Fish & Shellfish Immunology 51 (2016) 64–69

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

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

Full length article

Immunolocalization of immune cells and cell cycle proteins in the *bulbus arteriosus* of Atlantic salmon (*Salmo salar* L.)

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ARTICLE INFO

Article history:
Received 14 April 2015
Received in revised form
2 February 2016
Accepted 5 February 2016
Available online 10 February 2016

Keywords:
Atlantic salmon
Bulbus arteriosus
Caspase
CD3
HIF1α
Immunohistochemistry
MHC II
TNFα

ABSTRACT

The *bulbus arteriosus* is the most anterior chamber of the teleost heart. The present study aimed to establish the presence, and to provide semi–quantitative information on the abundance, of several immune and cell–cycle proteins in the *bulbus arteriosus* of healthy Atlantic salmon (*Salmo salar* L.). Using immunohistochemistry, lymphocyte–like cells were identified in the *bulbus arteriosus* using antibodies to CD3ε and MHC class IIβ. Few PCNA positive cells were identified in post–smolt fish as compared to moderate levels of staining in fresh water fry. Interestingly no staining was evident in adult fish (1–3 kg), thus there was a loss of cells expressing cell–cycle regulatory proteins with ontogeny/progressive life –history stages. Eosinophilic granulocytes (EGCs) were identified in the *bulbus arteriosus* using TNFα and HIF1α antibodies. Anti–caspase 3 immune–reaction identified a strong endothelial cytoplasmic staining in the *bulbus arteriosus*. Taken together, the immunolocalization of immune–related molecules (CD3, MHC class II and TNFα), cell–cycle regulatory proteins (PCNA and HIF1α) and apoptosis markers (TUNEL, caspase 3) suggest that the *bulbus arteriosus* may have an immune component within its functional repertoire.

1. Introduction

The teleost heart consists of four serially arranged main chambers: (1) the *sinus venosus*, (2) the atrium, (3) the ventricle and (4) the *bulbus arteriosus*. In addition to these conventionally described chambers, the *conus arteriosus* and a distinct atrio–ventricular segment have also been identified in a few teleost species [1–8]. The *bulbus arteriosus* is a white–colored, thick walled, fibro–elastic cylindrical pear–, or onion–shaped structure. It is the most anterior chamber of the teleost heart, and connects the ventricle to the ventral aorta which lies outside the pericardial cavity and takes blood to the gills. It is enlarged proximally and tapers distally to give a highly variable shape to the *bulbus arteriosus* [7,9–12]. The *bulbus* wall is organized into the following layers: the endocardium and the endocardial ridges that form the inner layer, the middle layer and the outer sub–epicardial layer [7]. The full length of the *bulbus arteriosus* is occupied by longitudinal columns that are thicker at the base and attenuate towards the ventral aorta [7]. The cells of the endocardial ridge may be squamous to columnar in appearance, depending upon the species [13]. The endocardial cells contain moderately dense bodies with secretory function, as seen in the Antarctic teleosts where endothelial cells are suggested to be involved in the production of anti–freeze mucins [7,14]. The middle layer of the *bulbus arteriosus is* comprised of smooth muscle cells and variable amounts of elastin or collagen fibers interspersed with elastin, as seen in the common eel [15]. The outer sub–epicardium is a thin layer and contains collagen, elastin, fibroblasts, vessels and nerves [13]. The *bulbus arteriosus* provides passive dampening (windkessel function), whereby it reduces the pulse pressure associated with the ventricular contraction and improves cardiac efficiency [1,8,13,16–18].

Previously, we have demonstrated the presence of immune cells in healthy and diseased atria and ventricles of the Atlantic salmon (*Salmo salar* L.) heart using different antibody markers [19,20].

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http://dx.doi.org/10.1016/j.fsi.2016.02.006
1050-4648/

However, the immunolocalization of immune cells in the *bulbus arteriosus* of Atlantic salmon heart was not previously studied. The characterisation of the immunological status of tissues in healthy fish is required to compare and better understand the immunopathological responses seen in disease states. Therefore, the present study aimed to determine whether cells expressing cell–cycle regulatory proteins (PCNA and HIF1α), selected immune–molecules (CD3, MHC class IIβ and TNFα) and apoptosis–related molecules (TUNEL, caspase 3) could be detected in the *bulbus arteriosus* of healthy Atlantic salmon smolts. In addition, PCNA immunostaining was performed using fresh water fry, smolts and adult Atlantic salmon.

2. Materials and methods

2.1. Heart samples for immunohistochemistry

This study used healthy Atlantic salmon hearts from our previously published study [19]. For most of the immunolabelling, 5–8 hearts were used. For proliferative cell nuclear antigen (PCNA) staining, the numbers per life stage are listed in Table 1. All fish were euthanized in accordance with national research animal regulations. The necropsy was performed in addition to histological observation of the hearts and other vital organs to identify any other abnormalities or signs of overt disease. The healthy hearts were fixed in 10% neutral phosphate buffered formalin solution and processed by a standard paraffin wax protocol (dehydrated, embedded in paraffin and 3 μm thick sections cut on a Shandon Finesse ME microtome (Shandon Lipshaw, Pittsburgh, PA, USA)). Sections were processed for immunostaining using different antibodies according to our previous studies [19–21]. The slides were evaluated by light microscopy using an Olympus microscope BX51 equipped with Cell^{B} software (Olympus Corporation, Tokyo, Japan).

2.2. Antibodies

The antibodies used to stain the *bulbus arteriosus* included those to caspase 3 (1:500, sc–7148, Santa Cruz Biotechnology, Santa Cruz, CA), hypoxia inducible factor (HIF1α, 1:200, sc–8711, Santa Cruz Biotechnology, Santa Cruz, CA), proliferative cell nuclear antigen (PCNA 10, 1:150, Dako, Glostrup, Denmark), CD3ε (1:400), major histocompatibility complex (MHC) class IIβ chain (1:1000) and tumor necrosis factor–α (TNFα, 1:500) [19–23]. The specificity of these antibodies has been described elsewhere [19–22].

2.3. Immunohistochemistry on the bulbus arteriosus of Atlantic salmon

Immunohistochemistry was performed on mid–sagittal sections of the *bulbus arteriosus* following the protocol described by Yousaf et al. [19]. Briefly, the *bulbus arteriosus* sections were deparaffinized in xylene, followed by immersion in graded ethanol baths and rehydration in dH_2O. Antigen retrieval was performed using 10 mM citrate buffer, pH 6.0 containing 0.1% Tween 20 (Sigma–Aldrich, Norway) by autoclaving (121 °C for 20 min). The endogenous peroxidase activity was inhibited by incubating slides in 3% H_2O_2 (Sigma–Aldrich, Norway) in methanol. The sections were blocked in 5% bovine serum albumin (BSA) (Sigma–Aldrich, Norway) in phosphate buffer saline (PBS) (Sigma–Aldrich, Norway) for 1 h at room temperature. After removal of the BSA, primary antibodies were applied in 1.5% BSA in PBS overnight at 4 °C. The following morning, slides were washed three times with PBS with 0.1% Tween 20 (PBST) (Sigma–Aldrich, Norway), pH 7.4 for 5 min each. After washing, slides were incubated with horseradish peroxidase (HRP) conjugated secondary antibodies such as donkey anti–goat Ig (sc–2020, Santa Cruz biotechnology) or goat anti–rabbit Ig (sc–3837, Santa Cruz biotechnology) in 1.5% BSA in PBS for 60 min at room temperature. Slides were again washed three times with PBST and incubated either with 3, 3′–diaminobenzidine (DAB; Sigma–Aldrich, Norway) or 3–amino–9–ethylcarbazole (AEC; Sigma–Aldrich, Norway) for 5 min in the dark and then washed with dH_2O. Slides were counterstained in haematoxylin for 10 s. All incubations were performed in a closed–lid humidity chamber and negative controls included primary antibody replaced with 1.5% BSA in PBS. A semi–quantitative scoring system was used as described in previous studies [19,20].

2.4. TUNEL staining for detection of apoptotic cells

To identify apoptotic cells, terminal deoxynucleotidyl transferase biotin–dUTP nick end labeling (TUNEL) was performed, following the instructions in the ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (CHEMICON® Int. Inc. USA). Briefly, the mid–sagittal heart sections including the *bulbus arteriosus* were unmasked with freshly made IHC Select® Proteinase K (20 μg ml^{-1}, Millipore, Norway) for 15 min at room temperature and washed twice in dH_2O for 2 min. The endogenous peroxidase was quenched with 3% H_2O_2 (Sigma–Aldrich, Norway) in PBS for 5 min at room temperature and washed twice with dH_2O for 5 min. Slides were then carefully incubated with equilibration buffer at 75 μl 5 cm^{-2} for 10 s, followed by incubation in working strength TdT enzyme at 55 μl 5 cm^{-2} in a humidified chamber at 37 °C for 1 h. The slides were next dipped in working strength stop/wash buffer, agitated for 15 s and then incubated for 10 min at room temperature. After washing three times, the slides were then incubated in anti–digoxigenin conjugate at 65 μl 5 cm^{-2} for 30 min at room temperature, and then washed ×4 with PBS for 5 min. Sections were next dipped in peroxidase substrate at 75 μl 5 cm^{-2} for 3–6 min at room temperature, counter stained with 0.5% (W:V) methyl green (Sigma–Aldrich, Norway) for 10 min and washed three times in dH_2O. Finally, the slides were washed in 100% *N*–butanol (Sigma–Aldrich, Norway). Positive controls (provided with the kit) and negative controls (replacing TdT with equilibration buffer) were performed. The apoptotic cells were identified by both positive staining and morphological signs of apoptosis.

Table 1
Proliferative cell nuclear antigen (PCNA) immunostaining in Atlantic salmon *bulbus arteriosus*.

Number of fish	Weight	Physiological status	Staining
7	0.5–5 g	Fresh water (fry)	++
5	100 g	Sea water	+/−
8	1–3 kg	Sea water	−

No staining = −, focal (few scattered cells) staining = +, focal to moderate staining = ++.

3. Results

3.1. Immunohistochemistry

Anti–CD3ε and anti–MHC class IIβ immunostaining identified foci of mononuclear lymphocyte–like cells in the *bulbus arteriosus* of healthy Atlantic salmon (Fig. 1A,B,C and D). Few apoptotic cells were identified in the *bulbus arteriosus* using TUNEL staining (Fig. 1E and F), however anti–caspase 3 immunostaining identified a strong endothelial cytoplasmic staining in the *bulbus arteriosus* (Fig. 1G and H).

Using the PCNA–10 antibody, moderate levels of staining in the middle, endothelial and epithelial layers of the *bulbus arteriosus*