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Research and Development of Antibody Production Using Zebrafish against Human LGR3

Natsuki Nukada[1], Eriko Avşar-Ban[1], Hisayoshi Ishikawa[1] and Yutaka Tamaru[1,2,3]*

[1]*Department of Life Sciences, Mie University, Japan*
[2]*Department of Bioinfomatics, Mie University Life Science Research Center, Japan*
[3]*Laboratory of Applied Biotechnology, Mie University Industrial Technology Innovation Institute, Japan*

*Corresponding author
Yutaka Tamaru, Department of Life Sciences, Graduate School of Bioresources, Mie University, 1577 Kurimamachiya, Tsu, Mie, Japan, Tel:/Fax: +81-59-231-9560; Email: ytamaru@biomie-u.ac.jp

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Abstract

Membrane-associated proteins such as leucine-rich repeat-containing G-protein-coupled Receptors (LGRs) are structurally complicated and conserved among mammals. Therefore, there are several problems that specific antibodies against LGRs have never been made in experimental mammals such as mice and so on caused by their immune tolerance. To overcome these problems, we have attempted to produce polyclonal antibodies against LGRs using teleosts that are located at the origin of vertebrates and have the acquired immune system in addition to the innate immune system. In this study, we have developed antibody production using zebrafish (*Danio rerio*) by oral immunization. The recombinant human LGR3 (hLGR3) was expressed in *Escherichia coli* transformed with an expression vector for the leucine-rich repeat region of hLGR3. After the transformants having the recombinant protein as an antigen were feed to 10-50 zebrafish, theier blood was collected all together and analyzed. As a result, specific antibodies against the leucine-rich repeat region of hLGR3 were detected by the method of dot blotting.

ABBREVIATIONS

DAB: 3,3'-Diaminobenzidine, Tetrahydrochloride; GPCR: G-protein-coupled receptor; His-tag: Histidine Tag; Ig: Immunoglobulin; IPTG: Isopropyl β-D-1-Thiogalactopyranoside; LGR: Leucine-rich repeat-containing G-protein-coupled Receptor; PBS: Phosphate-Buffered Saline; PBST: PBS containing 0.1 % Tween20; PVDF: Polivinylidene Difluoride; TF: Trigger-Factor tag

INTRODUCTION

Membrane-associated proteins such as G-protein-coupled receptors (GPCRs) are important targets for drug discovery [1]. However, since Leucine-rich repeat-containing G-protein-coupled Receptors (LGRs) are structurally complicated and conserved among mammals, the production of specific antibodies against LGRs is often difficult [2]. Accordingly, for example, the method of DNA immunization has been developed [3]. cDNA of a target protein introduced into mammalian cells by an expression vector and the vector was directly administered to immune animals. Although this method enabled to produce many kinds of anti-GPCR antibodies, it took a lot of time and costs to select and prepare specific antibodies. In order to overcome these problems, we focused on a teleost, zebrafish (*Danio rerio*), as a new antibody producer. A secreted type of Immunoglobulin heavy chain (IgH) in zebrafish is generally composed of four CH domains (CH1-CH4) that are similar to mammalian IgM and show both stable and flexible V-D-J regions [4]. Furthermore, there exist four complementarity determining regions that show not only most extremely significant and changeable but also side-by-side and crossed by each other such as three flame-work regions and that show relatively less the flexible regions of a teleost IgH.

In this study, we tried to make zebrafish antibodies against human LGR3 (hLGR3), a thyroid-stimulating hormone-receptor-containing LGR family protein, because good antibodies against this kind of GPCRs have never been made in mammals. Therefore, we developed the method of induced polyclonal antibodies using zebrafish fed by transformant *E. coli* expressed in the leucine-rich repeat region of hLGR3 as an antigen (Figure 1).

MATERIALS AND METHODS

Fish

Zebrafish were maintained at 27.5±1 °C under light condition of 14 h light period and 10 h dark period. In this study, 50 zebrafish were used in a test group and 6 test groups were tested for each experiment.

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Antigen preparation

To construct the recombinant plasmid for expression of the leucine-rich repeat region of hLGR3 added with a His-tag region at its N-terminal side. The hLGR3 gene was cloned into pET15b vector (Novagen) to construct a plasmid named pET15b hLGR3. Expression plasmid was introduced into *E.coli* (BL21) by transformation. *E.coli* BL21 cells were grown in LB medium containing 100 µg/ ml ampicillin at 37°C overnight with shaking. Bacteria were induced by treatment with 1 mM IPTG for 4 h at 25°C. The bacteria were collected at 2,500 g for 10 min, and the expression of recombinant proteins was confirmed by SDS-PAGE and Western blotting analysis. For Western blotting analysis, the proteins in a gel were transferred to a PVDF membrane. After transferred membranes were blocked for 2 h in 5% (w/ v) skim milk of PBST. After blocking, the membrane was incubated for 2 h with anti-His mouse IgG antibody (GE Healthcare) at a dilution of 1: 3,000 with PBST. Then, it was incubated with anti-mouse IgG HRP conjugate antibody (Cell Signaling) at a dilution of 1: 3,000 with PBST for another 2 h. Finally, the membrane was observed by developing with Diaminobenzidine (DAB).

Immunization

Zebrafish were given immunization food mixture (*E. coli* carrying a gene encoding the leucine-rich repeat region of hLGR3: TetraMin fish food = 1: 4) added with ampicillin (final concentration 40 mg/ g) as about 2 mm width. A booster immunization was given after 10 days from first immunization. The antiserum was collected from 10 to 50 zebrafish after 6 days from the booster immunization.

Antigen for dot blotting analysis preparation

The gene encoding the leucine-rich repeat region of hLGR3 was cloned into pCold TF vector (TaKaRa) to construct a plasmid named pCold TF-hLGR3 for the detection of anti-hLGR3 antibody in zebrafish IgM by dot blotting analysis. In addition, the TF-tag region contains a His-tag region. The pCold TF-hLGR3 plasmid was introduced into *E. coli* (BL21) by transformation. *E. coli BL21* cells were grown in LB medium containing 100 µg/ ml ampicillin at 37°C overnight with shaking. The transformants were induced by treatment with 0.1 mM IPTG for 24 h at 15°C. The cells were collected at 2500 g for 10 min, and the protein expression was confirmed by SDS-PAGE and Western blotting analysis. For Western blotting analysis, the proteins in a gel were transferred to a PVDF membrane. After transfer membranes were blocked for 2 h in 5% (w/ v) skim milk of PBST. After blocking, the membrane was incubated for 2 h with anti-His mouse IgG antibody (GE Healthcare) at a dilution of 1: 3,000 with PBST. Then, it was incubated with anti-mouse IgG HRP conjugate antibody (Cell Signaling) at a dilution of 1: 3,000 with PBST for another 2 h. Finally, the membrane was observed by developing with DAB.

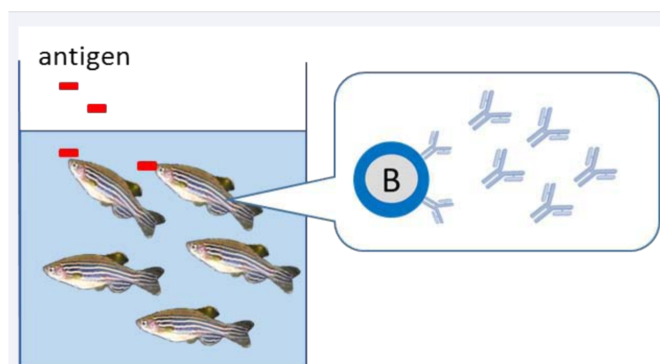

Figure 1 Scheme of oral immunization zebrafish. Zebrafish were given antigen that the target protein as an antigen was expressed in *E. coli* for acquired immune response.

Dot blotting analysis

The antiserum was collected from zebrafish after immunization to detect whether zIgM immunocrossreacted with the recombinant hLGR3 using dot blotting. First, the recombinant hLGR3 protein (50 to 500 ng) was solid-phased on PVDF membranes and dry-up overnight. The membrane was washed three times with PBST and then blocked with 5% (w/v) skim milk of PBST for 2 h. After washing with PBST, 10-µl mouse serum or 10-µl zebrafish antiserum at a dilution of 1: 100 with Can Get Signal Solution I (TOYOBO) was incubated for 2 h. Mouse serum and unimmunized zebrafish serum were also used as negative controls. After further washing steps, the membranes were incubated with anti-zebrafish IgM HRP-linked antibody at a dilution of 1: 3,000 with Can Get Signal Solution II (TOYOBO) as a secondary antibody for 1 h. Finally the membrane was observed by developing with Chemi-Lumi One (Nacalai).

RESULTS AND DISCUSSION

Antigen preparation

The recombinant protein having the leucine-rich repeat region of hLGR3 was successfully expressed in *E. coli* transformed pET15b-hLGR3 plasmid, with a molecular weight of around 40 kDa. The identity of expressed protein was further confirmed by Western blotting using anti-His-tag antibody. As a result, it was suggested that the expressed protein as an antigen was specifically detected with the anti-His-tag antibody (Figure 2). Therefore, since the recombinant protein obtained from the pET15b-hLGR3 plasmid was expected to contain a His-tag at its N-terminal region, the transformants was used for antibody production in zebrafish.

Antigen for dot blot analysis expression

The recombinant protein having a Trigger Factor (TF) tag, a His-tag, and the leucine-rich repeat region of hLGR3 was successfully expressed in *E. coli* transformed pCold TF-hLGR3 plasmid, with a molecular weight of around 80 kDa. In addition, the product of the recombinant protein was concentrated in the supernatant of lysate. The identity of expressed TF-hLGR3 protein was further confirmed by Western blotting using anti-His-tag antibody. As a result, the recombinant protein was specifically detected with the anti-His-tag antibody (Figure 3). Therefore, these results suggested that the recombinant protein contained both His-tag and TF regions.

Detection of antibody in zebrafish serum

Dot blotting showed significant response and the detection of the spot on 100 ng of the recombinant TF-hLGR3 protein compared with negative controls (Figure 4A). According to the

results obtained by quantifying the fluorescence intensity, the intensity was saturated at over 250 ng hLGR3 antigen, suggesting that a linear relationship to detection antigen at lower 100 ng the recombinant protein (Figure 4B). Thus, dot blotting clearly indicated that the zIgM from immunized zebrafish antiserum was immunocrossreacted with the recombinant TF-hLGR3 protein.

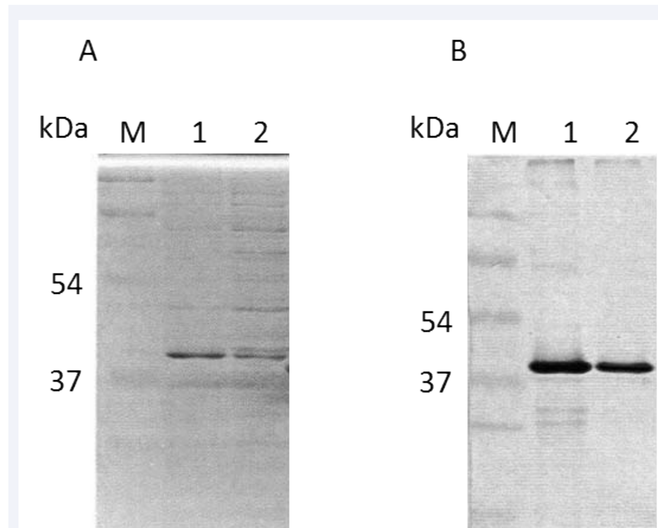

Figure 2 Identification of the recombinant hLGR3 proteins for an antigen. (A) SDS-PAGE and **(B)** Western blot analysis. Western blotting was performed with anti-His mouse IgG antibody (1: 3,000). Lane M, prestained protein marker; lanes 1 and 2, the recombinant proteins of *E .coli* with pET15b hLGR3. Whereas the protein in lane 1 is induced by 1 mM IPTG, the protein in lane 2 is un induced as a control.

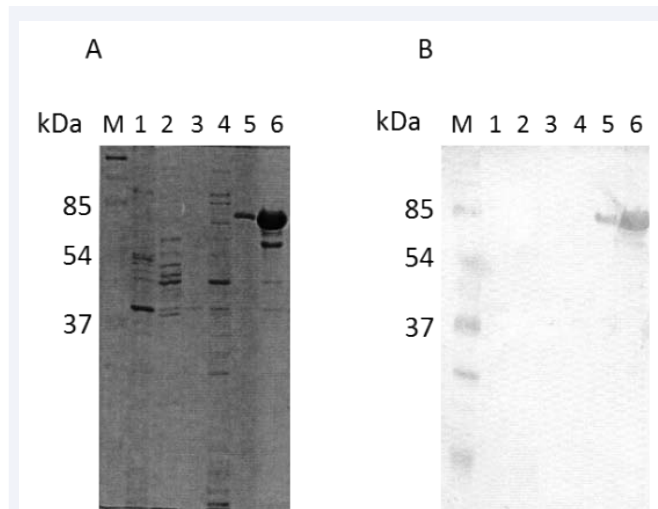

Figure 3 Identification of the recombinant TF-hLGR3 proteins for dot blotting. (A) SDS-PAGE and **(B)** Western blot analysis. Western blotting was preformed with anti-His mouse IgG antibody (1: 3,000). Lane M, prestained protein marker; lane 1, insoluble fraction; lane 2, soluble fraction; lane 3, insoluble fraction before induction; lane 4, soluble fraction before induction; lane 5, insoluble fraction after induction; lane 6, soluble fraction after induction. Whereas the proteins in lanes 1 and 2 were prepared from *E. coli* BL21, lanes 3-6 proteins were done in *E. coli* with pCold TF-hLGR3.

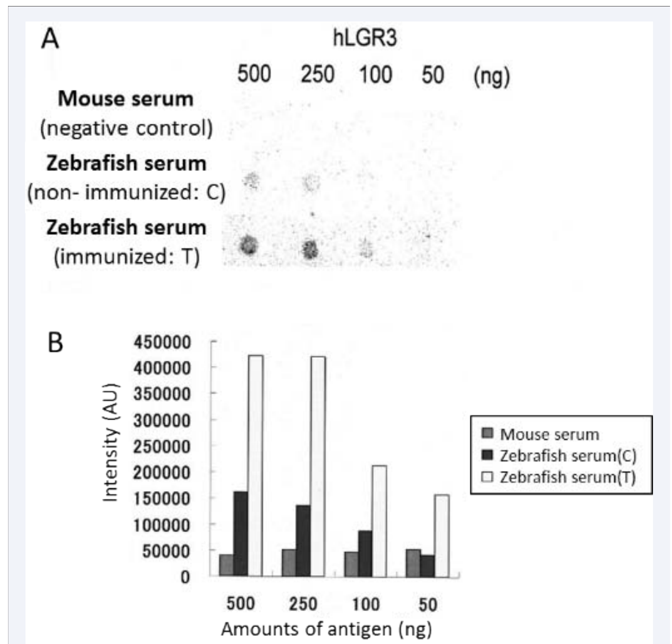

Figure 4 Detection of zebrafish IgM antibody with the leucine-rich repeat region of hLGR3 by dot blotting. (A) Dot blot analysis and **(B)** Fluorescence intensity. The top row on the blots was amounts of hLGR3 protein, from 50 to 500 ng/ a dot. Immunological detection was done with mouse serum and non-immunized zebrafish serum as negative controls, and immunized zebrafish serum. Each dot signal was counted from the density on the membrane.

CONCLUSION

In this study, we succeeded to produce antibodies using zebrafish against the leucine-rich repeat region of hLGR3. It has so far been difficult to obtain antibodies by general methods with mammals. It is also expected that these researches not only lead to drug discovery support but also are useful as diagnostic agents and research tools for detection and identification of cultured cells that might express the target molecules [5]. In this study, we used hLGR3 protein containing a leucine-rich repeat region in the extracellular and seven-transmembrane conformation as an antigen. Although GPCRs including LGRs currently exist about 800 types, only dozen of conformation has been clarified. Therefore, antibodies specifically binding to GPCRs could contribute to form a complex and to improve the crystallization and stabilization of the structure [6]. Furthermore, since such target molecules as GPCRs are often difficult to express themselves, even polyclonal antibodies against GPCRs are urgently needed. On the other hand, when antibodies against the membrane-associated proteins conserved and highly homologous between species of interest as human and mice are generated, the antibodies having higher titration and specificity could not be obtained because of the immune tolerance. Therefore, we tried to induce and produce specific antibody using zebrafish against the leucine-rich repeat region of hLGR3 by oral immunization. Animal intestine is in a unique environmental because of constantly contacting antigens such as some foods and coexisting with great deal of intestinal bacteria. In regard to intestinal immunity, one research was reported to mitigate the cedar pollen allergy by eating rice everyday accumulated T-cell epitope of the allergens has been

promoted [7]. Another research showed 17 strains of clostridia were isolated from intestinal bacterial flora [8]. Thus, zebrafish living in fresh water might have highly antibody production system at their intestine as port of foreign substances.

Further work is needed to analyze the immunological system in zebrafish and to show whether bacterial hosts carrying another antigens and how oral vaccination work. Zebrafish are an ideal model for developmental research as a valuable tool for immunological studies. Our immunological studies in zebrafish raise the developmental possibility of anti-GPCR antibodies for diagnostic agent and antibody production.

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