Resource conservation using whole body autophagy: Self-digestion of shedded gut lining cells in the small intestine

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Abstract
To retain valuable resources, organisms adopt several strategies including coprophagy. Cells covering the outer skin and internal digestive lumen are actively recycled to maintain their integrity. In present study, we suggested that the small intestine can consume dead cells in a manner similar to how it consumes protein from the diet. We examined the eluates from five segments of the mouse small intestine and cecum and 2 segments of the large intestine, and detected immunoreactivity with eukaryotic caveolin-1 and β-actin antibodies only in the cecum and 2 segments from the large intestine. Bacterial agitation of the mouse intestine with Shigella disrupted the architecture and absorptive function of the small intestine. Small intestine eluates were immunoreactive with murine caveolin-1 and contained heme as determined by dot blot analysis. We concluded that the body conserves resources in the small intestine by disposing of and recycling shedded cells.

Keywords: caveolin-1, small intestine, autophagy, heme, resource conservation

Introduction

Strategies for preserving valuable organismal resources are well-recognized phenomena in nature; examples include coprophagy of undigested food and placenta feeding in mammals (Martin, 2011). Cells covering the outer skin and internal digestive lumen are actively recycled to maintain their integrity and protect the body cavity from harsh environments (Williams et al., 2015) Intestinal enterocytes form a barrier between the gut lumen and animal body. Various biological mechanisms maintain the barrier function of the small intestine. It has been reported that an estimated 1,400 mature mouse enterocytes (EC) are shed from a villus tip in each 24 h period, equating to 2×10^10 cells/day in the small intestine (Lodish, 2000; Bullen et al., 2006). In humans, the extent of daily shedding of ECs has been estimated at 10^9-10^10 cells (Lodish, 2000; Bullen et al., 2006). Assuming the weight of a murine hepatocyte is 3.5×10^6 g and that daily shedding of ECs in humans may account for a total weight of 35-350 g, then the daily loss from the small intestine is approximately 14-140 g, which is very high considering the daily consumption of food by the average human and the volume of human ECs (1,400 µm^3) is less than that of human hepatocytes (3,400 µm^3). It has been reported that apoptosis and anoikis initiate enterocyte shedding. Interestingly, mice lacking apoptotic machinery (bcl-2 or bax) have an apparently normal villus structure, indicating that alternative mechanisms are used to dispose of exfoliated enterocytes (Bullen et al., 2006; Pritchard et al., 1999). Interestingly, a decade ago, the Fujita group suggested that shed (effete) cells from the ileum are damaged by intraepithelial lymphocytes and subsequently phagocytosed by subepithelial macrophages (Iwanaga et al., 1993). More recently, Hausmann proposed that the life cycle of intestinal enterocytes is terminated by apoptosis and/or shedding (Hausmann, 2010). We postulated that the small intestine can consume dead cells in a manner similar to how it consumes protein from the diet. To test this hypothesis, we examined the eluates from five segments of the mouse small intestine and cecum and 2 segments of the large intestine, rather than the tissue itself, to detect tissue-derived proteins by immunoreacting with antibodies specific to mouse proteins.

Materials and Methods

Mice C57BL/6 mice (5 weeks old) purchased from Charles River Laboratories (Orient Bio, Inc., Seongnam, Korea) were housed under a standard specific pathogen-free environment with a 12 h dark/light cycle and free access to water and food. All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Review Committees of Ajou University (Permission number: 2013-0006). All mice used in the study were over 8 weeks of age. Virulent Shigella flexneri 2a (YSH6000) was a generous gift from Dr. Chihiro Sasakawa (University of Tokyo,
Japan). Mice were infected intragastrically by gavage with $5 \times 10^9$ colony-forming units per mouse for 3 h. Following that, the small intestine of the mouse was separated using surgery. The small intestine was cut into five parts, and the large intestine was cut into cecum and colon 1/2. The cut parts were clearly washed with a PBS. The washed solution was collected.

**Western blotting and antibodies**

Cells were lysed with radioimmunoprecipitation assay buffer containing 1× phosphate-buffered saline, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 0.1 mg/mL phenylmethylsulfonyl fluoride, 30 µL/mL aprotinin, and 1 mM sodium orthovanadate and protease inhibitor cocktail. The cell lysates were centrifuged, and the resulting supernatants were collected. Proteins were separated by 8-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. Each membrane was blocked in Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% non-fat dry milk for 1 h at room temperature, followed by overnight incubation with a primary antibody in TBST containing 1% non-fat dry milk at 4°C. Anti-caveolin-1 and anti-β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Membranes were washed with TBST and incubated with goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG secondary antibody for 2 h. Signals were measured using a chemiluminescence system (GE Healthcare, Little Chalfont, UK).

**Detection of Heme level by Chemiluminescence**

Each eluate sample was added to radioimmunoprecipitation assay buffer containing 1× phosphate-buffered saline, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate, 0.1 mg/mL phenylmethylsulfonyl fluoride, 30 µL/mL aprotinin, and 1 mM sodium orthovanadate and protease inhibitor cocktail. The cell lysates were centrifuged, and the resulting supernatants were collected. Dot blot was performed on PVDF membranes and incubated with chemiluminescence (CL) which detects the presence of heme-associated horseradish peroxidase activity. Signals were measured using a chemiluminescence system (GE Healthcare).

**TUNEL assay**

TUNEL analysis was conducted as previously described with an *In Situ* Cell Death Detection Kit® (Roche, Basel, Switzerland).

![Fig. 1. Immunoreactivity of eluates from small and large intestine to murine antigens.](image-url)

(A) Segmental eluates were probed with antibodies against cav-1, HO-1, and β-actin (B) Quantification of Cav-1 band intensity (C) Quantification of HO-1 band intensity (D) Quantification of β-actin band intensity Segmental eluates were probed with antibodies against cav-1 and β-actin basally. Loading of the samples was quantitatively validated with Ponceau S staining. The statistical significance of the results was analyzed using one-way ANOVA with Bonferroni correction.
(Jeon et al., 2014) The paraffin slides with embedded lung tissue were deparaffinized with xylene, rehydrated in graded ethanol, and rehydrated with running water for 5 min. The tissues were denatured for 10 min in boiling 10 mM citric acid (pH 6.0), and allowed to stand at room temperature for 10 min. The sections were post-fixed in ethanol-acetic acid (2:1), followed by rinsing. The sections were incubated with proteinase K (100 μg/mL), rinsed, incubated in 3% H$_2$O$_2$, permeabilized with 0.5% Triton X-100, rinsed again, and incubated in the TUNEL-reaction mixture. The sections were rinsed and visualized using Converter-POD with 0.05% 3,3-diaminobenzidine. The slides were air-dried overnight at room temperature, and coverslips were mounted using Permount® (Fisher Scientific, Waltham, MA, USA).

Statistical analysis
Analysis and data graphing were done with Prism 4.0 (GraphPad Software, San Diego, CA, USA). Data are expressed as means±SEM of at least three independent experiments. Statistical analysis was performed by one-way ANOVA for multiple group comparisons followed by Bonferroni correction. A value of $p<0.05$ was considered statistically significant.

Results and Discussion
Based on previous reports estimating the daily weight loss in the small intestine (Lodish, 2000; Bullen et al., 2006; Iwanaga et al., 1993), the purpose of this study was to carry out lost cells in the small intestine, cecum and colon1/2. This study showed that the small intestine can consume dead cells in a manner similar to how it consumes protein from the diet. Caveloin-1 is found in caveolae, which are 0-100 nm cell surface plasma membrane invagination (Jin et al., 2009) Because caveolin-1 and caveolae are present in nearly all cell types, the presence of caveolin-1 can indicate the presence of a cell (Lee et al., 2015). Additionally, the intracellular functions of caveolin-1 and caveolae are diverse (Lee et al., 2018). β-actin is a cytoskeletal protein related to cell structure and

Fig. 2. Measurement of intracellular cav1 and heme level in time-dependent. (A) Segmental eluates were probed with antibodies against eukaryotic cav-1 (B) Relative band intensity of Fig 1A (C) Segmental eluates were probed with antibodies against eukaryotic HO-1 (D) Relative band intensity of Fig. 1C. Segmental eluates were probed with antibodies against eukaryotic cav1. Heme levels in each segment were analyzed using a chemiluminescence (CL) assay after bacterial infection, which detected heme-associated horseradish peroxidase activity. SI, small intestine; cav-1, caveolin-1. The statistical significance of the results was analyzed using one-way ANOVA with Bonferroni correction.
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Motility and is ubiquitously expressed. Specifically, many studies have considered β-actin as a housekeeping gene (Lin et al., 2012). Therefore, in our study, cav-1 and β-actin antibodies were applied to determine whether cells remained in the elutes. As shown in Fig. 1A-D, immunoreactivity with eukaryotic caveolin-1 antibody was detected only in the cecum and 2 segments from the large intestine. Thus, this protein was not detected in eluate from small intestine, compared to eluate from cecum and large intestine, which indicated that few cell was in eluate from small intestine. Likewise, mouse β-actin antibodies also reacted with eluates only from the cecum and large intestine. Reportedly, dead cells shed from the lining of the large intestine are in part disposed of by bacterial feeders, which produce vitamins and help digest fiber (Cummings et al., 1997). Some dead cells were detected with antibodies against eukaryotic epitopes. To understand this result, we agitated the lining of the small intestine with virulent Shigella flexneri 2 by intragastrical administration (Chang et al., 2013). As shown in Fig. 2, after 3 h of bacterial infection, we detected undisposed cells in the eluates from the small intestine by western blotting with caveolin-1 antibodies. However, immunoreactivity against caveolin-1 disappeared in the small intestine after 24 h. This indicates that proteins isolated from dead cell were reabsorbed into the small intestine during diet. Diets in experiments are considered as a variable factor such as the size and type of food, which can lead to inconsistent results. Therefore, we examined elutes from the small intestine after bacterial infection (Fig. 3). Specifically, the eluate of segment 5 from the small intestine after 3 h of infection contained caveolin-1-positive clumps with DAPI-stained nuclei (Fig. 3A). An emerging field in biology considers the number of cells as a novel factor for scaling of biological structures and phenomena (Phillips et al., 2007). Depending on the number of enterocytes that are shed daily and their weight, eliminating them entirely would constitute a considerable loss of valuable resources from the body. Shedded enterocytes may be disposed in the same way as the food that an animal eats every day, in addition to by apoptosis. Bacterial agitation not only facilitates apoptosis or shedding of enterocytes, but also impedes barrier function, including the villi function (Chang et al., 2013). At an early time point (1 h after bacterial infection), we observed no caveolin-1 immunoreactivity in the small intestine, even though the gut lining underwent partial injury, indicating that the intestine’s absorptive function was still operational. We also measured heme levels in the eluates from each segment (Fig. 2A). Heme is a highly conserved molecule ubiquitously distributed in organisms from all three domains, Prokaryote, Archaea, and Eukaryote (Warren and Smith, 2009). Heme may have originated from the dead bodies of intestinal bacteria or damaged enterocytes. Basal level of heme was faintly observed in the distal parts of both the small and large intestines. One hour after bacterial infection, heme levels were somewhat increased in the distal part of the small intestine and cecum. However, heme levels were dramatically increased after 3 h of bacterial infection in the small and large intestine. Careful examination of images for DNA staining revealed that the level of prokaryotic DNA was negligible in the eluates, suggesting that enterocytes damaged by bacterial infection are responsible for the increased heme levels. Heme oxygenase (HO)-
Catalyzes degradation of heme to formation of bilirubin and carbon monoxide (CO) (Lee et al., 2020). Heme offers severe cellular oxidative damage by promoting ROS formation, lipid peroxidation, DNA and protein damage (Lee et al., 2015). To overcome its toxicity, HO-1 upregulation can be accompanied in heme. In line with this, we measured HO-1 and confirmed that HO-1 level was dependent to heme (Fig. 1A). Additionally, destruction of villus architecture was obvious at 1-3 h and recovered 24 h after bacterial infection (Fig. 3B). Hence, we reasoned that the absorptive function of the villi was not greatly affected by bacterial infection, which was accompanied by a minor increase in terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining 1 h after bacterial agitation (Fig. 3C). The villous architecture divided from the small intestine by bacterial infection was visually confirmed. Nevertheless, we did not distinctly observe protein compared to that in the cecum and colon 1/2, indicating that the small intestine can consume proteins from dead cells. In agreement with this, this study suggests that the body conserves resources by disposing of shedded cells in the small intestine. Therefore, our study may provide new insights into evolutionary biology. Collectively, given the results of our studies in a murine model, we suggest that the body conserves resources in the small intestine by disposing of shedded cells (Fig. 4).

Conflicts of interest

The authors declare no conflict of interest.