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Cathepsin D in pancreatic acinar cells is implicated in cathepsin B and L degradation, but not in autophagic activity

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ABSTRACT

Cathepsin D (CD) is the major lysosomal aspartic protease and is widely distributed in the cells of various mammalian tissues. CD participates in various physiological events such as regulation of programmed cell death, activation of enzymatic precursors, and metabolic degradation of intracellular proteins through macroautophagy.

To investigate the role of CD in pancreatic acinar cells, which constitute the exocrine pancreas, we generated and examined mice specifically deficient for CD in pancreatic acinar cells. CD deficient mice showed normal pancreatic development and autophagic activity, although LC3-II, which is a marker of the autophagosome, accumulates in both physiological and pancreatitis conditions. Moreover, CD deficiency leads to accumulation of matured cathepsin B (CB) and cathepsin L (CL) which are members of the cysteine protease family. We therefore conclude that CD in pancreatic acinar cells is implicated in CB and CL degradation but not in autophagic activity.

1. Introduction

Cathepsin D (CD) is a major intracellular aspartic protease of the pepsin superfamily. It is expressed in endosomes and lysosomes, where it is involved in several physiological functions such as protein degradation, apoptosis, autophagy [1,2], cell growth, and tissue homeostasis [3]. Additionally, it is associated with several pathophysiological conditions such as cancer [4], Alzheimer's disease [5], atherosclerosis [6], and neuronal ceroid lipofuscinosis [7]. Although CD is found in almost all mammalian cells and has a typical lysosomal localization [8], its organ-specific roles are not well understood.

CD matures by multiple proteolytic cleavages of preprocathepsin D [9]. In humans, the 52 kDa pro-CD is localized to lysosomes [10], where 44 amino acids are removed from the amino terminus, producing a 48 kDa single-chain intermediate active form. This proteolytic cleavage is either performed by lysosomal cysteine proteases and/or by autocatalysis of CD itself [11,12]. Cleavage of the intermediate 48 kDa single-chain form produces the active, mature double-chain enzyme which is composed of heavy (34 KDa) and light (14 KDa) chains linked by non-covalent interactions. It is believed that lysosomal cysteine proteases perform this cleavage, as processing is partially inhibited by leupeptin [13].

Recent advances in understanding the effect of CD in *vivo* were obtained by analysis of CD-deficient mice. Although CD-deficient mice have no obvious phenotype at birth and grow normally, they develop anomalies later in life. At the age of 2 weeks, CD-deficient mice exhibit abnormal weight loss accompanied by progressive atrophy of intestinal mucosa. This is followed by massive intestinal necrosis, thromboembolia, and significant loss of lymphocytes in the spleen and thymus. CD-deficient mice die in an anorexic state at 4 weeks old [3,14]. Increased apoptosis observed in the thymus, thalamus, and retina indicates that CD is required in certain epithelial cells for tissue remodeling and renewal, possibly by regulating essential growth factors [14]. Accumulation of autofluorescent, ceroid lipopigment material occurs in sheep with an

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enzymatically inactive form of CD [15] and in mice expressing a truncated CD polypeptide [3,14]. Accumulation of this material leads to neuronal ceroid lipofuscinoses (NCLs), a group of inherited neurodegenerative lysosomal storage diseases.

Autophagy is a highly conserved degredation pathway by which excess, unneeded, and old intracellular macromolecules are discarded. Macromolecules to be discarded are delivered to lysosomes, where they are degraded into active monomers and subsequently reused to maintain cellular metabolic turnover and homeostasis [2]. Processing of macromolecules into free monomers is performed by enzymes of the hydrolase family, the most important of which is the cathepsin family of proteases such as CD, cathepsin B (CB) and cathepsin L (CL).

In this study, we generated mice lacking CD in the pancreas and analyzed both normal and acute pancreatitis conditions. Although autophagic activity as estimated by the expression level of the selective autophagy substrate p62 is normal, CD deficiency results in accumulation of LC3-II, an autophagosome marker, and mature CB and CL, which are lysosomal cysteine proteases.

2. Materials and methods

2.1. Animal protocol and experimental design

Mice were kept under specific pathogen-free conditions with free access to food and water in a 12 h (h) light/dark cycle. C57BL/6N mice were purchased from the CREA Japan. All animal experiments were performed with approval of the Kumamoto University Institutional Animal Care and Use Committee.

2.2. Generation of CD deficient mice in the pancreatic acinar cells

We generated mice where the second exon of CD was flanked by *lox*P sites. Mice homozygous for this modification are denoted by $CD^{f/f}$. $Spink3^{cre/+}$ [15] and $Ptf1a^{cre/+}$ [16] mice were generated previously. In $Ptf1a^{cre/+}$ mice, cre recombinase is expressed all pancreatic cells [16], while in $Spink3^{cre/+}$, cre recombinase is expressed in the acinar cells, but not in islet and ductal cells of the pancreas [15]. All mouse lines were backcrossed at least 6 times to C57BL/6N mice before use in experiments.

2.3. Cerulein pancreatitis

Induction of acute pancreatitis by cerulein was performed as previously described [17].

2.4. Histological analysis

For histological analysis, pancreatic tissue was fixed overnight in 15% formalin (Wako, Osaka), embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) stain.

2.5. Transmission electron microscopy (TEM)

Pancreatic tissues were fixed with 1% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Slices of these fixed tissues were postfixed with 2% OsO_4, dehydrated in ethanol and embedded in Epok 812 (Okenshoji Co. Tokyo). Ultrathin sections were cut with an ultramicrotome (ultracut N or UC6: Leica, Tokyo), stained with uranyl acetate and lead citrate, and were examined on a Hitachi HT7700 or JEOL JEM-1230 electron microscope.

2.6. Trypsin activity in the pancreas

Pancreatic trypsin activity was measured as previously described [17].

2.7. Cathepsin B activity in the pancreas

Z-Phe-Arg-AMC (Peptide Institute, Osaka) was added to give a final concentration of 100 μM and the reactions were incubated at 37 °C for 30 min. Fluorescence (excitation at 380 nM; emission at 460 nM) was then measured.

2.8. Western blot analysis

Western blots were performed as previously described [17]. Primary antibodies used were as follows: antibodies recognizing CB, CD, and CL were obtained from R&D systems (Minneapolis). Antibodies recognizing extracellular signal-regulated kinase 1/2 (ERK1/2), p62, and LC3B were obtained from Cell Signaling Technology (Tokyo). Anti-trypsinogen antibodies were obtained from Nordic Immunology (Tilburg, Netherlands). Anti-amylase antibodies were obtained from Santa Cruz Biothecnology. Blots were developed with Chemi-Lumi One Super reagents (Nacalai Tesque). Densitometric Analysis was performed by ImageJ software (http://rsbweb.nih.gov/ij/), and ERK1/2 was used as a loading control.

2.9. Reverse transcriptase RT-PCR analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Tokyo). cDNA was synthesized using the ReverTra Ace qPCR RT Kit (Toyobo, Tokyo). For the detection of Ctsb (Mm01310506), Ctsl (Mm00515597), and actin (Mm00607939) mRNA, TaqMan Gene Expression Assays on the AB 7500 Real Time PCR System (Applied Biosystems, Tokyo) were used.

2.10. Statistical analysis

Data in graphs are expressed as means ± standard error of mean (SEM) from 4 or more experiments per group, and each experiment was performed at least twice. Statistical analysis was performed by unpaired Student's t test, or one-way analysis of variance (ANOVA) test, as appropriate, using GraphPad Prism 6 (GraphPad Software, Inc., San Diego). $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

It is reported that CD is expressed in multiple mouse organs including lung, heart, kidney, liver, and intestine, but not in the pancreas. Thus, we measured pancreatic CD expression in both mouse embryos and adults. An accumulation of the pro- and heavy chain forms of the soluble lysosomal enzyme CD were detected in pancreas at both 4- and 8-weeks-old (Fig. 1).

To analyze the role of CD in pancreatic acinar cells, we generated mice harboring a *loxP*-flanked allele of CD (*CDf/f*) and specifically disrupted CD expression in pancreatic acinar cells by mating with *Spink3-cre* ($Spink3^{cre/+}$) transgenic mice, which specifically express cre recombinase in pancreatic acinar cells [15]. We used this approach because mice lacking CD in all tissues die at approximately day 26 because of progressive atrophy of the intestinal necrosis and profound destruction of lymphoid organs [3]. *CDf/f* mice were used as a control and $CDf/f;Spink3^{cre/+}$ mice homozygous for targeted alleles of CD as the deficient mouse. Western blot analysis of protein isolated from the pancreas at 8 weeks of age revealed that CD protein was present in *CDf/f* mice under both fed