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Pigment-producing granulomatous myopathy in Atlantic salmon: A novel inflammatory response

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ABSTRACT

Melanin comprises a complex group of pigmented polymers whose primary function is ascribed to dermal solar protection, but may also have an interesting role in innate immunity. In ectothermic vertebrates, melanogenesis is reported in leukocyte populations, but it is not known if this occurs in connection with inflammatory reactions. Melanin accumulations in ectopic locations, in particular muscle, represent a serious quality problem in salmon production. Here, we investigated such changes for the expression of dopachrome tautomerase and tyrosinase as well as some important immune genes and pathogens. Furthermore, the nature of the pathological changes was addressed by morphological methods. Gene transcripts encoding key enzymes in melanogenesis, suggesting a *de novo* melanin synthesis in pigmented muscle, were found. MHC class II transcripts were up-regulated and there was no indication of bacterial or viral infection. The histological examination revealed granulomatous inflammation with distribution of MHC class II positive cells and T cells, analogous to the pattern found in mammals. Importantly, in contrast to mammals pigmented cells were contributing in the inflammation. We demonstrate that melanin production occurs in granulomatous inflammation in salmon, revealing a close and hitherto unreported link between the pigmentary and immune systems.

1. Introduction

Melanocytes are melanin-producing cells, and in mammals, they derive from ectoderm [1]. The functions of melanin synthesis and deposition in dermal tissues have generally been attributed to the need for solar protection. Nevertheless, there is an increasing interest to investigate the relationship between the pigmentary and the immune systems [2]. There are indications that melanin plays a role in immune functions such as antimicrobial defense, suggesting that immune modulation exerted by the pigmentary system might be an important and underestimated entity [2,3]. Melanocytes respond to cytokines, including interferons, interleukins and tumor necrosis factor [3–5]. Furthermore, they have been shown to produce several inflammatory mediators, suggesting participation in the inflammatory response [4,6]. Interestingly, in humans skin coloration correlates with susceptibility to certain infectious diseases. This connection was indicated as early as in the beginning of the 19th century by Dr. William Charles Wells [7]. During the Vietnam War, Allen et al. [8] found that the incidence of bacterial pyoderma among American troops was significantly higher in Caucasians than African Americans. Melanin and melanocytes are not only confined to the skin, but are also found in ectoderm-derived cells in extracutaneous locations, i.e. the brain, meninges, choroid of the eye and in the inner ear, where protective roles have been suggested [2]. Increased focus on the immunological role of mammalian melanocytes invites comparison to non-mammalian species and their extracutaneous melanocytes, which seem to be closely linked to the immune system [6,9–11].

In contrast to mammals and birds, ectothermic vertebrates possess a second group of melanin-producing cells originating from

Abbrevations: Dct, dopachrome tautomerase; Tyr, tyrosinase.

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mesenchymal, hematopoietic stem-cells [12]. These cells are commonly termed melanomacrophages in the literature of ectothermic vertebrates. A well-established Atlantic salmon (*Salmo salar*) cell-line (SHK-1 cells), classified as head–kidney derived macrophages, expresses transcripts of the tyrosinase gene family and produces melanosomes [13,14]. The principal genes related to melanogenesis include the tyrosinase gene family, and the expression of tyrosinase-related protein 2/dopachrome tautomerase (TYRP-2/Dct) is considered to be specific for melanocytes and their precursors [15]. Dct-expression is present in important lymphatic organs of fish including the head, kidney and the spleen, where melanomacrophages are found dispersed throughout the tissue [14,16]. These cells are thought to be part of the antigen-presenting cell system [16,17].

Inflammatory responses in fish show several similarities to those of mammals [9]. However, it has been noted that, in contrast to mammals, chronic inflammation in fish may appear with abundant pigmentation [18,19]. The presence of melanin in ectopic locations of farmed salmon, especially in the muscle fillet, represents a considerable quality problem for the salmon industry affecting up to 20% of the fillets at the processing plants [20]. The presence of possible melanin-producing leukocytes in salmon indicates that melanin may play an active role in inflammation in fish, and establishes a collaborative relationship between the pigmentary and immune systems. This would be a unique finding, as the leukocytes of mammals do not use melanin production in their defense responses and such an inflammatory response has not been described previously. The purpose of this work was to investigate the possible *de novo* production of melanin at the site of inflammation, and further to characterize pathological changes and underlying molecular mechanisms associated with pigmentation of the fillet, using and combining both morphological and gene-transcriptional approaches.

2. Materials and methods

2.1. Samples from fish

The fish sampled were vaccinated Atlantic salmon with abnormal pigmentation of the muscle fillet. They originated in four commercial farms and had routinely been intraperitoneally injected with oil-adjuvant vaccines before transfer to sea water, following standard procedures [21]. The fillets were collected from slaughter-sized salmon harvested at four different locations - Hjelmeland (8 fillets), Eggebønes (10 fillets), Romsdalsfjord (4 fillets) and Frøya (8 fillets).

Tissue samples were collected from the areas of brownish discoloration (Fig. 1A,B). In addition to the pigmented sample, non-pigmented control samples of apparently-normal muscle were obtained from the same fillet at various distances from the pigmented areas. Pigmented and non-pigmented tissue samples were processed for histology and immunohistochemistry (IHC). Macro-dissected tissues from the second and third sampling, i.e. a total of 28 samples from 14 fillets, were analyzed by quantitative real-time reverse transcriptase polymerase chain reaction (RT-qPCR). In addition, tissues from the last sampling were analyzed using laser-assisted micro-dissection.

2.2. Gene expression analysis

Macro-dissected tissue samples (3 × 3 × 3 mm) intended for RT-qPCR were immediately transferred to RNAlater® after sampling and kept at 4 °C until processed. RNA was extracted using an RNeasy Mini® kit (QIAGEN, Hilden, Germany), and concentrations and purity were measured spectrophotometrically with

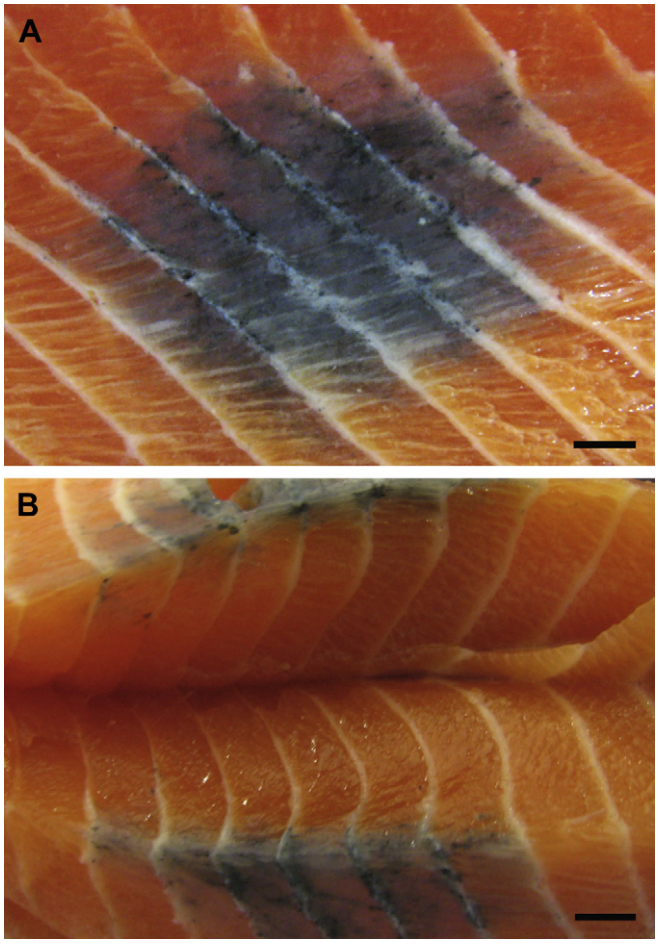

Fig. 1. Gross pathological changes of investigated Atlantic salmon muscle. A. An area with brown-black pigmentation, or a pigment spot. B. Section through a typical pigmented area. Bar = 5 mm.

a Biospec-Nano (Shimadzu Corporation, Kyoto, Japan). The cDNA synthesis was performed immediately after extraction using ~600 ng RNA/reaction, Oligo(dt)-primer and Omniscript® kit (QIAGEN). Real-time PCR reactions were set up using Platinum® Quantitative PCR Supermix-UDG (Invitrogen AS, Oslo, Norway) and run on a Cromo 4™ (Bio Rad Laboratories AB, Oslo, Norway). The following genes were analyzed by real-time RT-PCR – tyrosinase (Tyr), dopachrome tautomerase (Dct), major histocompability complex class II (MHC class II), interferon α (IFNα), cluster of differentiation 3 (CD3ζ), membrane bound and secretory immunoglobulin M (mIgM, sIgM). When possible, primers and probe were designed to span across intron sections [Table 1]. The level of expression was normalized using elongation factor $1A_A$ ($EF1A_A$) as the reference gene [22].

For laser-capture micro-dissection, tissue samples (10 × 10 × 3 mm) of pigmented muscle from eight individuals were immediately transferred to liquid nitrogen and stored at −70 °C until further processing. Cryo-sections, 14 μm in thickness, were cut with a cryostat and mounted on special membrane slides, as described previously [23]. The sections were air dried for 1 h, washed twice in xylene to remove fat and stained with RNase-free hematoxylin before air-drying for 1 h. Subsequently, a representative part of melanomacrophage accumulations (Fig. 2A,B) were dissected [23]. Up to eight successive dissected sections, with a total area ranging between 0.3 and 0.5 mm^2, were cut and collected in tubes. From each individual, neighboring non-