Erebosis, a new cell death mechanism during homeostatic turnover of gut enterocytes

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学 位 論 文 の 内 容 要 旨

Erebosis, a new cell death mechanism during homeostatic turnover of gut enterocytes

腸細胞の恒常的ターンオーバーにおける新規細胞死、エレボーシス

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Summary of thesis content

Research Background and Introduction

Maintaining tissue homeostasis is important for organisms throughout their lifetime, and this often results in a constant flux of cells. Old differentiated or damaged cells die while stem cells proliferate to replace them.

In the monolayer intestine of the fruit fly *Drosophila melanogaster*, the foremost nutrient absorptive enterocytes are the major differentiated cell type. Under stress conditions such as tissue damage or infection, it has been reported that enterocytes die by apoptosis and are extruded to the luminal side of the gut epithelium. Intestinal stem cells receive proliferative signals and divide to compensate the loss of cells. The posterior midgut, especially the region called R4, has been described as the region with the fastest turnover from as short as four days. However, it has been difficult to detect apoptotic cells in the *Drosophila* midgut. By blocking apoptosis, we tried to inhibit the proliferative signal but this did not lead to a decrease in mitotic cell numbers.

In this study, we tried to elucidate the possibility of another cell death mechanism that regulates cell turnover in the intestine under physiological condition. To that end, fly genetics are an extremely powerful tool that allows tissue-specific gene expression or knockdown of genes of interest even in a time-dependent manner by using the yeast-derived Gal4/UAS/($Gal80^{ts}$) system.

We found that a certain subpopulation of enterocytes in mainly the posterior midgut contains the enzyme Ance, a *Drosophila* homolog of mammalian Angiotensin-converting enzyme (ACE). Although ACE is well studied in mammals and known for converting angiotensin I into angiotensin II as well as to inactivate bradykinin, comparatively little is known about the function of Ance in *Drosophila*. *Ance* expression has been described in several larval tissues. Unlike ACE, Ance does not contain a membrane anchor but is secreted.

Results

Immunostaining of the *Drosophila* midgut reveals that the number of Ance+ cells remain constant over time. Ance+ enterocytes are located basally in the gut epithelium and often possess an enlarged nuclear area but a reduced nuclear height [Figure 1]. In extreme cases, the nucleus was hardly detectable. Moreover, staining for nuclear lamin was reduced and Phalloidin labeling demonstrated loss of the cytoskeleton component F-actin in Ance+ cells. Further analysis of Ance+ enterocytes showed less amount of cell adhesion molecules. The adherens and septate junction components armadillo (β-catenin) and Discs large are decreased in Ance+ cells, respectively. This finding was supported by a transgenic fly line expressing cadherin-GFP.

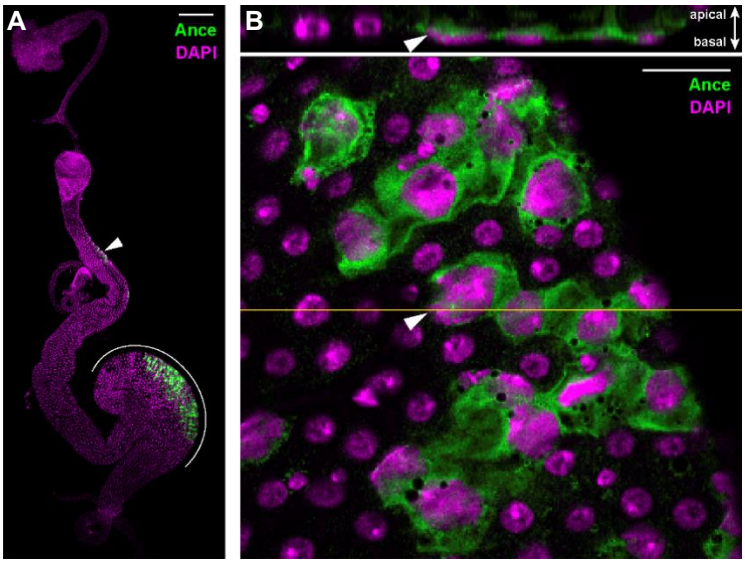

Figure 1: Ance+ enteroyctes in the midgut of *Drosophila melanogaster*.
A) Ance+ cells are mainly present in the posterior midgut region R4 (white line). B) Ance+ enterocytes are located basally exhibiting a larger nuclear area and shorter nuclear height than neighboring cells. Scale bars (A) 200µm, (B) 20µm.

While trying to manipulate enterocytes in the midgut using the pan-enterocyte driver *Myo1D-Gal4*, we noticed that the fluorescence of *Myo1D*-driven GFP shows a complementary pattern to Ance staining. Due to the absence or reduction of signals, we decided to call this phenomenon “erebosis” which is based on the ancient Greek word for deep darkness έρεβος [erebos].
Co-expression of GPF and nuclear RFP reveals that *Myo1D-Gal4* indeed drives expression in Ance+ enterocytes but astonishingly, they seem to lose GPF. Deeper analysis depicted erebosis as a multistep process that can be demonstrated by the gradual loss of fluorescent proteins. Early erebotic cells (1) lose cytoplasmic GFP and later nuclear GFP as intermediate erebotic cells (2). Finally, late erebotic cells (3) lose nuclear RFP and eventually, the nucleus becomes undetectable [Figure 2]. We could confirm the loss of GFP in erebotic cells of cultured midguts by time labs Using this knowledge, we were able to perform immuno-electron-microscopy comparing GFP+ (normal enterocytes) and GFP- enterocytes (erebotic cells). Among others, the analysis indicated that compared to neighboring enterocytes, erebotic cells contain less mitochondria. A transgenic fly line expressing mito-mCherry confirmed this result. Additionally, immunostaining revealed a strong reduction of Golgi and ER in erebotic enterocytes.

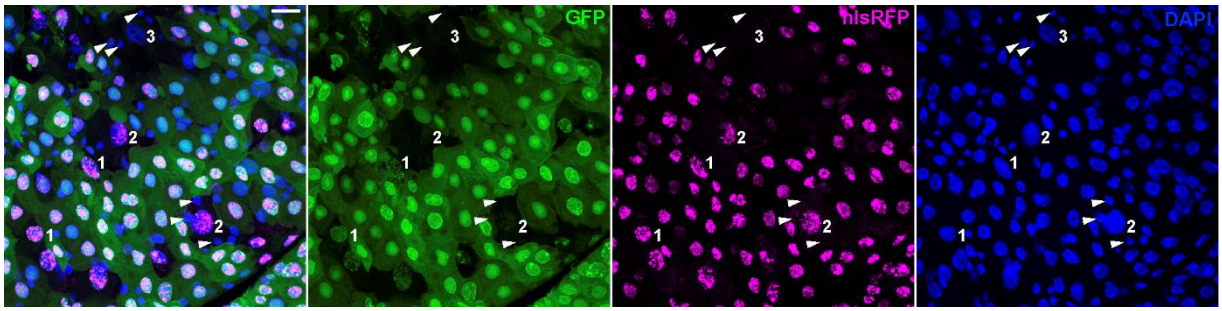

Figure 2: Erebosis is a multistep process losing cellular components. *Myo1D>GFP, nlsRFP*
1) early erebosis. 2) intermediate erebosis. 3) late erebosis. Arrow heads indicate progenitor cells. Scale bar: 20µm

Taken the above findings together, the question arose whether erebotic cells are alive. Subsequently, we examined whether erebotic cells die by one of the commonly described cell death types: apoptosis, necrosis and autophagic cell death. Immunostaining for cleaved caspase 1 (cDCP1) was not elevated in erebotic cells suggesting that executional caspases are not activated. Inhibition of apoptosis using several different approaches did not alter the presence of erebotic cells. Accordingly, erebosis was still observed in *dpf*^{K1} *(Apaf-1)* mutant flies and mutant clones of the *H99 (Δ rpr, hid, grim)* deletion or the initiator caspase *Dronc*. Neither midgut-specific expression of RNAi against *p35* nor microRNAi for *rpr*, *hid* and *grim* did lead to a change in the percentage of erebotic cells. Next, we used propidium iodide (PI) which can only enter cells with breached plasma membrane as it occurs upon necrosis. PI did not label erebotic cells. Last, we investigated whether erebotic cells undergo autophagic cell death using a transgenic fly line that expresses mCherry-Atg8a. Erebotic enterocytes did not show any increase in autophagosomes and midgut-specific inhibition of autophagy by knockdown of different *Atg* genes did not eliminate erebotic cells.
However, when labeling DNA nicks using TUNEL staining, we found that late erebotic cells become TUNEL positive and this correlates with the loss of GFP and the decrease in nuclear height. Neither TUNEL staining nor erebosis was affected by the inhibition of the caspase-activated DNAse *Drep4*, consistent with the idea that erebosis is independent of apoptosis. A dye labeling cellular ATP revealed that erebotic cells contain less amounts of ATP. Additionally, there is less tubulin in erebotic enterocytes and the nucleolus component Fibrillarin is lost.

The remarkable decrease of cell organelles and structural components makes it difficult to assume erebotic cells as metabolically alive albeit showing no characteristic features of apoptosis, necrosis or autophagic cells death. Further experiments suggest that the stress and immune related JNK and IMD pathways are not involved. We could show that erebosis is different from the recently described cell shedding upon infection. On the contrary, we observed erebosis even in flies that were raised under sterile conditions. Thus, we hypothesize erebosis as a new cell death mechanism.
Since cells seem to incorporate secreted Ance upon erebosis, we examined a possible role of Ance in the erebotic process. Although *Myo1D*-driven Ance RNAi decreased Ance protein signals, the knockdown did not affect erebosis and neither did Ance overexpression. Additionally, the number of mitotic cells was comparable in control and Ance mutants indicating Ance has no effect on gut cell turnover. However, Ance seems to have an erebosis-unrelated function in the intestine, suggested by a change in waste excretion upon enterocyte-specific inhibition of Ance.
Since loss of cells is often compensated by proliferation of stem cells, we next examined intestinal stem cells and progenitors in the proximity to erebotic enterocytes. They often reside close to erebotic cells and occasionally beneath them [Figure 2]. We were able to observe two young enterocytes beneath a legacy of an erebotic cell suggesting enterocytes undergoing erebosis are eventually replaced by new enterocytes.

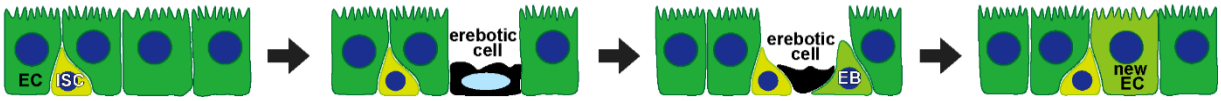

Figure 3: Erebosis is a novel cell death mechanism regulating homeostatic turnover of gut enterocytes.
Enterocytes (EC) that undergo erebosis flatten at the basal side of the gut epithelium. Intestinal stem cells (ISCs) and enteroblast (EBs) reside in close proximity to erebotic enterocytes. Eventually, erebotic cells are replaced by new enterocytes.

Discussion and Impact

We discovered a previously uncharacterized cell death mechanism that regulates the homeostatic turnover of enterocytes in the intestine of the fruit fly *Drosophila melanogaster*, named erebosis.
Cells undergoing erebosis demonstrate flat nuclei that occasionally become undetectable. Erebotic cells lose cytoskeleton and cell adhesion components but for a yet unknown reason accumulate Angiotensin-converting enzyme (Ance). Moreover, erebotic enterocytes show a decrease of cell organelles such as mitochondria, Golgi and ER but they do not exhibit characteristic features of apoptosis, necrosis or autophagic cell death. We could mainly observe erebosis in a subregion of the posterior midgut (R4) where cell turnover is known to be the fastest. Assuming erebosis is a slower and less inflammatory cell death, erebotic cells might be of structural importance preventing the breach of the gut barrier function. Thus, we hypothesize that erebosis is a novel molecularly coordinated cell death mechanism mediating homeostatic cell turnover in the gut.