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Cell proliferation and apoptosis in the olfactory epithelium of the shark *Scyliorhinus canicula*

Sara Ferrando [a,*], Lorenzo Gallus [a], Chiara Gambardella [a,b], Laura Ghigliotti [a], Silvia Ravera [a], Mauro Vallarino [a], Marino Vacchi [c], Grazia Tagliafierro [a]

[a] Department of Biology, University of Genoa, Genoa, Italy
[b] Department of Animal Biology and Marine Ecology, University of Messina, Messina, Italy
[c] ISPRA, c/o National Antarctic Museum, University of Genoa, Genoa, Italy

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ABSTRACT

To date, no study has been published on cell renewal in the olfactory epithelium of Chondrichthyes. Our work aimed at detecting proliferating cells (by Proliferating Cell Nuclear Antigen – PCNA immunohistochemistry) and apoptotic cells (by terminal uridine deoxynucleotidyl transferase nick end labeling method) in the olfactory epithelium of the shark *Scyliorhinus canicula*. PCNA immunoreactivity and mitotic figures were localized almost exclusively at the basal and apical thirds of the epithelial thickness. Double immunofluorescence for PCNA and OMP (a marker of mature olfactory neurons) showed that PCNA immunoreactivity is lacking in mature olfactory neurons, with the exception of crypt neurons. Crypt neurons, a cell type peculiar to fish, often showed PCNA immunoreactivity in the nucleus and may be involved in repair processes. The role of PCNA in mature crypt neurons requires further investigation to be clarified. Apoptosis was observed in sensory neurons and in basal cells. Our data highlight the presence of cell proliferation at different levels within the epithelium and the occurrence of apoptosis in both mature and proliferating cells.

1. Introduction

The olfactory epithelium (OE) of vertebrates includes three main cell types: sensory cells, basal cells (BCs) and supporting cells (SCs). The sensory cells of this primary sensory epithelium are olfactory receptor neurons (ORNs), one of the only neural populations continuously renewed throughout the entire lifespan of an organism (Graziadei and Monti Graziadei, 1978; Farbman, 1992). Different types of ORNs are present in the OE of vertebrates, characterized by various apical specializations (microvilli, cilia, apical crypt) and by differential expression of receptors and molecules involved in the transduction cascade (Thornill, 1967; Holl, 1973; Schild and Restrepo, 1998; Hansen et al., 1999; Hansen and Finger, 2000; Asan and Drenckhahn, 2005). These neurons are held in place and separated from one to another, by SCs, which carry out both epithelial and glial functions (Weiler and Farbman, 1998; Beites et al., 2005). BCs are proliferating cells, involved in neuroepithelial renewal.

In adult mammals, under normal conditions, BCs give rise to ORNs, while SCs seem to be a self-renewing population (Weiler and Farbman, 1997; Beites et al., 2005). The limited data available in teleosts are unclear on this point: some data suggest that BCs could give rise to both ORNs and SCs because cell proliferation happens only in the BCs and not in the upper layers of the OE (Byrd and Brunjes, 2001). Other studies, describing exceptional cell proliferation during recovery after chemical injury, reported cellular proliferation at all epithelial levels (Bettini et al., 2006, 2009). No data are present regarding the cellular renewal of the OE in cartilaginous fish. The aim of this work was to investigate, for the first time, cell renewal in the OE of a cartilaginous fish, the small-spotted catshark *Scyliorhinus canicula*. In particular, cell proliferation has been evaluated by detecting the presence and distribution of Proliferating Cell Nuclear Antigen (PCNA) immunoreactivity (ir). The synthesis of this protein begins in the late G1 phase and peaks during the S phase of the cell cycle (Foley et al., 1991); its immunolocalization has already been used to detect cell proliferation in retinas of several elasmobranch species, including *S. canicula* (Ferreiro-Galve et al., 2010). The identification of mature olfactory neurons was provided using the olfactory marker protein (OMP) (Margolis, 1972; Farbman and Margolis, 1980). The presence of OMP ir in the OE of *S. canicula* has been previously assessed (Ferrando et al., 2007a) and it has been used as a

* Corresponding author at: LIBiOM, Department of Biology, University of Genoa, Viale Benedetto XV, 5, I-16132, Genoa, Italy. Tel.: +39 010 3537015; fax: +39 010 3538047.
E-mail address: sara.ferrando@unige.it (S. Ferrando).

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marker in many previous studies (e.g., Miragall and Monti Graziadei, 1982; Schwob et al., 1992; Kondo et al., 2010) about cell renewal in the OE.

In order to give a more complete description of cell renewal, the presence and distribution of apoptosis have been evaluated by the terminal uridine deoxynucleotidyl transferase nick end labeling (TUNEL) method.

2. Materials and methods

2.1. Sampling

Six adult specimens (4 males and 2 females) of *S. canicula*, with lengths ranging between 48 and 53 cm, were collected in the Irish Sea (NE Atlantic). Three specimens (1 male and 2 females), with lengths ranging between 39 and 46 cm, were collected in the Ligurian Sea (NW Mediterranean Sea) by bottom trawlers. Given their size and gonadal morphology, these specimens were immature or maturing individuals (Ivory et al., 2004). The specimens were brought on board, deeply anesthetized with 0.01% ethyl 3-aminobenzoate methanesulfonate salt (Sigma–Aldrich, MO, USA; dilution 1:1000 in sea water), rapidly decapitated and then dissected to collect the olfactory organs. No differences were highlighted during our work between the different stages or between the two populations (Mediterranean and Atlantic) and the data from all the specimens will be described and treated together.

2.2. Histological and immunohistochemical methods

Olfactory rosettes were fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered solution (PBS, pH 7.4) at 4 °C, Paraplast (Bio-Optica, Italy)-embedded and sectioned at 5-μm thickness. Histological observations were performed on hematoxylin and eosin-stained sections (Bio-Optica, Italy). Cell proliferation was immunodetected by the rabbit anti-PCNA polyclonal antiserum FL-261 (1:200 in PBS, Santa Cruz Biotechnology, USA), which is raised against the entire human PCNA gene (261 aa). As secondary antiserum, the EnVision System anti-rabbit HRP (DAB) (Dako Cytomation, Denmark) was used.

Double immunofluorescence for PCNA and OMP was performed using rabbit anti-PCNA polyclonal antiserum FL-261 (1:200 in PBS, Santa Cruz Biotechnology, USA) and goat anti-OMP polyclonal antiserum (1:800 in PBS, Wako Chemicals, VA, USA). As secondary antisera, a chicken anti-rabbit Alexa 594 and a chicken anti-goat Alexa 488 (both 1:800 in PBS, Molecular Probes, Invitrogen Corporation, CA, USA) were used. To verify the data obtained, another antiserum against PCNA was used: goat anti-PCNA polyclonal antiserum C-20 (1:200 in PBS, Santa Cruz Biotechnology, USA), raised against an epitope mapped to the C-terminus of human PCNA. The chicken anti-goat Alexa 488 (1:800 in PBS, Molecular Probes, Invitrogen Corporation, CA, USA) was used to detect this second anti-PCNA antiserum. Double immunofluorescence method for PCNA FL-261 and PCNA C-20 was performed using the two antisera at the concentrations reported above. A chicken anti-rabbit Alexa 594 and a chicken anti-goat Alexa 488 (both 1:800 in PBS, Molecular Probes, Invitrogen Corporation, CA, USA) were used as secondary antisera.

Relationships between PCNA immunostaining and various phases of the cell cycle were considered as described in Foley et al. (1991). Briefly, no staining corresponds to the G0 phase, weak nuclear staining corresponds to the G1 phase, intense nuclear staining corresponds to S phase or initial G2, intense nuclear and cytoplasmic staining corresponds to late G2, and speckled cytoplasmic staining corresponds to mitosis.

2.3. TUNEL method

Apoptosis was assessed in histological sections based on morphological characteristics, such as cell shrinkage, chromatin condensation with hematoxylin staining, as well as fluorescein-conjugated TUNEL staining (Roche, France). The fluorochrome used was FITC (excitation wavelength 494 nm, emission wavelength 518 nm). Before the TUNEL reaction was initiated, the histological sections were incubated for 20 min at 37 °C with Proteinase K 20 μg/ml in Tris–HCl (pH 8). Sections were then rinsed and incubated for 60 min at 37 °C with a freshly prepared solution of terminal deoxynucleotidyl transferase (TdT) from calf thymus and nucleotide mixture in reaction buffer. Negative control was performed by using nucleotide mixture without TdT enzyme.

2.4. Microscopy

Sections were examined using a BX60 Olympus light and epi-fluorescence microscope and visualized through an Olympus CCD Color-ViewII Camera (Olympus, Japan) with analySIS software (Soft Imaging System GmbH, Germany). Alternatively, sections were observed with a Leica DMRB light and epi-fluorescence microscope, equipped with differential-interference contrast filters. Images were acquired with a Leica CCD camera DFC420C (Leica, Switzerland).

2.5. Image analysis

Micrograph measurements and nuclei counts were performed using ImageJ 1.33 open source software (Rasband, 1997–2009). To quantify proliferating and apoptotic cells, labeled nuclei were counted by dividing the epithelium into apical and basal regions, with a hypothetical line in the middle of the epithelial thickness.

In the present study, cells in the S, G2 (according to Foley et al., 1991) and mitotic phases were considered as proliferating and thus counted. The total number of nuclei was determined by hematoxylin staining.

Each olfactory rosette was sectioned, and three slices were considered for the PCNA-immunoreactive nuclei count. The slices were separated by 0.2 cm in the thickness of the olfactory rosette; the central one was roughly in the dorso-ventral middle plane of the olfactory rosette (Fig. 1). One secondary lamella from each slice (thus three secondary lamellae for each fish) was randomly considered and examined from the proximal part to the distal apex. About 12,000–14,000 total nuclei were counted for each fish. The same method has been used for the TUNEL-labeled nuclei count and for CNs. In each lamella considered (three for every fish), the epithelial thickness was measured in the middle zone of the lamella.

2.6. Electrophoresis separation and Western blot analysis

Olfactory rosettes were frozen at −80 °C for Western blot analysis. All operations to obtain *S. canicula* mass homogenate were carried out at 4 °C. Samples were homogenized by a Politron system in 1 ml of homogenizing medium containing 10 mM Tris–HCl (pH 7.4) and a protease inhibitor cocktail (Ravera et al., 2007). The homogenate was stored at −80 °C, and the protein concentration was determined using the Bradford assay (Bradford, 1976). Denaturing electrophoresis was performed using a Laemmli protocol (Laemmli, 1970) with minor modifications. In these experiments, 50 μg of homogenate was loaded in the gel. Electrophoresis was carried out using a Mini Protean III (BioRad, Hercules, USA) apparatus (60 mm × 80 mm × 1.5 mm), in which both faces of the gel sandwich were immersed in buffer. The separating gel was a gradient from 10% to 14% (w/v) of polyacrylamide and 0.1% SDS (pH 8.8). The stacking gel contained 4.5% (w/v) polyacrylamide and 0.1% SDS. The samples were incubated with 8% SDS (w/v) in 125 mM Tris–HCl (pH 6.8) and 1.25% (v/v) DTT for 15 min to facilitate denaturation. Then, the samples were boiled for 5 min and a second solution, containing 40% (w/v) sucrose and 0.008% (w/v) Bromophenol blue, was added. The run was performed at 4 °C and 50 mA for each gel for 120–150 min with running buffer (0.05 M Tris (pH 8.0); 0.4 M glycine; 1.8 mM EDTA; 0.1% SDS). Protein molecular weight (MW) markers were purchased from Fermentas (Canada). After the run, the gel was stained with a Silver Blue stain (Candiano et al., 2004) or the proteins were transferred onto nitrocellulose (NC) membranes (Hybond ECL; GE Healthcare, Sweden) in a mini-transblot device (Bio-Rad Laboratories, CA, USA) at 400 mA for 2 h. The sheets were blocked overnight at 4 °C, in phosphate-buffered saline (PBS) plus 5% BSA. After washing with PBS, NC was incubated and stirred with the antiserum against PCNA FL-261 (Santa Cruz Biotechnology, CA, USA) diluted 1:200 in PBS plus 3% BSA, for 1 h at 20 °C. After extensive washing with PBS plus 0.15% Tween, the binding of Ab was revealed by the ECL detection system (Roche, Germany) using an anti-rabbit IgG as secondary antibody (Amersham Pharmacia Biotech, Sweden), diluted 1:7500 in PBS plus 1% BSA.

3. Results

3.1. Gross morphology

The olfactory organs of *S. canicula* were situated in cartilaginous capsules, positioned ventral-laterally in the head. They were roundish structures comprising numerous primary lamellae connected by a central raphe (Fig. 1a and b). Each primary lamella bore secondary lamellae on each side (Fig. 1c and d).

3.2. Histology

The OE of *S. canicula*, lining the olfactory lamellae, showed a typical morphology that has already been described (Franceschini and Ciani, 1993; Ferrando et al., 2006a,b; Ferrando, 2008). Briefly, the pseudostratified epithelium comprised ciliated SCs, BCs, light cells (LCs) and two types of sensory neurons, the microvillous ORNs and the crypt neurons (CNs) (Fig. 2a–c). The LCs have been observed in freshwater teleosts (Ruzhinskaya et al., 2001) and Chondrichthyes (Ferrando et al., 2006a; Ferrando, 2008); a role in ionic regulation has been suggested for the LCs, according to the related morphology features and to a characteristic localization of Na^+/K^+ATPase (Ruzhinskaya et al., 2001; Ferrando et al., 2006a; Ferrando, 2008). The BCs displayed two nuclear morphologies: