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Kinetic exclusion assay of monoclonal antibody affinity to the membrane protein Roundabout 1 displayed on baculovirus

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ABSTRACT

The reliable assessment of monoclonal antibody (mAb) affinity against membrane proteins in vivo is a major issue in the development of cancer therapeutics. We describe here a simple and highly sensitive method for the evaluation of mAbs against membrane proteins by means of a kinetic exclusion assay (KinExA) in combination with our previously developed membrane protein display system using budded baculovirus (BV). In our BV display system, the membrane proteins are displayed on the viral surface in their native form. The BVs on which the liver cancer antigen Roundabout 1 (Robo1) was displayed were adsorbed onto magnetic beads without fixative (BV beads). The dissociation constant (K_d, ~10^{-11} M) that was measured on the Robo1 expressed BV beads correlated well with the value from a whole cell assay (the coefficient of determination, $R^2 = 0.998$) but not with the value for the soluble extracellular domains of Robo1 ($R^2 = 0.834$). These results suggest that the BV–KinExA method described here provides a suitably accurate K_d evaluation of mAbs against proteins on the cell surface.

Monoclonal antibodies (mAbs) are important tools in the fight against cancer and autoimmune disorders [1–5]. The main protein targets in cancer immunotherapy are membrane proteins such as growth factor receptors and overexpressed differentiation antigens [6]. For effective immunotherapy, it is desirable to obtain mAbs with a higher affinity, with a dissociation constant (K_d) of less than 10^{-9} M being highly desirable [7,8]. However, the determination of mAb affinity to membrane proteins is not an easy task, mainly due to the difficulty of preparing membrane proteins having the proper conformation [6]. Classically, the extracellular part of membrane protein is purified and immobilized on the sensor chip of a surface plasmon resonance (SPR) device, so the results obtained from the solubilized extracellular portion of the antigen do not necessarily correlate with the antibody's affinity to the native form of the antigen on the cell surface [9]. SPR spectroscopy has evolved so as to be able to use membrane proteins in a lipid-reconstituted or detergent-solubilized form for the characterization of the intact membrane protein–ligand interaction [10,11]. However, the purification and solubilization of a membrane protein remain challenging [6,12], and there is no single approach that is applicable to all membrane proteins. An intact cell-based SPR method was reported [13], but this method does not provide the equilibrium K_d value, which is indispensable for the quantitative evaluation of mAbs. The biolayer interferometry assay, a label-free optical method like SPR, is another means for affinity determination. Abdiche and coworkers reported that the use of a "sink" method to abolish any rebinding of analyte to ligand-coated tips gave a more accurate kinetic rate constant than SPR [14]. However, the application of this method for intact cell-based measurement of the affinity of mAbs to membrane proteins has not yet been reported. Radioisotope-labeled antibodies can be used for the determination of antibody affinity to cell surface antigens [15,16], but there are problems with modifications and the loss of binding activity in the process of labeling [17]. The classical enzyme-linked immunosorbent assay (ELISA) using antigen

Abbreviations used: mAb, monoclonal antibody; K_d, dissociation constant; SPR, surface plasmon resonance; ELISA, enzyme-linked immunosorbent assay; KinExA, kinetic exclusion assay; BV, budded baculovirus; Robo1, Roundabout 1; FBS, fetal bovine serum; hRobo1, human Robo1; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; sRobo1, soluble form of hRobo1 protein; CHO, Chinese hamster ovary; HA, hemagglutinin; TBS, Tris-buffered saline; EDTA, ethylenediaminetetraacetate; TCM, titrant concentration multiplier; FN3, third fibronectin type III; JM, juxtamembrane.

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expressed cells attached to a plate has been reportedly used for antibody affinity determination [18]. However, ELISA has a detection limit of approximately 0.1 nM and, thus, is not applicable to an evaluation of the K_d values of high-affinity antibodies [19]. Recent studies [20,21] have reported the determination of antibody affinity to cell surface antigens using the kinetic exclusion assay (KinExA). The KinExA method is able to measure unmodified molecules in solution with high sensitivity [22]. This method is used as an immunoassay to determine the concentration of free antibody in a mixture of antibody, whole cells and antibody–cell complexes.

We and other investigators have reported that membrane proteins such as cell surface receptors [23–26], a transporter [27], and enzymes [28] are expressed on the budded baculovirus (BV) in the biologically active form. This BV display is also useful for the generation of antibodies against membrane proteins that are typically difficult to obtain in a sufficient amount [6]. By means of BV-displayed whole protein, we have obtained reactive antibodies to a cancer antigen, Roundabout 1 (Robo1) [29]. Robo1, first identified as an axonal guidance receptor, is a single-pass membrane protein that consists of five immunoglobulin-like domains and three fibronectin-like domains in its extracellular portion. Robo1 is recognized as a good candidate for immunotherapy for liver cancer, small cell lung cancer, and other cancers [30,31]. Although the BV display is a simple and easy way to prepare most of the membrane proteins, the use of it for antibody kinetic study is insufficient because of its unusually big size of approximately 300 nm, which also causes difficulties for SPR measurement and the quantitative evaluation of expressed antigen concentration.

Here, we describe a novel and simple means of determining membrane protein antibody affinity by combining the BV display technique and KinExA method. The BV that is adsorbed on magnetic beads permits the mAbs to be determined at a sub-nanomolar K_d affinity to a membrane protein. The K_d measured from the BV display was shown to be in good correlation with a whole cell assay, making BV a useful alternative to whole cells for affinity determination by KinExA. Moreover, the method described in this article implies a broad application to the development of neutralizing mAbs against a variety of viruses.

Materials and methods

Reagents

PMMA beads were obtained from Sapidyne Instruments (Boise, ID, USA). Alexa Fluor 647-conjugated anti-mouse IgG (H + L) antibody, goat anti-mouse IgG (H + L) antibody, and horseradish peroxidase-conjugated anti-mouse IgG (Fc fragment-specific) antibody were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Control IgG (Hyb3423) was obtained from the Institute of Immunology (Tokyo, Japan). Block Ace was purchased from Dainippon Sumitomo Pharma (Osaka, Japan). ProClin 300 and RPMI-1640 were purchased from Sigma–Aldrich Japan (Tokyo, Japan). Protein G–Sepharose was purchased from GE Healthcare Japan (Tokyo, Japan). Ultra-low IgG fetal bovine serum (FBS), Flp-In System, and Lipofectamine 2000 Transfection Reagent were purchased from Life Technologies Japan (Tokyo, Japan). Ni–NTA Agarose was purchased from Qiagen (Hilden, Germany). Magnosphere MX200/carboxyl beads were obtained from JSR Life Sciences (Ibaraki, Japan). TMB Soluble Reagent and TMB Stop Buffer were purchased from ScyTek Laboratories (Logan, UT, USA).

Anti-human Robo1 mAbs

The recombinant baculovirus expressing human Robo1 (hRobo1) described below was immunized directly into gp64 transgenic mice, and then isolated spleen cells were fused with myeloma cells as described previously [27]. Anti-hRobo1 mAb producing hybridomas were selected by their reactivity to Robo1-expressing stable cell lines as determined by cell ELISA and flow cytometry. The binding sites of each mAb were determined using purified proteins of a partial domain of Robo1 expressed in an *Escherichia coli* expression system as described previously [32]. To obtain mAbs in culture supernatant, these hybridomas were cultured with 10% ultra-low IgG FBS that contained RPMI-1640 medium. After 2 weeks of culture, anti-Robo1 mAbs were purified from the culture supernatant using Protein G–Sepharose affinity chromatography. The purity of the mAbs was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis, and the concentration of the mAbs was determined by ultraviolet analysis.

Preparation of hRobo1-expressing BV

The hRobo1-expressing budded baculovirus (Robo1/BV) was generated as described previously [27,29,30]. Briefly, hRobo1 cDNA was polymerase chain reaction (PCR)-amplified from Alexander cells and inserted into the pBlueBac 4.5–TOPO vector. The recombinant baculovirus was collected from Sf9 culture medium by centrifugation at 40,000 *g* for 40 min and resuspended with phosphate-buffered saline (PBS).

Preparation of a soluble form of hRobo1

The soluble form of hRobo1 protein (sRobo1) was affinity-purified using Ni–NTA Agarose from the culture supernatant of Sf9 cells infected with recombinant baculovirus containing a gene fragment encoding the extracellular domain of hRobo1 (1–861 aa) with V5 and 6 × His tags at its C terminus.

Preparation of human Robo1-expressing cell lines

A Chinese hamster ovary (CHO) cell line stably expressing hRobo1 fused with a hemagglutinin (HA) tag (Robo1/CHO) was generated using the Flp-In System as described previously [30]. Robo1 fused to HA (Robo1–HA), with the tag encoded at the C terminus, was inserted into the pcDNA5/FRT vector and was co-transfected with the pOG44 vector to Flp-In–CHO cells using Lipofectamine 2000 Transfection Reagent. A highly expressing Robo1–HA clone was selected from 1 mg/ml hygromycin-resistant cells.

Preparation of BV adsorbed magnetic beads (BV beads)

Magnosphere MX200/carboxyl beads (40 mg) were washed three times with PBS and suspended in 1.5 ml of PBS. Then 1.8 mg of BV was added to the suspended beads to 1.2 mg/ml in final. The solution was kept rotating with a tube rotator for 16 h, and then the beads were washed three times with PBS and blocked with reaction buffer, 40% Block Ace, and 0.05% ProClin300 in 10 mM Tris-buffered saline (TBS).

Enzyme-linked immunosorbent assay

BV ELISA, sRobo1 ELISA, and cell ELISA were performed as described previously [27]. For BV ELISA and sRobo1 ELISA, the BV was coated at 20 μg/ml in saline and sRobo1 was coated at 1 μg/ml in saline on high-binding polystyrene 96-well plates. For cell ELISA, cells were dissociated with 2 mM ethylenediaminetetraacetate (EDTA) in PBS and 10^5 cells/well were plated on poly-D-lysine-coated 96-well plates. The plates were centrifuged at 2000 rpm for 1 min, and supernatants were discarded. In the case of cell fixation ELISA, 4% paraformaldehyde in PBS was added and incubated at room temperature. After 10 min of incubation, the fixed cells were