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Short communication

Regeneration of coelomocytes after evisceration in the sea cucumber, *Apostichopus japonicus*

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ABSTRACT

Sea cucumber, *Apostichopus japonicus*, is one of the most important holothurian species cultured in China. Severe evisceration induced by various natural and artificial factors commonly occurs during transport and culture of *A. japonicus*. Evisceration causes higher mortality and lower yield. Along with the visceral regeneration process, sea cucumbers also regenerate coelomocytes in order to recover immune function. In this study, evisceration of *A. japonicus* was induced by intracoelomic injection of 0.35 M KCl. Regeneration of coelomocytes was investigated by time course cell counting as well as detection of DNA replication by the EdU labeling technique. Coelomic fluid volume was restored to the pre-evisceration level within 2 h after evisceration. Total coelomocyte count (TCC) reached a peak at 6 h post-evisceration, followed decreased and then increased with a slight fluctuation, restored to the pre-evisceration level at 35 d post-evisceration. The change in different subtypes of coelomocytes was consistent with that of total coelomocytes. However, there were some variations in the regeneration of coelomocyte subtypes. At the end of the study, only the counts of amoebocytes and morula cells recovered to the pre-evisceration level. DNA replication assay showed EdU-positive cells accounted for 9.5% before evisceration and 4.7% at 6 h post-evisceration. However, the percentage of EdU-positive cells significantly increased, reaching 18.6% at 3 d after evisceration, then declined. Therefore, we analyzed the observed increase in coelomocytes at 6 h post-evisceration, which may be due to coelomocyte migration from the water-vascular system into the coelom rather than *de novo* cell proliferation.

1. Introduction

Sea cucumber, *Apostichopus japonicus*, is classified in the Phylum Echinodermata, Class Holothuroidea, Order Aspidochirotida, Family Stichopodidae, and Genus *Apostichopus*. *A. japonicus* is considered an economically important organism in East Asia due to its nutritional and medical value [1]. Sea cucumbers have a special defense mechanism against environmental changes or pathogenic infection. When a sea cucumber encounters a predator or adverse environmental conditions (hypersaline stress, absence of dissolved oxygen, etc.), the animal will eject all of internal organs including intestine, respiratory tree and gonads [2]. This is known as autotomy or evisceration. Subsequent to evisceration, the animal is capable of regenerating the full complement of internal organs and resuming norm functions [3]. This type of autotomy and regeneration is also seen in brittle star limbs. While autotomy is not observed in sea stars, they also have powerful regeneration properties. Likewise, sea urchins also have regeneration properties in their wound healing pathways. Thus, echinoderms including sea cucumbers, have emerged as important models to study cellular and molecular mechanisms of post-traumatic tissue regeneration [4]. Until now, more studies have been conducted to investigate histological and cellular aspects of regeneration, including cell division, differentiation and migration within all major organs and appendages [5–8]. Recently, the molecular mechanisms that drive fast and complete regeneration in sea cucumbers have been studied, Myc was the first transcription factor discovered in echinoderm regeneration by RNA interference-mediated gene knockout experiments [9–12]. However, there is little known about regeneration of the echinoderm immune system.

Sea cucumbers, like other echinoderms, lack a developed blood circulatory system. Instead, they possess coelomic fluid that fills the coelomic cavity. Coelomic fluid has been known to serve in circulation and homeostasis [13]. Coelomocytes are the cell type shown to be involved in cellular immune functions such as phagocytosis of pathogens, encapsulation and cytotoxicity. Coelomocytes are indispensable in biodefense and wound healing in echinoderms [14]. After sea cucumber evisceration, coelomocytes would also be expulsed together with

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visceral organs. This causes a transient state of hypo-immunity. Along with the visceral regeneration process, sea cucumbers need to regenerate their coelomocytes [15], thereby recovering immune function. To date, regeneration of the immune system, including humoral immunity within coelomic fluid and cellular immunity of coelomocytes, have remained uninvestigated. Thus, the regeneration mechanism of immunity is still unknown. In this study, time-course regeneration of coelomocytes in *A. japonicus* after evisceration was investigated. The results are crucial in understanding regeneration of immunity in echinoderms and potentially other animals as well. In addition, this information may provide valuable insights toward development of better treatment options for human hemorrhage disorders.

2. Materials and methods

2.1. Animal collection, maintenance, and sampling procedure

Normal, healthy *A. japonicus* (60.1 ± 10.2 g, mean ± SD) animals were collected from a local aquatic farm (Dalian, Liaoning Province, China) and acclimated in an indoor aquarium with well aerated seawater at 17–19 °C for 1 w prior to experiments. To study the regeneration of coelomocytes, evisceration was induced by intracoelomic injection of 1.2 mL of 0.35 M KCl. Nine individuals of *A. japonicus* at different time points [pre-evisceration, 1 min (minutes post-evisceration), 2 hpe (hours post-evisceration), 6 hpe, 12 hpe, 36 hpe, 3 dpe (days post evisceration), 5 dpe, 7 dpe, 14 dpe, 21dpe, 35 dpe] were sampled to study coelomic fluid volume, total coelomocyte density (TCD), differential coelomocyte density (DCD), total coelomocyte count (TCC), and differential coelomocyte count (DCC). During the whole experiment, the *A. japonicus* pre-eviscerated and after 21 d post-evisceration, were fed once each day at 3.0% of their body weight with a commercial diet (Haijie, Qingdao, China), until the intestine was regenerated after 21 dpe [5].

2.2. Time-course regeneration of coelomic fluid post-evisceration

Coelomic fluid volume at pre-evisceration and different time points post-evisceration was detected to illustrate the regeneration characterization of coelomic fluid. *A. japonicus* at different time points (nine individuals in each group) were sampled and allowed to stand for 20 min on paper to desiccate the seawater accumulated in the respiratory tree [16]. Microscopic examination demonstrated that coelomocytes were not found in fluid accumulated in respiratory tree under microscope observation. After desiccation, the ventral surface of *A. japonicus* was opened longitudinally with a surgical knife and coelomic fluid was collected in a corning tube and the volume was measured.

2.3. Time-course regeneration of coelomocytes post-evisceration

Classification of coelomocytes and cell counting at pre-evisceration and different time points post-evisceration were conducted to explain the regeneration characterization of coelomocytes. Nine *A. japonicus* specimens were randomly selected at different time points and coelomic fluid was collected as described above. 1 mL coelomic fluid mixed (1:1) with Modified Alsever's solution (27 mM sodium citrate, 336 mM sodium chloride, 115 mM glucose, 9 mM EDTA, pH 5.6) as anticoagulant was used to study the regeneration characterization of coelomocytes.

The total coelomocyte density (TCD) and differential coelomocyte density (DCD) were measured using a hemocytometer (25 × 16 squares) under the light microscope. The total coelomocyte count (TCC) and differential coelomocyte count (DCC) were calculated by the coelomic fluid volume multiplied by TCD or DCD.

$TCD = A \times B \times 10^4$; $DCD = C \times B \times 10^4$; $TCC = A \times B \times 10^4 \times D$; $DCC = C \times B \times 10^4 \times D$

where A represents sum of total cell number in the 25 medium squares, B represents dilution times, C represents sum of differential cell number in the 25 medium squares, D represents coelomic fluid volume.

2.4. Detection of DNA replication with EdU labeling

To observe the proliferation of coelomocytes of *A. japonicus* after evisceration, EdU labeling assay was performed. Three individuals of *A. japonicus* at different time points [pre-evisceration, 6 hpe (hours post–evisceration), 36 hpe, 3 dpe (days post–evisceration), 5 dpe, 7 dpe] were sampled to study the proliferation of coelomocytes. 200 μL EdU (1 mg/mL in sterilized saline) was injected into the coelom of *A. japonicus* 24 h before anatomical sampling except for the 6 hpe group which was injected immediately after evisceration. Coelomocytes were collected as described above, settled onto glass slides and incubated at room temperature in a wet chamber for 15 min. The slides were then fixed in 4% paraformaldehyde diluted in PBS, for 15 min and stored at −20 °C for later use.

The EdU labeling assay was performed as previously described [17]. Briefly, Monolayers were washed two times in PBS with 5 min intervals and 2 mg/mL glycine for 10 min, then 0.5% Triton® X-100 in PBS was used to treat the samples at room temperature for 10 min. After two washes with PBS, the EdU was detected using the Cell-Light™ Apollo® 567 Stain Kit (RiboBio, Guangzhou, China) under the fluorescence microscopy (Leica) and the percentage of EdU-positive cells was calculated by microscope counting.

2.5. Statistical analysis

All experimental data were expressed as the mean ± SD (standard deviation), and subjected to one-way analysis of variance using the statistical software program SPSS version 24.0 (SPSS Inc., IL, USA). When a statistically significant difference was determined via the one-way analysis of variance ($p < 0.05$), we conducted a post-hoc analysis using Dunnet's test to compare all post-evisceration groups to pre-evisceration group.

3. Results

3.1. Time-course regeneration of coelomic fluid post evisceration

The coelomic fluid volume of *A. japonicus* at pre-evisceration and different time points post-evisceration was detected. The results showed that coelomic fluid was almost evacuated post evisceration and then increased rapidly. The maximum value occured at the 6 h post-evisceration (about 23 mL), then it slightly fluctuated until the 35th day (Fig. 1). Statistical analysis showed that except 1min post-evisceration group, there was no significant difference between the groups of post-evisceration and pre-evisceration ($p > 0.05$).

3.2. Classification of A. japonicus coelomocytes

The coelomocytes of *A. japonicus* were tentatively classified into seven types based on microscopic observation of living cells (Fig. 2). Type-1 were the morula cells, which were spherical in shape, and ranged in diameter from 6 to 13 μm. Small spherules that completely filled the inside of the cells were of uniform size, measuring 0.5–2 μm diameter. Type-2 were the spherule cells, which were spherical in shape, ranged in diameter from 5 to 10 μm, and contained granules of uniform in size that completely filled the cells. Type-3 were the amoebocytes. The cells showed various shapes, demonstrated a great difference in size, and in contained numerous cytoplasmic filopodia giving the impression of the petals of a flower. Type-4 were the lymphoid cells, which were spherical in shape, ranged in diameter from 5