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Short communication

Revising lysenin expression of earthworm coelomocytes

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ABSTRACT

Lysenin is a species-specific bioactive molecule of *Eisenia andrei* earthworms. This protein is a potent antimicrobial factor; however its cellular expression and induction against pathogens are still not fully understood. We developed a novel monoclonal antibody against lysenin and applied this molecular tool to characterize its production and antimicrobial function. We demonstrated by flow cytometry and immunocytochemistry that one subgroup of earthworm immune cells (so called coelomocytes), the chloragocytes expressed the highest amount of lysenin. Then, we compared lysenin expression with earlier established coelomocyte (EFCC) markers. In addition, we determined by immunohistology of earthworm tissues that lysenin production is only restricted to free-floating chloragocytes. Moreover, we observed that upon *in vitro Staphylococcus aureus* but not *Escherichia coli* challenged coelomocytes over-expressed and then secreted lysenin. These results indicate that among subpopulations of coelomocytes, lysenin is mainly produced by chloragocytes and its expression can be modulated by Gram-positive bacterial exposure.

1. Introduction

Earthworm's coelomic fluid exerts a wide spectrum of biological functions including immunity against environmental pathogens. Humoral factors of this compartment control the growth of commensal and pathogenic microorganisms. Coelomic fluid has been a subject of intensive research since many years in earthworms (Bilej et al., 2000; Kauschke et al., 2007; Valembois et al., 1986; Wang et al., 2010).

The most extensively characterized factors are the unique fetidin/lysenin group of earthworm's coelomic fluid. First, fetidin is described as a glycoprotein with antibacterial and haemolytic properties (Lassegues et al., 1997). In addition, another bioactive molecule, designated as lysenin, has been cloned (Sekizawa et al., 1997). Lysenin was described primarily as a smooth muscle contraction factor (Sekizawa et al., 1996a). Membrane biological studies proved that lysenin binds specifically to the sphingomyelin compartments of the cell membrane (Shogomori and Kobayashi, 2008). Initially, lysenin and fetidin were considered as separate molecules exhibiting similar molecular weights (Cooper and Roch, 2003). However, recently it is known that both lysenin and fetidin belong to the same multimolecular family, that consist of four members such as lysenin, lysenin related protein 1, lysenin related protein 2 (fetidin), and lysenin related 3 (Bruhn et al., 2006). Lysenin/fetidin molecules were found abundantly in the coelomic fluid; however their exact production site and function in earthworms remained elusive.

Chloragogenous tissue covers the gut of earthworms. Chloragocytes, thus forming this tissue, are considered as a functional homologue cell type for vertebrate hepatocytes (Jamieson, 1981). Chloragocytes can be separated into two distinctly localized cell populations such as the peripheral chloragocytes found on the gut wall, while the other one termed as central chloragocytes settled inside of the typhlosolis (Ohta et al., 2000). It is suggested that lysenin expression is coupled mainly with the central chloragocytes demonstrated by in situ hybridization and polyclonal antibody-based immunohistochemistry (Sekizawa et al., 1996b; Ohta et al., 2000).

Previously we have developed a group of monoclonal antibodies (mAbs) against earthworm coelomocytes and with the aid of these molecular tools we characterized three subpopulations of free-floating coelomocytes (Engelmann et al., 2005). Furthermore, recently we paid more attention to characterize the functional status of coelomocytes (Engelmann et al., 2011). For this purpose we have developed a mAb against lysenin to characterize its cytotoxic nature. In addition, we aimed to revise lysenin expression profile in free-floating coelomocytes, its tissue localization and expression patterns upon bacterial challenge.

Abbreviations: EFCC, *Eisenia* coelomocyte clusters; LBSS, *Lumbricus* balanced salt solution.

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2. Materials and methods

2.1. Earthworm culture

Eisenia andrei earthworm species were maintained at room temperature and fed with manure complemented soil. Prior to coelomocyte isolations earthworms were placed onto moist tissue paper for depuration.

2.2. Preparation of mouse anti-lysenin monoclonal antibody

Lysenin (PeptaNova GmbH, Sandhausen, Germany) were dissolved in distilled water at 1 mg/ml concentrations and used for immunizing 8 weeks old female BALB/c mice. Mice were injected with lysenin protein (10 μg) mixed in complete Freund's adjuvant (CFA, Sigma–Aldrich Co., St. Louis, MO, USA) into each hind footpad followed with two intraperitoneal (i.p.) boosts in incomplete Freund's adjuvant (IFA, Sigma) 3 weeks intervals. Finally, one booster was injected in mice selected, according to its antibody titer and isotype, 3 days prior to splenocyte/myeloma-cell fusion. Hybridomas were developed as we described earlier (Engelmann et al., 2005). Hybridoma supernatants were screened by enzyme linked immunosorbent assay (indirect ELISA, for technical details please see the Supplementary Material) using lysenin, coelomocyte lysate as target antigen, and bovine serum albumin (BSA, Sigma) as control antigen. Selected hybridomas were cloned three times by limiting dilution.

2.3. Extrusion of coelomocytes

Coelomocytes were isolated as we described earlier (Engelmann et al., 2005) and living cell numbers were evaluated by trypan-blue exclusion method. Cell viability was above 95%. Coelomocytes of individual worms were analyzed further by immunocytochemistry, immunofluorescence, and flow cytometry. For technical details please see the SM.

2.4. In vitro bacterial challenge

Isolated, pooled coelomocytes (6×10^6/ml) were incubated with heat-inactivated *Staphylococcus aureus* (OKI II2001) and *Escherichia coli* (ATCC 25922) at room temperature by end-over-end rotation for 6 h (Bacterial strains were obtained from Dr. Béla Kocsis MD, PhD, Department of Medical Microbiology and Immunology, Clinical Center, University of Pécs). Coelomocytes (10^6) and bacteria (10^7) were mixed in 1 ml final volume in 12×75 mm tubes (Falcon, BD Labware). Following phagocytosis, coelomocyte samples were washed in LBSS, centrifuged at 1000 rpm for 5 min (Engelmann et al., 2005). Then supernatant was kept at −80 °C for further analysis and the pellet was applied for the preparation of coelomocyte lysates.

2.5. Preparation of coelomocyte lysate

Coelomocytes were lysed in RIPA buffer (50 mM Tris/HCl; pH 8.0, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) Na-deoxycholate, 5 mM EDTA, 0.1% SDS) complemented with Protease Inhibitor Cocktail (Sigma) on ice for 15 min and then centrifuged by 13,000 rpm (15 min, 4 °C). Total protein concentrations of the coelomocyte lysates and supernatants were measured with BCA Reagent Kit (Pierce, Rockford, IL, USA). Immunoreactivity of supernatants and coelomocyte lysates were analyzed by ELISA and Western blot assays (further technical details can be obtained in SM).

2.6. Statistical analysis

Statistical analysis was performed with Microcal Origin (Microcal Software Inc., Northhampton, MA USA). The results in the figures are representative values from four independent experiments. All results are presented as mean with standard error shown as error bar. The effect of treatments was analyzed by one way-ANOVA. $p < 0.05$ was denoted as statistically significant.

3. Results and discussion

3.1. Lysenin expression of isolated coelomocytes

Coelomocytes are multi-tasking cellular mediators of immune response in earthworms. In addition to their cellular immune functions (e.g. phagocytosis, encapsulation) they participate in the humoral immune mechanisms by producing haemolytical and antimicrobial factors (Bilej et al., 2000; Cooper et al., 2002). Most of these proteins belong to the fetidin/lysenin multiprotein family characterized mainly by membrane biologists. However, the exact role of lysenin in the immune response of earthworms is not so well understood (Kobayashi et al., 2004).

Previously, we observed the cytotoxicity mediated by earthworm coelomocytes produced factors (Engelmann et al., 2011). Now we intend to uncover more details about the cytotoxic mechanisms (Macsik et al., in preparation). For this purpose we raised a monoclonal antibody against lysenin (a-EFCC5) and first we initiated to characterize its expression profile in coelomocytes. Antibody positive cells have large granular cytoplasm and relatively small nucleus revealed by immunocytochemistry (Fig. 1A). Immune positive reaction is localized mainly in the intracellular granules. This morphology marks one characteristic subgroup of coelomocytes, the free-floating chloragocytes (sometimes denoted as eleocytes). Other cell types with larger nucleus and smaller cytoplasm (effector coelomocytes, presumably hyaline amoebocytes) were negative for a-EFCC5 staining (Fig. 1A). Using the previously generated mAbs against coelomocyte subgroups we revealed co-expression with lysenin positive coelomocytes (Fig. S1). A-EFCC3 positive coelomocytes are tend to attach together and form aggregates, while EFCC5 positive cells are mainly solitary (Fig. 1B, *Figs.* S1 and S2). By means of flow cytometry, circulating coelomocytes of the body cavity has several subgroups (Engelmann et al., 2005; Fuller-Espie et al., 2010; Vernile et al., 2007). In our experiments, we could identify three physically distinct subpopulations of coelomocytes denoted as R1, R2, and R3 (Fig. 1C). R1 and R2 populations resemble effector coelomocytes such as hyaline and granular coelomocytes, while R3 subgroup is the highly granular and autofluorescent eleocyte population (Plytycz et al., 2006) (Fig. 1C). EFCC5 positivity could be observed in all three subgroups with various intensities compared with appropriate isotype control antibodies (Fig. 1D). R3 group majorly consisting eleocytes had the highest percentage (39.5 ± 10.2%) of EFCC5 antibody positive cells (Fig. 1D and Table S1).

3.2. Solitary free-floating chloragocytes are restricted for lysenin expression

Chloragocytes are usually very abundant, highly granular coelomocyte subpopulation of the coelomic cavity. Their origin is a matter of debate, however most authors agreed that free-floating chloragocytes derived from the chloragogenous tissue of the gut (Jamieson, 1981). These cells are harboring nutrient factors such as glycogens, lipids and participate mainly in general metabolism. Moreover, they are able to produce haemoglobin and metal-sequestering cysteine-rich proteins as well (Fischer, 1993; Morgan