Archives of Biochemistry and Biophysics 526 (2012) 219–225

Contents lists available at SciVerse ScienceDirect

Archives of Biochemistry and Biophysics

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

Anti-EGFR biparatopic-SEED antibody has enhanced combination-activity in a single molecule

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ARTICLE INFO

Article history:
Available online 10 March 2012

Keywords:
Bispecific
Biparatopic
C225
hu425
Antibody combination
SEED antibody

ABSTRACT

Certain combinations of non-competitive anti-EGFR antibodies have been reported to produce new effects on cells compared to either antibody used separately. New and enhanced combination-activity includes increased inhibition of signaling, increased receptor internalization and degradation, reduced proliferation of tumor cell lines and induction of complement-dependent cytotoxicity (CDC) effector function. To test requirements and mechanisms to elicit enhanced combination-activity with different EGFR binding domains, we created an anti-EGFR biparatopic antibody. A biparatopic antibody interacts through two different antigen-binding sites to a single antigen. A heterodimeric antibody with one binding domain derived from the C225 antibody and one binding domain derived from the humanized 425 (hu425) antibody was built on the strand-exchange engineered domain (SEED) scaffold. This anti-EGFR biparatopic-SEED antibody was compared to parental antibodies used alone and in combination, and to the corresponding monovalent anti-EGFR-SEED antibodies used alone or in combination. We found that the anti-EGFR biparatopic-SEED had enhanced activity, similar to the combination of the two parental antibodies. Combinations of monovalent anti-EGFR-SEED antibodies did not produce enhanced effectiveness in cellular assays. Our results show that the anti-EGFR biparatopic antibody created using the SEED scaffold has enhanced combination-activity in a single molecule. Furthermore, these data suggest that the potential to cross-link the two different epitopes is an important requirement in the mechanism of enhanced combination-activity.

Introduction

Monoclonal antibodies that target the epidermal growth factor receptor (EGFR) have shown efficacy in the clinic for treatment of cancer. Cetuximab (C225 antibody), was the first anti-EGFR monoclonal antibody on the market and is registered for the treatment of colorectal and head and neck tumors (Imclone/BMS and Merck KGaA). C225 recognizes an epitope in domain III of the extracellular domain of EGFR and directly competes with ligand binding to inhibit signaling [1]. There is also accumulating evidence that cetuximab treatment may stimulate the immune system through the effector functions of the Fc region that mediate activation of antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), or tumor antigen-targeted cytotoxic T lymphocyte responses (TA-targeted CTL) [2]. Matuzumab (EMD72000/humanized 425 (hu425) antibody) is another antibody that inhibits EGFR activity but acts through binding to a different epitope on domain III. Matuzumab does not directly block binding of the ligand, but instead prevents structural changes that are required for high affinity-ligand binding and receptor dimerization [3]. Several studies have shown that cetuximab and matuzumab can simultaneously bind to EGFR, and when used in combination can synergize and mediate more potent effects *in vitro* than each antibody used separately. These effects included greater inhibition of signaling and tumor cell proliferation, increased EGFR down-regulation and degradation, enhanced apoptosis, and activation of CDC [4–6]. Furthermore, observations of synergistic activity have been extended to other non-competitive pairs of EGFR antibodies that bind domain III [7–9]. Experimental evidence using various approaches suggests that the combination of antibodies simultaneously binding two epitopes causes extensive cross-linking of cell surface receptors, creating clusters or lattices visible by confocal microscopy [7,8]. The complexes subsequently undergo endocytosis, in which recycling is blocked, promoting increased receptor down-regulation through the lysosomal degradation pathway [9]. In addition to the enhanced potencies shown *in vitro,* a formulation of two non-competitive anti-EGFR antibodies called Sym004 had activity in a variety of pre-clinical tumor models and is being tested for activity in solid tumors in Phase I clinical trials [10].

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http://dx.doi.org/10.1016/j.abb.2012.03.005

However, co-development of two antibodies for separate administration, or for formulation as a single dose, can be complicated and expensive to manufacture and to administer, and thus may result in increased treatment costs that may be prohibitive [11]. An alternative approach is to combine the EGFR binding domains of two non-competitive antibodies into one molecule to create a single biparatopic antibody. The heterodimeric strand-exchange engineered domain (SEED)[1] platform is ideal for creating such a dual-binding molecule, and has been shown previously to retain the desirable characteristics of a therapeutic antibody such as long half-life and immune effector functions [12,13]. Accordingly, we used the SEED platform to build a biparatopic antibody comprised of one EGFR binding domain derived from cetuximab and one from matuzumab and compared its activity to the combination of the two parental antibodies. In addition, we created the two corresponding monovalent anti-EGFR-SEED antibodies specific for each epitope, and together these molecules were used to test the mechanisms by which two different anti-EGFR binding domains result in new and enhanced activity.

We found that the anti-EGFR biparatopic-SEED antibody has activity similar to the combination of the two parental antibodies. Furthermore, the combination of the two monovalent anti-EGFR-SEED antibodies did not produce the additional activity seen with the biparatopic-SEED antibody or the combination of the two parental antibodies. These results suggest that the combination effect requires cross-linking of each epitope and showed that the anti-EGFR biparatopic-SEED antibody has this enhanced combination-activity in a single molecule.

Materials and methods

A431 cell membrane preparation

A431 cells were disrupted by nitrogen cavitation in a buffer containing 250 mM sucrose, 25 mM tris, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA and protease inhibitors (Sigma). The cells were pressurized with 900 psi of N_2 gas for 30 min, after which the lysate was centrifuged at 1000*g* for 10 min at 48 °C. The supernatant was collected and centrifuged at 100,000*g* for 1 h at 48 °C. The resulting pellet was re-suspended in binding buffer (50 mM HEPES, pH 7.5, 130 mM NaCl, 5.1 mM KCl, 1.3 mM $MgSO_4$) with a dounce homogenizer. The protein concentration of the samples was determined using the BioRad protein assay reagent and the samples were stored frozen at −80 °C for future use.

Competition binding on A431 membranes

Radioligand binding assays were performed in 100 μl of 50 mM HEPES, pH 7.5, 130 mM NaCl, 5.1 mM KCl, 1.3 mM $MgSO_4$, 0.5% BSA (Sigma A3059; assay buffer) and 0.1 nM ^{125}I-EGF (Perkin Elmer) in 96-well plates (Corning 3365). Competing antibodies and SEED proteins were diluted in assay buffer and mixed with ^{125}I-EGF prior to the addition of 2 μg of membrane prepared from A431cells. Non-specific binding was determined in the presence of a 100 nM concentration of unlabeled EGF. The reactions were incubated for 90 min at 37 °C, with shaking, and terminated by filtering through GF/C filters (Millipore, Multiscreen MSFCN6B), pre-treated with 0.5% PEI (polyethylenimine). The filters were washed five times with cold binding buffer (50 mM HEPES, pH 7.5, 130 mM NaCl, 5.1 mM KCl, 1.3 mM $MgSO_4$) and counted on a gamma counter. Data were analyzed using GraphPad Prism software.

[1] *Abbreviations used:* SEED, strand-exchange engineered domain; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor.

Biacore kinetic analysis

Purified goat anti-human IgG Fc (Jackson Immuno Research Laboratories) was immobilized onto the CM5 chip using amine coupling chemistry. Biacore CM-5 chips, ethanolamine, NHS/EDC coupling reagents and buffers were obtained from Biacore (GE Healthcare). The immobilization steps were carried out at a flow rate of 30 μl/min in HEPES buffer (20 mM HEPES, 150 mM NaCl, 3.4 mM EDTA and 0.005% P20 surfactant). The sensor surfaces were activated for 7 min with a mixture of NHS (0.05 M) and EDC (0.2 M). The goat anti-human IgG Fc was injected at a concentration of ~30 μg/ml in 10 mM sodium acetate, pH 5.0, for 7 min. Ethanolamine (1 M, pH 8.5) was injected for 7 min to block any remaining activated groups. An average of 12,000 response units (RU) of capture antibody was immobilized on each flow cell.

Kinetic binding experiments were performed using the same HEPES buffer (20 mM HEPES, 150 mM NaCl, 3.4 mM EDTA and 0.005% P20 surfactant) and was equilibrated at 25 °C. Kinetic data was collected by injecting test and control antibodies at concentrations ranging from 1 to 0.1 μg/ml for two minutes at a flow rate of 30 μl/min, followed by a buffer wash for 30 s at the same flow rate. For rigorous kinetic characterization of test antibody and control complexes, six concentrations of analyte ranging from 0 nM (buffer only) to 40 nM were used. Human EGFR-1 (R&D Systems recombinant Human EGF Receptor (1095-ER)) was bound at 40, 20, 10, 5, 2.5 and 0 nM for 3 min followed by a dissociation step for 10 min at the 30 μl/min flow rate.

The data were fit using a 1:1 Langmuir binding model with the BIA evaluation software. Kinetic rate constants were determined from the fits of the association and dissociation phases, and the KD was derived from the ratio of these constants.

EGF-stimulated phosphorylation assay

EGF-stimulated protein phosphorylation was measured in MDA-MB-468 cells either without (control) or with prior addition of antibodies. MDA-MB-468 cells were seeded into 6-well plates at 1×10^6 cells/well and incubated in complete medium (RPMI with 10% FBS) for 6–7 h at 37 °C/5% CO_2, then the cells were incubated in medium without serum for another 18 h. Serum-starved cells were first incubated for 50 min with 100 μg/ml of each antibody tested (or 50 + 50 μg/ml for antibody combinations), and then incubated with EGF (10 ng/ml) for an additional 10 min. Cells were washed 2 times with cold PBS then lysed with 500 μl of lysis buffer (150 mM NaCl, 50 mM Tris/Cl pH 8.0, 1 mM EDTA, 1% Triton X-100 plus complete protease and phosphatase inhibitors cocktail) for 30 min on ice. Cell lysates were collected into tubes and sonicated twice for 30 s. Aliquots from each sample were taken for measurement of protein concentration (Pierce BCA kit detergent compatible) and the remaining sample was used for Western Blot analysis.

Western blot

4X LDS sample buffer (Invitrogen) and 10X Sample Reducing Agent (Invitrogen) were added to each cell lysate sample. Samples were heated at 70 °C for 10 min before loading onto 4–12% Bis-Tris protein gels (Invitrogen). The gels were run at 200 V for 1 h, and proteins transferred onto nitrocellulose membrane using the iBlot transfer apparatus (Invitrogen) for 7 min. The membrane was incubated in Blocking Buffer (5% nonfat dry milk in Tris-Buffered Saline (TBS)) for 1 h at room temperature or overnight at 4 °C. The blocked membrane was then incubated in diluted primary antibody overnight at 4 °C. Primary antibodies used: monoclonal anti-EGFR (Sigma E3138); monoclonal anti-Actin (Sigma A1978);