Developmental and Comparative Immunology 55 (2016) 203–210

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

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

Investigation of variable lymphocyte receptors in the alternative adaptive immune response of hagfish

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ARTICLE INFO

Article history:
Received 21 July 2015
Received in revised form
1 October 2015
Accepted 1 October 2015
Available online 9 October 2015

Keywords:
Variable lymphocyte receptors
Hagfish
Hemagglutinin of avian influenza virus
Adaptive immune response

ABSTRACT

Jawless vertebrates have an alternative adaptive immune system mediated by variable lymphocyte receptors (VLRs), VLRA, VLRC and VLRB. In investigation on the adaptive immunity of hagfish, avian influenza virus hemagglutinin (H9N2-HA1) was used as a model antigen, with mRNA expression levels of VLRA, VLRC and Ikaros were up-regulated in the first week post-immunization. CD45 was up-regulated after the first week; and expression of VLRB progressively increased over the course of the trial. The transcriptional/translational activation of VLRB in blood was verified. The VLRBs cloned from these transcripts showed diversity in their leucine-rich repeats (LRRs). The production of specific VLRB increased in a time- and dose-dependent manner, detected by an anti-VLRB antibody (11G5). The plasma VLRB could distinguish H9N2-HA1 from unrelated proteins, but not from other HA1 subtypes. Together, our findings show that VLRs play a major role in the alternative adaptive immune system of hagfish by responding to specific foreign substances, such as H9N2-HA1.

1. Introduction

In jawed vertebrates, B-cell receptors (BCRs) and T-cell receptors (TCRs) play a pivotal role in the antigen recognition step of adaptive immunity, which is characterized by antigen-specific interactions and memory (Cooper and Alder, 2006). On the other hand, the existence of BCR, TCR or major histocompatibility complex (MHC) molecules was not identified in agnathans until the middle of 2004, although many studies had tried to prove the existence of adaptive immunity in jawless vertebrates (Finstad and Good, 1964; Linthicum and Hildemann, 1970; Marchalonis and Edelman, 1968; Pollara et al., 1970). Recent studies have solved this enigma by showing that lampreys and hagfish (the extant jawless vertebrates) utilize variable lymphocyte receptors (VLRs) for antigen recognition (Pancer et al., 2004a, 2005).

VLRs, which are structurally unrelated to BCRs or TCRs, are members of the leucine-rich repeat (LRR) protein family (Pancer et al., 2004a). In jawless vertebrates, the VLRs are expressed by separate lymphocyte lineages (Guo et al., 2009; Pancer et al., 2004a). Three distinct types of lymphocytes (VLRA, VLRB and VLRC) were recently identified in both lampreys and hagfish (Kasamatsu et al., 2010; Li et al., 2013; Pancer et al., 2004a, 2005). VLRA and VLRC are phylogenetically and functionally related to the αβ and γδ T cells, respectively, of jawed vertebrates; they are expressed on the outer surface of T-cell-like lymphocytes and function as membrane-bound receptors. VLRB, in contrast, is expressed by B-cell-like lymphocytes and occurs as both membrane-bound and secretory forms (Alder et al., 2008; Flajnik and Kasahara, 2010; Guo et al., 2009; Hirano et al., 2013). All VLR proteins are structurally similar; they are composed of an N-terminal LRR (LRRNT), LRR1, as many as eight 24-residue variable LRR modules (LRRVs), a connecting peptide (CP), the C-terminal LRR (LRRCT), and an invariant threonine/proline-rich stalk region. These

Abbreviations: BCRs, B-cell receptors; BclA, *Bacillus anthracis* spore surface protein; CP, connecting peptide; ELISA, enzyme-linked immunosorbent assay; GLM, General Linear Models; GST, glutathione S-transferase; HEL, hen egg lysozyme; H9N2-HA1, hemagglutinin 1 subunit of avian influenza virus A H9N2; i.p., intraperitoneally; LRRs, leucine-rich repeats; LRRNT, N-terminal LRR; LRRVs, variable LRR modules; LRRCT, C-terminal LRR; mAbs, monoclonal antibodies; MBP, maltose binding protein; MHC, major histocompatibility complex; qRT-PCR, quantitative real-time PCR; s.e.m., standard error of the mean; TCRs, T-cell receptors; VLRs, variable lymphocyte receptors.

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http://dx.doi.org/10.1016/j.dci.2015.10.001
0145-305X/

LRR family proteins form characteristic horseshoe-shaped structures in which β-sheets forma concave surface that can recognize and specifically bind to antigens, as shown in studies of X-ray crystal structures of the VLR proteins (Herrin et al., 2008). Variations in the amino acid sequence and number of LRRV modules lead to a high level of diversity among the VLRs (Han et al., 2008). A huge VLR repertoire can be generated by somatic gene assembly of multiple LRR-encoding cassettes (Pancer et al., 2004a), with Monte Carlo simulations predicting that ~10^{14} unique VLRs can be created in this way (Alder et al., 2005). As these responses have only been recently discovered, there are relatively few studies relating to them in hagfish (*Eptatretus burgeri*).

The jawless vertebrates were thought to produce serum antibody-like proteins, with agglutination and neutralization activities in response to a specific antigen (Finstad and Good, 1964; Linthicum and Hildemann, 1970; Marchalonis and Edelman, 1968; Pollara et al., 1970). After the discovery of VLRB, the direct binding of VLRB with antigen was verified in experiments in which lampreys were immunized with hen egg lysozyme (HEL), and took place on its concave surface showing the highest sequence diversity (Velikovsky et al., 2009). Moreover, antigen-induced VLRBs were found to be secreted into the blood by plasma-like cells after proliferation, lymphoblastoid transformation, and differentiation of VLRB lymphocytes (Guo et al., 2009; Pancer et al., 2004a). In jawed vertebrates, several proteins such as Ikaros and CD45 have been found to be involved in lymphocytes activation (Georgopoulos et al., 1997; Zikherman et al., 2012). The Ikaros, which is a family of hematopoietic-specific zinc finger proteins, has been described as a central regulator of lymphocyte differentiation and is specifically required during the earliest stages of T- and B-cell specification (Georgopoulos et al., 1997). Meanwhile, CD45 is known to act as a positive regulator of B-cell antigen receptor signalling throughout the development and differentiation of B-cells (Zikherman et al., 2012). Ikaros and CD45 have been identified in lampreys and hagfish (Haire et al., 2000; Mayer et al., 2002a; Nagata et al., 2002; Uinuk-ool et al., 2005), but there are no published studies examining their involvement in the VLR response of hagfish.

To gain a better understanding of the adaptive immune responses of hagfish, we investigated the transcription profiles of VLRA, VLRB, VLRC, Ikaros and CD45 at various time points after fish were immunized with hemagglutinin 1 (H9N2-HA1), which is a major surface protein of avian influenza virus A subtype H9N2. To confirm that VLRB plays a crucial role for humoral response against foreign molecules, we examined the transcriptional and translational activation of VLRB in the plasma of immunized hagfish and characterized antigen-induced VLRB depending on the numbers of LRRV modules. Moreover, specific binding of VLRB with the antigen present in the plasma was also evaluated.

2. Materials and methods

2.1. Animals and immunization

Juvenile inshore hagfish (length 200–300 mm, weight 27.5–45 g; Bogyeong Hagfish Services) were maintained in aquaria at a constant water temperature (14–15 °C) and fed brewer's yeast. For immunization, H9N2-HA1 (A/Chicken/Hong Kong/G9/97; Sinobiological) in 100 μl of 0.67 × PBS (91 mM NaCl, 1.8 mM KCl, 2.8 mM Na_2HPO_4, 0.9 mM KH_2PO_4) was injected intraperitoneally (i.p.) into hagfish that had been anesthetized by immersion in ethyl 3-aminobenzoate methanesulfonic acid (0.1 g/L; Sigma). As previously reported, 0.67 × PBS was used because it is isotonic and non-toxic to hagfish (Alder et al., 2005). H9N2-HA1 was selected due to its diversity of subtype, well-known molecular structure and availability. Peripheral blood was collected in 0.67 × PBS/10 mM EDTA, layered onto a 28% Percoll (GE Healthcare) gradient and centrifuged at 400 × g, 20 min, 4 °C. Subsequently, hagfish leukocytes were collected and antisera were analyzed for reactivity to the antigen.

2.2. Production of monoclonal antibodies (mAbs) specific for the VLRB invariant region of hagfish

BALB/c mice (Koatech Inc) were immunized with a recombinant partial VLRB protein including the invariant stalk region, which was purified from bacterial lysates using a Ni-NTA affinity column (Qiagen). Fifty micrograms of the antigens were mixed 1:1 with Freund's complete (for the first immunization) or incomplete adjuvant (Sigma–Aldrich) for the second and third immunizations, and mice were injected i.p. every other week for 6 weeks. One week after the third injection, the mice were boosted by tail vein injection with 50 μg of adjuvant-free recombinant protein. Three days later, spleen cells were harvested from immunized mice and fused with Sp2/o myeloma cells (#CRL-1581; ATCC) using polyethylene glycol. The fused hybridoma cells were cultivated in 96-well plates on a feeder layer of mouse blood cells. Monoclonal antibodies (mAbs) for the recombinant VLRB were screened by enzyme-linked immunosorbent assay (ELISA) and Western blotting. The final clone (11G5) was selected as having the best specificity for the VLRB proteins of hagfish.

2.3. RNA extraction, cDNA synthesis and quantitative (q) RT-PCR

For gene expression analysis, total RNA was extracted from blood leukocytes using a QIAamp RNA blood mini kit (Qiagen) according to the manufacturer's instructions. One microgram of total RNA was treated with DNase I and reverse transcribed with a RevertAid First strand cDNA Synthesis kit (Thermo Scientific). To remove the surplus RNA from the original template, the reactants were incubated with RNase H (Life Technologies). Quantitative real-time PCR (qRT-PCR) was performed using TOPreal qPCR 2X PreMIX (SYBR Green with high ROX; Enzynomics) and a StepOne Plus Real-time PCR system (Life Technologies). The primers used for qRT-PCR were designed with the Primer Express Software (Life Technologies), and are listed with amplification efficiency in Supplementary Data 1. The amplification efficiencies and specificities of the primer sets were examined according to the protocol provided with the PCR system. The relative transcript levels in immunized hagfish were determined by comparison to the β-actin gene (internal reference) using the comparative Ct ($2-\Delta\Delta Ct$) method, as described in ABI Prism 7700 User Bulletin #2 (Life Technologies). We examined parallel cDNA samples in three individual experiments involving both naive and immunized animals.

2.4. Recombinant VLRB protein expression

The genes encoding various mature VLRBs were amplified from the mRNA of blood leukocytes by extension PCR. The synthesized cDNA samples were cloned into the gateway vector pMGWA derived from the pET22b vector (Novagen) between the attR1 and attR2 sites with a maltose binding protein (MBP) tag containing an N-terminal cleavage site for affinity purification. *E. coli* cells were individually transformed with the VLRB-encoding pMGWA vectors and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration, 1 mM). The cells were further incubated at 25 °C for 10 h, harvested by centrifugation at 4000 × g, and suspended in lysis buffer (pH 7.4) containing 50 mM NaH_2PO_4, 300 mM NaCl and 1 mM EDTA. The mixtures were further