Developmental and Comparative Immunology 61 (2016) 154–160

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Differential expression of skin mucus C-type lectin in two freshwater eel species, *Anguilla marmorata* and *Anguilla japonica*

Shigeyuki Tsutsui*, Tatsuki Yoshinaga, Kaoru Komiya, Hiroka Yamashita, Osamu Nakamura

School of Marine Biosciences, Kitasato University, 1-15-1 Kitasato, Sagamihara, Kanagawa 252-0373, Japan

ARTICLE INFO

Article history:
Received 4 February 2016
Received in revised form
24 March 2016
Accepted 24 March 2016
Available online 26 March 2016

Keywords:
Lectin
Skin mucus
Expression profile
Anguilla species

ABSTRACT

Two types of lactose-specific lectins, galectin (AJL-1) and C-type lectin (AJL-2), were previously identified in the mucus of adult *Anguilla japonica*. Here, we compared the expression profiles of these two homologous lectins at the adult and juvenile stages between the tropical eel *Anguilla marmorata* and the temperate eel *A. japonica*. Only one lectin, predicted to be an orthologue of AJL-1 by LC-MS/MS, was detected in the mucus of adult *A. marmorata*. We also found that an orthologous gene to AJL-2 was expressed at very low levels, or not at all, in the skin of adult *A. marmorata*. However, we detected the gene expression of an AJL-2-orthologue in the skin of juvenile *A. marmorata*, and a specific antibody also detected the lectin in the juvenile fish epidermis. These findings suggest that expression profiles of mucosal lectins vary during development as well as between species in the *Anguilla* genus.

1. Introduction

The stratum corneum is a layer in the skin of land vertebrates, and it serves as a strong physical defensive-barrier against infection. In contrast, fish skin is not keratinized and is exclusively composed of live cells, despite the fact that pathogens thrive in marine and freshwater habitats. Therefore, fish skin, which is directly and continuously exposed to the water, is an important line of defense that prevents the entry of disease-causing organisms. Fish skin is coated with mucus, which is secreted by the mucous cells in the epidermis. Fish skin mucus contains a diverse array of defense molecules such as immunoglobulins, lysozymes, proteases and anti-bacterial peptides (Ingram, 1980; Alexander and Ingram, 1992), which provide immune competence to the mucus.

Lectins are a group of proteins and glycoproteins, excluding antibodies and enzymes, that can bind to carbohydrates (Barondes, 1988). Lectins are universally found in all kingdoms including bacteria, plants, and animals. So far, animal lectins have been classified into at least 15 families (Vasta and Ahmed, 2008). Although animal lectins have a wide variety of biological functions, which can be intracellular, extracellular or at the cell surface (Vasta and Ahmed, 2008), the main function of lectins might be to as a self-defense factor in innate immunity. For example, several animal lectins opsonize antigens, agglutinate microorganisms, activate the complement system, and induce apoptosis (Ni and Tizard, 1996).

Lectins have also been identified from the skin mucus of several fish species, and play important defensive roles on the body surface (Ingram, 1980; Alexander and Ingram, 1992). To date, 7 families of lectins including galectins, C-type and Lily-type lectins, have been found in the skin mucus of 8 fish species (Muramoto and Kamiya, 1992; Muramoto et al., 1999; Tasumi et al., 2002, 2004; Tsutsui et al., 2003; Okamoto et al., 2005; Tsutsui et al., 2007, 2009, 2011a, 2011b). Among these, *Anguilla japonica* lectin (AJL)-1 and AJL-2 are lactose-specific lectins that were identified from the skin mucus of adult Japanese eel *A. japonica* (Tasumi et al., 2002, 2004). AJL-1 belongs to the galectin family, while AJL-2 is classified into the C-type lectin family. These lectins play important roles in the skin surface immunity of *A. japonica*; AJL-1 shows agglutination activity against a pathogenic bacterium *Streptococcus difficile* (Tasumi et al., 2004), while AJL-2 agglutinates *Escherichia coli* and suppresses its growth (Tasumi et al., 2002). Recently, we reported that a gene orthologous to AJL-2 is widely conserved among the freshwater eels of the genus *Anguilla* (Tsutsui et al., 2015).

So far, 19 species/subspecies of fish have been classified into the genus *Anguilla* (Ege, 1939; Castle and Williamson, 1974; Watanabe et al., 2009), which are widely distributed throughout the world (Ege, 1939). The anguillid species are the only migratory fish in the

* Corresponding author.
E-mail address: tsutsui@kitasato-u.ac.jp (S. Tsutsui).

http://dx.doi.org/10.1016/j.dci.2016.03.027
0145-305X/

Anguilliformes family, which are exposed to both seawater and freshwater environments. For example, after migrating from their freshwater habitats to their spawning areas in oceanic waters, these fish breed far offshore. The characteristic leptocephalus larvae are transferred by sea currents to coastal regions, where they metamorphose into the juvenile fish, called "glass eel," which then grow in rivers (Schmidt, 1922; Tsukamoto, 1992, 2006; Aoyama, 2009; Tsukamoto et al., 2011; Aoyama et al., 2014). Therefore, we propose that the freshwater eels are a good model species to study the differences between the defense mechanisms present on the fish skin surface in freshwater and seawater.

Based on their growth habitats, the genus *Anguilla* can be divided into tropical and temperate species. The giant-mottled eel *A. marmorata*, which is a tropical eel, is known to share its spawning area at least partly with *A. japonica* in the West Mariana Ridge in the Pacific Ocean (Kuroki et al., 2009), and has the widest distribution area in the genus (Minegishi et al., 2008). In contrast, *A. japonica* is a panmictic species that is distributed in colder waters in East Asian countries such as Japan, Korea, China and Taiwan. In the present study, we selected the two species *A. marmorata* and *A. japonica* as representatives of tropical and temperate eels, and compared the expression of orthologues of AJLs at both transcriptional and translational levels. Furthermore, the comparison was made at juvenile and adult stages in these species since they may have defense mechanisms specific for seawater and freshwater environments, respectively. The current study focuses on the differences in lectin-based defense mechanisms between the two species, in perspective of their ecological and biological properties.

2. Materials and methods

2.1. Eels

Five individuals each of *A. marmorata* and *A. japonica*, which were raised in a commercial fish pond in Japan, were purchased from the farm. They were morphologically well-developed adults, referred to as "yellow-phase eel stage," and the average weights were 375 ± 137 g for *A. marmorata* and 177 ± 21 g for *A. japonica*. All the individuals were put under anesthesia with 2-phenoxyethanol or by icing before being sampled.

Wild juveniles, or glass eel (about 5 cm in total length), of *A. marmorata*, which had been captured at the estuary of the Iran River, Taiwan in January 2015, were kindly gifted by Dr. Yu-San Han, National Taiwan University, Taiwan. The *A. japonica* glass eels were collected at the estuary of the Sagami River, Japan in February 2014, as part of the "Eel River Project" (Aoyama et al., 2012) with the permission of the Fishery Division of the Kanagawa Prefecture Government.

2.2. Comparison of adult skin mucus components between the two species

Skin mucus was collected by scraping the body surface of adult eels of each species with a spatula. The mucus was homogenized using equal volumes of 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM $CaCl_2$, and 10 mM $MgCl_2$ (TBS (+)), and centrifuged at 15,000*g* for 15 min at 4 °C. Protein concentrations in the supernatants were measured by the Quick Start protein assay kit (Bio-Rad) using bovine serum albumin as a standard. These samples were mixed with equal volumes of SDS-sample buffer including 2-mercaptoethanol and boiled for 5 min, and then analyzed by SDS-PAGE on a 15% gel. Protein bands were visualized by staining with Coomassie brilliant blue R-250.

2.3. Purification of lactose-specific lectin from *A. marmorata* skin mucus

Isolation of skin mucus lectins from *A. marmorata* was carried out by the method described in Tasumi et al. (2002). In brief, skin mucus extract from the adult *A. marmorata* was incubated with lactose-agarose (about 1.2 cm^3, Seikagaku Biobusiness) at 4 °C overnight with shaking. After washing with 30 ml of TBS (+), 1 ml of TBS (+) containing 200 mM lactose was added to the lactose-agarose.

For comparison, lactose-binding lectins of adult *A. japonica* were also purified by the same method. The eluted fractions derived from the two species were compared by SDS-PAGE.

2.4. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of *A. marmorata* lectin

Affinity purified lactose-binding lectin from *A. marmorata* skin mucus was analyzed by LC-MS/MS. The band on the SDS-PAGE gel was excised and digested with trypsin (Wako) in the gel at 37 °C for 20 h. Digested peptides were loaded on an LC-MS/MS system that consisted of an HPLC system (Nanospace SI-2; Shiseido Fine Chemicals) and an ion trap mass spectrometer (LCQ Deca; Thermo Finnigan).

Individual spectra obtained by the MS/MS system were processed using the TurboSEQUEST software (Thermo Quest), and the generated peak list files were used to query SwissProt using the Mascot program (http://www.matrixscience.com).

2.5. Comparison of gene expression of the skin mucus lectins between the two species

To amplify a cDNA fragment of the skin mucus C-type lectin, forward and reverse primers (5′-TTGCATGTCGCTGAGAAGAAGACG-3′ and 5′-AATGTCGTTCCAGTGTTTTTGGCC-3′ respectively) were designed. The primers were designed based on a sequence that is conserved between the AJL-2 lectin (Tasumi et al., 2002) and the genomic sequence of its orthologue in *A. marmorata*, as determined in our previous study (Tsutsui et al., 2015). On the basis of highly conserved sequences between cDNA of AJL-1 (Tasumi et al., 2004) and congerin II, an orthologue in conger eel *Conger myriaster* (Ogawa et al., 1999), forward (5′-ACTCATMCAGCTGTGAGGA-3′) and reverse (5′-TACTCWGTGAGTTTGCACAGA-3′) primers were synthesized to amplify galectin cDNA fragment.

As the first step, we verified that these primers can amplify the lectin genes of both species equally by genomic PCR, according to the method described in Tasumi et al. (2016). Genomic DNA extracted from both the species (Tsutsui et al., 2015) was adjusted to a concentration of 10 ng/μl. PCR was done with an initial step at 95 °C for 3 min, followed by 30 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s. A thermal-cycler (Bio-Rad) was used for the PCR, which was done in a total volume of 20 μl, using Ex taq (Takara), and 1 μl of genomic DNA isolated above was used as the template. PCR products were electrophoresed on an 1.8% agarose gel, and the amplicons were stained with ethidium bromide.

As the next step, we extracted total RNA from the skin of five individuals of both the species using Isogen (Nippon Gene) and synthesized cDNA using SMARTer™ RACE cDNA amplification kit (Clontech) from 1 μg of total RNA, following manufacturer's instructions. The eels were euthanized with overdose of 2-phenoxyethanol. Thirty cycles of PCR of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s following the first step of 95 °C for 3 min was performed with Ex taq in a total volume of 20 μl, which included 1 μl of cDNA. The β-actin cDNA fragment was also amplified with a primer pair of 5′-ATGGAAGATGAAATCGCC-3′ and 5′-