

# UNIVERSITY OF PÉCS

Biological Doctoral School

Regulation Biology Program

## Coelomocytes of *Eisenia fetida*: structure, function, origin

*PhD thesis*

**Ildikó Somogyi**

Supervisors:

**Edit Pollák, PhD**  
**László Molnár, PhD**



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# INTRODUCTION

The coelomic fluid and hemolymph or the blood in some phyla (Nemertean, Annelids) of invertebrates play a crucial role in some physiological processes: transportation of nutrients, metabolic intermediates and end-products, respiratory gases and signalling molecules, e.g. hormones. These body fluids have defined composition; containing characteristic cell types which take part in blood coagulation, wound healing and immune response. The cells of invertebrate body fluids are analogous in function with vertebrate blood cells, therefore to ascertain of their structure and function contribute to the understanding of the evolution of some physiological processes (blood coagulation, wound healing and immune response).

Coelomocytes of oligochaetes have already been observed with light- and electron microscopical methods, so three cell types namely amoebocytes, granulocytes and eleocytes, have been distinguished based on their morphology. Morphological variability of the cells in each coelomocyte group suggests the existence of functional subpopulations of coelomocyte types, however, no experimental evidences are known yet. Amoebocytes move by pseudopodia, phagocytose foreign materials and their cytoplasm are rich in lysosomes. Cytoplasm of granulocytes contains various granules, electron dense organelles and vacuoles. The structure of eleocytes resembles to chloragocytes, their cytoplasm contains glycogen particles, lipid droplets and granules with variable density like chloragosomes of chloragocytes.

These days the origin of earthworm coelomocytes is not clear yet. They can stem from somatopleure and/or splanchnopleure. Chloragogenous tissue is situated around the midgut and the large yellow cells attach to the periintestinal blood vessels and capillaries. Chloragocytes develop from splanchnopleure. There are some smaller cells so-called hyaloblasts with light cytoplasm and large nucleus among the chloragocytes, these cells are believed to be the precursors of certain coelomocytes.

Some earlier study suggested the contribution of coelomocytes to the homeostatic regulation e.g. in blood coagulation, immune reactions and in regeneration of lost body parts. Annelids are the first animals in the phylogenetic tree in which not only the cellular but also humoral immune response are developed. During the cellular immune response coelomocytes play a role in phagocytosis, inflammatory processes, graft rejection and coagulation of coelomic fluid. During humoral immune response these cells synthesize and secrete lysosyme, agglutinine, phenoloxidases, peroxidases and antimicrobial factors (fetidin, lysenin,

eiseniapore, coelomic cytolytic factor). Cytotoxic molecules increase the intracellular calcium concentration in target cells, which participate in exocytosis, enzyme-function, regulation of gene expression, cell proliferation, apoptosis and cell growing, therefore the coelomocytes can induce and influence important physiological processes by these signal molecules.

The oligochaetes have enormous regeneration capability, they are able to renew ablated body parts and segments from the regeneration blastema. The formation of the regeneration blastema and the origin of its cells are not known exactly. There are some experimental evidences that the nervous system mediates the blastema formation in which coelomocytes (amoebocytes, granulocytes and eiecocytes) accumulate in high number. While the influence of the nervous system on the blastema formation and differentiation is well documented and generally accepted, the putative role of coelomocytes (transportation of bioactive compounds, formation of new tissues by transdifferentiation) has not been verified yet by experimental evidences.

There is a growing evidence of the communication between the immune and nervous system by bioactive compounds (cytokines, neuropeptides) in both invertebrates and vertebrates. Several neuropeptides have endogenous messenger function in the immune system and participate in the regulation of immune response. Some neuropeptides, for example oxytocin/antidiuretic hormone, angiotensin appeared early in evolution and are conservative molecules. They appeared earlier than the nervous system, suggesting that these molecules were signal molecules at first and mediated the intercellular communication.

In the central nervous system of earthworms (cerebral ganglion, ventral nerve cord) there are several neurosecretory cells which synthesise signal molecules like steroids and peptides. About 30 neuropeptides have been isolated from earthworms, which are grouped into well known peptide families based on the structure and physiological effects of the molecules. There are some peptides which can only be found in invertebrates (e. g. CAPA-peptides), and conservative peptides which can be find both in vertebrates and invertebrates. In the last few years annetocin from oxytocin/antidiuretic hormone family was isolated from oligochaetes and PACAP (pituitary adenylate cyclase activating peptide) from vasoactive intestinal peptide/secretin/glucagon family were revealed in the central nervous system of some species of Lumbricidae. PACAP plays a role in immune processes, inhibits the pro-inflammatory effect and stimulates anti-inflammatory processes, namely delays the inflammatory processes. It participates in the differentiation of neurons and regeneration of injured axons. PACAP can play a similar role in invertebrates, but it has not been studied yet.

## **AIMS OF THE STUDY**

Based on previous data coelomocytes are involved in several important physiological processes of oligochaetes, sometimes they are essential for regulation. Nevertheless the identification of coelomocyte types (except the eleocyte) is not clear and our knowledge about function of cell population is not complete.

To receive more complex data on the cytology and physiology of coelomocytes we are going to focus on

1. the identification of major coelomocyte types and their possible subpopulations based on their morphological and histochemical characterization by conventional light and electron microscopical methods, carbohydrate- lipid- and enzyme histochemistry and immunocytochemistry;
2. the investigation of the structural and compositional changes of coelomocytes in ‘control’ animals keeping at standard laboratory conditions and in those ones which are exposed to various physiological stresses (stimulation of the immune system, regeneration) to establish their function;
3. the identification of stem cells of coelomocytes and follow their differentiation with the detailed characterization of developmental stages.

## **MATERIALS AND METHODS**

### **Animals**

For our experiments adult sexually mature earthworms (*Eisenia fetida* Sav., Lumbricidae, Oligochaeta) were collected from our standard laboratory breeding stock. The coelomocytes were harvested by mechanical or chemical irritation of the selected animals using extrusion buffer as a medium. To cytotoxic studies a coelomic cell lysate (CCL) was prepared with ultrasound. In Western blot experiments isolated tissues of BALB/c mice (*Mus musculus*) were used as controls.

### **Histology and histochemistry**

Serial sections of selected segments and smears prepared from the suspension of coelomocytes were stained with May-Grünwald Giemsa and/or hematoxylin-eosin. Complex carbohydrates were identified with Periodic acid-Schiff reaction, neutral lipids with Sudan Black and acidic cytoplasm granules with Acridine Orange in vivo staining.

### **Phagocytotic activity assay**

Formaline-fixed erythrocytes of sheep suspended in 1 µl Ringer solution were injected to coelomic cavity of intact animals. On the 1, 3 and 5 days of survival segments of the experimental animals were isolated, fixed and to localize erythrocytes processed for haematoxylin-eosin histological staining.

Phagocytosis and cellular distribution of silver nano-particles (AgNP) were studied with conventional electron microscopic methods and X-ray microanalysis.

### **Conventional electron microscopy and enzyme-cytochemistry**

Coelomocytes of intact and regenerated animals were fixed in the mixture of 1% paraformaldehyde and 2,5% glutaraldehyde, then postfixed in OsO<sub>4</sub>.

To localize and characterize acid phosphatase (AcP) activity regenerated body-tails were isolated and fixed in buffered glutaraldehyde solution, then cryostat sections were cut from the tissue samples. AcP activity was demonstrated on frozen specimens with Gömör acid phosphatase technique.

Cytotoxic effect of coelomocytes' lysate was tested with Sp2 cells fixed in the mixture of 4 % paraformaldehyde and 0.3% glutaraldehyde.

Fixed samples were embedded in epoxy resin, ultra-thin sections were cut and contrasted with uranyl acetate and lead citrate, and than examined with a transmission electronmicroscope.

### **Immunohistological and immuncytochemical localization of PACAP isoforms and specific PACAP-receptor protein PAC1**

To localize PACAP27 and PACAP38 isoforms of PACAP peptide in coelomic cells, intact and regenerated segments of the animals were isolated and fixed in 4% paraformaldehyde. Pre-embedding labeling of PACAP-contatining structures was carried out with avidin-biotin horse radish peroxidase complex method (ExtrAvidin kit, Sigma) and visualized in 3,3'-diaminobenzidine (DAB) solution.

To identify PAC1-receptors regenerated segments were fixed in modified Karnovsky solution (2% paraformaldehyde and 2,5% glutaraldehyde) and embedded in epoxy resin. Ultrathin sections were etched, deosmificated and to prove the presence of PAC1-receptor proteins immunogold reactions were performedon the specimens.

### **Determination of concentration of PACAP isoforms with radioimmunoassay (RIA)**

To quantification of PACAP peptide content some samples were homogenized and centrifugated and the supernatants were used for radioimmunoassay (RIA). Each sample contained antiserum (anti-PACAP27/38), RIA tracer (radioactive iodine labelled PACAP fragments) and known concentrations of synthetic PACAP27/38 peptide or a same amount of our investigated samples, diluted in phosphate buffer. Following the incubation the antibody-peptide complexes were separated from the unconjugated antibodies and radioactivity of precipitate was measured by a gamma-counter.

### **Western blot**

For Western blot analysis the isolated coelomocytes mouse tissues were homogenized and centrifuged. Supernatants were analysed with SDS-PAGE gel electrophoresis (polyacrylamide gel, containing sodium-dodecyl-sulphate), the separated peptides were transported to a nitrocellulose membrane. The membranes were then incubated in anti-PAC1R solution as a primary antiserum. Horse-radish peroxidase conjugated IgG was used as secondary antibody, labelled bands were visualized with ECL (enhanced chemiluminescence) reagent and documented by a light-sensitive film.

### **Flow cytometry**

For flow cytometric analysis of AcP ativity of coelomocytes AcP-immuncytochemical reaction was carried out, then the labelled cells were fixed in paraformadehyde, washed in Lumbricus balanced salt solution (Ringer), and the labelling was detected by a flow cytometer.

To verify cytotoxic effect of CCL the Sp2 cells were loaded with Fluo3 AM for 1 h, then intracellular calcium concentration was measured with a flow cytometer.

## **RESULTS AND DISCUSSION**

This study reports on five distinct types of coelomocytes (eleocytes, amoebocytes, granulocytes, cell with basophil cells and coelomocytes with invaginated nucleus) identified in *E. fetida* by conventional light- and electron microscopy, carbohydrate-, lipid- and enzyme-histochemistry. While eleocytes, amoebocytes and granulocytes have already been identified in several lumbricid species, basophil cells and coelomocytes with invaginated nucleus are first described in this work. Based on the detailed cytological and cytochemical characteristics

of various coelomocyte types their putative subpopulations that can represent different developmental and physiological stages were identified, too.

**Eleocytes** can be identified based on their size, structure of nucleus and cytoplasm. Number and density of their granules, glycogen- and lipid content of the cytoplasm can change in wide range. Results of the light- and electron microscopical investigations prove that stem cells of eleocytes are chloragocytes; during the asymmetric cleavage of chloragocytes the apical daughter cells transform to eleocytes. The explanation can be the structural similarity of young eleocytes and chloragocytes. Eleocytes can release granules, reserved nutriments to extracellular space during their life-cycle, according to which the structure of their cytoplasm can change. An unknown cytological process was identified by ultrastructural investigations which can be an experimental evidence of the origin of nucleus free cell-fragments in the coelomic cavity. Dens granules (chloragosomes with high calcium-content) and lipid droplets accumulate in the apical part of chloragocytes, and then the apical part detaches along the intracellular membranes (cisterns of endoplasmic reticulum) from the basal part of cells. This process is similar to the formation of platelets. The separated cell-fragments are phagocytosed and digested by amoebocytes, releasing of their compounds which can contribute to the change of composition of coelomic fluid and the mediation of physiological processes in coelomic cavity. For this reason we can suppose that chloragocytes influence the function of coelomocytes indirectly.

Developmental stage of **amoebocytes** with phagocytotic activity can be seen from the size and number of pseudopods and the degree of phagocytotic activity. Their phagocytotic activity was demonstrated by injection of sheep erythrocytes to coelomic cavity in vivo and application of silver nanoparticles in vitro. Based on our results phagocytotic activity can be stimulated both in vivo and in vitro. Number and acid phosphatase activity of amoebocytes increase in regenerated animals. High acid phosphatase activity is a characteristic feature of phagocytotic cell with activated lysosomal system and digestion of phagocytosed structures (microorganisms, cell- and tissue debris). Considerable extracellular acid phosphatase activity was observed in neighbouring amoebocytes. Extracellular lysosomal enzyme can contribute to the digestion of injured tissue fragments. Since amoebocytes cluster in injured segments in regenerated animals and many cell- and tissue debris can be found in these cells, we suppose that amoebocytes provide intact surface for neoblasts to attach to by removing degenerated elements.

**Basophil and eosinophil granulocytes** with different composition of granules were identified with conventional staining methods. The size and the density of granules are

extremely variable. Their compositions are unknown, high PAS positivity of a part of them suggests the presence of complex carbohydrates. Exocytosis of granules refers to the influence of the activity of granulocytes or other cells by releasing bioactive compounds. Both cell-types contain few lysosomes apart from typical granules.

Few **basophil cells** can be found in smears of coelomocytes, which can be divided into two groups. Smaller cells can be stem cells of oligochaetes, so-called **neoblasts**, according to cytological characteristics (diameter, size and structure of nucleus, nucleus/cytoplasm ratio). Our results reveal at first that a large number of neoblasts accumulates in coelomic cavity, in injured segments and in regeneration blastema in regenerated animals. PAC1-receptor immunoreactive elements of these cells were demonstrated by immunocytochemistry, which suggests that the function of these cells is influenced by PACAP-like neuropeptides. The description of the function of large basophil cells needs further investigation.

**Cells with invaginated nucleus** were identified first in intact animals, the number of these cells increased in regenerated ones. They are star-shaped cells with large pseudopods. Since these cells can be found in high number in injured segments and phagocytose intensively, they can participate in elimination of tissue-debris and in the destruction of microorganisms in wound presumably.

Our immunohistochemical and RIA investigations demonstrated PACAP27 and PACAP38 (PACAP-immunoreactive molecules) in a part of coelomocytes. Increasing of PACAP-concentration in coelomocytes can be stimulated by injury, and the concentration of these peptides are higher in intact than regenerated segments. Based on our results we suppose that coelomocytes transport PACAP (and other bioactive compounds) to the place of injury and they release them, so they are involved in the regulation of the regeneration process influencing the function of other cells (neoblasts, other coelomocytes characterized by PAC1-receptor expression). The origin of the PACAP content of coelomocytes has not been identified yet but our previous results suggest that coelomocytes can take up the peptid from the blood, so they do not synthesize just transport it.

According to the comparison of our results and previous data we can determine that several coelomocyte-types can be found in the coelomic cavity of *E. fetida* and other oligochaeta earthworms, and there are several subpopulation with different forms and activities. Cells of the coelomic cavity can be in continuous alteration, so the transitional forms can be determined by the observation of specific cell markers.

We described that the **regeneration blastema** contained not only amoebocytes and eleocytes but granulocytes as well in high number, which could take part in the transportation

of bioactive compounds. In contrast with the previous hypotheses, according to which eleocytes can transdifferentiate to other cells during regeneration, we determined that the new tissues differentiated from totipotent neoblasts of the regeneration blastema.

Our *in vivo* and *in vitro* examinations revealed the role of amoebocytes in cellular immune response. Applying Sp2 cells in our model-experiments we proved the production of cytotoxic molecules by coelomocytes which participated in the humoral immune response. The cytotoxic molecules induce the death of foreign cells by increasing the intracellular calcium-concentration.

## **SUMMARY**

The coelomocytes of the earthworm (*E. fetida*) were studied with microscopic and molecular biological methods. Similarly to the previous findings three major types of coelomocytes, the eleocyte, the granulocyte and the amoebocyte were identified and characterized by conventional light- and electron microscopical techniques and histochemical methods. In addition, subtypes and possible developmental stages were described within these groups on the basis of their ultrastructure and cytochemical features.

Our results show that both eleocytes and granulated cell debris originated from haemoglobin-producing chloragogen tissue possessing also trophic and storage functions. Based on electron microscopic results, we have to assume that the discrete cytoplasmic segments split from the peripheral chloragocytes, while the eleocytes are apical daughter cells of dividing chloragocytes.

The role of coelomocytes was described in the regeneration. Based on the RIA, immunocytochemical and ultrastructural results we conclude that certain bioactive materials are transported to the regenerating part of the body by eleocytes and granulocytes. The phagocytotic activity and inducible acid phosphatase production were confirmed in the amoebocytes. Based on the above mentioned amoebocytes recognize, phagocytose and eliminate the damaged cells and cell debris. It was shown with light-and electron-microscopic immunocytochemistry, radioimmunoassay and Western blot analysis that the neuroprotective PACAP-like peptides (which were previously demonstrated in vertebrates as pituitary adenylate cyclase activating peptide) and their specific PAC1-like receptor occurred in the coelomocytes isolated from the regenerating segments. Our results show that the concentration of PACAP-like peptides increased in the coelomocytes of the regenerating animals, and the amount of these compounds show a decreasing rostrocaudal gradient. It may

indicate that coelomocytes uptake and transport these peptides to the regenerating segments. Contrary to previous data we found that the renewing tissues of the regenerating body part did not originate from the free-floating coelomocytes (eleocytes, amoebocytes and granulocytes) or dedifferentiating tissues, but they were formed from the mesodermal totipotent stem cells, so called neoblasts, that migrated to the site of the damage, attached to old tissues. Furthermore, cells of cancer cell lines were treated with lysate of coelomocytes. It can be established by our flow cytometric and ultrastructural studies that the coelomocyte lysate has cytotoxic effect, which may be evidence of active involvement in humoral immune response.

## **PUBLICATIONS**

### **Publications related to the thesis**

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(\*: according to the data of 2011)

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