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Construction and characterization of functional anti-epiregulin humanized monoclonal antibodies

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ABSTRACT

Growth factors are implicated in several processes essential for cancer progression. Specifically, epidermal growth factor (EGF) family members, including epiregulin (EREG), are important prognostic factors in many epithelial cancers, and treatments targeting these molecules have recently become available. Here, we constructed and expressed humanized anti-EREG antibodies by variable domain resurfacing based on the three-dimensional (3D) structure of the Fv fragment. However, the initial humanized antibody (HM0) had significantly decreased antigen-binding affinity. Molecular modeling results suggested that framework region (FR) residues latently important to antigen binding included residue 49 of the light chain variable region (VL). Back mutation of the VL49 residue (tyrosine to histidine) generated the humanized version HM1, which completely restored the binding affinity of its murine counterpart. Importantly, only one mutation in the framework may be necessary to recover the binding capability of a humanized antibody. Our data support that HM1 exerts potent antibody-dependent cellular cytotoxicity (ADCC). Hence, this antibody may have potential for further development as a candidate therapeutic agent and research tool.

1. Introduction

The epidermal growth factor (EGF) signaling system consists of at least seven ligands, that is, EGF, amphiregulin, transforming growth factor α, heparin-binding EGF, betacellulin, epiregulin (EREG), and epigen [1]. These ligands bind to the extracellular region of the EGF receptor (EGFR) and induce a conformational change in EGFR, leading to its dimerization and activation. Subsequently, activated EGFR stimulates many intracellular signaling pathways, such as the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/Akt pathways, and promotes proliferation, cell survival, and angiogenesis [1,2]. In many different cancer cells, EGFR ligands are produced either by the cancer cells themselves or by surrounding stromal cells, leading to constitutive EGFR activation [3]. Of the EGF ligands, EREG is produced as a transmembrane precursor and exerts mitotic activity in various primary cell types, such as rat hepatocytes, and various types of human cancer cells, particularly epithelial tumor cells [4,5]. Interestingly, EREG is expressed at relatively low levels in most adult normal tissues, but is highly expressed in various human cancers, including colon, breast, prostate, and ovary cancers [6–9]. Many studies have demonstrated the possible involvement of EREG in tumorigenesis and the oncogenic effects of cancer-specific overexpression of EREG. Thus, EREG is likely to be involved in the development of a variety of human cancers, and its potential use as a therapeutic target is being intensely investigated. To this end, several anti-EREG murine monoclonal antibodies (mAbs) have been successfully tested *in vivo* (unpublished data). However, one of the primary problems in developing monoclonal antibodies as drugs is the human anti-mouse antibody response (HAMA), which limits the administration of murine antibodies [10]. In this study, we have described the construction and expression of humanized anti-EREG antibodies with high-affinity targeted cytotoxicity and decreased immunogenicity through resurfacing the variable region and recombination.

2. Materials and methods

2.1. Materials

9E5, a human anti-EREG antibody, was a member of a panel of murine monoclonal antibodies (mAbs) generated using a subtractive immunization protocol in hybridoma cells and was provided

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as a kind gift by Dr. Kenji Yoshida (Forerunner Pharma Research, Tokyo, Japan), along with its sequence information. The nucleotide sequences of 9E5 heavy and light variable regions (VH and VL, respectively), synthesized by Hokkaido System Science (Sapporo, Japan), were inserted into the pUC57 vector.

2.2. Modeling the variable fragment (Fv) of 9E5

The 3D structure of the 9E5 variable region was constructed by homology modeling based on Ig VH and VL domains with highly matched amino acid sequences and known structures. The Protein Data Bank (PDB) was searched for antibody sequences with more than 70% similarity to the 9E5 variable region. Two separate BLASTP searches were performed for VH and VL. Candidate template structures were selected based on the IMGT database for residue numbering and CDR location [11]. For refining the modeling of the CDR conformations, CDR loop template structures were selected based on the framework region sequence identity of two sides of the CDR loop and the canonical structure type of the CDR loop in candidate template structures. Canonical structures were used to predict the backbone structures of CDRs L1–3, H1, and H2 [12]. Only CDR H3 and the neighboring side chains were remodeled de novo using a kinematic loop modeling algorithm in a Rosetta protocol. The complex models of 9E5 VH and VL were built with Rosetta Antibody [13]. The 200 lowest energy conformations from this run were extracted and subjected to energy minimization. The five lowest energy conformers were used in subsequent analyses. Differences between murine and humanized variants of HM0 antibodies were individually analyzed to investigate their possible influence on the CDRs.

2.3. Humanization of 9E5 antibodies

We chose human variable regions that were the most homologous in sequence to the murine variable regions as the human framework for humanized 9E5 antibodies. 9E5 VH and VL sequences were subjected to separate IgBLAST searches against the immunoglobulin GenBank database. A set of human data, which contained 1000 antibody sequences sharing the highest identities with 9E5, was selected for each variable region, and another set of murine data was chosen in the same way. Several systematic differences were found when comparing the murine and human selections, and these were collected as candidates for humanization, excluding the CDR residues. Candidates from differential regions were selected according to the analysis of the constructed model and the following criteria: (i) the candidates were located within the solvent accessible surface area according to CHIMERA [14]; (ii) surface residues of 9E5 variable regions (defined as having >30% relative solvent accessibility) [15] were found by aligning the sequences of antibodies of known structure to the sequence of 9E5 to identify homologous positions; (iii) the candidates could not form pivotal contacts comprising intermolecular hydrogen bonds between VH and VL or intramolecular hydrogen bonds that were important for retaining the conformation of CDRs and interaction with CDRs directly; and (iv) the candidates were only differential residues, not unique residues.

2.4. Construction, expression, and purification of antibodies

Humanized VH and VL gene sequences were subcloned into predigested expression vectors containing the human interleukin 2 (IL2) signal sequence [16] and encoding human IgG1, including the constant hinge, CH2, and CH3 regions. Humanized anti-EREG heavy and light chain expression plasmids were cotransfected into CHO-S host cells. Selection of cell lines was followed by puromycin selection (5–15 μg/mL) to obtain high and stable expression of humanized mAbs. Conditioned media containing humanized mAbs was harvested and passed through a 0.2-μm filter. The clarified supernatant was purified using Bio-Scale Mini UNOsphere SUPrA (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations. Eluted antibodies were neutralized with 1 M Tris–HCl (pH 9), and buffer exchange was carried out using phosphate-buffered saline (PBS) through dialysis of purified antibodies overnight. The concentration of the purified antibodies was determined by measuring the absorbance at 280 nm.

2.5. Cell lines and culture

Human colonic adenocarcinoma cell lines, including HCT116 (American Type Culture Collection [ATCC], CCL-247) and DLD-1 (ATCC, CCL-221), and a human gastric cancer cell line, AGS (ATCC, CRL-1739), were propagated and maintained according to the manufacturer's instructions.

2.6. Immunostaining

Cells were cultured on cover glass and fixed with ice-cold ethanol for 10 min. After three washes with PBS, nonspecific sites were blocked with 1 × PBS and 0.2% fish skin gelatin (PBS/FSG) for 10 min. The primary antibodies (9E5, HM0, and HM1) were diluted to 0.1 mg/mL in PBS/FSG, 50 μL of diluted primary antibodies was added to each well, and coverslips were incubated at room temperature (RT) for 1 h. Following three washes with PBS/FSG, the samples were incubated for 30 min with appropriate secondary antibodies (Alexa Fluor 488-labeled mouse/human anti-IgG). Images were taken using a Leica DM LB microscope (Leica Microsystems).

2.7. Flow cytometry

Cells were detached from culture plates with PBS containing 2 mM EDTA at RT for 10–20 min and centrifuged at 1000 rpm for 5 min. Detached cells were washed once with PBS and were then resuspended in FACS buffer (PBS containing 1% bovine serum albumin [BSA], 0.1 mM EDTA, and 0.01% NaN_3). The cells were counted, plated at 1×10^5 cells per well in 96-well plates (50 μL volume per well), and kept on ice for 30 min. After washing three times with ice-cold FACS buffer, the cells were then incubated in the presence or absence of 0.1, 0.5, or 1 μg/mL Alexa Fluor 488-labeled antibodies in 10 μL of ice-cold FACS buffer. After 1 h of incubation on ice in the dark, cells were washed three times with ice-cold FACS buffer. Subsequently, cells were resuspended in 200 μL FACS buffer and analyzed by Guava EasyCyte (Millipore). The experiment was performed in triplicate, and data were analyzed with GuavaSuite (Millipore).

2.8. Surface plasmon resonance (SPR)

SPR measurements were performed on a Biacore T200 instrument (GE Health Sciences). Antibodies were immobilized onto a CM5 chip using standard amine-coupling chemistry addressing flow cells individually (200 RU level). Human EREG-Fc (MW, 76 kDa), used as a ligand for antibodies, was serial diluted into HBS-EP + buffer (GE Health Sciences) to obtain 1.5 μM, 300, 60, 12, and 2.4 nM concentrations. Each hEpiregulin-Fc was titrated from low to high concentration samples, and single-cycle kinetic analysis was performed with an on-time of 120 s and an off-time of 600 s at 30 μL/min. Curve fitting and K_D determinations were performed with the Biacore T200 Evaluation software and were used to evaluate the data assuming a bivalent analyte model. The results were based on three independent experiment repeats.