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Determination of sialic acids in immune system cells (coelomocytes) of sea urchin, *Paracentrotus lividus*, using capillary LC-ESI-MS/MS

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ABSTRACT

Coelomocytes are considered to be immune effectors of sea urchins. Coelomocytes are the freely circulating cells in the body fluid contained in echinoderm coelom and mediate the cellular defence responses to immune challenges by phagocytosis, encapsulation, cytotoxicity and the production of antimicrobial agents. Coelomocytes have the ability to recognize self from non-self. Considering that sialic acids play important roles in immunity, we determined the presence of sialic acid types in coelomocytes of *Paracentrotus lividus*. Homogenized coelomocytes were kept in 2 M aqueous acetic acid at 80 °C for 3 h to liberate sialic acids. Sialic acids were determined by derivatization with 1,2-diamino-4,5-methylenediaoxy-benzene dihydrochloride (DMB) followed by capillary liquid-chromatography-electrospray ionization/tandem mass spectrometry (CapLC-ESI-MS/MS). Standard sialic acids; Neu5Ac, Neu5Gc, KDN and bovine submaxillary mucin showing a variety of sialic acids were used to confirm sialic acids types. We found ten different types of sialic acids (Neu5Gc, Neu5Ac, Neu5Gc9Ac, Neu5Gc8Ac, $Neu5,9Ac_2$, $Neu5,7Ac_2$, $Neu5,8Ac_2$, $Neu5,7,9Ac_3$, $Neu5Gc7,9Ac_2$, Neu5Gc7Ac) isolated in limited amounts from total coelomocyte population. Neu5Gc type of sialic acids in coelomocytes was the most abundant type sialic acid when compared with other types. This is the first report on the presence of sialic acid types in coelomocytes of *P. lividus* using CapLC-ESI-MS/MS-Ion Trap system (Capillary Liquid Chromatography-Electrospray Ionization/Tandem Mass Spectrometry).

1. Introduction

Sialic acids are a diverse family of monosaccharides widely expressed on all cell surfaces of vertebrates and higher invertebrates (such as starfish and sea urchin), and on some bacteria that interact with vertebrates [1]. The expression of sialic acids is highly conserved in deuterostomes, i.e., from echinoderms to humans [2]. Sialic acids are biosynthesized by almost all organisms as a 9-carbon carboxylated monosaccharide and found most frequently as the terminal sugars of cell surface glycolipids and glycoproteins [3–6]. Because of their terminal location and negative charge, sialic acids have numerous roles in many aspect of immunity [1]. These monosaccharides have the potential to contribute or inhibit many intermolecular and intercellular interactions [4,7]. Sialic acids also contribute to many features of the cell surface and frequently serve as ligands for receptor-mediated intercellular interactions, host cell-pathogen recognition processes and lymphocyte-endothelial cell interactions [8,9]. Many earlier studies removed sialic acids from immune cell surfaces using sialidases and observed changes in behavior of such cells [1]. Sialic acids are crucial in the development of vertebrates, as observed that mouse embryos engineered to deficiency of sialic acid expression die after they birth [10]. In pathogenic diseases, including some inflammations, particular microbes recognize cell surface sialic acids when invading host cells [11,12]. For example, binding of influenza A and B viruses to host cell is accomplished by the specific recognition of the host sialic acid linkage by the virus. Sialylated molecules on *Aspergillus fumigatus* conidia (fungal pathogen) are recognized by cultured mouse macrophages and lung epithelial cell lines and enhance the uptake of conidia into the endosomal system of the host cell [13]. Sialic acid residues can also mask recognition sites such as galactose residues on glycoproteins to prevent their in vivo removal by asiaologlycoprotein receptors [14]. In certain cancers, changes in sialic acid amounts, types and linkages have been associated with tumorigenesis and cancer metastasis [12]. More than 50 naturally occurring sialic acid [15–17] derivatives of the three main forms, N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc) and 2-keto-3-deoxy-nonulosonic acid (KDN) were identified [6,18]. The great structural diversity in the sialic acids originates from different

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substitutions on the amino group at carbon 5 and hydroxyl groups at carbons 4, 7, 8 and 9 by acetyl groups (O-acetylation) [3,19,20].

There have been many reports concerning the analysis of specific sialic acids in animals. Although small amounts of glycoconjugates with sialic acids have been reported in certain protostome animals such as insect [21–24], these sugars are found in largest amount and exhibit greatest structural variability in deuterostomia, predominantly in the echinodermata and hemichordata [25].

Echinoderms have a potential immune system for their survival [26]. Many species, living in coastal and estuarine waters, are directly exposed to potentially pathogenic microorganisms and have developed defence responses mainly based on immunocytes (coelomocytes) and humoral factors contained in the coelomic fluid [27–30]. In echinoids, sea urchin, *Paracentrotus lividus*, at least four main coelomocyte types including a subpopulation of macrophage-like cell, phagocytic cells that circulate in the coelomic fluid have been described: amoebocytes, vibratile cells, red and uncolored spherulocytes [26,30–35]. Cell-based immunity is carried by the coelomocytes, a morphologically heterogeneous population of free roaming cells that are capable of recognizing and neutralizing pathogens. Coelomocytes present diverse functions which include phagocytosis, encapsulation, clotting, cytotoxicity and wound healing [31,35].

The cell-based immunity system is able to distinguish between self and non-self by oversialylation and undersialylation of their cell surface (but so far not examined in echinoids) [36]. In terms of their immune systems, echinoderms represent the same basic types of responses that most multicellular (including vertebrates) animals do. The coelomocytes of echinoderms can recognize self from non-self and, if a foreign material (e.g., microorganism/pathogen) enters the body cavity, they can readily neutralize it and dispose of it [32,34,35]. Due to multifarious roles of sialic acids in immunity [1], sialic acid is an ideal points of investigation in coelomocytes of echinoids. Until recently, there has been no study on the determination and function of sialic acid and their types in immune system cells, particularly in the echinoderm coelomocytes.

2. Materials and methods

2.1. Sea urchins

Sea urchins of the species *P. lividus* were collected from the nearshore waters of the northern Izmir coastline, Foça, Turkey.

2.2. Coelomocyte collection for sialic acid analysis

Necessary quantities of the coelomic fluid with coelomocytes were immediately taken from the coelomic cavity without feeding sea urchins. As previously described [37,38], the coelomic fluid from each animal was collected by removing Aristotle's lantern, or the mouth structure, and aliquoted into 3 ml volumes and then diluted with isosmotic anticoagulant solution (20 mM Tris, 0.5 M NaCl, 70 mM EDTA pH 7.5) (ISO-EDTA). After centrifugation (1000 × *g* for 10 min at 4 °C), coelomocytes were washed three times in ISO-EDTA and then fixed in methanol either at −20 °C or −80 °C.

2.3. Coelomocyte preparation for microscopy

Coelomocytes were suspended in coelomocyte culture (CCM: 0.5 M NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM HEPES, pH 7.4). Cell suspensions (100 μl) were settled onto glass coverslips. Cells in fresh preparations were viewed on a Leica DM 4000B microscope using a 63× NA 1.4 apochromatic objective lens coupled to a DP71 camera (Olympus).

2.4. Materials

All chemicals and solvents were of the highest grade commercially available (LC-MS grade). Standart sialic acids; Neu5Ac, Neu5Gc and KDN (A9646, G9793 and 60714) were purchased from Sigma (Sigma-Aldrich Chemie GmbH Riedstrasse 2 D-89555 STEINHEIM). DMB (1,2-diamino-4,5-methylenediaoxy-benzene dihydrochloride) was obtained from Sigma (D4784). Sialic acids were determined by labeling with DMB. Mucin (purchased from Sigma-M-3895 Bovine Submaxillary Gland) was used in the present study as a model for sialic acid compounds. Salivary mucin is a collection of proteins that contain large amounts of O-glycosidically linked carbohydrate chains. Mucin isolated from bovine submaxillary gland using as a standard compound contains most members of sialic acid family [4,39]. The amino group of sialic acids in mucin is usually substituted with acetyl or glycolyl groups. Hydroxyl groups of sialic acids are also sometimes substituted with acetyl, lactyl and other groups [40].

2.5. Liberation of sialic acids from mucin glycoprotein and derivatization of standards

For the confirmation of molecular species of sialic acids, mucin (1 mg) was mixed with 20 μL 2 M aqueous acetic acid. The mixture (1 mg/20 μL) was kept at 80 °C for 3 h for releasing sialic acids. The solution (20 μL) containing sialic acids was mixed with 7 mM DMB, 0.75 M β-mercaptoethanol, and 8 mM sodium hydrosulfite in 1.4 M aqueous acetic acid. The mixture was incubated at 60 °C for 150 min in the dark. After cooling, a portion was analyzed using LC-ESI-MS/MS.

Standards; Neu5Ac, Neu5Gc and KDN (1.25 μg/mL) were directly derivatized with DMB and reaction mixture directly injected to LC-ESI-MS/MS system.

2.6. Liberation and derivatization of sialic acids from coelomocytes

Coelomocytes were homogenized by sonication (Bandelin-Sonorex) at 4 °C in 2 M aqueous acetic acid solution (1 mg of cells/5 μL). The solution was used for sialic acid analysis. Homogenizated 40 μL sample solutions (1 mg/5 μL) were kept for 3 h at 80 °C. After hydrolysis, the mixtures were derivatized with DMB in the same manner as above. The mixtures were centrifuged at 16,300 rpm for 10 min at 4 °C. Supernatant solution containing the derivatizated sialic acids was pipetted to HPLC vial insert (250 μL). The injection volumes of both samples were 0.5 μL for analyzing by CapLC-ESI-MS/MS with ion trap.

2.7. Capillary LC-ESI-MS/MS/Ion trap parameters

LC was performed using an Agilent 1200 Capillary HPLC system with an ODS capillary column (Agilent ZORBAX SB-C18 150 × 0.5 mm, 5 μm, USA) delivering 20 μL/min of the eluent. Elution was performed by isocratic mode using mixture of methanol–acetonitrile–water (7.5:5:87.5, v/v). The column was kept at 30 °C during the analysis. The samples were stored at 5 °C in temperature controlled autosampler board (Agilent G1377A). The column of LC system was connected to an electrospray ion source (ESI positive). All mass spectrometric measurements were performed on a HCT Ultra ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) source. Spectrometric conditions such as the ion optics voltages, nebulizer gas and dry gas flow rates, and the dry gas