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Matuzumab and cetuximab activate the epidermal growth factor receptor but fail to trigger downstream signaling by Akt or Erk

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Molecular inhibition of the epidermal growth factor receptor (EGFR) is a promising anticancer strategy, and monoclonal antibodies (mAbs) to EGFR are undergoing extensive evaluation in preclinical and clinical trials. However, the effects of anti-EGFR mAbs on EGFR signaling have remained unclear. We have now examined the effects of 2 anti-EGFR mAbs, matuzumab (EMD72000) and cetuximab (Erbitux), both of which are currently under assessment for treatment of various cancers, on EGFR signal transduction and cell survival in nonsmall cell lung cancer cell lines. Similar to EGF, matuzumab and cetuximab each induced phosphorylation of EGFR at several tyrosine phosphorylation sites as a result of receptor dimerization and activation of the receptor tyrosine kinase. In contrast to the effects of EGF, however, EGFR activation induced by these antibodies was not accompanied by receptor turnover or by activation of downstream signaling pathways that are mediated by Akt and Erk and are important for regulation of cell proliferation and survival. In addition, clonogenic survival assays revealed that matuzumab and cetuximab reduced the survival rate of H292 cells, in which they also inhibited the EGF-induced activation of Akt and Erk. Although we have examined only a few cell lines, our results indicate that the antitumor effects of matuzumab and cetuximab depend on inhibition of EGFR downstream signaling mediated by Akt or Erk rather than on inhibition of EGFR itself.

Key words: EGF receptor; signal transduction; matuzumab; cetuximab; nonsmall cell lung cancer

The epidermal growth factor receptor (EGFR, also known as ErbB1), a member of the ErbB family of receptor tyrosine kinases, is a 170-kDa plasma membrane glycoprotein composed of an extracellular ligand binding domain, a transmembrane region and an intracellular tyrosine kinase domain with a regulatory COOH-terminal segment.[1] Binding of ligand to EGFR induces receptor dimerization, activation of the receptor kinase and autophosphorylation of specific tyrosine residues within the COOH-terminal region of the protein.[1] These events trigger intracellular signaling pathways that promote cell proliferation and survival.[2,3]

EGFR is frequently overexpressed in many types of human malignancy, with the extent of overexpression being negatively correlated with prognosis.[4,5] Recognition of the role of EGFR in carcinogenesis has prompted the development of EGFR-targeted therapies that include both small-molecule tyrosine kinase inhibitors (TKIs) that target the intracellular tyrosine kinase domain and monoclonal antibodies (mAbs) that target the extracellular domain.[6–8] Among EGFR-TKIs, gefitinib and erlotinib have been extensively evaluated in nonsmall cell lung cancer (NSCLC), and sensitivity to these drugs has been correlated with the presence of somatic mutations in the EGFR kinase domain or with EGFR gene (*EGFR*) amplification.[9–16] Among anti-EGFR mAbs, cetuximab (Erbitux), a chimeric mouse-human antibody of the immunoglobulin (Ig) G1 subclass, has proved efficacious in the treatment of irinotecan-refractory colon cancer[17] and was recently approved by the U.S. Food and Drug Administration for the treatment of patients with head and neck squamous cell carcinoma.[18] Several clinical studies of anti-EGFR mAbs such as matuzumab (EMD72000, humanized IgG1) and cetuximab are ongoing for other types of cancer including NSCLC.[19–24] Anti-EGFR mAbs bind to the extracellular ligand binding domain of the receptor and are thereby thought to block ligand binding.[18,25] The antitumor effects of these mAbs are thus thought to be attributable to inhibition of EGFR signaling as well as to other mechanisms such as antibody-dependent cellular cytotoxicity.[18,26] However, the detailed effects of anti-EGFR mAbs on EGFR signaling have remained unclear.[27–30]

We have now examined in detail the effects on EGFR signal transduction of 2 anti-EGFR mAbs, matuzumab and cetuximab, both of which are used clinically, to provide insight into the mechanisms of their antitumor effects.

Material and methods

Cell culture and reagents

The human NSCLC cell lines NCI-H292 (H292), NCI-H460 (H460) and Ma-1 were obtained as previously described[31] and were cultured under a humidified atmosphere of 5% CO_2 at 37°C in RPMI 1640 medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum. Matuzumab and cetuximab were kindly provided by Merck KGaA (Darmstadt, Germany) and Bristol Myers (New York, NY), respectively; gefitinib was obtained from AstraZeneca (Macclesfield, UK); and trastuzumab (Herceptin; Genentech, South San Francisco, CA) was obtained from Chugai (Tokyo, Japan). Neutralizing antibodies to EGFR (clone LA1) were obtained from Upstate Biotechnology (Lake Placid, NY).

Immunoblot analysis

Cell lysates were fractionated by SDS-polyacrylamide gel electrophoresis on a 7.5% gel, and the separated proteins were transferred to a nitrocellulose membrane. After blocking of nonspecific sites, the membrane was incubated consecutively with primary and secondary antibodies, and immune complexes were detected with the use of enhanced chemiluminescence reagents, as described previously.[31] Primary antibodies to the specific intracellular phosphorylation sites of EGFR (pY845, pY1068 or pY1173), to Erk, to phospho-Akt and to Akt were obtained from Cell Signaling Technology (Beverly, MA); those to the extracellular domain of EGFR (clone 31G7) were from Zymed (South San Francisco, CA); those to the intracellular domain of EGFR (EGFR 1005) and to phospho-Erk were from Santa Cruz Biotechnology (Santa Cruz, CA); and those to β-actin (loading control) were from Sigma. Horseradish peroxidase-conjugated goat antibodies to mouse or rabbit IgG were obtained from Amersham Biosciences (Little Chalfont, UK).

Chemical cross-linking assay

Cells were incubated first with 1 mM bis(sulfosuccinimidyl) suberate (BS^3; Pierce, Rockford, IL) for 20 min at 4°C and then with

Abbreviations: EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; mAb, monoclonal antibody; NSCLC, nonsmall cell lung cancer; Ig, immunoglobulin; BS^3, bis(sulfosuccinimidyl) suberate; PE, R-phycoerythrin; PI3K, phosphoinositide 3-kinase.

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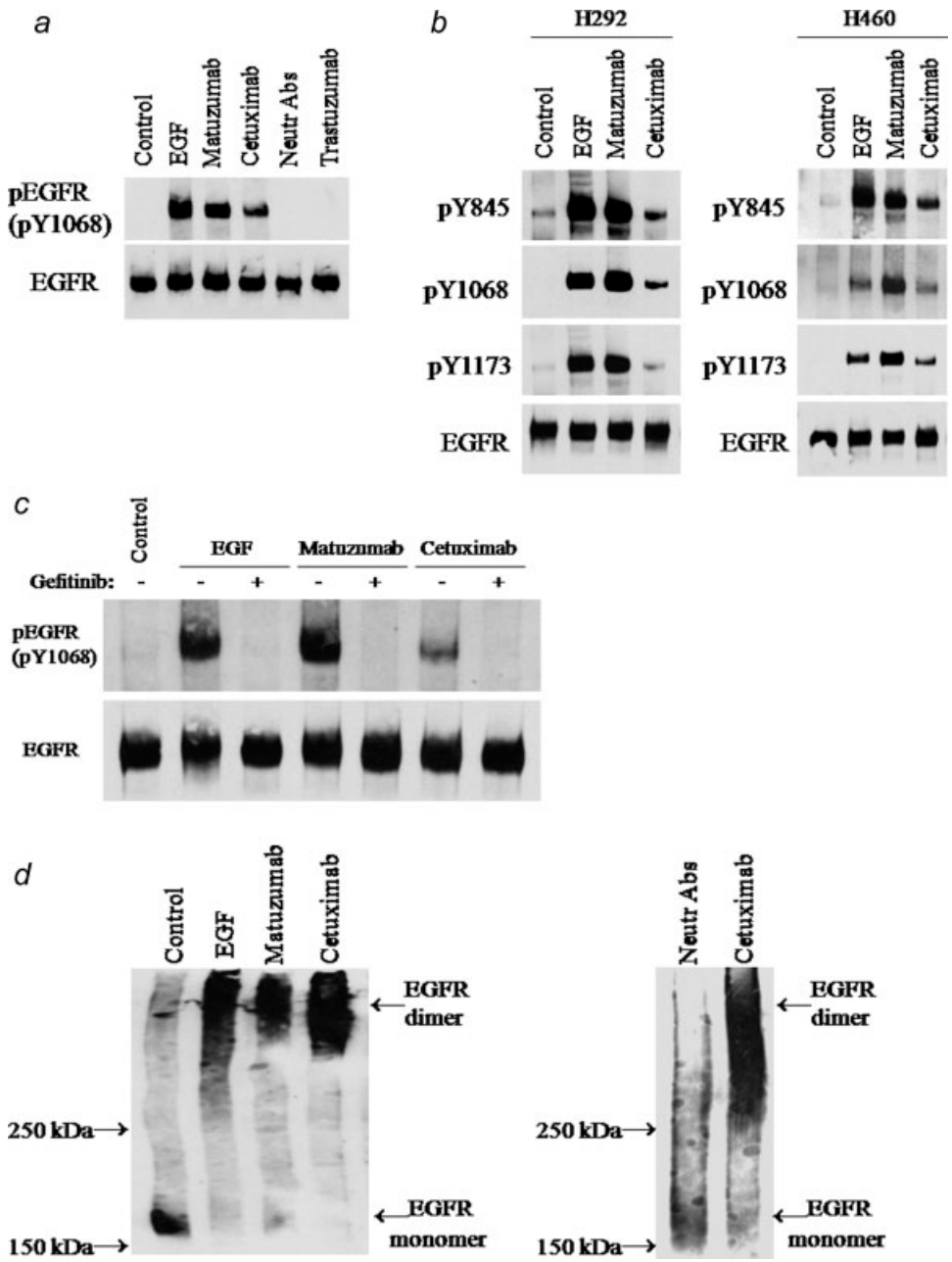

FIGURE 1 – EGFR phosphorylation induced by matuzumab or cetuximab as a result of receptor dimerization and activation of the receptor tyrosine kinase. (*a*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence (Control) or presence of matuzumab (200 nM), cetuximab (100 nM), neutralizing antibodies to EGFR (80 nM), trastuzumab (50 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR (pY1068) and to total EGFR (the extracellular domain). (*b*) H292 or H460 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y845-, Y1068- or Y1173-phosphorylated forms of EGFR and to total EGFR (the extracellular domain). (*c*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM), EGF (100 ng/ml) or gefitinib (10 μM), as indicated. Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR and to total EGFR (the extracellular domain). (*d*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM), neutralizing antibodies to EGFR (80 nM) or EGF (100 ng/ml). The cells were then washed and exposed to the chemical cross-linker BS^3 after which cell lysates were subjected to immunoblot analysis with antibodies to EGFR (the intracellular domain). The positions of EGFR monomers and dimers as well as of molecular size standards are indicated.

250 mM glycine for 5 min at 4°C to terminate the cross-linking reaction, as described previously.[31] Cell lysates were resolved by SDS-polyacrylamide gel electrophoresis on a 4% gel and subjected to immunoblot analysis with rabbit polyclonal antibodies to the intracellular domain of EGFR (EGFR 1005).

Immunofluorescence analysis

Cells were grown to 50% confluence in 2-well Lab-Tec Chamber Slides (Nunc, Naperville, IL), deprived of serum overnight, and then incubated with 200 nM matuzumab or EGF (100 ng/ml) for 4 hr at 37°C. They were fixed with 4% paraformaldehyde for

30 min at 4°C, permeabilized with 0.1% Triton X-100 for 10 min, and exposed to 5% nonfat dried milk for 1 hr at room temperature. The cells were stained with rabbit polyclonal antibodies to the intracellular domain of EGFR (EGFR 1005) for 1 hr at room temperature and then incubated for an additional 45 min with Alexa 488-labeled goat antibodies to rabbit IgG (Molecular Probes, Eugene, OR). Cell nuclei were counterstained for 5 min at room temperature with 4′,6-diamidino-2-phenylindole (Sigma) at 2 μg/ml. The chamber slides were mounted in fluorescence mounting medium (DakoCytomation, Hamburg, Germany), and fluorescence signals were visualized with a fluorescence microscope (Eclipse E800; Nikon, Kawasaki, Japan). Negative controls (secondary antibodies alone) did not yield any substantial background staining.

Flow cytometry

Cells were deprived of serum overnight and then incubated with 200 nM matuzumab or EGF (100 ng/ml) for 4 hr at 37°C. They were isolated by exposure to trypsin, and aliquots of $\sim 1.0 \times 10^6$ cells were incubated for 2 hr at 4°C either with an R-phycoerythrin (PE)-conjugated mouse mAb to EGFR (clone EGFR.1; Becton Dickinson, San Jose, CA), which does not interfere with the binding of EGF to EGFR,[32] or with a PE-conjugated isotype-matched control mAb (Becton Dickinson). The cells were then examined by flow cytometry (FACScalibur, Becton Dickinson) to detect the intensity of EGFR staining at the cell surface.

Clonogenic assay

Cells were plated in triplicate at a density of 200 per 25-cm^2 flask containing 10 ml of medium and were cultured for 7 days in the presence of the indicated concentrations of matuzumab or cetuximab. They were then incubated in medium alone for 7 days at 37°C, fixed with methanol:acetic acid (10:1, v/v), and stained with crystal violet. Colonies containing >50 cells were counted for calculation of the surviving fraction as follows: (mean number of colonies)/(number of inoculated cells × plating efficiency). Plating efficiency was defined as the mean number of colonies divided by the number of inoculated cells for untreated controls.

Results

Matuzumab and cetuximab induce EGFR phosphorylation in a manner dependent on the receptor tyrosine kinase activity

With the use of immunoblot analysis, we first examined the effects of the anti-EGFR mAbs matuzumab and cetuximab on EGFR phosphorylation in human NSCLC H292 cells, which express wild-type EGFR. Incubation of the serum-deprived cells for 15 min with EGF, matuzumab or cetuximab-induced phosphorylation of EGFR on tyrosine-1068 (Y1068), whereas treatment of the cells with neutralizing antibodies to EGFR or with trastuzumab, a mAb specific for HER2 (ErbB2), had no such effect (Fig. 1*a*). Furthermore, like EGF, matuzumab and cetuximab each induced phosphorylation of EGFR on Y845, Y1068 and Y1173 in H292 and H460 cells (Fig. 1*b*), the latter of which are also human NSCLC cells that express wild-type EGFR.

To determine whether the antibody-induced phosphorylation of EGFR requires the kinase activity of the receptor, we examined the effect of gefitinib, a specific EGFR-TKI. H292 cells were deprived of serum and then exposed to matuzumab, cetuximab or EGF for 15 min in the absence or presence of gefitinib. EGFR phosphorylation on Y1068 induced by EGF, matuzumab or cetuximab was completely blocked by gefitinib (Fig. 1*c*). These findings thus indicated that, like EGF, matuzumab and cetuximab each induce EGFR phosphorylation by activating the tyrosine kinase of the receptor.

Matuzumab and cetuximab induce EGFR dimerization

Ligand-dependent EGFR dimerization is responsible for activation of the receptor tyrosine kinase.[33,34] To examine whether matuzumab or cetuximab induces EGFR dimerization, we incubated serum-deprived H292 cells with the mAbs for 15 min and then exposed the cells to the chemical cross-linker BS^3. Immunoblot analysis of cell lysates with antibodies to the intracellular domain of EGFR revealed that matuzumab and cetuximab each induced EGFR dimerization to an extent similar to that observed with EGF, whereas only the monomeric form of the receptor was detected in control cells or in cells treated with neutralizing antibodies to EGFR (Fig. 1*d*). These data thus suggested that matuzumab and cetuximab activate EGFR through induction of receptor dimerization.

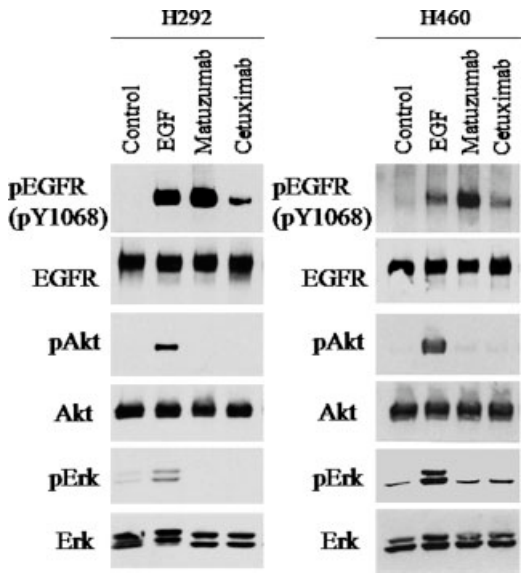

FIGURE 2 – Failure of matuzumab or cetuximab to activate Akt or Erk. H292 or H460 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to the Y1068-phosphorylated form of EGFR, to phosphorylated Akt and to phosphorylated Erk as well as with antibodies to total EGFR (the extracellular domain), Akt or Erk.

Matuzumab and cetuximab fail to induce signaling downstream of EGFR

EGFR signaling is transduced by 2 main pathways mediated by phosphoinositide 3-kinase (PI3K) and Akt and by Ras, Raf and Erk.[35,36] To determine whether EGFR phosphorylation induced by matuzumab or cetuximab is accompanied by activation of these pathways, we examined the levels of phosphorylated (activated) Akt and Erk in H292 and H460 cells treated with these antibodies for 15 min after serum deprivation. In contrast to the effects of EGF, neither matuzumab nor cetuximab induced the phosphorylation of Akt or Erk in H292 or H460 cells (Fig. 2). These results thus indicated that matuzumab and cetuximab induce EGFR activation but fail to activate the downstream Akt and Erk signaling pathways.

Matuzumab and cetuximab do not induce EGFR downregulation

Endocytic trafficking of EGFR is important for full activation of Erk and PI3K.[37] To examine further the defect in signaling downstream of EGFR activation by matuzumab or cetuximab, we determined the effects of these mAbs on receptor turnover. H292 or H460 cells were deprived of serum and then cultured with EGF, matuzumab or cetuximab for various times up to 24 hr, after which the levels of phosphorylated and total EGFR, Akt and Erk were measured. In both H292 and H460 cells treated with EGF, the amount of total EGFR decreased in a time-dependent manner

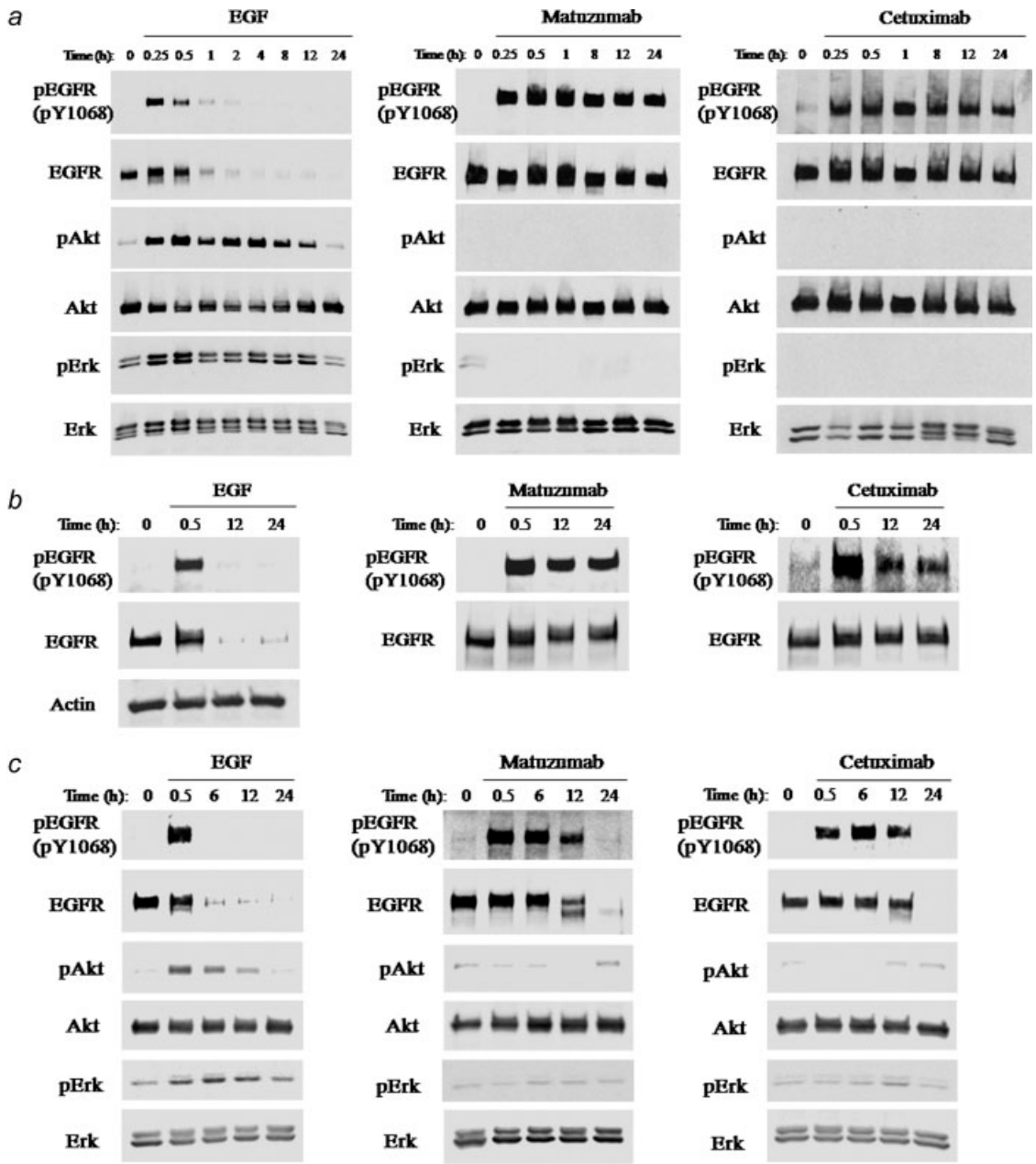

FIGURE 3 – Lack of EGFR turnover in cells treated with matuzumab or cetuximab. (*a*) H292 cells were deprived of serum overnight and then incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM), respectively. Cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (pY1068), Akt or Erk as well as with those to total EGFR (the extracellular domain), Akt or Erk. (*b*) H292 cells deprived of serum overnight were incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM). Cell lysates were subjected to immunoblot analysis with antibodies to theY1068-phosphorylated form of EGFR, to total EGFR (the intracellular domain) or to β-actin (loading control). (*c*) H460 cells deprived of serum overnight were incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM), after which cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (pY1068), Akt or Erk as well as with those to total EGFR (the intracellular domain), Akt or Erk. (*d*) H292 cells plated on chamber slides were deprived of serum overnight and then incubated for 4 hr in the absence or presence of matuzumab (200 nM) or EGF (100 ng/ml). The cells were fixed, permeabilized, and stained with antibodies to EGFR and Alexa 488-labeled secondary antibodies (green). Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (blue). Fluorescence signals were visualized with a fluorescence microscope, and the merged images are shown. Scale bar, 20 μm. (*e*) H292 cells were deprived of serum overnight and then incubated for 4 hr in the absence or presence of matuzumab (200 nM) or EGF (100 ng/ml). The cells were stained with either a PE-conjugated mAb to EGFR (right peaks) or a PE-labeled isotype-matched mAb (left peaks) and analyzed by flow cytometry. Representative histograms of relative cell number versus PE fluorescence are shown.

(Figs. 3*a*–3*c*), an effect that has been shown to be the result of receptor internalization and degradation.[30,38] In parallel with this EGFR downregulation, the extent of EGF-induced tyrosine phosphorylation of EGFR also decreased and was virtually undetectable by 4–6 hr (Figs. 3*a*–3*c*). The phosphorylation of Akt and Erk induced by EGF persisted for at least 12 hr but had declined by 24 hr in both cell lines (Figs. 3*a* and 3*c*). In contrast, the levels of phosphorylated and total EGFR in H292 cells treated with

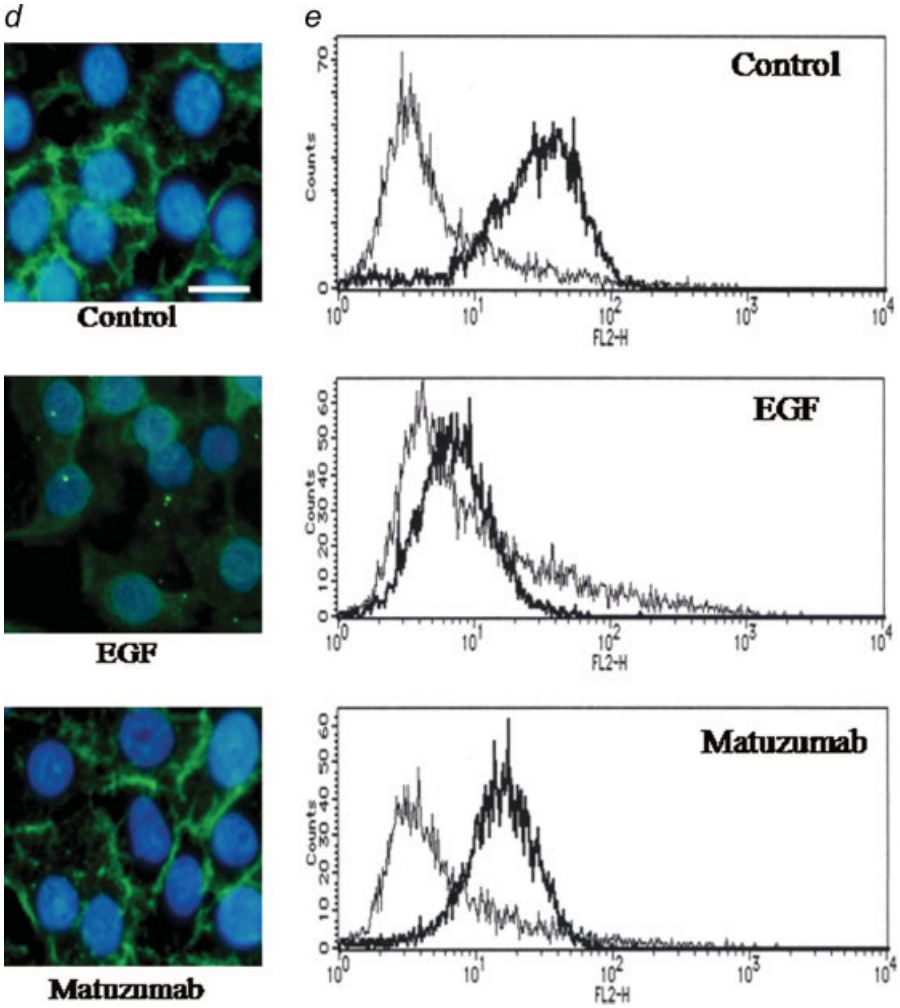

FIGURE 3 – CONTINUED

matuzumab or cetuximab for 24 hr were similar to those apparent after exposure to the antibodies for only 15 or 30 min (Figs. 3*a* and 3*b*). A marked delay in EGFR turnover was also apparent in H460 cells treated with matuzumab or cetuximab (Fig. 3*c*), although EGFR dephosphorylation and downregulation had occurred by 24 hr. Neither matuzumab nor cetuximab induced the activation of Akt or Erk or affected the total amounts of these proteins over a period of 24 hr in either cell line (Figs. 3*a* and 3*c*). We eliminated the possibility that the antibodies to the extracellular domain of EGFR used for the immunoblot analysis shown in Figure 3*a* bind only to the unoccupied form of EGFR (as a result of competition with EGF, matuzumab or cetuximab) by performing the immunoblot analysis shown in Figures 3*b* and 3*c* with antibodies to the intracellular domain of EGFR. These results thus suggested that downregulation of EGFR is impaired in cells treated with matuzumab or cetuximab, likely explaining the failure of these antibodies to activate downstream signaling by Akt and Erk.

To confirm that the inability of the anti-EGFR mAbs to induce EGFR downregulation is attributable to a failure to induce internalization-dependent receptor degradation, we treated serum-deprived H292 cells with matuzumab or EGF for 4 hr and then examined the expression of EGFR by immunofluorescence analysis (Fig. 3*d*) or flow cytometry (Fig. 3*e*). Whereas EGFR was localized at the cell surface in control cells, treatment with EGF resulted in internalization and a decrease in the fluorescence intensity of EGFR. In contrast, EGFR remained at the surface of cells treated with matuzumab. These data suggested that, in contrast to EGF-EGFR complexes, antibody-EGFR complexes remain at the cell surface and do not undergo internalization and degradation.

TABLE I – CHARACTERISTICS OF NSCLC CELL LINES

Cell line	*EGFR* mutation	*EGFR* copy number
H292	Wild type	Polysomy
H460	Wild type	Monosomy
Ma-1	del E746-A750	Gene amplification

Effects of matuzumab and cetuximab on EGF-induced signaling and cell survival

We next determined whether matuzumab or cetuximab inhibits ligand-dependent EGFR signal transduction. To examine also whether the effects of these antibodies are dependent on *EGFR* status, we studied 3 human NSCLC cell lines: 2 cell lines (H292, H460) that possess wild-type *EGFR* alleles and 1 (Ma-1) with an *EGFR* mutation in exon 19 that results in deletion of the residues E746–A750. Our recent fluorescence in situ hybridization analysis[31] revealed that *EGFR* copy number is increased (polysomy) in H292 cells and that H460 cells exhibit monosomy for *EGFR*. Ma-1 cells were also found to manifest *EGFR* amplification (Table I).[31] We treated serum-deprived cells of the 3 NSCLC lines with

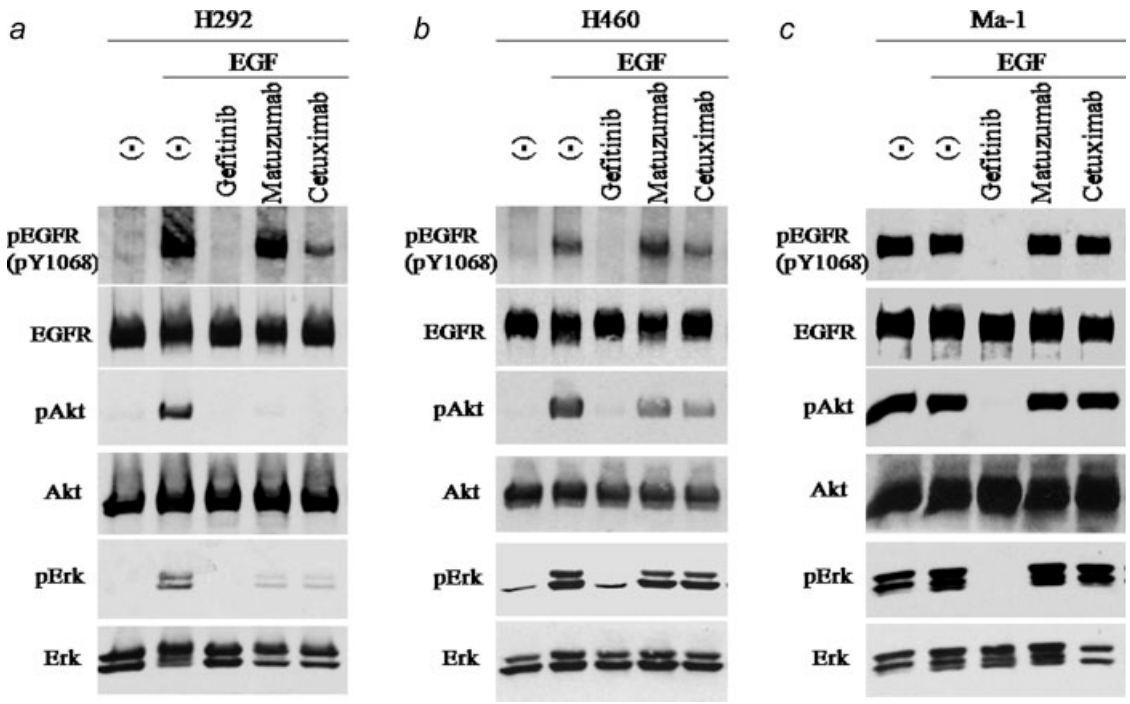

Figure 4 – Effects of matuzumab and cetuximab on EGF-induced EGFR signaling. H292 (*a*), H460 (*b*) and Ma-1 (*c*) cells were deprived of serum overnight and then incubated first for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or gefitinib (10 μM) and then for an additional 15 min in the additional absence or presence of EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies to phosphorylated forms of EGFR (pY1068), Akt or Erk as well as with those to total EGFR (the extracellular domain), Akt or Erk.

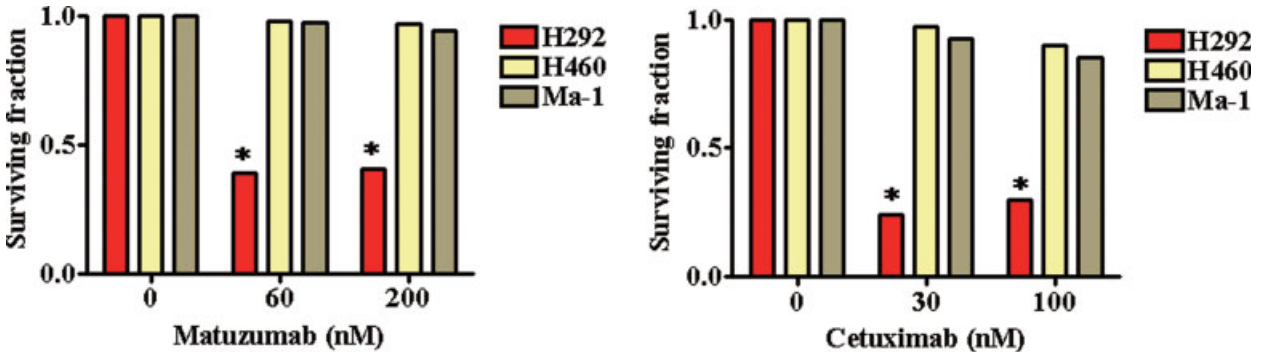

Figure 5 – Effects of matuzumab and cetuximab on cell survival. H292, H460 or Ma-1 cells were plated at a density of 200 cells per 25-cm^2 flask in triplicate and cultured for 7 days in the presence of the indicated concentrations of matuzumab or cetuximab. They were then incubated with medium alone for 7 days before determination of the number of colonies containing >50 cells for calculation of the surviving fraction. Data are means of triplicates from a representative experiment. *$p < 0.001$ *versus* the corresponding value for cells not exposed to mAb (Student's *t*-test).

matuzumab, cetuximab or gefitinib for 15 min and then stimulated them with EGF for 15 min. Gefitinib prevented the phosphorylation of EGFR, Akt, and Erk induced by EGF in H292 (Fig. 4*a*) and H460 (Fig. 4*b*) cells. The level of EGFR phosphorylation in EGF-treated H292 or H460 cells was not substantially affected by matuzumab or cetuximab, likely because these antibodies also induce EGFR phosphorylation. However, whereas matuzumab and cetuximab did not substantially affect EGF-dependent phosphorylation of Akt or Erk in H460 cells, they markedly inhibited these effects of EGF in H292 cells. As we showed previously,[31] EGFR, Akt, and Erk are constitutively activated in the *EGFR* mutant cell line Ma-1 cell (Fig. 4*c*). Furthermore, whereas gefitinib blocked the phosphorylation of each of these 3 proteins in Ma-1 cells, matuzumab and cetuximab did not.

Finally, we performed a clonogenic assay to determine whether cell survival is affected by the differences in EGF-dependent signaling among H292, H460 and Ma-1 cells after treatment with matuzumab or cetuximab (Fig. 5). Matuzumab and cetuximab each induced a marked reduction in the survival rate of H292 cells, consistent with the inhibition of EGF-dependent EGFR downstream signaling by these antibodies in these cells. In contrast, neither mAb affected the survival of H460 or Ma-1 cells, consistent with the lack of inhibition of EGF-dependent or constitutive EGFR downstream signaling by matuzumab or cetuximab in these cell lines. These results suggested that the effects of matuzumab and cetuximab on EGF-dependent or constitutive EGFR downstream signaling are correlated with their effects on cell survival in NSCLC cell lines.

Discussion

The effectiveness of treatment with anti-EGFR mAbs has been thought to be based on prevention of ligand binding to EGFR and consequent inhibition of EGFR activation.[18,25,26] Matuzumab and cetuximab have recently been developed as EGFR-inhibitory mAbs for clinical use.[17–22,25] A structural study revealed that cetuximab binds to the extracellular ligand binding domain (domain III) of EGFR,[25] and matuzumab is also thought to bind to domain III on the basis of its observed competition with EGFR ligands.[18] We have now shown that matuzumab and cetuximab induced phosphorylation of EGFR at several sites, including Y845, Y1068 and Y1173. These findings are consistent with previous observations that mAb 225, the mouse mAb equivalent to cetuximab, is able to induce EGFR dimerization and activation.[38,39] Cetuximab was also recently shown to induce phosphorylation of EGFR in head and neck squamous cell carcinoma cell lines[29] as well as in NSCLC cell lines including H292.[40] These in vitro results appear to contradict observations that matuzumab and cetuximab inhibit EGFR phosphorylation in vivo.[28,41,42] This apparent discrepancy may be due to the more complex cellular environment in vivo, including the presence of stromal cells that interact with tumor cells. We have also now shown that gefitinib, a specific EGFR-TKI, completely blocked EGFR phosphorylation induced by matuzumab or cetuximab, confirming that this effect of the antibodies is dependent on the intrinsic tyrosine kinase activity of EGFR. Furthermore, our cross-linking analysis showed that matuzumab as well as cetuximab activated EGFR through induction of receptor dimerization. Although recent structural analysis has revealed that cetuximab restricts the range of the extended conformation of EGFR that is required for ligand-induced receptor dimerization,[25] matuzumab and cetuximab likely induce EGFR dimerization in a manner dependent on their immunologically bivalent binding capacities, as was previously shown for mAb 225.[39] We found that neutralizing antibodies to EGFR did not activate EGFR, even though they also recognize the external domain of EGFR and compete with EGFR ligands for receptor binding.[43] The neutralizing antibodies did not induce EGFR dimerization, however, likely accounting for their inability to activate EGFR. This difference in the ability to induce EGFR dimerization between matuzumab and cetuximab on the one hand and the neutralizing antibodies on the other might be due to differences in the corresponding binding sites on EGFR.

To examine the mechanism by which matuzumab and cetuximab exert antitumor effects despite their induction of EGFR activation, we investigated the effects of antibody-induced EGFR activation on EGFR downstream signal transduction. We found that EGFR activation induced by matuzumab or cetuximab was not accompanied by activation of downstream signaling pathways mediated by Akt and Erk, both of which play an important role in regulation of cell proliferation and survival.[35,36] Moreover, we found that the antibody-EGFR complexes were not removed from the plasma membrane, in contrast to the rapid receptor turnover induced by EGF. In response to ligand binding, the ligand-EGFR complex is rapidly internalized and then either recycled back to the cell surface or proteolytically degraded.[44–46] The internalized EGFR interacts with various signaling proteins that are important for sustained activation of the major signaling pathways mediated by PI3K-Akt and Erk.[44,47] The activity of the PI3K-Akt and Erk pathways is thus greatly reduced in cells that are defective in internalization of ligand-EGFR complexes as a result of their expression of a mutant form of dynamin.[37] Furthermore, expression in glioblastoma cells of an EGFR chimeric protein that does not undergo internalization resulted both in a reduction in the extent of EGFR-dependent activation of Akt and Erk as well as in inhibition of tumor growth.[48] These observations thus suggest that inhibition of EGFR turnover by matuzumab or cetuximab is likely responsible for the failure of these mAbs to activate Akt and Erk.

We examined the effects of matuzumab and cetuximab on EGF-dependent EGFR signaling and on cell survival in 3 NSCLC cell lines of differing *EGFR* status. The inhibition of EGF-dependent activation of Akt and Erk by these antibodies appeared related to the inhibition of clonogenic cell survival in the 3 cell lines. With regard to NSCLC cell lines harboring wild-type *EGFR* alleles, matuzumab and cetuximab markedly inhibited EGF-dependent phosphorylation of Akt and Erk in H292 cells but not in H460 cells. Both antibodies inhibited cell survival in H292 cells but not in H460 cells. These results suggest that the antitumor effects of matuzumab and cetuximab depend on inhibition of EGFR downstream signaling such as that mediated by Akt and Erk rather than on inhibition of EGFR itself. Our present data are consistent with previous observations that cetuximab did not inhibit EGFR phosphorylation completely even in cells sensitive to this antibody.[27,30] It is possible that the difference in sensitivity to matuzumab and cetuximab between the 2 cell lines expressing wild-type EGFR in the present study is due to the difference in gene copy number, given that we found an increase in *EGFR* copy number in H292 cells compared with that in H460 cells.[31] A previous clinical study showed that *EGFR* copy number correlated with the response to cetuximab treatment in individuals with colorectal cancer.[49] *EGFR* copy number was not determined by fluorescence in situ hybridization in previous clinical studies of NSCLC patients treated with matuzumab or cetuximab.[19,22–24] Several clinical studies of the therapeutic efficacy of anti-EGFR antibodies in NSCLC patients are underway, and investigation of the potential of molecular markers including *EGFR* copy number to predict clinical response is warranted. Matuzumab and cetuximab failed to inhibit both activation of Akt and Erk and clonogenic cell survival in Ma-1 cells, which express a mutant form of EGFR that shows an increased sensitivity to EGFR-TKIs such as gefitinib and erlotinib.[9–16] We recently showed that cells expressing EGFR mutants exhibit constitutive, ligand-independent receptor dimerization and activation,[31] likely explaining the lack of effect of matuzumab or cetuximab on EGFR signaling or cell survival in such cells. However, previous studies showed that cetuximab exerted an antitumor effect in a cell line with an *EGFR* mutation, whereas several other cell lines with *EGFR* mutations were resistant to cetuximab.[27,30] Our results are consistent with clinical observations showing that the presence of an *EGFR* mutation is not a major determinant of a positive response to cetuximab in individuals with NSCLC or colorectal cancer.[22,50,51]

In conclusion, we have shown that EGFR turnover is impaired in cells treated with the anti-EGFR mAbs matuzumab or cetuximab, resulting in inhibition of EGFR downstream signaling. Although our study is limited by the small number of cell lines analyzed, our findings provide important insight into the mechanisms by which anti-EGFR mAbs exert their antitumor effects, and they suggest that it may be possible to predict the therapeutic efficacy of such mAbs by assessment of EGFR signal transduction.

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