Growth factors in asexually reproducing Catenulida and Macrostomida (Plathelminthes)?

A confocal, immunocytochemical and experimental study

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Summary. The occurrence and localization of immuno-reactivity (IR) to epidermal growth factor receptor (EGF-r), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), revealed by the present study, indicate growth-factor-like substances in the asexually reproducing flatworms, *Stenostomum leucops* and *Microstomum lineare*. IR to all three antibodies occurs in the nervous system. Differences in the number of positive cells and intensity of IR during asexual development of new individuals were observed. By confocal scanning laser microscopy, immunopositive growth cones of nerve fibres were seen in developing zooids, and weakly or unstained perikarya were observed in close contact with the positive nerve fibres. Antibodies to the growth factors EGF-r, EGF and bFGF as well as to the neuroactive substances 5-HT and RF-amide had a negative influence on the growth and asexual reproduction of cultured *S. leucops*. No significant differences in the influence of antibodies to growth factors and antibodies to the neuroactive substances were observed.

A. Introduction

The mitogenic polypeptides, epidermal growth factor (EGF) and basic fibroblastic growth factor (bFGF), were originally purified from male mouse submaxillary gland and bovine pituitary gland respectively (Cohen 1962; Bohlen et al. 1984, 1985). Recent data indicate that both EGF and bFGF have neurotrophic effects in addition to their mitogenic effect and play a significant role in the guiding of neuronal development (Morrison et al. 1988 a; Westermann et al. 1990 a).

The occurrence and influence of growth factors on lower invertebrates has hitherto gained little interest. Growth is usually associated with the early stages of development, but in asexually reproducing flatworms it remains through the whole life and is also manifested in the regenerative capacity. Recent studies indicate that the epidermal growth factor is a potent mitogen stimulating cell proliferation in intact planarians (Baguña et al. 1988). Furthermore, receptors for growth hormones are suggested to exist in the cell membranes of the proliferating cells (neoblasts), based on faint reactions to EGF-receptor antibodies in recent studies (Baguña et al. 1990).

A number of neuromediators of peptidergic and amnergic nature have been localized in the nervous systems of flatworms in different taxa by IC methods (for review see Reuter and Gustafsson 1989; Gustafsson and Reuter 1992). In the phylogenetically low-ranking microturbellarians, *Stenostomum leucops* and *Microstomum lineare*, the investigated immunoreactivity patterns differ significantly from each other (Reuter et al. 1986; Reuter and Palmberg 1983; Wikgren and Reuter 1985; Wikgren and Thorndyke 1990). Both these microturbellarians are characterized by their capacity to form chains of zooids and to regenerate (Palmberg and Reuter 1983). The development of the immunoreactivity to 5-HT, RF-amide and SCP₈ in *M. lineare* follows different, distinct sequential patterns, antigenicity to 5-HT in the postpharyngeal commissure indicating the initiation of the development of a new zooid (Reuter and Palmberg 1989). The order of the sequence in the appearance of new zooids is visualized in Diagram 1.

In addition to IR to 5-HT, FMRF/RF-amide and SCP₈, IR to the growth hormone releasing factor (GRF) occurs in all investigated flatworm taxa (Reuter et al. 1988; Reuter and Gustafsson 1989). In *S. leucops*, IR to GRF is observed in two cells peripherally to the brain (Wikgren and Reuter 1985), whereas in *M. lineare* it is detected in the nervous system both by light microscopic IC and by the immunogold technique on the ultrastructural level (unpublished observations).

Up to now, however, no investigations of the growth regulating factors have been made in flatworms. In the present study, the presence and localization of the bFGF, EGF and EGF-r are investigated in the asexually reproducing species *S. leucops* and *M. lineare* by the
IC method. Furthermore, the use of confocal scanning laser microscopy combined with the immunocytochemical wholmount technique (Johnston et al. 1990), opens a new approach for three dimensional studies of the development and differentiation of the asexually reproducing and regenerating microturbellarians (Palmberg and Reuter 1990). Moreover the influence of the antibodies to the EGF, bFGF and to the EGF-r on the asexual reproduction rate is investigated.

B. Materials and Methods

Specimens of *Stenostomum leucops* (Dugès, 1828) (Catenulida) were collected from a stock culture maintained in containers with tap water. Most specimens consisted of only one zooid, but species with two zooids were also obtained. Specimens of *Microstomum lineare* (Müller, 1774) (Macrostomida), consisting of chains of 2-4 zooids in different developmental stages, were caught in summer in shallow brackish water at Stortervo, Pargas (SW Finland).

**Immunocytochemistry.** The worms were fixed in "Stefanini's fluid", a mixture of formaldehyde and picric acid (paraformaldehyde 2 g, 0.1 M Na phosphate buffer 85 ml, picric acid 15 ml) at pH 7.0 for 1-3 h at +4°C, and rinsed overnight in a 0.1 M Na phosphate buffer (pH 7.4) containing 10% sucrose. They were then placed on glass slides, allowed to dry, and frozen at -70°C. Prior to staining, the worms were thawed and penetrated with Tris buffer saline (TBS) containing 1% bovine serum albumin (BSA) and 0.2% Triton X-100. For control, and for the preservation of the three-dimensional structure of the nervous system, fixed specimens were produced by an Olympus automatic photomicroscopic system, model PM 10ADS. For images recorded with the confocal microscope artificial colour, tables greengreen (means from black to bright green) and inverse (means negative image) were used and images were photographed from a computer screen (Film: Kodak T-Max).

**Examination.** The whole mounts were either examined by a Leitz Orthoplan microscope combined with filter-blocks I2 and N2 or by a confocal beam scanning laser microscope (CCM, designed and built in EMBL for the Centre for Biotechnology, Turku/Abo) connected to a Zeiss Axiosvert 10 inverted fluorescence microscope. When the Leitz Orthoplan microscope was used, the photographs were produced by an Olympus automatic photomicroscopic system, model PM 10ADS. For images recorded with the confocal microscope artificial colour, tables greengreen (means from black to bright green) and inverse (means negative image) were used and images were photographed from a computer screen (Film: Kodak T-Max).

**Experimental study.** Specimens of well-fed *S. leucops* were cultured for five days in media containing antibodies to EGF, bFGF or EGF-r and as controls antibodies to 5-HT, RF-amide and a medium without any antibody. The following antibody concentrations were used: 1:500, 1:1000, 1:2000 and 1:5000. In total 125 specimens, 5 specimens per vial, were cultured in 600 μl medium. In addition, each culture was supplied with liver pieces of equal size (0.005 gr) washed free from blood.

C. Results

I. General results

Immunoreactivity to antibodies to the growth factors EGF and bFGF and to EGF-receptor are revealed in the nervous systems of the investigated flatworm species. However, *S. leucops* reacted positively only to a-EGF-r and a-FGF, while *M. lineare* showed positive reactions to all three antibodies. The most intensive IR was obtained by a-EGF-r in both species. In *M. lineare*, specimens consisting of several zooids, the development of brain and pharynx innervation could be studied in great detail by a confocal scanning laser microscopy. The information obtained from the specimens of *S. leucops* consisting of only two zooids gave less details. No differences in the preservation of the three-dimensional structure of the nervous system was observed by confocal microscopy between freeze-dried and freshly prepared worms.

1. *Microstomum lineare*

EGF-receptor antibodies give strong positive reactions in cells and fibres of the nervous system. The overview image of the distribution of IR to EGF-r in four zooids obtained by ordinary fluorescence microscopy shows different intensities of IR in the zooids (Fig. 1 B). In early stages, i.e. zooids III and IV, the IR intensity is low. The strongest intensity is obtained in the young mature zooid II. In the oldest mature zooid I it is somewhat lower, but still stronger than in immature zooids. The