Fish & Shellfish Immunology 31 (2011) 571–577

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Cytochemical, immunocytochemical and ultrastructural observations on leukocytes and thrombocytes of fat snook (*Centropomus parallelus*)

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ARTICLE INFO

Article history:
Received 25 April 2011
Received in revised form
8 July 2011
Accepted 14 July 2011
Available online 23 July 2011

Keywords:
Cytochemistry
Immunocytochemistry
Ultrastructure
Leukocytes
Centropomus parallelus

ABSTRACT

The cytochemical, immunocytochemical and ultrastructural characteristics of leukocytes and thrombocytes in the peripheral blood of the fat snook (*Centropomus paralellus*) – a fish occurring in Brazil – were investigated. The cytochemical methods were performed to demonstrate four enzymatic reactions – o-toluidine-hydrogen peroxide, naphtol AS-MX phosphate, naphtol AS-BI phosphate and alpha-naphtil acetate to detect myeloperoxidase (MPO), alkaline phosphatase (ALP), acid phosphatase (ACP) and non-specific esterase (α-NAE), respectively – and two non-enzymatic ones – Periodic-Acid Schiff (PAS) and Sudan black B (SBB) to detect the occurrence of glycogen and phospholipids, respectively. Immunocytochemical method utilizing polyclonal rabbit antibody against mammal metalloproteinases (MMPs) 2 and 9 were done. Standard method for Electron Microscopy (EM) was applied for the ultrastructural study. The cytochemical reactions were positive in neutrophils for MPO, ACP, α-NAE, glycogen and phospholipids; in lymphocytes for ACP and α-NAE; in monocytes for ACP and α-NAE and in thrombocytes for ACP, α-NAE and glycogen. Only neutrophils were positive for MMPs 2 and 9, and none of the cells studied were positive for ALP. Ultrastructurally: 1) neutrophil showed a spherical shape with a spherical, indented or lobulated euchromatic nucleus, and cytoplasm containing granules of varied sizes and mitochondria of varied shapes and sizes. The nucleus/cytoplasm relation and the size of granules suggest neutrophil maturation in peripheral blood; 2) lymphocytes showed partially heterochromatic nucleus and minimal cytoplasm; 3) monocytes had long cytoplasmic projections, an indented nucleus, evident nucleolus and cytoplasm with granules of varied sizes and vacuoles; 4) thrombocytes were predominantly elliptical or roughly spherical in shape, had a partially heterochromatic nucleus and cytoplasm containing electron-dense granules, intricate canalicular system and vacuoles occasionally holding phagocytic material.

1. Introduction

The understanding of fish biology, in special the immune response, is very important for appropriate fish raising as well as for learning how fish cope with environmental challenges [1]. The immune system of these animals are similar to that of other vertebrates, which has an innate, non-specific response and a specific, acquired counterpart one, which identifies receptors and produces specific antibodies [2].

Over the late years, the research into the fish innate immune response has attracted increasing interest and has been viewed as playing a major role in immune response system and as a fundamental factor in the building up of acquired immune response. Recently, many reports have suggested many species of teleost fish as a model for the understanding of the interaction of the various factors participating in the immune response [1].

The main role of the innate immune system is to put up the first defense line against pathogenic organisms or foreign material, which are eliminated in the process of phagocytosis [3,4]. The main

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doi:10.1016/j.fsi.2011.07.019

cells involved in this process are granulocytes, monocytes and tissue macrophages [5–7]. In fish, however, thrombocytes have been also reported as involved in phagocytosis [5,8,9].

Over the late decades, several authors studied the roles of the cells that take part in the fish innate immune system and many of them studied the different aspects of the various cells circulating in the blood stream of the many species. In their research work, cytochemical, immunocytochemical and ultrastructural studies were performed in samples of blood cells from various species [10–13].

Currently, it is possible to establish correlation between the role and the type of a cell by determining the chemical nature of the different components inside each cell [12,14].

An important point regarding the capacity of peripheral blood leucocytes to migrate is that the higher the level of degradation of the components of the extracellular matrix, the easier for leucocytes to move through. In mammals, it is well established that such degradation is promoted by zinc and calcium dependent enzymes from the metalloproteinases (MMPs) family, usually described as comprising four main groups – colagenases, gelatinases, stromelysin and membrane metalloproteinases. These enzymes are normally secreted by activated monocytes, macrophages and neutrophils [10]. During embryo development such secretion is responsible for the degradation of the extracellular matrix in order to make possible cell migration, morphogenesis and tissue remodeling. The secretion of zinc and calcium dependent enzymes also mediates a number of biological processes [15–18].

Not many are the studies on MMPs in fish, but it has already been reported that MMP 2 and 9 breaks down connective tissue for cell migration and embryo development as it is noted in mammals [10,19–21].

Similarly, the ultrastructural analysis in the leukocyte is a useful tool for precise characterization of the cell type as well as provides a more detailed demonstration of intracellular components.

Given to the scientific relevance of knowing of the different aspects of the cell types involved in the fish innate immune response, and in the attempt to fill a knowledge gap, the present study determined the cytochemical, immunocytochemical and ultrastructural features of peripheral blood leukocytes and thrombocytes in the *Centropomus parallelus* (fat snook), a commercially interesting species as a source of nutritional food and sport fishing challenge [22–24].

2. Methods

Twenty healthy adult specimens of fat snook (size = 26 to 29 cm and weight = 144 and 202 g) were captured in the lagunar estuary area of Cananéia in the state of São Paulo, Brazil. The animals were anesthetized with benzocaine (Sigma Chemical Co, St Louis, MO, USA) following immediate collection of their peripheral blood samples taken with a 30 × 7 needle and syringe wetted with EDTA at 10%. After collection the blood samples were transferred to vacutainer vials containing EDTA at 10% to prevent coagulation. A little drop of blood sample was spread on glass slides to produce smears for cytochemistry and immunocytochemistry. The remaining blood was centrifuged and the respective buffy coat was processed for Electron Microscopy examination. The fish specimens were kept in water tanks for recovery. Some of the smears were stained according to Rosenfeld protocol in order to produce morphological guidance to help interpreting the cytochemical analysis of the respective blood sample.

2.1. Cytochemistry

The blood smears were processed using cytochemistry kits for human leukocytes available on the market, previously reported as used to study fish leukocytes [13,25,26]. The following kits produced by Sigma Chemical Company (St Louis, MO, USA) were used: Periodic-Acid Schiff (PAS) staining, Sudan black B (SBB) staining; o-toluidine-hydrogen peroxide kit for detection of myeloperoxidase (MPO); naphtol AS-MX phosphate kit for detection of alkaline phosphatase (ALP); naphtol AS-BI phosphate kit for detection of acid phosphatase (ACP) and alpha-naphtyl acetate for detection of non-specific esterase (α-NAE).

Cytochemical results were examined through an Olympus BX51 microscope, photographed with a CCD Olympus (Q color 5) camera, and the digital image was analyzed using Image-Pro Express software.

2.2. Immunocytochemistry

Metalloproteinases 2 and 9 were detected using the avidin biotinperoxidase complex (ABC) and diamine-benzidine (DAB) staining. The smears were fixed in formaldehyde vapor for 10 min and washed in tap water for 10 min and after in distilled water. After washing, the smears were incubated in hydrogen peroxide at 3% for 20 min to inactivate the endogenous peroxidase, washed again in tap water and in distilled water followed by incubation in skim-milk at 3% and phosphate buffered saline (PBS) for 30 min at room temperature. After, the smears were incubated with 100 μl rabbit polyclonal antibody (Ig) to human anti-MMPs 2 and 9 (Diagnostic BioSystem DBS, USA, dilution 1:100) overnight in humid chamber at 4 °C. The slides were then washed in PBS and incubated with secondary mouse biotinylated antibody (LSAB, Dako) to rabbit Ig (Diagnostic BioSystem DBS, USA, dilution 1:100) for 30 min at room temperature and then washed in PBS for 10 min before treatment with 2 drops of streptavidin-peroxidase conjugate (LSAB) for 30 min at room temperature. Staining was performed with DAB solution at 0.06% in PBS for 5 min. After washing the slides in tap water, counterstaining was performed with Carazzi's hematoxylin.

2.3. Electron microscopy (EM)

For the ultrastructural study, the blood samples were centrifuged at 2000 × g for 15 min and the buffy coat was immediately treated with Karnovsky fixative for three and a half hours at 4 °C, and washed in sodium cacodylate solution at 0.2 M and 7.2 pH at 4 °C for 2 h and subsequently treated with osmium tetroxide at 1% in 7.2 pH phosphate buffer solution at 0.2 M for 1 h at 4 °C and uranyl acetate at 0.5% for 12 h at 4 °C. Buffy coat serial ethanol dehydration and soaking in propylene oxide were carried out for embedding in epoxy resin (Sigma/Aldrich Corp, St Louis, MO, USA). Micrographs of ultrathin sections of the samples were obtained using an electron microscope LEO 906E.

3. Results

Recent morphological studies [27] examining leukocytes and thrombocytes in the peripheral blood of the fat snook have found only 3 types of leukocytes (granulocytic neutrophils, lymphocytes and monocytes) and thrombocytes. The present study has found the same types of leukocytes and thrombocytes. In addition the neutrophils were classified into young and mature ones. The cytochemical and immunocytochemical findings of the present study are shown in Table 1.

3.1. Cytochemistry

Cytochemical methods are important as tool for inferring the function of a cell by identifying types of macromolecules present in