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Structural and neurochemical changes during the posterior regeneration of *Eisenia fetida*

PhD Thesis

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INTRODUCTION

The experiments with earthworms have contributed to a better understanding of the basic processes of the regeneration. Due to their special anatomical organization, such as the segmental structure, their closed circulatory system, or the presence of free-flowing coelomocytes in the body cavity, they have enormous regenerative potential. The capability of the segment regeneration varies from species to species within the Annelids. Regeneration can occur with epimorphosis (blastemic regeneration) or morphallaxis (tissue rearrangement). In epimorphosis, totipotent stem cells, called neoblasts are activated and form the regenerative blastema. The process can be divided into five parts such as wound healing, blastema development, blastema patterning, differentiation and growth. In that case, when the new segment is formed by intact cells without neoblast, it is called morphallaxis. The reorganization capacity depends on the size and the anatomical position of the amputation and the environmental conditions. As a first step of the regeneration, the severed segment is contracted by rapid muscular contraction of the body wall. After wound healing the renewed segments will develop from the regeneration blastema which consists of several undifferentiated cell forms: epiblast, which come from body wall epithelium, myoblasts, that are derived from the dedifferentiation of body wall and alimentary canal musculature, endoblasts, that is continuous with the severed midgut epithelium and migrating mesodermal cells. The latter group includes various phagocytic coelomocytes, chloragocytes from splanchnopleura and neoblasts. Although the exact cellular and molecular biological background of the blastema formation is not known yet in the smallest detail, but it has been reported that the ventral nerve cord (VNC) has significant influence to initiate segmental regeneration in the posterior regeneration of annelids worms. Different experimental results were obtained in each annelid species, so there has been no clear evidence of the neural elements dependence or their inhibitory effects on regeneration yet.

The nervous system of the earthworms contains a large number of neurosecretory cells, which are mainly located in the supraesophageal ganglia (brain) and in the subesophageal ganglia. As a result of amputation, the nervous system can release trophic and growth factors and neurohormones, which activate repair mechanisms in peripheral tissues and induce cell proliferation in the area of blastema. Possible regeneration regulator is PACAP (Pituitary Adenylate Cyclase-Activating Peptide), which is a major regulator of cell growth and differentiation in vertebrates and plays an important role in the development of the nervous system.

It has been shown that PACAP is strongly upregulated following different types of nervous injuries, which implies its functional importance in regeneration processes. The PACAP molecule is widespread and its primary structure is the same in all mammalian species that shows great similarity to PACAP proteins isolated from lower vertebrates. The cell and tissue renewal are essential processes and their fundamental steps are very similar in different animal groups. Based on the neuroprotective effect of PACAP and on the several common mechanisms of vertebrate and invertebrate nervous regeneration processes, the question can be raised whether PACAP has any role in invertebrate nervous system regeneration.

GABA (Gamma-AminoButyric Acid) also can be taken into consideration as a potential regenerative factor. It is a well-known inhibitory neurotransmitter in the vertebral brain but it has also been described as a neuronal differentiation regulator. As a regulatory neurotransmitter, it participates in regenerative neurogenesis, regulates the proliferation and migration of neural cells and the differentiation of neuroblasts. In addition, GABA is believed to be responsible for the synaptic integration of newly formed neuronal cells. Most of these results are based on the researches on vertebral nervous system and there is only a few available information on the role of GABA in the regeneration of invertebrates, despite the relatively high number of GABA immunoreactive cells (GABA-IR) in both central and peripheral nervous systems in Oligochaeta. But these studies were mostly limited to the morphological changes of GABA-IR elements and the differentiation of the pattern of neural structures.

AIMS

According to the literature data, it is still not known what happens immediately after the removal of the sections, how the injured organs / tissues react to the surgical trauma; furthermore how to fit the old and the new structural elements during the regeneration of the segments and the organs in them; whether the same tissue pattern characterizes the organs of the regenerated and the original sections also. Neither is it known what kind of role have the circulating hormones and the mesodermal neoblasts and coelomocytes in the regeneration processes besides the local nervous system effects.

- For this reason, in the first part of our researches, after standard segment amputation we aimed to reveal the exact temporal process of tissue changes due to surgical trauma, in order to better understand the early processes of regeneration and the way in which regenerative blastema develops, and to discover the accuracy of the posterior regeneration (anatomical pattern loyalty).
- We aimed to study how the regeneration blastema is affected by transmitters / neurohormones released from the severed nervous system.
- Our aim was to identify transmitter-specific neurons and neuronal groups that, based on their number, position and their pathway, can be anatomical milestones that can be used to determine the accuracy of regeneration for further regeneration biology research.
- In order to distinguish the local nervous system effects affecting the posterior regeneration and the possible hormonal effects, it was necessary to eradicate the brain and the pharyngeal connectives known as the neurosecretory centers.
- We aimed to study the stimulating effect of the regeneration on the sessile and freely flowing coelomocytes close to the wound side and further in the neighboring segments.

MATERIALS AND METHODS

Experiments were carried out on adult sexually mature earthworms (*Eisenia fetida* Sav., Lumbricidae, Oligochaeta), which were collected from our standard laboratory breeding stock.

Experiments of the regeneration kinetics

Four different groups were defined with contains of 10 animals and different operations were carried out in each group. The first two segments were amputated in the first group, the brain was removed in the second group, the subesophageal connectives were isolated in each side of the ganglia in the third group and we used a control group without anterior amputation. Posterior amputation was applied after the clitellar region in the posterior border of the 25^{th} segment in each group.

Histology

The animals were used for histological investigation after 1, 3, 6, 24, 72 hours and one week of posterior amputation. The severed segments with the five previous intact segments and with the regenerated part were fixed with the suitable fixative solution. Cross sections and longitudinal sections were also created and the samples were stained with haematoxylin-eosin stain.

Ultrastructural investigation

The regenerated animals were fixed in the mixture of 4% paraformaldehyde and 5% glutaraldehyde in 4 °C, then post fixed in 2% OsO4. Fixed samples were embedded in epoxi resin, ultra-thin sections were cut and contrasted with uranyl acetate and lead citrate, than examined with a transmission electron microscope.

Immunohistochemistry

After preparation, the samples were fixed in Boer-solution at room temperature. Pre embedding immunolabellings were applied using anti-GABA (A2052) and PACAP27 (88121-5), PACAP38 (88111-3) primary antibody. The immunolabellings were visualized by 3,3'-diaminobenzidine (DAB). Most of the samples were used as whole-mount, others were embedded into epoxy resin and were sectioned in serial sections.

PACAP Radioimmunoassay (RIA)

To quantification of PACAP peptide content some samples were homogenized and centrifugated and the supernatants were used for 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.

RESULTS AND DISCUSSION

According to our histological experiments we have found extremely dynamic histological changes in the severed segment affecting all organs and tissues. After amputation, in the early stages of regeneration in great numbers of small-size, eosinophilic cytoplasmic cells, identified as dedifferentiated muscle cells, were accumulated near the peripheral tissue surfaces (body wall, epithelium of alimentary canal). These were partly derived from the body wall and partly from the alimentary canal. Between them, we found free-flowing coelomocytes types (eleocytes, granulocytes) and some chloragocytes. The results of our histological studies suggest, contrary to the previous assumption, that in the wound healing basically not the free-flowing coelomocytes play the main role, but rather the rapidly dedifferentiated muscle cells.

In earlier summary studies, the significance of totipotent stem cells, called neoblasts, was emphasized in the regeneration processes. However, recent research findings questioned the role of neoblasts in regeneration of Annelids. There are known regenerating earthworms, such as *Enchytraeus buchholzi*, in which neoblasts have not been found and were still capable of posterior regeneration. In addition, the latest results by time lapse video microscopy showed that some of the cells, identified as neoblast, migrated to the opposite direction from the severed segment during the regeneration. In our histological sections, neoblastic forms were found only in a small number, forming characteristic rows in the area between the regenerating VNC and the alimentary canal. These cells characterized by high nuclear-cytoplasm ratio developed process-like structures, and between them cellular contacts were detected. According to this, these cells were already considered to be neuroblasts. Therefore, these results suggest that neoblasts are thought to be differentiated to only neural structures.

After the amputation the released epithelial and muscle tissue factors and the discharged materials from the damaged nerve cells caused rapid degeneration of the severed ganglion neurons to the roots of the second and third segmental nerves. The localization of chromatolysis in neurons of severed ganglion coincided with neuronal degeneration of later regeneration phases. Since our histological sections were characterized by ganglion degeneration in the early regeneration phase, our results suggest that the neurological effects may not be the determinants in contrast to the generally accepted model.

According to our experiments, thinner fibers running in the central neuropil of the severed gangion, especially those on the dorsal side regenerated in large numbers, while the dorsal giant axons degenerated to the border of the segment. Thin fibers running on the dorsal side of neuropil of the ganglion (e.g. GABA-IR polysegmental fibers) were originated from relatively large pericaryons, because of this, after damaging fibers, was caused less trauma to the neurons that were able to regenerate their processes. Observations of immunostained whole-mount preparations of the regenerating VNC (GABA-IR structures) proved that the size of the first two or three regenerated ganglia, the number of segmental nerves and the pattern of GABA-IR neurons may differ from both the original and from the other regenerated ganglia. These results suggest that the survivor neural structures of the severed ganglion play a determinative role in the formation of the new ganglia. At the dorsal side of the ganglion the regenerating processes leaving the ganglion penetrate into myoblast and neoblasts located loosely behind the ganglion and as "anatomical scaffolds" guide the adherence of migratory neoblasts between the regenerating ganglion and the alimentary canal, and the compounds released from them (e.g., PACAP-like peptides, GABA) may regulate cell proliferation and differentiation processes.

In our GABA immunohistochemical observations, GABA-expressed neural elements were detected in all regeneration phases. The early appearance of GABA positive structures suggests that this substance may have a role in inducing regeneration, and supports the hypothesis that neural structures, with other regulatory factors, may play an important role in organizing tissue regeneration. In some cases, asymmetric distribution was also observed in the regenerated ganglion next to the incision, suggesting that there was a lack in the formation of the central neural pattern because of the amputation. In the regenerated ganglia further away from the operation, pattern information was restored. The occurrence of ganglia with asymmetric cell pattern in the already regenerated earthworms shows that bilateral organization of GABAergic cells is not necessary to the complete regeneration of the animal. Regeneration of GABA-IR neural elements located in the center is followed by segmental epimorphic regeneration of peripheral structures. Based on the results of our experiments we can conclude that GABA may play a significant role in regeneration processes. According to its early appearance and continuous expression, it can be concluded that GABA can participate in the modulation of the regeneration process and in the reorganization of the sensory and motor function. However, further molecular, histological and electrophysiological studies are required to reveal the complete and accurate mediator function and the detailed mechanisms of action.

Our presented results showed that the concentration of PACAP-like compounds increased in certain tissues of earthworms following injury, and PACAP-like immunoreactivity appeared in various tissues and cells of the regenerated segments. We have shown that the occurrence of PACAP-like molecules varies along a rostrocaudal gradient in coelomocytes of the regenerating earthworm. Based on these we can conclude that these materials can be transported (or even synthesized) by the coelomocytes in the regenerated segments. The role of coelomocytes in trophic and transport functions has already been demonstrated. Another study also raises the potential role of coelomocytes in hormone secretion, transport and stopping the hormone effect. PACAP-like compounds in the earthworm may not only participate in the regeneration process as a mediator but can also modify the behavior of the coelomocytes involved in phagocytosis or anti-inflammatory effects.

Increasing in the concentration of PACAP-like compounds in the regenerating body wall and alimentary canal (consisting of regeneration blastema and the neighbouring original tissues) indicates that these compounds could have influence on the regeneration of earthworms. Since numerous immunolabeled neoblasts were found in the regenerating segments and especially in the regeneration blastema, we suggest that PACAP-like substances could have stimulating effects both on cell cycle and differentiation of neoblast to neural direction. Our studies have shown that PACAP or PACAP-like peptides accumulate in the regeneration tissues of the earthworm. Based on these, we can assume that PACAP can also perform trophic function in these animals, similarly to vertebrates.

SUMMARY

The process of posterior segments regeneration of *Eisenia fetida* was investigated by anatomical, histological and immunhistochemical methods after the amputation of 25th posterior segments behind the clitellum. Based on our results a new model of the segment regeneration was proposed. We have shown that the dedifferentiated epithelial and muscle cells of the alimentary canal and the body wall played a determinative role in the wound closure, although coelomocytes were involved in the process as well. We found that the neurons of the VNC were destructed to the roots of segmental nerves 2 and 3, and the disorganization of the neuropil was occurred at the same time. In the injured segment and its adjacent segments phagocytic coelomocytes were accumulated, which contributed to the formation of new tissues by recognizing and eliminating damaged cells and tissue debris and releasing main biomolecules. In the regeneration blastema behind the severed VNC mainly myoblast, free coelomocytes and several basophil neoblasts were occurred. Most of these neoblast were located between the severed ganglion and the gut. The epithelium of the regenerating gut was formed from the original gut epithelium, the muscle layer of the original gut muscle. Similar histological transformations characterized the regenerating body wall. In the regeneration of the VNC the neoblasts played a role, which adhered to the anatomical scaffold-forming neural projections from the severed ganglion and to the wall of the capillaries. Presumably, this explains why the first (sometimes second and third) regenerating ganglion has abnormal anatomical and histological organization. Ergo the direct effects of the nervous system are important, but not determinant in the process of the segment regeneration. We have shown that after the removal of the posterior sections the activity of not only the severed but also the adjacent 3-5 segments were changed (accumulation of free-floating coelomocytes, development of phagocytic chloragocytes). The results of our extirpation experiments (removal of cerebral ganglion, circumpharyngeal connectives, prostomium with the first two body segments) suggested the importance of the hormonal (neuroendocrine) regulation of the regeneration and noted a potential role of the peripheral neurohemal organs (prostomium with the first two body segments) not yet known in earthworms, and the endocrine organs (clitellum, metanefridia) as well. We have analyzed in detail the role of GABA and PACAP-like peptides in controlling regeneration blastema and in influencing tissue differentiation, emphasizing that, like these compounds, multiple neurotransmitters and neurohormones could influence the segment regeneration.

PUBLICATIONS

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