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Immunization method for multi-pass membrane proteins using highly metastatic cell lines

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ABSTRACT

A novel method using metastatic breast cancer cell lines was established for producing monoclonal antibodies (mAbs) against multi-span membrane proteins. Grafting of metastatic cells (MCF7-14) into the mammary gland of BALB/cJ/nu/nu mice induced splenic hypertrophy ($1.6–3.0 \times 10^8$ cells/spleen [$n = 6$]). More than half of the mAbs against MCF7-14 cells reacted with the cell membrane. Inducing production of antibodies against the extracellular domain of multi-pass membrane proteins is difficult. Because the protein structure becomes more complex as the number of transmembrane domains increases, preparing antigens for immunization in which the original structure is maintained is challenging. Using highly metastatic MDA-MB231 cells as the host cell line, we produced mAbs against a 12 transmembrane protein, solute carrier family 6 member 6 (SLC6A6), as a model antigen. When SLC6A6-overexpressing MDA-MB231 cells were grafted into nude mice, the number of splenocytes increased to $2.7–11.4 \times 10^8$ cells/spleen ($n = 10$). Seven mAb-producing clones that not only recognized the extracellular domain of SLC6A6 but also were of the IgG subclass were obtained. Immunocytochemistry and flow cytometry analyses revealed that these mAbs recognized the native form of the extracellular domain of SLC6A6 on the cell surface. Our novel immunization method involving highly metastatic cells could be used to develop therapeutic mAbs against other multi-pass membrane proteins.

1. Introduction

Multi-pass membrane proteins, including G protein-coupled receptors and solute carriers (SLCs), are currently the most important class of therapeutic targets. Antibodies directed against these proteins are highly sought after for therapeutic, diagnostic, and research purposes. Despite substantial interest in these targets, the generation of high-quality antibodies against membrane proteins using conventional methods is challenging [1]. Conventional approaches include immunization with whole cells [2,3], reconstituted proteins, or peptides. However, these approaches are difficult to apply to some of the most important membrane protein targets because multi-pass membrane proteins are often expressed at low levels in cells, have high amino acid sequence homology with human antigens and their homologues in immunized animals, and are very unstable when purified.

In order to obtain antibodies reactive to the native extracellular structure of membrane proteins, immunization by injection of cultured cells expressing the antigen has generally been used [4]. However, it is usually necessary to inject a large number of cells (typically $10^6–10^8$ cells [4–400 mg protein] per animal) to induce antibody production, which increases the risk of anaphylactic shock and death upon boosting. Alternatively, various approaches to produce mAbs for multi-pass membrane proteins have been developed, such as those involving Ig-Fc-domain fused proteins, DNA immunization [5], antigen-comprising budding baculoviruses [6], and phage screening *in vivo* [7]. Although these methods have some advantages when used with membrane protein antigens that maintain their three-dimensional structure, their use involves complicated experiments, and their application is limited and does present some problems. In the case of Ig-Fc fused proteins, several complex steps are required, such as plasmid construction, effective overexpression and purification of the target proteins. Moreover, there is no guarantee that the protein structure will be maintained.

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DNA immunization requires effective electrotransfer of the target gene-encoding plasmid to animal tissues. Reconstruction of membrane proteins in budding baculoviruses involves highly complex experimental techniques and expensive equipment. Phage display systems require a reliable source of antibodies for each antigen, and the construction of libraries for screening is time consuming. Thus, for immunization with multi-pass membrane proteins, improved methods for obtaining IgG subclass antibodies are needed.

The SLC family of eukaryotic membrane transport proteins includes over 300 members organized into 52 subfamilies. SLCs control the uptake and efflux of various solutes, including amino acids, sugars, and drugs [8]. Recent research revealed that some SLCs, such as SLC35F2 [9], SLC5A5 [10], SLC22A5 [11], SLC45A3-BRAF fusion protein [12], and SLC44A4 (which is in the clinical study phase at Astellas Pharma Inc.) are highly expressed in prostate cancer patients and thus are potential targets of therapeutic antibodies.

In order to develop and evaluate a novel immunization method, we selected SLC family 6 member 6 (SLC6A6) as a model protein. Human SLC6A6 contains 12 transmembrane (TM) regions connected by intra- and extracellular loops, with the N- and C-termini located intracellularly. SLC6A6 is a Na^+- and Cl^--dependent taurine transporter and is induced by osmotic stress [13]. It has been reported that the concentration of taurine is increased in the serum of colorectal cancer patients [14] and is correlated with tumor cell density [15]. Thus, it is likely that the expression of SLC6A6 might be related to colorectal cancer. Polyclonal antibodies against the extracellular domain of SLC6A6 are available, but monoclonal antibodies (mAbs) against this protein are not available.

In this study, we developed an immunization method using metastatic human breast cancer cell lines. Here, we demonstrate that this method can be used for the production of IgG subclass mAbs against multi-pass membrane proteins such as SLC6A6.

2. Material and methods

2.1. Cell lines

Human breast cancer cell line MCF7 was obtained from the Institute of Development, Aging and Cancer, Tohoku University. MCF7-derived MCF7-14 cells are described elsewhere [16]. MDA-MB231 cells were purchased (ATCC HTB-26, Manassas, VA). All cell lines were maintained in RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS) and an antibiotic–antimycotic.

2.2. Construction of the expression vector

RNA was extracted from MCF7-14 cells using an RNeasy Mini kit (Quiagen, Tokyo, Japan) and converted into cDNA using SuperScript III RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA). The resulting cDNA was used as a template. Amplicons of the human SLC6A6 gene (GenBank accession No.: NM_003043.3) were generated by polymerase chain reaction (PCR). PCR was performed with KOD Plus polymerase (TOYOBO, Osaka, Japan) using the following gene-specific primers: SLC6A6 (1863 bp) (forward) 5′-AAAGGATCCA TGGCCACCAAGGAGAAGCTGC-3′ and (reverse) 5′-AATCTAGACATCA TGGTCTCCACAATGATGT-3′. The amplified fragment was digested with *Bam*HI and *Xba*I and inserted into the pEF6-myc/HisA vector (Invitrogen) using the same restriction enzymes. The myc/His-tagged cDNA (pEF6-SLC6A6-myc/His) was introduced into MDA-MB231 cells using FUGENE 6 (Promega, Tokyo, Japan) according to the manufacturer's instructions. MDA-MB231 cells stably transfected with pEF6-SLC6A6-myc/His were selected for 3 weeks using Blasticidin S (10 μg/mL). Proteins were isolated from lysates of confluent cell cultures, subjected to SDS–PAGE, and transferred to PVDF membranes. The membranes were blocked (5% skim milk in TBS-T [25 mM Tris, 150 mM NaCl, 0.05% [v/v] Tween 20, pH 7.4]) and incubated with 1 μg/mL of anti-c-myc mAb (clone 9E10) (Santa Cruz, Dallas, TX) for 1 h, followed by incubation with the corresponding HRP-conjugated secondary antibody (1:200, BETHYL, Montgomery, TX) in TBS-T. Antibody complexes were detected using the Immobilon™ detection system (Millipore, Billerica, MA).

2.3. mAb production

We previously reported a method for grafting breast cancer cells into the fourth mammary gland of BALB/cJ/nu/nu mice [16]. All experiments were performed in compliance with both institutional guidelines and national laws and policies. MCF7-14 or MDA-MB231-SLC6A6-derived mouse spleen lymphocytes were fused in a standard manner with P3X63-Ag8 mouse myeloma cells (ATCC CRL-1580) using 50% (w/v) polyethylene glycol 4000 (Sigma). The fused cells were selected in HAT medium (Invitrogen). Culture supernatants were collected from the 96-well plates showing growth of the hybridoma cells and then analyzed for reactivity with intact colon cancer cells.

2.4. Titration of anti-sera (cell ELISA)

MCF7 and MCF7-14 cells were seeded at a concentration of 3000 cells/well in 96-well plates. After culturing, the cells were fixed with 10% (v/v) neutral buffered formalin solution (Wako, Osaka, Japan) for 10 min at room temperature. The plates were then washed with phosphate buffered saline (PBS[−]) and blocked (5% skim milk in TBS-T) for 30 min, after which 100 μL of mouse serum diluted 20,000-fold with TBS-T was added to each well. The plates were washed and reacted with the corresponding HRP-conjugated secondary antibody (1:2,000, BETHYL). Orthophenylenediamine (Sigma) was diluted with 50 mM carbonate–citrate buffer (pH 5.0) to a final concentration of 0.5 mg/mL and mixed with a 1/10,000 volume of 35% (w/w) aqueous hydrogen peroxide (Wako).

Alternatively, the TMB One-Step Substrate System (DAKO, Carpinteria, CA) was used. The plates were reacted at room temperature for 10 min. After stopping the reaction by the addition of 25 μL of 3 N sulfuric acid (Wako), the absorbance of each well was measured with a plate reader (SpectraMaxPure384, Molecular Devices, Tokyo, Japan).

2.5. SLC6A6-Loop2 recombinant protein

The construct SLC6A6-Loop2, corresponding to amino acids 143–217 of the full-length gene, was amplified by PCR using following gene-specific primers: 5′-ATAGGATCCGGCCTGGGCCACAT ATCACCTG-3′ (forward) and 5′-TATGAATTCGCTTTCAGAGAGCCTGG GTGGTC-3′ (reverse). The PCR fragment was subcloned into the pET-32b expression vector (Novagen, San Diego, CA) downstream of the T7 promoter using the *Eco*RI and *Bam*HI restriction sites. The construct, which is fused to a Trx (Thioredoxin), S, and His (polyhistidine) tags at the N- and C-termini, was transformed into *Escherichia coli* BL21 (DE3) (Novagen). After induction with 1 mM isopropyl-1-thio-β-D-1-galactopyranoside (Melford Laboratories, Ipswich, UK) for 3 h, bacterial cells were harvested and lysed using 10 volumes of lysis buffer (10 mM Tris–HCl, 10 mM EDTA, 150 mM NaCl, 10 mM dithiothreitol [DTT], pH 8.0) and then mechanically disrupted by sonication. Cell debris was removed by centrifugation, and protein was then extracted from the inclusion bodies in wash buffer containing 1 M guanidine hydrochloride for 1 h at

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