Impact of Epidermal Growth Factor Receptor (EGFR) Cell Surface Expression Levels on Effector Mechanisms of EGFR Antibodies

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The epidermal growth factor receptor (EGFR) is a widely expressed Ag that is successfully targeted in tumor patients by mAbs or tyrosine kinase inhibitors. A clinical study in non-small cell lung cancer patients demonstrated a positive correlation between EGFR expression levels and the therapeutic efficacy of the EGFR mAb cetuximab. However, the impact of EGFR expression on the different mechanisms of action (MoAs) triggered by the EGFR mAb has not been defined. In this study, BHK-21 cells were stably transfected to express different EGFR levels, which were quantified by immunofluorescence and immunohistochemistry and compared with EGFR levels of clinical non-small cell lung cancer samples. These cells were used to systematically investigate the impact of target Ag expression levels on Fab- or Fc-mediated MoAs of EGFR mAb. A negative correlation between EGFR levels and potency of Fab-mediated MoA was observed. Interestingly, Ab-dependent cell-mediated cytotoxicity (ADCC) by NK cells, monocytes, or polymorphonuclear cells as well as complement-dependent cytotoxicity positively correlated with the number of EGFR molecules. In comparison with ADCC by mononuclear cells, polymorphonuclear cell-mediated ADCC and complement-dependent cytotoxicity required higher EGFR expression levels and higher mAb concentrations to trigger significant tumor cell killing. This correlation between EGFR expression levels and Fc-mediated MoA was confirmed in an independent panel of human tumor cell lines carrying diverse genetic alterations. Furthermore, RNA interference-induced knockdown experiments reinforced the impact of EGFR expression on tumor cell killing by EGFR mAb. In conclusion, these results suggest that EGFR expression levels may determine distinct patterns of MoAs that contribute to the therapeutic efficacy of EGFR mAb. The Journal of Immunology, 2012, 189: 5230–5239.

Monoclonal Abs against tumor-expressed target Ags offer the possibility to recruit the innate immune system to combat malignant tumor growth (1). The cellular arm of innate immunity comprises immune effector cells, such as monocytes/macrophages, polymorphonuclear cells (PMNs), and NK cells, whereas the complement system constitutes an integral part of the humoral arm (2). Cellular attack mechanisms are triggered by Abs’ Fc regions through interaction with FcRs, expressed on immune effector cells, to mediate Ab-dependent cell-mediated cytotoxicity (ADCC) or Ab-dependent cell-mediated phagocytosis (3). Fixation of the C1q component of the classical complement pathway to target Ag-bound Fc is typically required to induce complement-dependent cytotoxicity (CDC) against target cells (4, 5). Additionally, tumor-directed Abs are able to elicit effector mechanisms via their Fab regions, including blockade of ligand binding, inhibition of signaling, or downregulation of target Ags (6). Today, Ab-based therapies constitute an increasingly important and effective armamentarium in the clinical management of tumors (7, 8). However, uncertainty exists about which mechanisms of action (MoAs) are most relevant for the clinical efficacy of therapeutic Abs. In solid tumors, overexpression of the transmembrane tyrosine kinase receptor epidermal growth factor receptor (EGFR) is often observed (e.g., in colorectal, lung, or head and neck cancer). Thus, EGFR has been validated as a therapeutic target in oncology using tyrosine kinase inhibitors or mAbs (9, 10). However, experiences from clinical studies and from animal models indicate that contribution of the EGFR-signaling pathway to tumor growth is variable.

Although clinical benefit is provided by therapeutic EGFR-directed mAbs, such as cetuximab or panitumumab, either as single agents or in addition to chemotherapy when compared with supportive care or chemotherapy alone, many patients fail to respond to these therapies (10). Meanwhile, progress has been made in identifying molecular biomarkers that predict response or resistance to EGFR inhibitors. For example, in colorectal cancer (CRC), resistance to EGFR inhibitors has been associated with activating mutations of downstream mediators, like Kirsten rat sarcoma viral oncogene homolog (KRAS), v-raf murine sarcoma viral oncogene homolog B1 (B-RAF), phosphoinositide-3-kinase (PI3K), and phosphatase and tensin homolog (PTEN), as well as with the expression of EGFR ligands, such as epiregulin and amphiregulin.
Based on these findings, guidelines for the application of the EGFR-directed mAbs cetuximab and panitumumab in CRC have been implemented that restrict these therapeutics to patients with KRAS wild-type–bearing tumors (12). Although activating mutations in downstream signaling molecules explain why Fab-mediated effector functions of EGFR mAbs are not operating against these tumor cells, the impact of these mutations on Fe–mediated MoAbs is less obvious. In a previous study, expression of oncogenic KRASG12V was linked to C/EBPβ-dependent downregulation of EGFR expression, leading to diminished ADCC and CDC activities of EGFR mAbs against these tumor cells (13). Biomarker analyses of the phase III FLEX study in non-small cell lung cancer (NSCLC) demonstrated a positive impact of higher EGFR expression on the efficacy of chemotherapy plus cetuximab, suggesting quantitative analysis of EGFR expression by immunohistochemistry (IHC) as a predictor for cetuximab therapy in NSCLC (14).

In this study, the impact of EGFR cell surface expression levels on distinct MoAbs of EGFR mAbs was investigated. Thus, analyses of Fab-mediated MoAbs revealed a negative correlation, whereas ADCC and CDC activities of EGFR Abs were positively correlated with EGFR cell surface expression levels.

### Materials and Methods

#### Study population and consent

Experiments reported in this article were approved by the Ethics Committee of the Christian-Albrechts-University in accordance with the Declaration of Helsinki. Blood donors were randomly selected from healthy volunteers, who gave written informed consent before analyses.

#### Cell lines

Cell lines A431, BHK-21, and SW480 (all from German Collection of Microorganisms and Cell Cultures), Panc1 (American Type Culture Collection, Manassas, VA), and Panc89 (from Dr. T. Okabe, Tokyo, Japan) were kept in RPMI 1640 medium. Cell lines A549 (European Cell Culture Collection, Salisbury, U.K.), A1207 (originally established by Dr. Aaronson, National Cancer Institute, National Institutes of Health, Bethesda, MD), Dif1 (15), and HeK293T (American Type Culture Collection) were cultivated in DMEM medium. Both cell culture mediums were supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

#### Generation of BHK cells stably transfected with human EGFR

BHK-21 cells were stably transfected with the plasmid pUSE-EGFR (Upstate Biotechnology), encoding the sequence for wild-type EGFR, by lipofection using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were put under selection by adding 1 mg/ml Geneticin. For all procedures, standard protocols were used according to the manufacturer’s manuals.

#### Immunohistochemical analyses of BHK-EGFR+ cell lines

BHK-EGFR+ cells were fixed in 4% PBS-buffered formalin, centrifuged, and embedded in paraffin; 1.5-μm sections were stained with anti-EGFR Ab (C225; chimeric IgG1; Erbitux; Merck, Darmstadt, Germany) for 10 min or were left untreated before stimulation with 100 ng/ml recombinant human EGFR 1 h. Subsequently, cells were washed with ice-cold PBS and pelleted by centrifugation. Cells were lysed using native lysis buffer (Cell Signaling Technology, Danvers, MA). Phosphorylation status of EGFR was assessed using 25 μg native protein and a specific ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer’s protocol.

#### Isolation of human effector cells

Human effector cells, such as mononuclear cells (MNCs) or PMNs, were isolated from peripheral blood drawn from healthy volunteers, as previously described (18). Monocytes and NK cells were isolated from the MNC fraction by negative selection and MACS cell separation (NK cell isolation Kit; Monocyte Isolation Kit II; Miltenyi Biotec, Auburn, CA).

#### Cytotoxicity assays

**ADCC assays.** ADCC assays were performed, as described (18), but without stimulation of PMNs. The EGFR Ab cetuximab (C225) was used for NK cell–mediated (E:T ratio = 10:1) or monocyte-mediated (E:T ratio = 40:1) ADCC experiments, whereas an IgA2 isotype class-switched version of the EGFR Ab C225-IgA2 (19) was chosen for PMN-mediated ADCC experiments (E:T ratio = 80:1). An irrelevant human IgG1 Ab and an irrelevant human IgG1 Ab (both from Alpha Diagnostic International, San Antonio, TX) were used as control Abs.

**CDC assays.** CDC assays were performed as described (20). freshly drawn human plasma (25% v/v), anticoagulated with 10 U/ml heparin, the Ab was added to the reaction mixture and incubated for 2 h at 37°C. After incubation, the percentage of cytotoxicity was calculated using the following formula: percentage lysis = (experimental cpm − basal cpm)/(maximal cpm − basal cpm) × 100.

#### Small interfering RNA–mediated knockdown experiments

Cells were seeded at a density of 1 × 10⁶ cells/well in six-well plates. On the day after transfection, the percentage of cytotoxicity was calculated using the following formula: percentage lysis = (experimental cpm − basal cpm)/(maximal cpm − basal cpm) × 100.

### SDS-PAGE and immunoblotting

Whole–protein extracts were prepared by lysing cell pellets in denaturing lysis buffer containing 1% SDS, 10 mM Tris (pH 7.4), 1% protease inhibitor mixture (Complete Protease Inhibitor Cocktail; Roche Applied Science, Mannheim, Germany). Ten micrograms of protein extracts was separated by denaturing SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). After blocking, membranes were probed with EGFR–specific primary Ab (Santa Cruz Biotechnology, Santa Cruz, CA), washed, and incubated with HRP-conjugated IgG as secondary Ab. Proteins were visualized by ECL (Thermo Fisher Scientific, IL). To determine similar transfer and equal loading, membranes were stripped and reprobed with an Ab specific for β-actin (Sigma-Aldrich, St. Louis, MO).

### Flow cytometric analyses

For indirect immunofluorescence, cells were stained as described previously (17). Relative fluorescence intensity was calculated using the following formula: mean fluorescence intensity EGFR Ab × mean fluorescence intensity control Ab. EGFR expression was quantified using the murine EGFR Ab m425 at saturating concentrations of 50 μg/ml and the QIFIKIT (DAKO, Denmark), according to the manufacturer’s instructions. An irrelevant murine Ab was used as control Ab. Samples were analyzed on a flow cytometer (Epicis Profile; Beckman Coulter, Fullerton, CA).

#### Phospho-EGFR–specific ELISA

BHK-EGFR+ cell lines #1, #2, #3, #4, #5, and #6 were seeded in six-well plates at a density of 0.8 × 10⁵ cells/well. On the following day, cells were preincubated with EGFR Ab cetuximab (C225; chimeric IgG1; Erbitux; Merck, Darmstadt, Germany) for 10 min or were left untreated before stimulation with 100 ng/mg recombinant human EGFR 1 h. Subsequently, cells were washed with ice-cold PBS and pelleted by centrifugation. Cells were lysed using native lysis buffer (Cell Signaling Technology, Danvers, MA). Phosphorylation status of EGFR was assessed using 25 μg native protein and a specific ELISA (R&D Systems, Minneapolis, MN), according to the manufacturer’s protocol.

### Synthesis of siRNAs targeting EGFR

Synthetic siRNAs targeting EGFR were purchased from Applied Biosystems/Ambion (Foster City, CA). Target sequences were as follows: EGFR siRNA #1 (ID s564), sense 5'-GAUAAGGAAUUGUGAUAU-3' and antisense 5'-CAAAGCCAAUACUAAUCG-3'; EGFR siRNA #2 (ID s564), sense 5'-CCUAAGUUCGACGAAU-3' and antisense 5'-CAUGUCUCCAGGUAA-3'; and EGFR siRNA #3 (ID s564), sense 5'-GAGUUCUUCCUCUAAAGA-3' and antisense 5'-UCUU UAGAGAAAGAGACuat-3'.
Data processing and statistical analyses

Data are displayed graphically and were analyzed statistically using GraphPad Prism 4.0. Curves were fitted using a nonlinear regression model with a sigmoidal dose response (variable slope). Statistical significance was determined by the two-way ANOVA repeated-measures test with the Bonferroni posttest. The results are displayed as mean ± SEM of at least three independent experiments. Correlations between EGFR Ab binding sites/cell and cytotoxic activity were calculated using the Pearson correlation test. The $p$ values were calculated, and null hypotheses were rejected at $p \leq 0.05$.

Results

Establishment of BHK-EGFR$^+$ cell lines displaying different EGFR cell surface expression levels

To systematically analyze the impact of EGFR expression levels on EGFR Ab–triggered cytotoxicity in an isogenic background, the BHK-21 cell line was stably transfected with human EGFR (= BHK-EGFR$^+$). Transfectants were categorized according to their EGFR cell surface expression levels, which were determined by indirect immunofluorescence and calibrated flow cytometry (Fig. 1A, 1B, respectively). Although the EGFR Ab did not bind to nontransfected BHK-21 cells (data not shown), various EGFR cell surface expression levels, ranging from 0.03 to $1.40 \times 10^6$ EGFR Ab binding sites, were observed in BHK-EGFR$^+$ cell lines #1 to #6. To compare EGFR expression levels on BHK-EGFR$^+$ cell lines with EGFR expression levels detected on clinical NSCLC samples, BHK-EGFR$^+$ cell lines were embedded in paraffin and immunohistochemically stained similarly to clinical NSCLC samples (Fig. 1C). Subsequently, the intensities of membranous DAB precipitates were determined by densitometric analyses. Increasing DAB intensities were detected from BHK-EGFR$^+$ cell lines #1 to #3, reaching a plateau in BHK-EGFR$^+$ cell line #4. Furthermore, NSCLC samples were subdivided by IHC score (1+, 2+, or 3+); IHC scores correlated with the measured DAB intensities (Fig. 1D).

Efficiency of Fab-mediated effector mechanisms correlates inversely with EGFR expression levels

To analyze the impact of EGFR cell surface expression levels on Fab-mediated effector mechanisms of C225, BHK-EGFR$^+$ cell lines #1 to #6 were compared with regard to concentration-dependent binding of C225 and inhibition of EGFR phosphorylation by C225. Comparisons among analyzed cell lines revealed that BHK-EGFR$^+$ #1 cells displayed the lowest EC$_{50}$ values for EGFR binding (0.03 $\mu$g/ml) and inhibition of EGFR phosphorylation (0.04 $\mu$g/ml), followed by BHK-EGFR$^+$ #2, BHK-EGFR$^+$ #3, BHK-EGFR$^+$ #4, BHK-EGFR$^+$ #5, and BHK-EGFR$^+$ #6 cells (EC$_{50}$EGFR binding = 1.96 $\mu$g/ml and EC$_{50}$EGFR phosph. = 3.48 $\mu$g/ml) (Fig. 2A–H). Together, dose-dependent binding patterns of C225 to BHK-EGFR$^+$ cells were reflected in C225-mediated inhibition of EGFR-induced phosphorylation of EGFR. Concentration-dependent inhibition of EGFR phosphorylation by C225 could be observed for all six cell lines displaying a negative correlation between EGFR Ab binding sites/cell and efficiency of EGFR phosphorylation inhibition (R$_{0.1 \mu$g/ml C225} = −0.8, R$_{1 \mu$g/ml C225} = −0.9, R$_{10 \mu$g/ml C225} = −0.6; Fig. 2I).

Higher EGFR cell surface levels positively affect EGFR Ab–triggered cytotoxicity

To investigate whether the extent of EGFR Ab–triggered cytotoxic activity depended on EGFR cell surface expression levels, dif-
different effector cells, such as NK cells, monocytes, or PMNs, as well as plasma as the source of human complement, were used in 
\[^{3}{}_{1}{}^{1}{\text{Cr}}\]-release assays with BHK-EGFR* cell lines #1 through #6. Relative binding to EGFR was calculated for all three cell lines by equating maximum relative fluorescence intensity values for each cell line with 100%. (B–G) Inhibition of EGF-induced phosphorylation of human EGFR in BHK-EGFR* cells #1 through #6 by C225 at the indicated concentrations was analyzed by phospho-EGFR (Tyr1068)-specific ELISA. (H) EC50 values (μg/ml) for analyzed BHK-EGFR* cell lines were calculated from data presented in (A)–(G). (I) Correlation between EGFR-Ab binding sites/cell and C225-mediated inhibition of EGF-induced EGFR phosphorylation was calculated for all six BHK-EGFR* cell lines at the indicated Ab concentrations. Data are mean ± SEM of three independent experiments. *p ≤ 0.05 without EGF versus with EGF stimulation, #p ≤ 0.05 without C225 versus with C225 inhibition.

**FIGURE 2.** Fab-mediated effector mechanisms are more effective against cells expressing low levels of EGFR. (A) Dose-response curves for C255 at the indicated concentrations were performed by indirect immunofluorescence using BHK-EGFR* cell lines #1 through #6. Relative binding to EGFR was calculated for all three cell lines by equating maximum relative fluorescence intensity values for each cell line with 100%. (B–G) Inhibition of EGF-induced phosphorylation of human EGFR in BHK-EGFR* cells #1 through #6 by C225 at the indicated concentrations was analyzed by phospho-EGFR (Tyr1068)-specific ELISA. (H) EC50 values (μg/ml) for analyzed BHK-EGFR* cell lines were calculated from data presented in (A)–(G). (I) Correlation between EGFR-Ab binding sites/cell and C225-mediated inhibition of EGF-induced EGFR phosphorylation was calculated for all six BHK-EGFR* cell lines at the indicated Ab concentrations. Data are mean ± SEM of three independent experiments. *p ≤ 0.05 without EGF versus with EGF stimulation, #p ≤ 0.05 without C225 versus with C225 inhibition.

Ab-mediated cytotoxicity is correlated with EGFR cell surface expression levels in genetically diverse tumor cell lines

Subsequently, we analyzed whether the results received with isogenic BHK-EGFR* cell clones can be transferred to human cell lines encompassing distinct genetic backgrounds. For this purpose, eight cell lines were compared by indirect immunofluorescence and grouped with regard to EGFR cell surface expression levels (Fig. 4A). Quantitative immunofluorescence was performed to calculate correlations between EGFR expression and cytotoxic...
EGFR EXPRESSION AND TUMOR CELL KILLING

FIGURE 3. Effective PMN- or complement-mediated cytolysis requires high EGFR cell surface expression levels and high EGFR Ab concentrations. In \[^{[\text{3}}\text{Cr}\text{-release assays, BHK-EGFR}^\text{+} \] cells were incubated in the absence or presence of EGFR-directed Ab, at increasing concentrations, with different effector sources, such as monocytes, NK cells, PMN, or plasma. Data were analyzed with regard to EGFR Ab binding sites/cell, as well as EGFR Ab concentrations used in the experiments. Means ± SEM from at least three independent experiments with different blood donors are presented. *p ≤ 0.05 EGFR Ab versus control Ab.

activity. EGFR Ab binding sites/cell ranged from 0.01 × 10^6 for HEK293T cells to 1.89 × 10^6 for DiFi cells (Fig. 4B).

Additionally, the impact of EGFR cell surface density on EGFR Ab–mediated cytotoxicity triggered by different effector sources was analyzed. \[^{[\text{3}}\text{Cr}-release assays were performed using NK cells, monocytes, or PMN effector cells, as well as human plasma, in the presence of saturating concentrations of EGFR Ab constructs (2 μg/ml C225-IgG1 for NK cells and monocytes, 10 μg/ml C225-IgA2 for PMNs, and 10 μg/ml H425-E3-IgG1 for plasma) (Fig. 4C). Similar to the results for BHK-EGFR^+ cells, complement- and PMN-mediated cytotoxicity against tumor cell lines (except HEK293T cells) strongly depended on high EGFR cell surface expression levels. A total of 1 × 10^6 EGFR Ab binding sites/cell (A1207, A431, or DiFi cells) was determined to be the threshold for the induction of cell lysis. In contrast, NK cells already triggered cell destruction against HEK293T cells expressing low levels of EGFR and reached their plateau of cytotoxic activity with PANC1 cells. Using isolated monocytes as an effector source in \[^{[\text{3}}\text{Cr}-release assays, no cytotoxic activity was detected against HEK293T cells expressing low levels of EGFR. However, starting with 0.08 × 10^6 EGFR Ab binding sites/cell (SW480 cells), increasing cytotoxic activity mediated by monocytes was observed in an EGFR Ab binding site–dependent manner, which reached a plateau at 1.51 × 10^6 EGFR Ab binding sites/cell, detected on A1207 cells expressing high levels of EGFR.

EGFR Ab binding sites/cell correlate positively with the extent of cytolysis

To determine whether the extent of cytotoxic activity could be linked to the level of EGFR Ab binding sites/cell, correlation coefficients (R) between both determinants were calculated for BHK-EGFR^+ cells, as well as for the analyzed tumor cell lines. Correlation was calculated for every effector source at the highest Ab concentrations by equating cytotoxic activity against BHK-EGFR^+ (Fig. 5, left panel) or DiFi cells (Fig. 5, right panel) with 100% (maximum lysis), which was related to the cytotoxic activity measured for all other BHK-EGFR^+ cells or (tumor) cell lines (indicated by individual symbols). In the case of BHK-EGFR^+ cell lines, a statistically significant positive correlation between EGFR Ab binding sites/cell and cytotoxic activity was observed for all analyzed effector sources, such as NK cells (R = +0.9), monocytes (R = +0.9), PMNs (R = +0.9), and plasma (R = +1). Further analyses of the (tumor) cell line panel also revealed a statistically significant positive correlation between EGFR Ab binding sites and Ab-mediated cytotoxic activity for monocytes (R = +0.9), PMNs (R = +0.8), and plasma (R = +1). Interestingly, Ab-triggered cytotoxicity mediated by NK cells did not correlate significantly (R = +0.4) with EGFR Ab binding sites/cell (Fig. 5, right panels).

RNA interference-induced EGFR knockdown affects cell lysis

In the next set of experiments, we examined whether siRNA-induced downregulation of EGFR mRNA expression in A431 cells expressing high levels of EGFR leads to impaired tumor cell lysis triggered by MNCs, PMNs, or complement. Knockdown of EGFR expression was induced using three EGFR-specific siRNAs. Control experiments revealed that transfection of A431 cells with 25 nM of EGFR-siRNA #2 for 72 h was the most effective (Fig. 6A). For ADCC and CDC experiments, A431 cells were transfected with 25 nM of EGFR-siRNA #2 for 48 h (Fig. 6B) or 72 h (Fig. 6BII–D). A549 cells, which demonstrated 10-fold lower EGFR expression on their cell surface in comparison with A431 cells, were also transfected with 25 nM of EGFR-siRNA #2 for 72 h and served as a control cell line in MNC-mediated ADCC experiments. Although transfection of A431 cells for 48 h achieved a strong downregulation of EGFR cell surface expression (reduction by ~70%), no differences could be detected between MNC-mediated tumor cell lysis and negative control siRNA-transfected cells (Fig. 6B). After 72 h of transfection, a knockdown of EGFR cell surface expression ~90% was achieved in A431 and A549 cells. Consequently, MNC-mediated ADCC activity was significantly decreased compared with control siRNA-transfected cells (Fig. 6BII, 6BIII). Furthermore, PMN- and complement-mediated...
lysis was significantly reduced in A431 cells transfected with EGFR-siRNA #2 for 72 h compared with control cells (Fig. 6C, 6D). To demonstrate that downregulation of EGFR by siRNA did not affect general tumor cell susceptibility, A431 cells were transfected with 25 nM negative control siRNA or EGFR-specific siRNA #2 for 72 h. These cells were analyzed in MNC-mediated ADCC experiments using an HLA class I-directed Ab, of which ADCC efficacy should not be affected by EGFR expression. Importantly, this Ab was similarly effective, irrespective of the siRNA treatment, confirming that EGFR-specific siRNA treatment specifically reduced EGFR cell surface expression and did not affect general susceptibility to ADCC (Fig. 6E). A nonbinding control Ab did not trigger ADCC (data not shown).

Discussion

Retrospective biomarker analyses of the largest EGFR Ab study in NSCLC demonstrated that patients with higher EGFR expression levels, as determined by quantitative IHC, obtained more therapeutic benefit from cetuximab therapy than did patients with lower EGFR expression (14). In the current study, the impact of EGFR cell surface expression levels on different effector mechanisms of EGFR mAbs, such as inhibition of EGF-triggered EGFR phosphorylation and ADCC or CDC, was analyzed systematically. For this purpose, BHK cells were transfected to express variable EGFR levels on their surface, which were in the range of those found on tumor cell lines and on clinical NSCLC samples. Interestingly, lower EGFR expression was linked to stronger induction of Fab-mediated MoAs but diminished Fc-mediated cytotoxic activity. In contrast, higher EGFR expression was accompanied by inefficient Fab-mediated MoAs but potent Fc-mediated killing.

Clinical experience with the HER-2/neu Ab trastuzumab in breast cancer patients suggested that Her-2/neu expression levels can be used as a predictive biomarker in trastuzumab-based breast cancer therapy (22, 23). Accordingly, previously reported results for trastuzumab revealed a relationship between HER-2/neu expression and Fc-mediated MoAs triggered by trastuzumab (24). However, data for Fab-mediated MoAs differed between that study for trastuzumab and our data for EGFR Abs using BHK-EGFR+ cell lines. Also, with regard to the therapeutic CD20 mAbs rituximab and ofatumumab, CDC and ADCC activity was demonstrated to correlate positively with CD20 cell surface expression levels (25, 26).

A positive correlation between EGFR expression levels, determined by quantitative IHC, and the clinical outcome of cetuximab therapy was reported in NSCLC (14); however, this remains controversial in CRC (27). Additional well-designed and -controlled studies are required to confirm the contribution of quantitative IHC as a biomarker for EGFR-directed Ab therapy. Furthermore, to our knowledge, EGFR expression levels have not been quantitatively compared between different tumor entities. It can be hypothesized that, due to the preponderance of Fab-mediated MoAs, tumors expressing low levels of EGFR may be more susceptible to developing resistance against EGFR mAbs in the presence of oncogenic mutations, such as KRAS, than are tumors with higher EGFR expression levels, which are controlled more effectively by immunological effector mechanisms. For example, oncogenic KRAS mutations impaired the clinical efficacy of therapeutic EGFR Abs in metastatic CRC (mCRC) (12), but they were not predictive in NSCLC (28). However, it remains to be elucidated by quantitative IHC and functional analyses whether EGFR expression levels differ significantly between these tumor entities and whether EGFR-triggered Fc-mediated MoAs are more active in NCSLC than in CRC.

Previous studies investigating the impact of EGFR expression levels on MoAs of EGFR mAbs were limited to NK cells, and conflicting results were reported. For example, one study using a panel of various tumor cell lines suggested that NK cell–mediated
ADCC triggered by cetuximab depended on EGFR cell surface expression levels (29). However, in another study using lung cancer cell lines, no positive linear correlation was reported between EGFR expression levels and NK cell–mediated ADCC (30). As shown in Fig. 5, we observed a positive correlation between EGFR cell surface expression levels and NK cell–mediated cytotoxicity in BHK-EGFR+ cell lines but not in the tumor cell line panel. Regulation of NK cell cytotoxicity is complex and involves interactions between a variety of NK cell receptors and cognate ligands, which may not function properly across species barriers. Some of these ligands exert their functions by binding to activating or inhibitory NK cell receptors to enforce or hamper activation and degranulation of NK cells and NK cell–mediated ADCC, respectively (31). It can be assumed that mismatches exist between ligands (e.g., HLA I, MICA, MICB, ULBP) expressed on the hamster cell line BHK and activating (e.g., NKG2D) and inhibitory receptors (e.g., killer cell Ig-like receptors) expressed on human NK cells. As a consequence, EGFR cell surface expression is expected to be more preponderant in an isogenic BHK-EGFR+ cell line panel than in different human tumor cell lines, which express different sets of compatible activating or inhibitory NK cell receptor ligands, leading to a greater variation in NK cell activation.

Fc-mediated effector mechanisms of EGFR mAbs also include activation of the complement system (20, 32). Thus, complement activation triggered by combinations of two noncompetitive EGFR- or EGFRvIII-directed mAbs or individual CDC-optimized (K326A/E333A) variants was observed in vitro (20, 33). Additionally, complement activation induced by cetuximab treatment was suggested to play a crucial role in tumor growth inhibition in vivo (34). However, the incompatibility of membrane-bound complement-regulatory proteins expressed on human tumor cell lines with murine complement components limits the interpretation of data received from such mouse xenograft models. Data generated in the current study delineate activation of the complement system to be a potentially important effector mechanism of EGFR Abs against tumors expressing high levels of EGFR but not against those expressing low levels of EGFR.

Despite the widespread clinical application of the two EGFR Abs cetuximab and panitumumab, uncertainty exists about their relevant MoAs in vivo (32). In mouse xenograft models, activation of ADCC was proposed to be an important MoA of EGFR mAb in the early stages of tumor development. However, in advanced stages, EGFR mAb’s therapeutic efficacy was reported to depend on Fab-mediated effector functions (35). Importantly, the significant role of Fc-mediated effector mechanisms of therapeutic EGFR mAbs on tumor cell destruction was reinforced by observations that distinct genetic polymorphisms of FcγRIIa (131 H/R) and FcγRIIIa (158 V/F) were associated with higher response rates to cetuximab therapy in mCRC patients (36–38). Additionally, based on immunohistochemical analyses of immune cell infiltrates in primary tumors of mCRC patients treated with a cetuximab-based chemotherapy, tumors with stronger CD56-positive staining correlated with response to cetuximab treatment and with a significantly longer progression-free survival compared with tumors with no CD56 staining. As a result of these findings, the investigators suggested that CD56+ NK cells represent a key effector cell population among leukocytes that mediated cetux-

**FIGURE 5.** Ab-mediated cytotoxicity correlates positively with the extent of EGFR cell surface expression. Correlation between the extent of EGFR cell surface expression and EGFR Ab–mediated cytotoxic activity was calculated for each effector source by equating cytotoxic activity against BHK-EGFR+ #6 (left panels) or DiFi cells (right panels) at the highest Ab concentration with 100% (maximum lysis). Maximum lysis was related to cytotoxic activity measured for all other BHK-EGFR+ or tumor cell lines (indicated by individual symbols) at the highest Ab concentrations. Data are mean ± SEM from at least three independent experiments with different blood donors.
imab’s clinical efficacy (39). However, correlations of cetuximab’s efficacy with the FcγRIIIa (H/H) polymorphism were also observed. FcγRIIIa is widely expressed on myeloid cells, such as monocytes, macrophages, and PMNs but not on NK cells (40). Based on data presented in Figs. 3 and 4, it can be concluded that monocytes represent a potent effector cell population against tumor cells expressing low and high levels of EGFR, whereas PMNs represent a potent effector cell population against tumor cells expressing high levels of EGFR. In tumor samples from patients, myeloid cells often constitute the predominant leukocyte infiltrate, particularly if tumors contain necrotic areas (39, 41, 42). Tumor-promoting and tumor-inhibitory roles have been suggested for both monocytes/macrophages and PMNs based on in vivo studies in mice (43, 44).

Our study has inherent methodological limitations that need to be considered in the interpretation of the results. For example, NK cell assays were performed against xenogeneic (BHK) or allogeneic (tumor) cell lines. This is expected to result in a decrease in killer cell Ig-like receptor-mediated NK cell inhibition and an overestimation of NK cell activity compared with syngeneic tumor cell killing. Furthermore, PMN- and complement-mediated killing was performed with IgA2 or Fc-engineered EGFR Abs, respectively, because unmodified IgG1 Abs did not trigger tumor cell lysis by these effector sources (data not shown). Additionally, results from our studies against EGFR cannot easily be transferred to other target Ags, because incompletely defined target Ag characteristics determine whether these molecules are suitable for ADCC or CDC recruitment (45). For example, among different B cell-related Ags, HLA class II was particularly effective (46), whereas, for example, CD19 Abs required Fc engineering to trigger significant ADCC (47).

The presented data raise the critical question of how Fc-mediated effector functions can be enhanced against tumors expressing low levels of EGFR. For example, a T cell-engaging bispecific Ab (BiTE) was demonstrated to mediate effective killing of KRAS-mutated tumors in vivo (48). Furthermore, the activation of EGFR mAb-triggered MoAs may be improved by combinations of noncompetitive EGFR mAbs to artificially enhance the Ag density on the tumor cell surface. Thus, combinations of EGFR mAbs were demonstrated to enhance CDC (20), as well as EGFR internalization (49), a concept that is currently under evaluation in clinical studies. Alternatively, Fc engineering approaches improved Fc-mediated MoAs, such as ADCC or CDC, against target cells with low levels of EGFR expression (21, 50). Thus, ADCC

![Diagram](image.png)

FIGURE 6. RNA interference-induced knockdown of EGFR expression is accompanied by decreased ADCC and CDC activity. A431 cells were seeded into six-well plates and grown overnight. (A) On the following day, cells were transfected with 25 nM control siRNA or three EGFR-specific siRNAs for 48 h (AI) or with increasing concentrations (5–50 nM) of a control siRNA or the EGFR-specific siRNA #2 for 72 h (AII). Whole-protein extracts were prepared, separated by SDS-PAGE, and immunoblotted against EGFR and β-actin, which served as a loading control. (B) Strong RNA interference-induced knockdown of EGFR cell surface expression also affected MNC-mediated tumor cell lysis. A431 cells were transfected with 25 nM control siRNA or EGFR-specific siRNA #2 for 48 h (BI) or 72 h (BII). As a control cell line, A549, which harbors lower EGFR levels than do A431 cells, was transfected with control siRNA or EGFR-specific siRNA #2 for 72 h. After transfection, indirect immunofluorescence was performed using 50 µg/ml of C225 or control Ab and polyclonal rabbit FITC-conjugated F(ab')2 fragments against human IgG.

Additionally, ADCC experiments with C225 or control Ab (both at 10 µg/ml) and MNCs (E:T ratio 80:1) were performed. Low levels of EGFR expression also impaired PMN-mediated ADCC (C) and CDC (D) activity. A431 cells were transfected with 25 nM control siRNA or EGFR-specific siRNA #2 for 72 h. PMNs (E:T ratio 80:1) or plasma served as effector sources in [51Cr]-release assays using C225 or control Ab (both at 10 µg/ml). (E) Downregulation of EGFR by siRNA did not generally affect tumor cell susceptibility. A431 cells were transfected with 25 nM negative control siRNA or EGFR-specific siRNA #2 for 72 h. MNCs (E:T ratio 80:1) served as the effector source in [51Cr]-release assays using an HLA class I Ab at increasing concentrations. Data are mean ± SEM of triplicate wells from at least three independent experiments with different donors. **p ≤ 0.01.
activity triggered by cetuximab against KRAS-mutated tumor cells was significantly enhanced by protein- or glyco-engineering of cetuximab’s Fc region (17). Interestingly, a glyco-engineered EGFR Ab induced clinical responses in individual CRC patients carrying KRAS-mutated tumors in a phase I clinical trial (51).

In conclusion, the current study presents novel insights into the impact of EGFR cell surface expression on the efficiency of EGFR-directed mAbs. The observations that higher EGFR levels are associated with better clinical outcome and enhanced Fc-mediated MoAbs compared with direct-killing mechanisms support the concept that Fc-mediated mechanisms may be critical for the clinical efficacy of cetuximab in NSCLC. If confirmed by additional studies—for example, on the impact of FcγR polymorphisms in the population expressing higher levels of EGFR—approaches to further enhance Fc-mediated killing appear logical.

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Disclosures

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