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Cytochemical identification of turbot myeloperoxidase-positive granulocytes by potassium iodide and oxidized pyronine Y staining

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ABSTRACT

Cytomorphological and cytochemical staining are important methods for the identification of cell types, in particular in fish which often lack biological tools such as specific antibodies. Myeloperoxidase (MPO) is usually used as an intracellular marker of neutrophil accumulation in tissues and a marker of neutrophil activity in plasma. In this study, we reported a potassium iodide and oxidized pyronine Y (KI-PyY) staining method for rapid and highly sensitive detection of MPO-positive cells in turbot blood, peritoneum, and tissues. MPO-positive cells, which mostly represented neutrophils, were stained brown and clearly distinguished from other cells, such as lymphocytes, monocytes, and macrophages, which were stained pink. Following bacterial stimulation, the proportions of neutrophils were 27.49% and 38.05% in peripheral blood leukocytes and peritoneum, respectively, judging by the stained MPO. Kidney granulocytes contained abundant MPO-positive cells which were probably immature neutrophils with low expression of MPO. It is noteworthy that MPO-positive cells were detected in the tissue sections of kidney, spleen, and gut, with distribution profiles specific to each tissue. However, the cell morphology was not distinct in the stained tissue sections. These results indicate that the KI-PyY staining method is highly sensitive, applicable to different types of samples, and will be useful for the study of neutrophils in different compartments of fish.

1. Introduction

Granulocytes are a type of granule-containing white blood cells composed mainly of neutrophils (West et al., 2017). Neutrophil granulocytes are the first immune cells to arrive at a site of infection and constitute an essential part of the innate immune system (Arazna et al., 2013). The immune function of neutrophils depends on their capacity of phagocytosis, production of reactive oxygen species (ROS) such as hydrogen peroxide, and release of extracellular chromatin fibres called neutrophil extracellular traps (NETs) (Burrows et al., 2001; Brinkmann et al., 2004).

Myeloperoxidase (MPO), which was originally named verdoperoxidase due to its intense green color, is a highly cationic, heme-containing, glycosylated enzyme found mainly in the primary (azurophilic) granules of neutrophils, making up approximately 5% of the total dry cell weight (Schultz and Kaminker 1962). It is a lysosomal protein that is released from the granules during degranulation (Kinkade et al., 1983). With its strong peroxidase activity, MPO consumes most of the hydrogen peroxide produced by neutrophils (Toumi et al., 2006). MPO has a heme pigment, which causes the green color in secretions rich in neutrophils, such as pus and some forms of mucus. MPO is usually used as an intracellular marker of neutrophil accumulation in tissues and a marker of neutrophil activity measured in plasma (Nichols and Hazen, 2005; Toumi et al., 2006).

MPO as well as other peroxidases in granulocytes have been detected by enzyme cytochemistry. In the past for a long time, benzidine, 4-chloro-1-naphthol, tetra-methylbenzidine, 4-aminoantipyrine and α-naphthol have been most commonly used for MPO staining (Tan et al., 2009; Singh et al., 2011). However, the derivatives of these chromogenic reagents are potentially carcinogenic (Latger-Cannard et al., 2010). In 1984, Pereira introduced the non-toxic potassium iodide (KI) method for the staining of MPO (Pereira, 1984). The technique has recently been improved, so that the positively stained particles are clear and easy to identify (Chen, 1987; Wang and Wang, 1989; Chen et al., 2002).

Turbot (*Scophthalmus maximus*) is one of the most important fish species in Chinese and European aquaculture. It is also good for studying fish MPO because it lacks blood eosinophils (Burrows et al.,

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2001). In other fish species and also in mammals, eosinophils contain a peroxidase that can interfere with the identification of neutrophil MPO (Castro et al., 2008). In this study, we aimed to develop a quick staining method for the identification of neutrophils in turbot. We optimized the pyronine Y-KI method and applied the method to the identification of MPO-positive cells in the peripheral blood leukocytes (PBL), peritoneal fluid, and various tissues of turbot.

2. Materials and methods

2.1. Fish

Clinically healthy turbot (*Scophthalmus maximus*) (average 250 g) were purchased from a local fish farm and acclimatized in the laboratory for two weeks before experimental manipulation. The fish were fed daily with commercial dry pellets and maintained at 22 °C in aerated seawater. For sample collection, fish were euthanized with tricaine methanesulfonate (Sigma, St. Louis, USA). Live animal study was approved by the Ethics Committee of Institute of Oceanology, Chinese Academy of Sciences.

2.2. Bacteria

Escherichia coli DH5α (Tiangen, Beijing, China) was cultured in Luria-Bertani broth (LB) medium at 37 °C to an OD_{600} of 0.8. The cells were washed and resuspended in PBS before intraperitoneal injection.

2.3. Isolation of granulocytes from head kidney

Isolation of turbot granulocytes was performed as reported previously with modifications (Chi and Sun, 2016). Briefly, head kidney was aseptically collected from turbot and placed into a 50 mL test tube containing 30 mL of L15 cell culture medium (HyClone, Logan, USA). The tissue was minced through a 100 μm nylon Falcon cell strainer (BD Falcon, Lexington, USA). The cells were isolated using a specific gravity of 1.070 g ml^{-1} percoll (GE Healthcare, Uppsala, Sweden), and the buffy coat and red blood cell pellet were removed and discarded. The granulocytes suspended in the percoll gradient were collected and re-suspended in L15-medium. Haemocytes were removed using another specific gravity of 1.090 g ml^{-1} percoll. The granulocytes were washed twice with phosphate buffer saline (PBS) and re-suspended in L15 medium. The cells were counted and determined for viability with trypan blue staining as reported previously (Palić et al., 2005; Chi and Sun, 2016). The granulocytes were seeded on cover slips in 24-well cell culture plates (Costar, Tewksbury, USA) containing L15 medium supplemented with 1% fetal bovine serum (FBS) (HyClone, Logan, USA) and maintained at 22 °C for 1 h. The granulocytes were fixed for 15 min in 4% paraformaldehyde (Sigma St. Louis, MO, USA) or fixed for 1 h in 2.5% glutaraldehyde (Sigma).

2.4. Visualisation by scanning electron microscopy (SEM)

The methods has been described previously (Chi and Sun, 2016), Briefly, the purified granulocytes from head kidney were fixed with 2.5% glutaraldehyde on cover slips, and then fixed in using repeated incubations with 1% osmium tetroxid 1% tannic acid, dehydrated with a graded ethanol series. critical-point dried. Lastly, coated with 2 nm platinum and observed by scanning electron microscopy (Hitachi S-3400N, Japan).

2.5. Preparation of PBL climbing slides

Turbot were injected intraperitoneally with 10^6 CFU of *E. coli*. At 12 h post-injection, blood samples were taken from the caudal vein of fish and diluted (1:1) with L15 medium supplemented with 1% (W/V) FBS (Hyclone, Logan, USA) and 20 IU ml^{-1} heparin. The cell suspension was centrifuged at 680*g* for 15 min at 4 °C, and the sediment was washed twice with PBS containing 20 IU ml^{-1} heparin. The cells were laid over a discontinuous Percoll (GE Healthcare, Uppsala, Sweden) gradient at the densities of 1.020 g cm^{-3} and 1.090 g cm^{-3} and centrifuged at 840*g* for 30 min. The leukocyte fraction was collected and washed twice with PBS containing 5% (V/V) FBS (Hyclone, Logan, USA). The cells were collected by centrifugation and re-suspended in PBS. The cells were counted in a haemocytometer and assessed for viability with trypan blue staining. Fifty microliters (1×10^6 cell/ml^{-1}) of cell suspension was sedimented onto slides for 1 h and then fixed for 15 min in 4% paraformaldehyde.

2.6. Isolation of peritoneal cells

Turbot were injected intraperitoneally with 10^6 CFU of *E. coli*. At 12 h post injection, the peritoneal cells were isolated by lavaging the turbot with L15 medium, and the cells were centrifuged and washed with PBS as previously reported (Havixbeck et al., 2016). The cells were adjusted to 10^6 cells ml^{-1} and sedimented on slides and fixed as described above.

2.7. Preparation of tissue section slides

Freshly dissected tissues (spleen, kidney, and gut) were fixed with 4% paraformaldehyde for 18–24 h and embedded in parafin wax. Tissue sections (6 μm in thickness) were placed onto slides and allowed to dry overnight at 37 °C for overnight, followed by an immersion in xylene and subsequent hydration in a series of graded ethanols. Some sections were stained with haematoxylin and eosin (H & E) for histological observation, and other sections were stored at 4 °C for KI and oxidized pyronine Y (KI-PyY) staining.

2.8. KI-PyY staining

To prepare the working solution of KI-PyY staining, the following solutions were prepared first: KI solution was prepared by dissolving 500 mg KI (Sinopharm, Beijing, China) in 50 mL distilled water and stored in a dark bottle at room temperature or at 4 °C; oxidized pyronine Y solution was prepared by dissolving 250 mg pyronine Y (Biotopped, Beijing, China) in 50 mL 50% ethanol; 3% H_2O_2 was prepared by adding 50 μL 30% H_2O_2 (Guacheng Tianjin, China) to 50 mL ddH_2O and stored in a dark bottle at room temperature or at 4 °C. The working solution was prepared just before use by mixing 1 mL PBS with 100 μL 3% H_2O_2, 50 μL KI solution, and 100 μL oxidized Pyronine Y solution. For KI-PyY staining, the working solution was added the cell climbing slides or tissue section slides prepared above and allowed to stand for 2 min. The solution on the surface of the slides was removed, and the slides were dried with filter paper and observed under a light microscope.

2.9. Statistical analysis

For MPO-positive cell detection, ten fish were used for each assay, and the proportions of MPO-positive cells in PBL, peritoneal, and purified head kidney neutrophils were calculated from 15 fields of microscopic observation for each fish. Statistical analysis was performed with SPSS 17.0 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. MPO-positive cells in PBL

Microscopic examination of H & E-stained turbot leukocytes revealed the presence of lymphocytes, monocytes, and granulocytes (Fig. 1A). The granulocytes appeared like neutrophils with characteristic multi-lobulated nucleus. KI-PyY staining showed that the PBL of