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A novel anti-EGFR monoclonal antibody inhibiting tumor cell growth by recognizing different epitopes from cetuximab

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ABSTRACT

The epidermal growth factor receptor (EGFR) overexpressed in many epithelial tumors is an attractive target for tumor therapy since numerous blocking agents of EGFR signaling have proven their anti-tumor activity. Here we report a novel monoclonal antibody (mAb), A13, which was generated from mice immunized with human cervical carcinoma A431 cells. In addition to binding to soluble EGFR with affinity of $K_D \approx 5.8$ nM, mAb A13 specifically bound to a variety of tumor cells and human placenta tissues expressing EGFR. A13 efficiently inhibited both EGF-dependant EGFR tyrosine phosphorylation in cervical and breast tumor cells and also *in vitro* colony formation of EGFR-overexpressing lung tumors. Competition and sandwich ELISAs, competitive surface plasmon resonance, and domain-level epitope mapping analyses demonstrated that mAb A13 competitively bound to the domain III (amino acids 302–503) of EGFR with EGF, but recognized distinct epitopes from those of cetuximab (Erbitux®). Our results demonstrated that anti-EGFR mAb A13 interfered with EGFR proliferation signaling by blocking EGF binding to EGFR with different epitopes from those of cetuximab, suggesting that combination therapies of mAb A13 with cetuximab may prove beneficial for anti-tumor therapy.

1. Introduction

The epidermal growth factor receptor (EGFR, *erb*B1, HER1), one of four member of the ErbB family of tyrosine kinase growth factor receptors, is a 170 kDa membrane-spanning glycoprotein comprising an extracellular ligand-binding domain, a transmembrane domain, and an intracellular cytoplasmic protein domain with tyrosine kinase activity (reviewed in (Holbro and Hynes, 2004; Burgess, 2008)). Binding to EGFR by its natural ligands, mainly epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), results in activation of the intracellular tyrosine kinase by phosphorylation, which ultimately leads to cell proliferation and differentiation, reduced apoptotic capacity, angiogenesis and metastatic phenotypes (Holbro and Hynes, 2004; Burgess, 2008).

Deregulations of EGFR signaling, such as EGFR overexpression, mutation/truncation, and activation by abnormal autocrine growth factor loops, have been implicated in the majority of epithelial solid tumors including lung, colon, breast, prostate, brain, head and neck, ovarian, and renal carcinoma and correlate with poor clinical prognosis, making it an attractive target for the developments of anti-cancer therapeutic agents (Mendelsohn and Baselga, 2006; Burgess, 2008). Two major classes of agents have been developed targeting EGFR, namely monoclonal antibodies (mAbs) and small molecule tyrosine kinase inhibitors (TKIs). mAbs, such as cetuximab (IMC-225, Erbitux®), matuzumab (EMD72000), panitumumab (ABX-EGF), and IMC-11F8, interact with the extracellular domain of the receptor preventing its activations by its ligands, whereas the TKIs, such as gefitinib (Iressa), erlotinib (Tarceva), and sorafenib (Nexavar), act by inhibiting the intracellular tyrosine kinase activity (Mendelsohn and Baselga, 2006; Burgess, 2008). Though the two agents finally interfere with EGFR-mediated intracellular signaling, clinical data have highlighted substantial differences between the two agents, in terms of toxicity (e.g., mAbs less cytotoxic than TKIs) and activity (e.g., mAbs more active in colorectal and head and neck tumors, whereas TKIs more active mainly in lung cancer) (Imai and Takaoka, 2006).

Even though numerous anti-EGFR mAbs are now in clinic or clinical trials, their specific indications are different from one another. For example, cetuximab and panitumumab were recently marketed for colon, head and neck, and/or lung cancers, covering limited ranges of solid tumors (Imai and Takaoka, 2006; Burgess, 2008).

Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; mAb, monoclonal antibody; TGF-α, transforming growth factor-α; SPR, surface plasmon resonance.

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Considering a variety of the EGFR-associated solid tumors, it is urgent to develop more anti-EGFR mAbs which can be applied to other solid tumors. Further, different properties of mAbs, such as epitope, affinity, and isotype, can result in distinct direct and/or indirect effects on the inhibition of EGFR signaling, providing further insight into the target receptor-mediated cellular signaling (Johns et al., 2004; Zhou et al., 2007).

Here we report a novel anti-EGFR mAb, A13, generated by immunizing mice with human A431 cervical carcinoma cells that overexpress EGFR (Parker et al., 1984; Ferrer et al., 1996). mAb A13 specifically bound to soluble EGFR (sEGFR) and various sets of EGFR-expressing tumor cells and inhibited EGFR activation and colony formation of tumor cells, by competitive binding to EGFR with EGF, but not with cetuximab.

2. Materials and methods

2.1. Cell lines and reagents

Adherent human cancer cell lines, cervical carcinoma A431 (Korean cell line bank (KCLB), Korea), breast carcinoma SK-BR-3 (KCLB), breast carcinoma MDA-MB-231 (KCLB), lung non-small cell carcinoma NCI-H727 (ATCC, Manassas, VA), pancreatic carcinoma BxPC3 (ATCC), were grown in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin (Gibco Invitrogen, Carlsbad, CA) (Park et al., 2007). Non-adherent human acute promyelocytic leukemia, HL60 (ATCC) were cultured in RPMI1640 (Welgene, Korea) supplemented with 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Park et al., 2007). Chimeric anti-EGFR cetuximab (Erbitux®, Merck) mAb, human sEGFR (Sigma, St. Louis, MO), and human EGF (Pepro Tech, Rocky Hill, NJ) were provided by commercial suppliers. A human anti-tetanus mAb, hTT2, which was manufactured in the Green Cross Corp. (Yongin, Korea) was employed as a negative control mAb. All of the other chemicals and solvents used were of analytical grade.

2.2. Generation of mAb against human EGFR

Male Balb/c mice were immunized 3 times intraperitoneally at 3 weeks intervals with 1×10^7 cells of human cervical epidermal carcinoma A431 cells in PBS (Sato et al., 1983; Gill et al., 1984; Parker et al., 1984). Hybridomas were generated by cell fusion of spleen cells from A431-immunized mice and SP2/0 myeloma cells following the reported method (Ferrer et al., 1996; Kim et al., 2001; Song et al., 2008). The culture supernatants of hybridomas were screened by ELISA for sEGFR binding activity. Briefly, ELISA plate (Nunc Immuno Module, Maxisorp, Roskilde, Denmark) was coated with 2 μg/ml of sEGFR in PBS (pH 7.4) at 4 °C overnight. After washing with PBST (PBS containing 0.05% (w/v) Tween 20) and blocking with PBSB (PBS plus 1% (w/v) bovine serum albumin (BSA)), 100 μl of hybridoma culture media were added and incubated for 2 h at 25 °C. The bound mAb was detected using peroxidase-conjugated goat anti-mouse IgG (Fab specific) (Sigma) with the substrate of 3,3′,5,5′-tetramethylbenzidine (TMB) (KPL, Gaithersburg, MD). The absorbance was measured at 405 nm with a VersaMax microplate reader (Molecular devices, Crawley, UK). Clones screened by positive binding to sEGFR were further evaluated by analyzing their binding activity for the cell lysates of A431, SK-BR-3, and HL60 cells, which were prepared according to the method of Parker et al. (1984). The concentrations of EGFR in the cell lysates were determined by EGFR quantification ELISA kit (Calbiochem, San Diego, CA), according to the manufacturer's instructions. The cell lysates were diluted to 10 μg/ml and immobilized at 4 °C overnight in ELISA plates. Then ELISA was performed as described above. Positively selected mAb was purified from the cell supernatant by protein A-sepharose 4 fast flow (Pharmacia, Uppsala, Sweden) (Song et al., 2008). Cloning of mAb gene from hybridoma by reverse transcription (RT)-PCR and then sequence analysis were performed as essentially described before (Song et al., 2008).

2.3. Binding of mAb to cell surface expressed EGFR

Binding activity of mAb to cell-surface expressed EGFR in various cells was analyzed by a flow cytometry (FACScan, Becton-Dickinson, Mountain View, CA), as specified in figure legends. HL60 cells, which has not been known to express EGFR (Stegmaier et al., 2005), were employed as a negative control. Briefly, the grown cells (1×10^7 cells) washed with PBSB were incubated with 10 μg/ml of mouse mAb for 1 h on ice. After washing with PBSB, the cells were stained with FITC conjugated goat anti-mouse IgG (Fab specific) (Sigma) in PBSB for 40 min in ice before analysis by flow cytometry.

2.4. Immunohistochemistry

Human placenta tissue slide which overexpresses EGFR was purchased from Spring Bioscience (Fremont, CA) and the experimental procedures were followed by the manufacturer's instructions (Jungbluth et al., 2003). All paraffin embedded samples were deparaffinized and rehydrated. The paraffin in the slides was removed by immersing the slide in xylen and the peroxidases in the tissue was inactivated by boiling in 10 mM citrate buffer, pH 6.0 and then immersing in the 3% H_2O_2. mAb was diluted to 1 mg/ml in PBS, incubated on the slide for 1 h at 25 °C, and washed with PBS. Mouse anti-EGFR 31G7 mAb (Zymed, South San Fransisco, CA), known to stain epidermal cells of skin, colon, testis, methothelium, kidney, placenta and prostate, was included as a positive control. N-Histofine Simple Stain MAX PO (MULTI) (Nichirei Corp., Tokyo, Japan) was incubated for 30 min and washed with PBS. Stable DAB peroxidase substrate (KPL) was reacted on the slide and washed with tap water. Then the slide was counterstained with Contrast Blue (KPL) and washed with tap water. The slide was immersed in ethanol and then xylen and mounted with Permount (Fisher Scientific, Fairlawn, NJ).

2.5. Detection of EGFR tyrosine phosphorylation by Western blotting

To analyze of inhibition of EGFR tyrosine phosphorylation by mAbs, 1×10^5 cells of MDA-MB-231 and A431 were plated in 24 well culture plate (Nunc) and serum-starved for 48 h before addition of EGF (16 nM) with or without mAbs (100 μg/ml). After 30 min, the cells were washed and lysed in Triton X lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 2 μg/ml leupeptin and aprotinin, 1 mM Na_3VO_4, 1 mM PMSF). Equal amount of protein (100 μg/ml) was separated by SDS-PAGE (10% gel) and then Western blottings were performed with mouse anti-phosphotyrosine-HRP (Zymed) and 4CN 2-Component Membrane Peroxidase Substrate Kit (KPL).

2.6. Soft agar assay

Colony formation was analyzed by soft agar assay in the absence and presence of mAb using anchorage-dependent, lung tumor NCI-H727 as a model tumor cells, following the procedures of Lewis et al. (1993). Briefly, a bottom layer consisting of 1 ml of culture medium containing 0.5% SeaKem GTG agarose (BMA, Rockland, ME) was first solidified in 12-well culture plate. Then 1 ml of 0.3% agarose solution containing 2×10^4 cells with or without 100 μg of mAb A13