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Technical note

Development and validation of a cell-based fluorescent method for measuring antibody affinity

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ABSTRACT

Monoclonal antibodies have become essential tools for diagnostic and therapeutic purposes. Antibody affinity is one of the critical factors influencing the therapeutic success of tumor-targeting antibodies. Therefore, developing an accurate and reliable method for determining antibody affinity is crucial. In this study, we describe a fluorescent cell-based immunosorbent assay that can accurately measure antibody affinity (K_D) in the nanomolar range. This method involves the addition of fluorescently labeled antibodies to antigen-positive and antigen-negative cell lines fixed on 96-well plates. The fluorescent signals from nonspecific binding to negative control cell lines is subtracted from the specific binding to the antigen-positive cell lines. The K_D values obtained by this method were comparable with values obtained by the flow cytometry and radioactive (I^{125}) scatchard assay. Our results demonstrate that this modified cell-based fluorescent method allows for a convenient and efficient identification of therapeutically relevant leads.

1. Introduction

Monoclonal antibodies (mAbs) have emerged as novel therapeutics for the treatment of cancer and chronic diseases (Alewine et al., 2015; Modjtahedi et al., 2012). To function as an efficacious and safe therapeutic agent, mAbs are required to display optimum affinity for the target antigen. Therefore, determining antibody affinity with an accurate and reliable method is crucial, especially when applying that method within a therapeutic context. The affinity of an antibody binding to the antigen is expressed as the dissociation equilibrium constant K (K_D), which is determined by the ratio of dissociation to association rate constants (k_{off}/k_{on}). Currently, most common techniques employed to assess K_D are radioimmunoassay, surface plasmon resonance (SPR), flow cytometry, enzyme-linked immunosorbent assays (ELISA), and kinetic exclusion assays (KinExA) (Salimi-Moosavi et al., 2012).

To be successful therapeutic candidates mAbs need to recognize native epitopes on target antigens. The cell-based fluorescent assay (e.g. cell-based ELISA) offers a rapid and sensitive way to measure antibody affinity utilizing a monolayer of cells adhering to a 96-well plate to comprise the solid phase of a conventional assay, in which the protein of interest is immobilized (Donald et al., 1997). Advantages of a cell-based fluorescent assay include its use of intact cells expressing target antigens in their native conformation, excluding the need to purify target antigen, high throughput analysis using 96-well plates, conservation of costly reagents due to the small assay volume, and relative inexpensiveness of using a plate reader. Despite these advantages no attempt has been made yet to utilize a direct fluorescent method for assessing antibody affinity and validate it by comparison to other established methods.

In this paper, we describe the development of a simple and rapid fluorescent cell-based method that allows for efficient and accurate measurement of antibody equilibrium dissociation constant (K_D) in the nanomolar range using a standard fluorescence microplate reader. We validated the K_D values obtained by this method with those obtained by the I^{125} scatchard assay and flow cytometry. We also demonstrated that competitive binding assays can be performed using the fluorescent cell-based assay.

2. Materials and methods

2.1. Antibodies

Development, production, and purification of the Mel-14 monoclonal antibody targeting human chondroitin sulfate proteoglycan 4 (CSGP4), the D2C7 monoclonal antibody targeting human wild-type epidermal growth factor receptor (EGFRwt) and human mutant EGFR variant III (EGFRvIII), and the NZ-1 monoclonal antibody targeting human podoplanin have been previously described (Carrel et al., 1982; Zalutsky et al., 2012; Kato et al., 2006). The purity of Mel-14 (mouse IgG2a), D2C7 (mouse IgG1), and NZ-1 (Rat IgG2a) were determined to be greater than 90% by SDS-PAGE (data not shown).

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2.2. Cell lines

H350 is a human melanoma cell line expressing CSPG4 maintained in our laboratory. The human embryonic kidney cells, HEK293 (ATCC, Manassas, VA), which lack expression of CSPG4, and the murine Swiss 3T3 fibroblast cell line, NR6 (kindly provided by Dr. Harvey Herschman, University of California, Los Angeles, CA), which lacks expression of murine or human EGFRwt, EGFRvIII, or human podoplanin, were used as negative controls. NR6EGFRwt is the murine NR6 cell line transfected with human EGFRwt (Carol et al., 1997). All cells were cultured in an incubator at 37 °C, 5% CO_2, and passaged at confluence with Accutase Cell Detachment Solution (BD Biosciences, San Jose, CA). HEK293 cells were cultured in DMEM media supplemented with 10% heat-inactivated fetal calf serum (FBS) (Thermo Fisher Scientific, Waltham, MA). All other cell lines were grown in 1x MEM ZINC Option media with 10% FBS (Thermo Fisher Scientific).

2.3. Dissociation of xenograft

D-341 MED, medulloblastoma xenograft tissue was obtained from mice under sterile conditions from the Duke animal facility, and prepared for cell culture in a laminar flow hood using sterile techniques. The tumor was finely minced and digested with 100 μg Liberase (Roche, Indianapolis, IN) at 37 °C for 20 min. The dissociated cells were filtered through a 70-μm cell strainer (BD Biosciences), and red blood cells were removed with ACK Lysing Buffer (Thermo Fisher Scientific). The tumor cells were then washed with phosphate-buffered saline, (1x PBS, pH 7.4), and treated with Ficoll-Paque Plus (GE Healthcare Bio-Sciences, Pittsburgh, PA) to remove residual red blood cells, debris, and dead cells. The cells were finally washed twice with 1x MEM Zinc Option media before they were transferred to tissue culture-treated flasks. The cells were cultured and passaged until sufficient numbers were obtained.

2.4. Labeling of antibodies

Mel-14, D2C7, and NZ-1 antibodies were directly conjugated with Alexa Fluor 488 (AF488) fluorescent dye using the amine-reactive agent tetrafluorophenyl esters (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, AF488 dye was added to the purified antibody at a 10:1 M ratio and incubated in 0.1 M Na_2HCO_3, pH 8.2 buffer for 1 h at room temperature. The excess dye was removed by overnight dialysis using Slide-A-Lyzer cassettes (Thermo Fisher Scientific). Protein concentrations were calculated from measurements of absorbance at 280 nm and 494 nm obtained using Nanodrop 1000 (Thermo Fisher Scientific). The fluorophore to protein ratio was calculated to be for 4.6 for Mel-14-AF488, 4.0 for NZ-1-AF488, and 1.2 for D2C7-AF488.

2.5. Affinity measurement by flow cytometry

To test antigen specificity, flow cytometry analysis was performed by incubating 66.7 nM of Mel-14-AF488, NZ-1-AF488, and D2C7-AF488 antibodies, or the corresponding AF488 labeled isotype control antibodies with both the antigen-positive and antigen-negative cell lines. To measure antibody affinity by flow cytometry, the cells were first prepared as described below. 3×10^5 cells (H350, HEK293, NR6, NR6EGFRwt, or D-341 MED) were resuspended in 500 ul of 1x PBS containing 5% FBS (5%FBS-PBS). Serially diluted Mel-14-AF488, D2C7-AF488, and NZ-1-AF488 antibodies were added to their corresponding antigen-positive cell lines at concentrations ranging from 100 pM to 500 nM. Cells were incubated for 45 min at 4 °C, washed 3 times with 1x PBS, and analyzed on a Becton Dickinson FACSCalibur instrument (BD Biosciences). Mean fluorescent intensity values were obtained from the histograms and were used to plot the binding curves. A standard approach of nonlinear regression using the one-site binding hyperbola available in Graphpad Prism 5 (Graphpad Software, La Jolla, CA), was used to fit the curves, and K_D values were calculated by the program.

2.6. Radioactive scatchard affinity analysis

Mel-14, D2C7, and NZ-1 antibodies were conjugated with I^{125} as previously described (Kato et al., 2010). Then, antigen-positive cells (H350 for Mel-14, NR6EGFRwt for D2C7, and D-341 MED for NZ-1) and antigen-negative cells (HEK293 for Mel-14, NR6 for D2C7, and NZ-1) were seeded in 24-well plates at 2×10^5 cells/well in triplicates, and cultured overnight. The next day, the cells were fixed in 0.25% glutaraldehyde (Sigma, St. Louis, MO) for 5 min at room temperature, washed three times with incubation buffer (115 mM PO_4 [$KH2PO_4 + K_2HPO_4$], 0.05% BSA, 0.05% gelatin), and blocked with the same buffer at room temperature for 1 h. I^{125} labeled antibodies were serially diluted from 10 pM to 80 nM with the incubation buffer. Diluted antibodies were added to the corresponding triplicate wells plated with their antigen-positive cells (Ag Pos wells) and antigen-negative cells (Ag Neg wells). The same series of antibody dilutions, in the same concentrations as the antibody solutions added to the wells, were prepared and added directly to 2 ml screw-cap tubes at 200 μl/tube in triplicates. These tubes were used as standard samples. The cell plates and the standard sample tubes were incubated at 4 °C overnight. The next day, the free radioactive antibodies were removed from the plates. Then the plates were washed four times with the incubation buffer. After that, 500 μl 2N sodium hydroxide was added to each well, and the plates were incubated overnight at 37 °C. The following day, the cells were completely resuspended in 500 μl 2N sodium hydroxide and transferred into new 2 ml screw-capped tubes. The test tubes (containing intact cells and cell debris bound to antibodies) as well as standard sample tubes were then placed in a gamma counter. The readout values (counts per min, cpm) were recorded. Eq. (1) presented below was used to calculate the average amount of antibodies bound to cells (molar bound) at each antibody concentration. Molar bound values were plotted against the molar input values, to determine the average amounts of antibody added to each well. The curves were fitted with one-site binding hyperbola in Graphpad Prism 5, and K_D values were calculated by the Prism 5 program.

$$\text{Molar bound(M)} = \frac{\sum \text{cpm of 3 Ag Pos wells} - \sum \text{cpm of 3 Ag Neg wells}}{\sum \text{cpm of 3 standard samples tubes}} * \text{molar input} \quad (1)$$

2.7. Affinity measurement by cell-based fluorescent assay

Antigen-positive and negative cells were seeded in duplicates in flat clear bottom black 96-well plates (Corning, Corning, NY) at 2×10^4 cells/well, and cultured overnight. For each antibody, its antigen-positive and antigen-negative cell line pair was cultured on the same plate to avoid differences between individual 96-well plates. The next day, the cells were fixed in 0.25% glutaraldehyde (Sigma) for 5 min at room temperature, and washed three times with 1x PBST buffer (1x PBS, 5% FBS, 0.05% Tween 20). The wells were then blocked with 5% FBS-PBS buffer for 1 h at room temperature. Serially diluted Mel-14-AF488, D2C7-AF488, and NZ-1-AF488 antibodies ranging from 40 pM to 400 nM were added to the corresponding antigen-positive and antigen-negative cells. The plates were incubated at 4 °C in dark for 1 h, and then washed six times with 1x PBST buffer. Then 5% FBS-PBS buffer was added to each well at 100 μl/well. The excitation and emission wavelengths on the Tecan Infinite 200Pro plate reader (Tecan, Morrisville, NC) were set at 490 nm and 525 nm, respectively. Gain, the amplification factor for the photomultiplier tube, was set to optimal so that its value was calculated automatically by the instrument according to the