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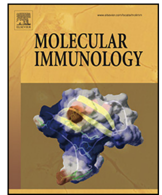

Induction of apoptosis-like cell death by coelomocyte extracts from *Eisenia andrei* earthworms

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ABSTRACT

Earthworm's innate immunity is maintained by cellular and humoral components.

Our objective was to characterize the cytotoxicity leading to target cell death caused by earthworm coelomocytes. Coelomocyte lysates induced strong cytotoxicity in tumor cell lines. Transmission electron microscopy revealed cell membrane and intracellular damage in cells treated with coelomocyte lysates. Using TUNEL-assay, within 5 min of incubation we detected DNA fragmentation. Moreover, we found phosphatidylserine translocation in target cell-membranes. Furthermore, we detected dose-dependent Ca^{2+} influx and decrease of mitochondrial membrane potential in coelomocyte lysate-treated cells. Interestingly, caspase 3/8 activation was undetectable in exposed tumor cells. One such cytotoxic molecule, lysenin identified in earthworms binds to sphingomyelin and causes target cell lysis in vertebrates. Pretreatment with our anti-lysenin monoclonal antibody rescued the majority but not all target cells from coelomocyte induced death. These data suggest that, not only lysenin but also other factors participate in the caspase-independent apoptosis induced by coelomocytes.

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1. Introduction

Metazoans exhibit various immune mechanisms against environmental pathogens. In earthworms cellular immune functions are maintained by coelomocyte (immune cells with mesodermal origin) subpopulations located in the coelomic cavity (Vetvicka and Sima, 2009). Based on their morphological characteristics three main populations of earthworm coelomocytes can be defined such as hyaline-, granular amoebocytes and eleocytes (free-floating chloragocytes) (Cooper, 1996). By means of flow cytometry three subgroups of coelomocytes (R1, R2 and R3) are distinguished, which correspond to these previously identified major populations (Engelmann et al., 2002; Vernile et al., 2007). With respect to function; hyaline and granular amoebocytes are capable of phagocytosis and encapsulation; however, granular amoebocytes engulf less foreign particles than hyaline cells (Cooper and Stein, 1981; Engelmann et al., 2005a,b). Eleocytes have no phagocytic activities but they exert metabolic functions as well as produce bioactive molecules (Dales and Kalaç, 1992; Kauschke et al., 2007; Vaillier et al., 1985). In addition coelomocyte-mediated cellular cytotoxicity is demonstrated in xenogeneic and allogeneic cultures (Cooper et al., 1995; Suzuki and Cooper, 1995).

Regarding to the humoral constituents of the coelomic fluid several proteins exhibiting cytotoxic, antibacterial, proteolytic and mitogenic activities have been described in this compartment (Hrženjak et al., 1992; Procházková et al., 2006; Roch et al., 1991). One such protein family (lysenin, lysenin-related proteins and fetidin) has antibacterial, hemolytic and smooth muscle contraction activities (Kulma et al., 2010). Additionally, a pattern recognition receptor (namely coelomic cytolytic factor, CCF) has been cloned and characterized sharing functional homology with the mammalian tumor necrosis factor alpha (TNF-α) (Bilej et al., 2010). This extensive literature reveals the existence of immune molecules in the coelomic fluid and presumes that earthworm coelomocytes can participate in the production of these humoral factors. Recent results suggest that the cytotoxic factors of coelomic fluid cause apoptosis of tumor target cells. The proposed apoptotic cell death was demonstrated by DNA fragmentation assay, and acridine-orange/ethidium bromide staining (Yanquin et al., 2007).

Abbreviations: CCF, coelomic cytolytic factor; CCL, coelomocyte lysate; LBSS, Lumbricus balanced salt solution.

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Previously we characterized earthworm cytotoxicity focusing on certain mechanisms in order to reveal possible relationships to coelomocytes. Our results showed clearly that cytotoxic components of coelomic fluid originated from coelomocytes of *Eisenia* earthworms (Engelmann et al., 2004). In the present study we aimed to investigate the molecular mechanisms of cytotoxicity originated from coelomocytes applying various mammalian tumor cell lines. To specify the apoptotic, necrotic or alternative cell death mechanisms various methodological approaches were performed such as WST cell-viability assay, transmission electron microscopy (TEM), TUNEL-staining, Annexin V labeling, measurement of caspase activity, assessment of Ca^{2+} influx and mitochondrial membrane potential. Moreover, we interested in whether this observed cytotoxicity is mediated by the identified cytotoxic molecules (e.g. lysenin).

2. Materials and methods

2.1. Extrusion of coelomocytes

Breading stocks of *Eisenia andrei* were maintained at standard laboratory conditions in a seminatural soil completed with horse manure. Prior to isolations earthworms were placed onto moist filter paper for depuration. Coelomocytes were isolated as we described earlier (Engelmann et al., 2004, please see the Supplementary Information) and cell numbers were evaluated by trypan-blue exclusion method.

2.2. Culture conditions of mammalian cell lines

HeLa (ATCC CCL2), Sp2/0-Ag14 (CRL-1581) and Jurkat (TIB152) tumor cell lines were cultured in DMEM (Sigma, St. Louis, MO, USA) or in RPMI (Sigma) medium in 5% CO_2 humidified atmosphere at 37 °C. The culture media was supplemented with 10% heat-inactivated FCS (Lonza-BioWhittaker, Basel, Switzerland) and antibiotics (streptomycin 50 μg/ml and penicillin 50 U/ml).

2.3. Preparation of coelomocyte lysate (CCL) and control tumor (Jurkat) cell lysates

Coelomocytes were sonicated in *Lumbricus* Balanced Salt Solution (LBSS) buffer using Cole Palmer Ultrasonicator (2 min on 4 °C, Vernon Hills, IL, USA) and centrifuged at 13,000 rpm (15 min, 4 °C). Control lysates from Jurkat cultures were performed similarly. Total protein concentrations were measured with BCA Reagent Kit (Pierce, Rockford, IL, USA). Supernatants of cell lysates (100 μg) were used to treat HeLa, and Sp2 cells in the following assays.

2.4. Treatment of tumor cell targets

Cultured Sp2 (5×10^5/well) and HeLa cells were incubated with CCL in various conditions (dilutions and incubation periods are detailed in the appropriate sections). DMEM medium, LBSS buffer, and Jurkat cell lysate were applied as negative controls. In the various methodological approaches anisomycin (1 μg/ml) and etoposide (50 pg/ml) exposures were performed in overnight Sp2 and HeLa cell cultures and served as positive controls.

2.5. WST cell viability assay

Initial cell viability was measured by WST-1 assay (for technical details please see the Supplementary Information).

2.6. Transmission electron microscopy

Control and exposed Sp2 cells were fixed in a mixture of 0.3% glutaraldehyde and 4% paraformaldehyde for electron microscopic post-embedding morphological observations. The fixative was dissolved in 0.1 M phosphate buffer (pH 7.4) and the specimens were incubated for 2 h at room temperature. Samples were post-fixed with 1% osmium tetroxide for 1 h at 4 °C. For post-embedding structural observations, the cells were dehydrated and embedded into epoxy resin (Durcupan ACM, Sigma). Serial ultrathin (60–70 nm) sections were cut with a Reichert ultramicrotome. Grids were then washed in drops of distilled water, counterstained in uranyl acetate and lead citrate. Observation and documentation were carried out with a JEOL-1200 transmission electron microscope.

2.7. Annexin V-FITC/propidium iodide (PI) staining

Control and exposed Sp2 cells were washed in Annexin V binding buffer. Annexin V-FITC (BD Biosciences, San Jose, CA, USA) staining was performed according to the manufacturer's instructions for 30 min at room temperature. Before flow cytometry we added propidium iodide (1 μg/ml, Sigma) to the samples.

2.8. TUNEL assay

HeLa cells were cultured on sterile 8-well Falcon culture slides (Corning Life Sciences, Amsterdam, Netherlands) in DMEM medium. After treatments target cells were washed with PBS. TUNEL assay was performed according to the manufacturer's protocol (DeadEnd Fluorometric TUNEL System, Promega, Madison, WI, USA). Before microscopic evaluation samples were counterstained with DAPI (Invitrogen Molecular Probes, Eugene, OR, USA) and slides were mounted with EverBrite Mounting Medium (Biotium, Hayward, CA, USA) Fluorescent signals were observed with Olympus BX61M microscope and AnalySIS software (Olympus Hungary, Budapest).

2.9. Detection of caspase activation

Following treatments Sp2 cells 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 then lysates centrifuged at 13,000 rpm for 15 min for debris-free supernatant. Protein concentrations were measured with BCA Reagent Kit (Pierce). SDS-PAGE and Western blot was performed as we described earlier (Opper et al., 2013, for details please see SI). Alternatively, exposed Sp2 cells were washed in PBS and stained with of NucView 488 caspase 3 substrate (Biotium) for 30 min at room temperature according to the company instructions. Caspase 3 substrate signals were measured in FL1 channel by flow cytometry.

2.10. Calcium influx measurements

Sp2 cells were suspended in DMEM (Sigma) or Ca^{2+}-free PBS at 10^7 ml^{-1} concentration and loaded with 1% Fluo3-AM for 30 min at room temperature with gentle shaking (Minta et al., 1989; Opper et al., 2010). After washing target cells were resuspended at 10^6 ml^{-1} concentration in DMEM or Ca^{2+}-free PBS. CCL along with controls were added to the samples after measuring the basal calcium levels for 50 s. Fluo3-AM fluorescent signal was measured in FL1 channel by flow cytometry.

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