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The function of rhamnose-binding lectin in innate immunity by restricted binding to Gb3

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ABSTRACT

L-Rhamnose-binding lectins (RBLs) have been isolated from various kinds of fish and invertebrates and interact with various kinds of bacteria, suggesting RBLs are involved in various inflammatory reactions. We investigated the effect of RBLs from chum salmon (*Oncorhynchus keta*), named CSL1, 2 and 3, on the peritoneal macrophage cell line from rainbow trout (*Oncorhynchus mykiss*) (RTM5) and an established fibroblastic-like cell line derived from gonadal tissue of rainbow trout (RTG-2). CSLs were bound to the surface of RTM5 and RTG-2 cells and induced proinflammatory cytokines, including IL-1β1, IL-1β2, TNF-α1, TNF-α2 and IL-8 in both cells by recognizing globotriaosylceramide (Gb3). In addition, CSLs had an opsonic effect on RTM5 cells and this effect was significantly inhibited by L-rhamnose, indicating that CSLs enhanced their phagocytosis by binding to Gb3 on cell surfaces. This is the first finding that Gb3 plays a role in innate immunity by cooperating with natural ligands, RBLs.

1. Introduction

Lectins are a group of sugar-binding proteins that recognize specific carbohydrate structures and agglutinate various cells by binding to cell-surface glycoconjugates. Animals produce a variety of lectins, which are classified into several lectin families depending on their sequence similarities and sugar-binding specificities; C-type, S-type (galectins), I-type (siglecs and others), P-type (phosphomannosyl receptor), Pentracins, rhamnose-binding lectin (RBL), calreticulin and calnexin, ERGIC-53 and VIP-36, discoidins, fucolectins and fibrinogen-type [1]. The RBL family has been found in over 25 species of fish, which belong to the orders Clupeiformes, Salmoniformes, Osmeriformes, Siluriformes, Perciformes and Cypriniformes. RBLs have been found not only in fish but also in sea urchin [2], penguin wing oyster (*Pteria penguin*) [3], and ascidian (*Botryllus schlosseri*) [4]. Most RBLs are composed of two or three tandemly repeated characteristic carbohydrate-recognition domains (RBL CRDs), which consist of about 95 amino acid residues. RBLs are classified into 5 subgroups based on their domain architecture, hemagglutination activity for human erythrocytes and sugar specificity against lactose [5]. Type I is composed of three tandemly repeated domains. Type II is composed of two tandemly repeated domains and an additional region. Types III and IV are composed of two tandemly repeated domains, but hemagglutination activity and sugar specificity are different among these subgroups. Type V is composed of one domain and homodimerizes by disulfide bond formation. RBLs have two characteristic peptide motifs, -(AN)YGR(TD)- (YGR-motif) and -DPCXGT(Y)KY(L)- (DPC-

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Abbreviations: BSA, bovine serum albumin; ConI, conger eel lectin I; CRD, carbohydrate recognition domain; CSL, chum salmon egg lectin; FAC, frontal affinity chromatography; HEPES, 2-[4-(hydroxyethyl)-1-piperazinyl] ethanesulfonic acid; Gb3, globotriaosylceramide; GEM, glycolipid-enriched membrane; LPS, lipopolysaccharides; LTA, lipoteichoic acid; MBP, mannose-binding protein; PA, pyridylaminated; PBS, phosphate-buffered saline; PMS, 1-methoxy-5-methylphenazinium methylsulfate; *p*NP, *p*-nitrophenyl; PRP, pattern recognition proteins; RBL, rhamnose-binding lectin; RTG-2, an established fibroblastic-like cell line derived from gonadal tissue of rainbow trout; RTM5, the peritoneal macrophage cell line from rainbow trout; RT-PCR, reverse transcription and polymerase chain reaction; Stx, Shiga toxin.

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motif), at the N- and C-terminal region of each domain, respectively [2,6,7]. The RBL CRD can be characterized by highly conserved 8 half-Cys residues at homologous positions as well as the conserved motif. The pairing of the disulfide bonds have been determined with Spanish mackerel (*Scomberomorous niphoninus*) egg lectin (SML) [8]. Recently, solution structure of RBL CRD in mouse latrophilin-1, which is a target receptor for α-latrotoxin, has been determined [9]. RBL CRD adopts a unique α/β fold with long structured loops important for monosaccharaide recognition.

RBLs have a variety of functions in fish eggs, such as prevention of polyspermy, regulation of carbohydrate metabolism, cross-linking of carbohydrate-rich proteins of the fertilization envelope and mitogenesis [10]. RBLs play an important role as germline-encoded pattern recognition proteins (PRP) in innate immunity. RBLs agglutinate Gram-negative and Gram-positive bacteria by recognizing the structures of lipopolysaccharides (LPSs) and lipoteichoic acid (LTA) on their surfaces, respectively [11,12]. RBLs bind to distinct serotypes of LPS and show much higher binding activities to smooth-type LPSs of *Escherichia coli* K-12 and *Shigella flexneri* 1A than their corresponding rough-type LPSs, indicating that the chemical structure of O-antigens is important for LPS-binding of RBLs. RBLs also bind to glycolipids and glycoproteins of the microsporidian *Glugea plecoglossi* which is a pathogen of ayu (*Plecoglossus altivelis*) [13]. RBLs are mainly localized in the tissues related to the immune system, such as mucous cells of gills, goblet cells of intestine, spleen, thrombocytes, lymphocytes, monocytes and neutrophils [13,14]. However, RBL was also isolated from spores of the microsporidian fish parasite, *Loma samonae*, which was located in gill tissue [15]. In addition, it has been shown that the receptor of RBL from amago (*Oncorhynus rhodurus*) was expressed on peritoneal macrophages after inflammatory stimulation [16]. These facts suggest that RBLs are involved in the innate immunity and inflammatory reaction, but it has not been fully understood yet.

It has been reported that RBLs bind to globotriaosylceramide (Gb3: Galα1 → 4Galβ1 → 4Galβ1 → Cer) [13,17]. Gb3 acts as functional receptor for toxins, such as Shiga toxin (Stx) from *Shigella dysenteriae* serotype 1 and *Escherichia coli* O157: H7, and pierisin-1 from cabbage butterfly [18,19]. These toxins are holotoxins composed of an enzymatically active A subunit and five B subunits participating in the binding to Gb3 [20,21]. The A subunit is a cytotoxic chain that inhibits RNA *N*-glycohydrolase activity and cleaves a specific adenine residue on the 28S ribosomal RNA in the cytosol, thereby inhibiting protein synthesis [18]. Stx has also been shown to up-regulate the expression of cytokines, such as TNF-α, IL-1β and IL-6, and a number of CC and CXC chemokines from human primary blood monocytes and transformed monocytic cell lines *in vitro* [22–26]. However, the non-toxic mutant Stx1 lacking *N*-glycosidase activity and Stx1 B subunits did not exhibit either cytotoxicity or cytokine inducing activity [25,27,28]. These results demonstrated that the enzymatic activity of the A subunit was essential for cytokine production. However, a number of recent studies revealed that the B subunit also had a biological effect on the target cells. Stx1 B subunit and anti-Gb3 antibody triggered apoptosis of Burkitt's lymphoma B cell lines [29]. Gb3 is located in glycolipid-enriched membrane (GEM) domains [30,31], known as lipid rafts [32]. Some gangliosides in GEM are associated with the transducer molecules that are activated by gangliosides. Gb3 is associated with Src family kinases such as Lyn and Yes by the binding of Stx to Gb3 in human renal tubular cell line (ACHN) and human B cells [30,31]. Stx1-B binding to Gb3 induces the clustering of lipid raft, leading to the activation of the Src family protein tyrosine kinase by aggregation-mediated kinase autophosphorylation [30,31,33]. The binding to Gb3 itself may transduce a signal via GEM and the contribution of Gb3 in various biological activities may differ in each cell line. It has been suggested that Gb3 could be involved in the elimination process of B cells [34,35]. B cells expressing Gb3 are more sensitive to apoptotic stimuli than germinal center B cells, suggesting a role of Gb3 in clonal deletion [35].

In this study, we show that multiple RBLs from chum salmon (*Oncorhynchus keta*) eggs, CSL1, 2 and 3, induced proinflammatory cytokines, including IL-1β1, IL-1β2, TNF-α1, TNF-α2 and IL-8, by recognizing Gb3 on the surface of the peritoneal macrophage cell line (RTM5) from rainbow trout (*Oncorhynchus mykiss*) and an established fibroblastic-like cell line (RTG-2), derived from gonadal tissue of rainbow trout. In addition, we show that CSLs have an opsonic effect on RTM5 by binding to Gb3. To our knowledge, this study is the first to show that Gb3 plays a role in innate immunity by cooperating with natural ligands, RBLs.

2. Materials and methods

2.1. Materials

A cell counting kit (WST-1) from Dojin (Kumamoto, Japan) and a celldesk LF from Sumilon (Tokyo, Japan) were used for the experiments. SuperScript™ III One-Step RT-PCR with Platirum® Taq, ribonucleoside vanadyl complex and proteinase K were purchased from Invitrogen (Carlsbad, CA, USA), Daiichi Pure Chemicals (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Pyridylaminated (PA-) and *p*-nitrophenyl (*p*NP-) oligosaccharides were obtained from Takara Bio (Kyoto, Japan), Seikagaku Kogyo (Tokyo, Japan) and Sigma (St. Louis, MO, USA), and their structures were numbered as described [36]. Mouse anti-Gb3 antibody (IgG2b) and Alexa fluor® 594 goat anti-mouse antibody (IgG) were purchased from Seikagaku Kogyo and Molecular Probes (Eugene, OR, USA), respectively. Permafluor from Beckman Coulter (Marseille, France) was used as mounting medium. A gel filtration column PC200S was a gift from Shiseido (Tokyo, Japan). Fluorescent microsphere (1 μm of diameter) and Hoechst 33258 were purchased from Polysciences Inc. (Warrington, PA, USA) and Dojin, respectively.

2.2. Isolation of CSLs

RBLs, CSL1, CSL2 and CSL3 were isolated from the eggs of chum salmon as described previously with modifications [12]. Briefly, chum salmon eggs (2 kg) were homogenized with 20 mM Tris–HCl buffer (pH 8.0) containing 0.5 M NaCl and centrifuged at 15,000 × *g* for 30 min at 4 °C. The supernatant was mixed with L-rhamnose-Sepharose 4B gel (180 ml) and the suspension was incubated at 4 °C overnight. Unabsorbed substances were removed by washing the gel with the buffer. The gel was packed in a glass column (2.5 cm × 22.5 cm), washed with the buffer and the adsorbed substance was eluted with 0.2 M L-rhamnose in the buffer. The fractions with significant absorption at 280 nm were collected, dialyzed against distilled water and lyophilized. The lectin fractions were dissolved in 50 mM sodium phosphate buffer (pH 6.9)/0.25 M NaCl and applied to gel-filtration HPLC using a PC200S column (4.6 mm × 250 mm) at 0.5 ml/min using the same buffer. The eluate was monitored by absorption at 280 nm. Each lectin fraction was collected separately, dialyzed against distilled water and lyophilized.

2.3. Cell culture

RTM5 cells, an established macrophage cell line derived from the abdominal cavity of rainbow trout [37], and RTG-2 cells, an established fibroblastic-like cell line (ATCC, CCL 55) derived from gonadal tissue of rainbow trout, were used. Cells were grown in RPMI 1640 supplemented with 10% FBS and L-glutamine (2 mM) at its optimum grow temperature, 20 °C.