulein injections was larger than from mice with nine cerulein injections (Fig. 2 D). Together with EM examination, these results suggest that vacuoles are autophagic in origin.

To analyze the role of autophagy in acute pancreatitis, we established conditional knockout mice in which the Atg5 gene was deleted in pancreatic acinar cells. We obtained an EL (rat elastase I promoter/enhancer)-Cre mouse line (EL-Cre2) in which Cre was specifically expressed in the pancreas under the control of EL (Fig. 3, A and B). This line was crossed with Atg5\textsuperscript{floxed/floxed} (Hara et al., 2006) to produce Atg5\textsuperscript{floxed/floxed},EL-Cre2 mice (Fig. 3 C). Cre-mediated excision of exon 3 of the Atg5 gene was detected in the pancreas but not in other organs of Atg5\textsuperscript{floxed/floxed},EL-Cre2 (Fig. 3 D). Southern blot analysis using genomic DNA from the pancreas showed that recombination occurred in about half of the pancreatic cells (Fig. 3 E). As the pancreas contains not only acinar cells but also endocrine and duct cells, this ratio suggests that recombination occurred in most Atg5\textsuperscript{floxed} alleles of the acinar cells. The expression of Cre in acinar cells was also assessed by expression of lacZ in mice obtained by mating EL-Cre2 mice with a reporter line for monitoring Cre expression, R26R (Soriano, 1999). All acinar cells examined were stained with X-gal, suggesting that recombination occurred in all Atg5\textsuperscript{floxed} alleles of the acinar cells (unpublished data). To further demonstrate the inactivation of Atg5 in acinar cells, we analyzed induction of autophagy under starving conditions. Atg5\textsuperscript{floxed/floxed},EL-Cre2 mice and Atg5\textsuperscript{floxed/floxed} mice were starved for 24 h and examined for induction of autophagy. As Atg5 was present in nonacinar cells, autophagy may be induced in these cells.

Atg5\textsuperscript{floxed/floxed},EL-Cre2 mice were born healthy with no signs of developmental anomalies and grew without displaying any noticeable pathological phenotype after 2 mo (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200712156/DC1). There were no apparent abnormalities in blood biochemistry, such as total protein, glucose, total cholesterol, amylase, and lipase (unpublished data). Hematoxylin and eosin (H&E) staining and EM analysis revealed that acinar cells were normal (Fig. S1, B and C). The levels of amylnase and trypsinogen were the same in the pancreas of Atg5\textsuperscript{floxed/floxed},EL-Cre2 mice (Fig. S2). In addition, we examined whether the lack of Atg5 affected CCK-induced calcium signaling in acinar cells. Type 2 and type 3 inositol 1,4,5-trisphosphate receptors (IP3R2 and IP3R3) are the major Ca\textsuperscript{2+} release channels responsible for secretagogue-induced Ca\textsuperscript{2+} signaling in pancreatic acinar cells (Futatsugi et al., 2005). Basal levels of IP3R1, -2, and -3 in both Atg5\textsuperscript{floxed/floxed} and Atg5\textsuperscript{floxed/floxed},EL-Cre2 mice at 2 mo of age
pancreatic acinar cells show similar [Ca\textsuperscript{2+}] increases as those of Atg5 fl ox/fl ox mice in response to 100-pM CCK8 stimulation (Fig. S3 B). Both Atg5 fl ox/fl ox and Atg5 fl ox/fl ox;EL-Cre2 mice at 2 mo of age were used in the following experiments.

were the same as revealed by Western blot assay (Fig. S3 A). We next examined Ca\textsuperscript{2+} signaling induced by pancreatic exocrine secretagogues CCK octapeptide (CCK8) in fura-2–loaded enzymatically isolated pancreatic acinar cells. Again, Atg5-deficient pancreatic acinar cells show similar [Ca\textsuperscript{2+}] increases as those of Atg5 fl ox/fl ox mice in response to 100-pM CCK8 stimulation (Fig. S3 B). Both Atg5 fl ox/fl ox and Atg5 fl ox/fl ox;EL-Cre2 mice at 2 mo of age were used in the following experiments.

### Figure 4. Absence of cerulein-induced acute pancreatitis in acinar cell–specific Atg5-deficient mice.

Atg5 fl ox/fl ox (A and B) or Atg5 fl ox/fl ox;EL-Cre2 (C and D) mice were injected with saline or cerulein 12 times, and pancreatic sections were analyzed by H&E staining (A and C) and EM analysis (B and D). Insets show higher magnifications of areas indicated in A–D. EM analysis demonstrating the absence of autophagy in Atg5 fl ox/fl ox;EL-Cre2 mice. (E) Histological changes were quantified using a histological score by a pathologist. There was statistically significant difference in pathological changes between Atg5 fl ox/fl ox and Atg5 fl ox/fl ox;EL-Cre2 mice. Unpaired t tests were used to calculate p-values. P < 0.05 was considered a significant difference. (F) Increase of LC3-II in Atg5 fl ox/fl ox but not in Atg5 fl ox/fl ox;EL-Cre2 after cerulein treatment. (G) Serum amylase activity in cerulein-induced acute pancreatitis. Open bars, saline injection (n = 4); black bars, cerulein injection (n = 4). The asterisk indicates the statistical difference between amylase levels in Atg5 fl ox/fl ox and Atg5 fl ox/fl ox;EL-Cre2 mice [*; P < 0.05]. Error bars represent SEM. Bars: (A and C) 50 \mu m; (B and D) 2 \mu m.
Saline injection did not cause any pathological changes in both Atg5flox/flox and Atg5flox/flox;EL-Cre2 mice as revealed by histochemical and EM analyses (Fig. 4, A and C). We then induced acute pancreatitis by cerulein in Atg5flox/flox and Atg5flox/flox;EL-Cre2 mice. Atg5flox/flox mice showed typical signs of acute pancreatitis, such as severe acinar cell degeneration, edema in connective tissue, and infiltration of inflammatory cells with H&E staining (Fig. 4 B). EM analysis revealed numerous autophagosomes and a decrease of zymogen granules in Atg5flox/flox mice (Fig. 4 B). In contrast, Atg5flox/flox;EL-Cre2 mice displayed normal histochemical characteristics except very mild edema in a small restricted region (Fig. 4 D). In fact, autophagosomes were not observed, and zymogen granules appeared to be intact in Atg5flox/flox;EL-Cre2 mice (Fig. 4 D). We quantified histological changes using a histological score (Hughes et al., 1996) by a pathologist and showed that there was statistical significant difference in pathological changes by a pathologist between Atg5flox/flox and Atg5flox/flox;EL-Cre2 mice (Fig. 4 E). In accordance with histological and EM examination, LC3-II increased significantly in Atg5flox/flox mice but not in Atg5flox/flox;EL-Cre2 mice after cerulein injection (Fig. 4 F). In addition, the serum amy- lase level in Atg5flox/flox;EL-Cre2 mice with cerulein was significantly lower than in Atg5flox/flox mice (Fig. 4 G). There was a slight increase in serum amylase when Atg5flox/flox;EL-Cre2 mice were treated with cerulein (Fig. 4 G). This may be caused by stimulation of secretion of digestive enzymes by cerulein itself. Collectively, these results indicate that typical acute pancreatitis is not induced in the absence of autophagy.

The mechanism for much milder acute pancreatitis in the absence of autophagy could be the low level of trypsinogen activation. To test this hypothesis, we analyzed trypsinogen activation by cerulein in primary cultured acinar cells using a highly sensitive method for detection of trypsin activity (Ohmuraya et al., 2006). Trypsinogen activation was observed in 90% (190/211 cells) and 33% (67/202 cells) of acinar cells from Atg5flox/flox and Atg5flox/flox;EL-Cre2 mice, respectively (Fig. 5, A and B). We also
used acinar cells isolated from Atg5-deficient newborn mice (Atg5−/−) in which Atg5 was completely inactivated in all cells (Kuma et al., 2004). Trypsinogen activation was observed in 91% (182/201 cells) and 5% (11/217 cells) of acinar cells from Atg5−/− and Atg5−/− mice, respectively. Trypsinogen activation in some acinar cells may be caused by the presence of an alternative pathway for trypsin activation.

We then measured trypsin activity in pancreatic homogenates from Atg5fl/−:EL-Cre2 and Atg5fl/−:EL-Cre2 mice treated with cerulein or saline. In saline controls, there was no difference in the levels of trypsin activities between Atg5fl/−:EL-Cre2 mice (0.25 ± 0.09 mU/mg; n = 3) and Atg5fl/−:EL-Cre2 mice (0.16 ± 0.03 mU/mg; n = 3). After cerulein injection, trypsin levels significantly increased in Atg5fl/−:EL-Cre2 mice (1.97 ± 1.10 mU/mg; n = 3) but mildly increased in Atg5fl/−:EL-Cre2 mice (0.52 ± 0.41 mU/mg; n = 3; Fig. 5 C). Activation of trypsinogen into active trypsin results in the production of trypsinogen activation peptide (TAP), which corresponds to the N-terminal region of the trypsinogen. TAP was quantified in pancreatic homogenates from Atg5fl/−:EL-Cre2 and Atg5fl/−:EL-Cre2 mice treated with cerulein or saline. In saline controls, there was no difference in the levels of TAP between Atg5fl/−:EL-Cre2 mice (2.36 ± 0.22 ng/mg; n = 3) and Atg5fl/−:EL-Cre2 mice (2.08 ± 0.18 ng/mg; n = 3). After cerulein injection, TAP levels significantly increased in Atg5fl/−:EL-Cre2 mice (18.54 ± 2.04; n = 3) but not in Atg5fl/−:EL-Cre2 mice (5.65 ± 1.19; n = 3; Fig. 5 C). All of these data suggest that trypsinogen activation is considerably suppressed by reduced autophagy.

Our findings established that autophagy is induced by supramaximal stimulation of cerulein and is directly related to trypsinogen activation and onset of acute pancreatitis. This is the first example that autophagy plays a destructive role in the early stage of disease development. Although the mechanism for autophagy induction by cerulein is not yet clear, insufficient recruitment of zymogene granule membranes under supramaximal stimulation may account for it. In physiological conditions, digestive enzymes are targeted to the secretory compartment, and lysosomes are targeted to the cell lysosome (Gorelick et al., 1992). In autophagy, autophagosomes containing intracellular components fuse with endosomes and lysosomes to form autolysosomes (Dunn, 1999). Thus, trypsinogen could be hydrolyzed to trypsin by a lysosomal enzyme such as cathepsin B in autolysosomes. Thus, we propose the autophagy theory for activation of trypsin in acute pancreatitis, and this can explain both the colocalization hypothesis and autoactivation hypothesis.

Materials and methods

**Ceruline-induced pancreatitis**

After overnight fasting, mice were given hourly intraperitoneal injections of saline or saline containing 50 μg/kg ceruline (Sigma-Aldrich) for 6, 9, and 12 h. 1 h after the last injection, mice were killed, and serum and pancreas samples were rapidly obtained.

**Histological analysis**

Tissue was fixed overnight in 10% formalin, embedded in paraffin, sectioned, and stained with H&E. For EM analysis, the pancreas was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h. Conventional EM was performed as previously described (Yamamoto et al., 1991).

**Fluorescence microscopic analysis**

The pancreas from GFP-LC3 mice was dissected, fixed with 4% PFA, and sectioned, and GFP fluorescence was observed using a fluorescence microscope (IX81; Olympus) equipped with a CCD camera (ORCA ER; Hamamatsu; Mizushima et al., 2004).

**Trypsin assay in acinar cells**

Isolated acinar cells were cultured with 10 nM ceruline, and trypsin activity was examined by using a synthetic trypsin substrate, CBZ-Ile-Pro-Arg. The fluorescence signal for the substrate was determined using a microplate reader (SpectraMax M5; Molecular Devices). The total protein concentration was determined using BCA protein assay kit (Pierce Biotechnology). Trypsin activity was expressed as nanograms of trypsin/milligrams of total protein.

**Measurement of trypsin activity**

Measurement of trypsin activity was performed as described previously (Towatari et al., 2002).

**Western blot analysis**

Conventional Western blot analysis was performed as previously described (Ohmuraya et al., 2005). Rabbit anti-LC3 antibody (MBL International), goat anti-amylase antibody (Santa Cruz Biotechnology, Inc.), rabbit anti-trypsinogen antibody (Nordic Immunological Laboratories), and rabbit anti–IP3R1-3 antibody (Chemicon) were used at 1:1,000, 1:1,000, 1:1,000, and 1:2,000 dilutions, respectively.

**Generation of pancreatic acinar cell–specific Cre recombinase expression mice**

pMintCre contained five fragments: NLS derived from SV40, splice donor and splice acceptor derived from mouse En2, the cre recombinase gene, and the polyadenylation signal derived from SV40. The elastase I gene is selectively expressed in pancreatic acinar cells (Hammer et al., 1987). The DNA fragment containing EL was obtained by PCR using the primers 5′-TGGTGGGAGACATCCCAA-CAACA-3′ for s1 and 5′-TGTGGAGAAGATGACACTGC-3′ for s2. A fragment containing EL was digested and inserted into pMintCre to generate pEL-Cre (Fig. 3 A). Fragments excised from the pEL-Cre plasmid were used and separated for microinjection. Transgenic founder mice (EL-Cre) were backcrossed with C57BL/6 mice.

**RNA analysis**

Total RNA was isolated from each organ at 2 mo after birth with Sepasol-RNA (Nacalai Tesque). RT-PCR analysis was performed using the primers 5′-AATGTCTTCTGCTCCGGG-3′ and 5′-GATTTCCTGCCTCTGGTGTAGC-3′ for cre recombinase (563 bp) and 5′-GGAAAGCTGTGGGCCTGATG-3′ and 5′-CTGGTCTGTGGCCTGATC-3′ for G3PDH (382 bp).

**Generation of pancreatic acinar cell–specific Atg5-deficient mice**

Mice bearing an Atg5 flox allele (Atg5fl/−) were crossed with a transgenic line, EL-Cre2. These mice caused deletion of theloxRflanked exon 3 of the Atg5 gene. The deleted allele was detected by PCR with the primers 5′-CAGGGAATGTTGCTCTCCAC-3′ for D (512) and 5′-GTACTGCTAAATGCTGTGACCTG-3′ for B (short2). Southern blot analysis was performed as described (Hara et al., 2006).

**Measurement of [Ca2+]i**

CCK-8 was obtained from the Peptide Institute, and Fura-2AM was obtained from Dojindo. Measurement of intracellular Ca2+ concentration in pancreatic acinar cell suspensions was performed as previously described (Futatsugi et al., 2005) using an Aquacosmos ratio (Hamamatsu).

**Online supplemental material**

Fig. S1 shows the pancreas of Atg5fl/− and Atg5fl/−:EL-Cre2 mice. Fig. S2 shows Western blot analysis using antiamylase and antitrypsinogen antibodies at embryonic day 18.5, at 0.5 d after birth, and at 2 mo. Fig. S3 shows Western blot analysis of IP3R subtypes in the pancreas and [Ca2+]i changes induced by CCK8 receptor stimulation in pancreatic cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200712156/DC1.
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