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Octopus arm regeneration: Role of acetylcholinesterase during morphological modification ☆

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ABSTRACT

The ability to regenerate whole-body structures has been long studied in both vertebrate and invertebrate animal models. Due to this regeneration capability here we propose the use of the Cephalopod *Octopus vulgaris* as a model of regeneration. We investigated the involvement of acetylcholinesterase (AChE) in the octopus arm regeneration. AChE has been demonstrated to have non-cholinergic functions in various cell types and to be involved in the regulation of cell proliferation, differentiation and apoptosis. In order to follow cell replacement in the octopus arm, we first assessed the expression of specific markers involved in cellular proliferation (AgNOR and PCNA). We showed that the activity of the enzyme AChE is related to the proliferation stage of the arm regenerative process. In the very initial stages of regrowth when most of the proliferation activity was at the level of the 'blastema' the cholinesterase activity was very low. AChE activity climbed slowly during the subsequent phase of cellular multiplication and, by the onset of morphogenesis, the activity rose sharply and active myogenesis was observed. AChE activity decreased then till reaching basal level at the time when the process of histogenesis occurred and the reestablishment of all the structures became evident. Interestingly AgNOR and AChE assay showed a similar trend in particular during the stages when the morphogenesis was mostly dependent upon cell proliferation. We suggest that AChE protein may have an important influence in the process of regeneration and that it could be considered as a potential target to promote or regulate the regenerative process.

1. Introduction

A regenerative process implies the renewal, restoration, and growth of cells, tissues, and organs that have been physically or functionally lost. Several vertebrates and invertebrates have been used as 'model system' to study conserved and convergent mechanisms of plasticity and regeneration pathway.

We are testing the hypothesis that acetylcholinesterase (AChE) plays a major role in the regenerative process of the octopus arm. Several studies in both vertebrates and invertebrates have been pointing toward the role of AChE in the regeneration process. The abundance of acetylcholine within the regenerating tissue has been correlated inversely with the activity of the AChE (Ellman et al., 1961; Karnovsky and Roots, 1964; Lenicque and Feral, 1976; Singer et al., 1960). The importance of AChE in regeneration lie in that during the early formative phases of growth the development can be most dependent upon the nerve. It seems that acetylcholine can be used by the nerve as an agent to control the early events of regeneration (Singer et al., 1960). In *Aplysia* AChE has been shown to promote neurite growth of adult neurons (Srivatsan, 1999; Srivatsan and Peretz, 1997). In *Planaria* it has been found to be connected with the regeneration stages (Lenicque and Feral, 1976). Also in *Triturus* it has been found that the activity of the enzyme changes in location and intensity during the whole regeneration process (Singer et al., 1960). Recently it has been proposed that AChE may contribute to various physiological processes through its involvement in the regulation of cell proliferation, differentiation, apoptosis and survival. AChE was also found to be highly expressed in proliferating myoblast during muscle regeneration. This process is always accompanied by cell apoptosis and therefore it was hypothesized that the AChE expression in myoblasts reflected the development of the apoptotic apparatus (Pegan et al., 2010). AChE participates in apoptosis in two ways: by promoting or suppressing cell death. Both direct and indirect evidence reported the involvement of AChE in regulation of cell proliferation and apoptosis (Bytyqi et al., 2004; Jiang and Zhang, 2008; Kehat et al., 2007; Robitzki et al., 1998; Soreq et al., 1994).

Regeneration of arms in cephalopods and in particular in Octopods has been the subject of several studies due to their high regenerative

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power (Callan, 1940; Dingerkus and Santoro, 1981; Feral, 1988; Lange, 1920; May, 1933; Voight, 1992). However, the morphological modification and molecular pathway of the regenerative process in the octopus arm remain to be elucidated.

Detailed information on the proliferative activities of cells relevant to events of regeneration is important to understand the mechanisms underlying regeneration. In this study immunohistochemical staining for proliferating cell nuclear antigen (PCNA) and argyrophilic nucleolar organizer regions (AgNORs) have been utilized to detect proliferating cells. Reported literature show that AgNOR expression is strictly related to the cell duplication rate in both cancer (De Vico et al., 1994, 1996; Madewell, 2001) and regenerative tissues (Tuccari et al., 1999) and it is believed to be a useful method for examination of nucleolar structure and variations in nucleolar activity (Egan and Crocker, 1992; Sirri et al., 2000). PCNA is a nuclear protein which serves as a cofactor for DNA polymerase-delta. It is involved in the coordination of cell cycle progression and DNA replication (Hall et al., 1990; Jónsson et al., 1998).

Due to the octopus typical cholinergic innervation here we are testing the hypothesis that AChE plays a major role in the arm regenerative process (Barlow, 1971; D'Este et al., 2008; Florey, 1963; Florey and Winesdorfer, 1968; Loe and Florey, 1966; Messenger, 1996, 2001; Rohrbach and Schmidtberg, 2006; Talesa et al., 1995; Welsch and Dettbarn, 1972). The identification of differences in the activity and localization of AChE activity during regeneration can provide useful information on latent basic pathways which could be unlocked to promote regeneration. In this context, AgNOR and PCNA can be helpful instruments to evaluate cell types involved in the regenerative process.

2. Materials and methods

2.1. Animals and treatment

Specimens of *Octopus vulgaris* of both sexes were collected from the Ligurian coast during the spring/summer period and placed in 80×50×45 cm marine aquaria. The tanks were filled with artificial sea water (ASW) and kept at a temperature of 18 °C at 12 h light/dark cycle. Water cleaning and oxygenation were assured by a pump-filter and aeration system which continuously circulated the water through biological filters. All relevant chemo/physical water parameters were constantly checked to prevent the occurrence of unhealthy or stressing conditions for the animals. Animals were left to adapt to captivity for at least 10 days before experimentation. The experimental animals were selected on the basis of the following criteria: healthy shape (all the arms and body parts had to be intact, the animal showed normal reflexes and voluntary movements such as arm extension, walking, etc.), regular eating and motivation to attack a prey (for a description of the behavior of *O. vulgaris* in captivity see for example: Boycott, 1954; Hochner, 2008).

Experimental animals (6 females) of around the same body weight (500–800 g) were used. Animals were anaesthetized in 2% ethanol in ASW (Andrews and Tansey, 1981; Boyle, 1981; Crook and Walters, 2011; O'Dor et al., 1984) for about 5 min, until clear change in body patterns confirmed that the cephalopod underwent the physiological process of anesthesia. Small portions of the arm tip (1–2 cm) were cut with fine scissors from all the arms (ranging between 35 and 38 cm), in a transverse plane perpendicular to the longitudinal axis of the arm and immediately fixed in 4% formaldehyde in ASW. Following surgery, each animal was placed in the experimental tank where it slowly (about 2–5 min) recovered from anesthesia. The animal did not display behavioral modification after the operation, and in all the animals the amputated arms regenerated as in the natural environment (Crook and Walters, 2011). Arm samples from intact arms were used both for anatomical description of non-regenerating arms and as controls for the detection of specific markers involved in the regeneration process. The regenerating arm tips (which include the entire regenerating arm portion plus a small piece of the intact arm) were collected at different days of regeneration and further processed for microscopical investigations. At the Day 134 all the animals were sacrificed by immersion in 2% ethanol until respiration ceased and the heart stopped pumping. We focused on 3, 11, 17, 21, 28, 42 and 55, 108 and 134 days of regeneration when, substantial changes in the arm morphology could be observed (Lange, 1920).

2.2. Histology and immunohistochemistry

Three arms from three different animals for each regenerating day (3–55) were employed. Samples were fixed for 4 h in 4% formaldehyde in ASW at 4 °C and then embedded either in paraffin wax or OCT compound, serially sectioned at 5–10 μm and collected on Superfrost slides (Bio-Optica). For basic anatomical description longitudinal sections were processed using a common hematoxylin and eosin staining to characterize the morphology of normal and regenerating tissue. Sections were examined and photographed with bright field microscopy (Nikon eclipse 80i upright microscope). Image acquisitions and analysis were performed with the Nis Elements (Nikon) and with ImageJ software. All the quantification analyses were performed by two people "blinded" to the stage of regeneration at which samples were taken.

2.2.1. AgNOR and PCNA assay

Longitudinal sections of control and regenerated arms from 3 animals were serially sectioned (5 μm) from the paraffin wax block, dewaxed in several baths of xylene and hydrated through graded alcohols to ultrapure water. Sections were processed for AgNOR (Ploton et al., 1986) and PCNA (proliferating cell nuclear antigen) evaluation.

Microscopical assessment of sections treated for both AgNORs and PCNA was performed at about 100 μm from the base of the regrowth (proximal part) and at about 100 μm from the arm tip (distal part). The specific activity of muscular cells was also evaluated. The number of dots of AgNORs (Days 3–55) per nucleus was assessed in all the exanimated animals on one focal plane with a 100× objective lens in at least 100 nuclei per specimen, as recommended by Crocker et al. (1989). The enumeration of each silver stained dot per cell counted at the microscope was performed carefully focusing through the section thickness at very high magnification (100×). On each slide, two operators independently defined the mean AgNOR number. According to the recommendations of Crocker et al. (1989) and De Vico et al. (1994), when a dot aggregation could not be resolved in individual NORs by focusing, the cluster was considered as one discrete AgNOR.

PCNA positive nuclei were scored assessing PCNA positivity per 100 cells (PCNA Index). Values were expressed as Mean ± SD.

2.2.1.1. AgNOR. The staining solution was obtained instantly by rapidly mixing one part of solution A (2% gelatine solution dissolved in ultrapure water, to which formic acid is then added to make a final 1% solution) with two parts of solution B (50% silver nitrate solution in ultrapure water). Re-hydrated sections were immersed in 0.01 M sodium-citrate monohydrate buffer pH 6.0 and autoclaved at 120 °C for 20 min. Subsequently the sections were allowed to cool down to room temperature and then treated with silver staining for 25 min at room temperature in a dark place.

2.2.1.2. PCNA. After dewaxing of the tissue sections, endogenous peroxidase was blocked by incubation in H_2O_2–methanol (4:1) for 20 min. Sections were hydrated and incubated with sodium citrate buffer (pH 6.0) in a microwave for 5 min at 575 W, in order to unmask the antigens and epitopes. Subsequently the sections were allowed to cool down to room temperature and washed several times in PBS. Sections were then treated with 10% goat serum,