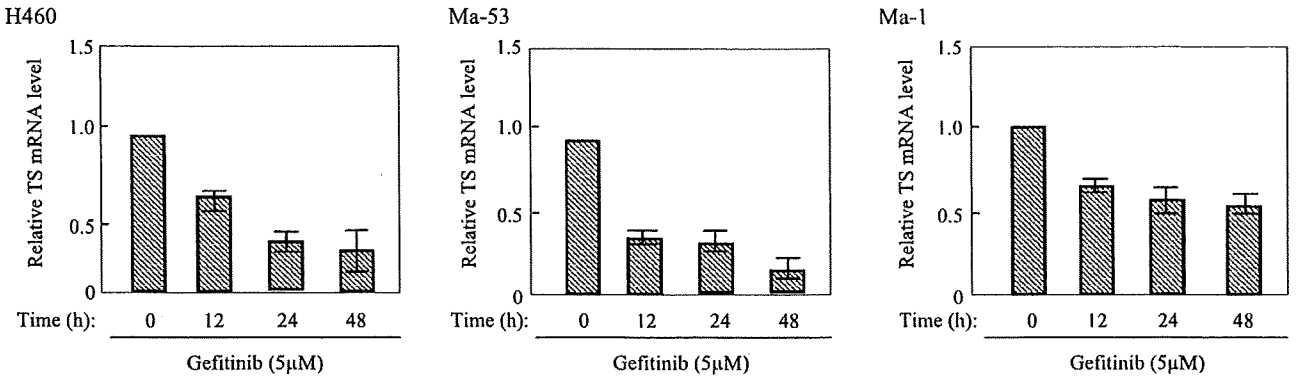

Figure 5. Down-regulation of TS mRNA by gefitinib in NSCLC cell lines. H460, Ma-53, or Ma-1 cells were incubated with gefitinib (5 μmol/L) for the indicated times in medium containing 10% serum, after which total RNA was extracted from the cells and subjected to reverse transcription and real-time PCR analysis of TS mRNA. The amount of TS mRNA was normalized by that of glyceraldehyde-3-phosphate dehydrogenase mRNA. Mean ± SE of values from three separate experiments.

achievable concentrations of gefitinib in tumor tissues of treated humans (34). These observations suggest that a gefitinib dose of 50 mg/kg in mouse xenograft models is appropriate for mimicking the therapeutic dose in humans.

EGFR-TKIs have been shown previously to act synergistically with radiation or cytotoxic agents such as cisplatin, paclitaxel, and irinotecan (38–40). These cytotoxic agents and radiation have been shown to increase the phosphorylation level of EGFR, possibly reflecting the activation of prosurvival signaling, and this effect is blocked by EGFR-TKIs, resulting in the synergistic antitumor effects of the combination therapies. Such a synergistic effect of 5-FU and gefitinib was attributed to 5-FU-induced EGFR phosphorylation in colorectal cancer cells (41). In contrast, we found that 5-FU had no effect on the level of EGFR phosphorylation in NSCLC cell lines. Further examination of different concentrations of 5-FU and different exposure times also failed to reveal an effect of 5-FU on EGFR phosphorylation in these cells (data not shown). These findings indicate that NSCLC cell lines respond differently to 5-FU than do colorectal cancer cells and that the synergistic antiproliferative effect of 5-FU and gefitinib in NSCLC cells is not mediated at the level of EGFR phosphorylation.

Our results indicate that the synergistic interaction of 5-FU (or S-1) and gefitinib is attributable, at least in part, to down-regulation of TS expression by gefitinib. The active metabolite of 5-FU, FdUMP, forms a covalent ternary complex with 5,10-methylenetetrahydrofolate and TS, resulting in inhibition of DNA synthesis (42). TS is thus an important therapeutic target of 5-FU. The amount of TS in neoplastic cells has been found to increase after exposure to 5-FU, resulting in the maintenance of free enzyme in excess of that bound to 5-FU (43–47). Such an increase in TS expression and activity has been viewed as a mechanistic driver of 5-FU resistance in cancer cells (48–50). The development of a new therapeutic strategy that reduces TS expression would therefore be of interest. Indeed, preclinical studies have shown that the down-regulation of TS by antisense oligonucleotides or other means enhances the efficacy of 5-FU (51–54). Down-regulation of TS would be expected to enhance the cytotoxicity of 5-FU as a result of the decrease in the amount of its protein target (55). Consistent with these preclinical data, an inverse relation between TS expression and 5-FU sensitivity has been shown in various human solid tumors (27, 28, 56–60). We have now shown that gefitinib alone induced down-regulation of TS expression, suggesting that this effect of gefitinib contributes to its synergistic interaction with 5-FU (or S-1) in NSCLC cell lines.

We further explored the molecular mechanism by which gefitinib induces down-regulation of TS expression in NSCLC cells. Given that EGFR signal transduction has been shown to be involved in activity of E2F-1 that regulates the expression of several genes including TS (61, 62), which controls the expression of several genes including that for TS, we examined the possible effects of gefitinib on E2F-1 expression and on the abundance of TS mRNA. Gefitinib induced down-regulation of E2F-1 in NSCLC cell lines harboring wild-type *EGFR*, consistent with previous observations (63), as well as in those expressing mutant *EGFR*. In addition, gefitinib reduced the amount of TS mRNA in NSCLC cells, consistent with the notion that the suppression of TS expression by gefitinib is attributable to inhibition of gene transcription as a result of down-regulation of E2F-1. For our experiments examining the effects of gefitinib on TS and E2F-1 expression, we used a drug concentration of 5 μmol/L. The concentration of gefitinib in tumor xenografts was shown previously to be 5 to 14 times that in the plasma concentration of the mouse hosts (34). Daily oral administration of gefitinib (250 mg) in patients also gave rise to a drug concentration in tumor tissue that was substantially higher (mean, 42-fold) than that in plasma concentration (34). We showed previously that the maximal concentration of gefitinib in the plasma of patients with advanced solid tumors had a mean value of 0.76 μmol/L at a daily dose of 225 mg (64). Based on these data, we considered that a gefitinib concentration of 5 μmol/L was appropriate for our

analyses of TS and E2F-1 expression. Together, our present findings suggest that down-regulation of E2F-1 and consequently that of TS by gefitinib is responsible, at least in part, for the synergistic antitumor effect of combined treatment with S-1 and gefitinib.

Somatic mutations of *EGFR* have been associated with sensitivity to EGFR-TKIs in patients with advanced NSCLC (13–16). However, although most NSCLCs with *EGFR* mutations initially respond to EGFR-TKIs, the vast majority of these tumors ultimately develop resistance to the drug. In the present study, the synergistic effect of combination chemotherapy with S-1 and gefitinib was observed even in *EGFR* mutant cells. Our findings thus suggest that the addition of S-1 (or 5-FU) to EGFR-TKIs might overcome chemoresistance to EGFR-TKIs and that exploration of the effect of such combination therapy in cells resistant to EGFR-TKIs is warranted. *EGFR* mutations appear to be largely limited to lung cancer, with few such mutations having been detected in other types of cancer (65–67). 5-FU is widely used as an anticancer agent and is considered a key drug in chemotherapy for solid tumors such as gastrointestinal and cervical cancer (68–70). Our present results show that gefitinib suppressed the expression of TS in NSCLC cell lines regardless of the absence or presence of *EGFR* mutations, suggesting that the addition of EGFR-TKIs to a 5-FU-containing regimen might increase the effectiveness of such treatment for solid cancers without *EGFR* mutations. Oral combined chemotherapy with drugs, such as S-1 and gefitinib, may also prove to be of low toxicity and therefore maintain quality of life. Our preclinical results provide a basis for future clinical investigations of combination chemotherapy with S-1 and EGFR-TKIs in patients with solid tumors.

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British Journal of Cancer (2008) 98, 749 – 755

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Enhancement of the antitumor activity of ionising radiation by nimotuzumab, a humanised monoclonal antibody to the epidermal growth factor receptor, in non-small cell lung cancer cell lines of differing epidermal growth factor receptor status

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The expression and activity of the epidermal growth factor receptor (EGFR) are determinants of radiosensitivity in several tumour types, including non-small cell lung cancer (NSCLC). However, little is known of whether genetic alterations of *EGFR* in NSCLC cells affect the therapeutic response to monoclonal antibodies (mAbs) to EGFR in combination with radiation. We examined the effects of nimotuzumab, a humanised mAb to EGFR, in combination with ionising radiation on human NSCLC cell lines of differing *EGFR* status. Flow cytometry revealed that H292 and Ma-1 cells expressed high and moderate levels of EGFR on the cell surface, respectively, whereas H460, H1299, and H1975 cells showed a low level of surface EGFR expression. Immunoblot analysis revealed that EGFR phosphorylation was inhibited by nimotuzumab in H292 and Ma-1 cells but not in H460, H1299, or H1975 cells. Nimotuzumab augmented the cytotoxic effect of radiation in H292 and Ma-1 cells in a clonogenic assay *in vitro*, with a dose enhancement factor of 1.5 and 1.3, respectively. It also enhanced the antitumor effect of radiation on H292 and Ma-1 cell xenografts in nude mice, with an enhancement factor of 1.3 and 4.0, respectively. Nimotuzumab did not affect the radioresponse of H460 cells *in vitro* or *in vivo*. Nimotuzumab enhanced the antitumor efficacy of radiation in certain human NSCLC cell lines *in vitro* and *in vivo*. This effect may be related to the level of EGFR expression on the cell surface rather than to *EGFR* mutation.
British Journal of Cancer (2008) **98,** 749–755. doi:10.1038/sj.bjc.6604222 www.bjcancer.com
Published online 5 February 2008

Keywords: epidermal growth factor receptor; non-small cell lung cancer; nimotuzumab; monoclonal antibody; genetic alteration; radiosensitisation

Epidermal growth factor receptor (EGFR) is a receptor tyrosine kinase that is abnormally upregulated and activated in a variety of tumours (Baselga, 2002). Deregulation of receptor tyrosine kinases as a result of overexpression or activating mutations is frequently associated with human cancers and leads to the promotion of cell proliferation or migration, inhibition of cell death, or the induction of angiogenesis (Gschwind *et al*, 2004). The epidermal growth factor receptor has thus been identified as an important target in cancer therapy (Baselga and Arteaga, 2005). Several agents, including small-molecule inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKIs) and monoclonal antibodies (mAbs) specific for EGFR, have been designed to block EGFR signalling selectively (Ettinger, 2006; Harari and Huang, 2006; Imai and Takaoka, 2006). Among EGFR-TKIs, gefitinib and erlotinib have been extensively evaluated in non-small cell lung cancer (NSCLC), and sensitivity to these drugs has been associated with the presence of somatic mutations in the EGFR kinase domain or with *EGFR* amplification (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004; Cappuzzo *et al*, 2005; Mitsudomi *et al*, 2005; Takano *et al*, 2005). Various mAbs to EGFR are also undergoing preclinical and clinical trials of their efficacy as anticancer agents. However, biological markers able to predict the response to such antibodies have remained elusive.

The possibility of combining chemotherapy or radiation therapy with anti-EGFR mAb treatment has generated much interest, because the cellular targets for these agents and their mechanisms of action are different (Baumann and Krause, 2004). Studies have thus been undertaken to determine whether inhibition of EGFR signalling improves the response to chemotherapy or radiation therapy. Preclinical studies have shown that the anti-EGFR mAb cetuximab markedly increases the cytotoxic effect of chemotherapy or radiation therapy in various EGFR-expressing tumour cell lines (Huang *et al*, 1999; Milas *et al*, 2000; Buchsbaum *et al*, 2002; Prewett *et al*, 2002; Raben *et al*, 2005; Ettinger, 2006). A phase III clinical trial also showed that the combination of cetuximab with

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Revised 20 November 2007; accepted 7 January 2008; published online 5 February 2008

radiation therapy resulted in a significant improvement in local control and survival compared with radiation therapy alone, without an increase in radiation-induced side effects, in patients with locally advanced head and neck cancer (Bonner *et al*, 2006).

Nimotuzumab (also known as h-R3) is a humanised anti-EGFR mAb, which is currently undergoing clinical evaluation. In a preclinical study, nimotuzumab showed marked antiproliferative, proapoptotic, and antiangiogenic effects in tumours that overexpress EGFR (Crombet-Ramos *et al*, 2002). In early clinical trials, nimotuzumab has shown a longer half-life and a greater area under the curve (AUC) in comparison with other anti-EGFR antibodies (Crombet *et al*, 2003). A phase I/II trial showed that nimotuzumab was well tolerated and enhanced the curative potential of radiation in patients with advanced head and neck cancer (Crombet *et al*, 2004). Given that little is known of the antitumor action of nimotuzumab in NSCLC, we investigated the growth-inhibitory effects of this mAb alone and in combination with radiation in NSCLC cell lines with differing patterns of EGFR expression. We also examined whether genetic alterations of *EGFR* affect the antitumor action of combined treatment with nimotuzumab and radiation.

MATERIALS AND METHODS

Cell lines and reagents

The human NSCLC cell lines NCI-H292 (H292), NCI-H460 (H460), Ma-1, NCI-H1299 (H1299), and NCI-H1975 (H1975) were obtained as previously described (Okabe *et al*, 2007) and were maintained under a humidified atmosphere of 5% CO_2 in air at 37.0°C in RPMI 1640 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. Nimotuzumab was provided by Daiichi Sankyo Co Ltd (Tokyo, Japan), and gefitinib was obtained from AstraZeneca (Macclesfield, UK).

Flow cytometric analysis of surface EGFR expression

Cells (1.0×10^6) were stained for 2 h at 4°C with an R-phycoerythrin-conjugated mAb to EGFR (BD Biosciences, San Jose, CA, USA) or an isotype-matched control mAb (BD Biosciences). The cells were washed three times before measurement of fluorescence with a flow cytometer (FACScalibur; Becton Dickinson, San Jose, CA, USA).

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 (Okabe *et al*, 2007). Primary antibodies to phosphorylated EGFR (pY1068) were obtained from Cell Signaling Technology (Beverly, MA, USA), and those to EGFR were from Zymed (South San Francisco, CA, USA). Horseradish peroxidase-conjugated goat secondary antibodies were obtained from Amersham Biosciences (Little Chalfont, UK).

Clonogenic assay

Exponentially growing cells in 25-cm^2 flasks were harvested by exposure to trypsin and counted. They were diluted serially to appropriate densities and plated in triplicate in 25-cm^2 flasks containing 10 ml of medium supplemented with 1% fetal bovine serum in the absence or presence of 700 nM nimotuzumab. After incubation for 24 h, the cells were exposed to various doses of γ-radiation with a ^{60}Co irradiator at a rate of approximately 0.82 Gy min^{-1} and at room temperature. The cells were then washed with phosphate-buffered saline, cultured in drug-free medium for 10–14 days, fixed with methanol : acetic acid (10 : 1, v/v), and stained with crystal violet. Colonies containing > 50 cells were counted. The surviving fraction was calculated as (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 control cultures not exposed to nimotuzumab or radiation. The surviving fraction for combined treatment was corrected by that for nimotuzumab treatment alone. The dose enhancement factor was then calculated as the dose (Gy) of radiation that yielded a surviving fraction of 0.5 for vehicle-treated cells divided by that for nimotuzumab-treated cells (after correction for drug toxicity).

Antitumor activity of nimotuzumab with or without radiation *in vivo*

Animal experiments were performed in accordance with the Recommendations for Handling of Laboratory Animals for Biomedical Research, compiled by the Committee on Safety and Ethical Handling Regulations for Laboratory Animal Experiments, Kyoto University, and they met the requirements of the UKCCCR guidelines (Workman *et al*, 1998). Tumour cells (2×10^6) were injected subcutaneously into the right hind leg of 7-week-old female athymic nude mice. tumour volume was determined from caliper measurement of tumour length (L) and width (W) according to the formula $LW^2/2$. Treatment was initiated when tumours in each group achieved an average volume of approximately 170–200 mm^3. Treatment groups consisted of control, nimotuzumab alone, radiation alone, and the combination of nimotuzumab and radiation, with each group containing seven or eight mice. Nimotuzumab was administered intraperitoneally in a single dose of 1.0 mg per mouse; mice in the control and radiation-alone groups were injected with vehicle (physiological saline). Tumours in the right hind leg of mice were exposed to 10 Gy of γ-radiation with a ^{60}Co irradiator at a rate of approximately 0.32 Gy min^{-1} beginning 6 h after drug treatment. Growth delay (GD) was calculated as the time required for treated tumours to achieve a fivefold increase in volume minus the corresponding time required for control tumours. The enhancement factor was then determined as $(GD_{combination} - GD_{nimotuzumab})/(GD_{radiation})$.

RESULTS

Surface EGFR expression in NSCLC cell lines of differing *EGFR* status

We first examined the surface expression of EGFR in five NSCLC cell lines by flow cytometry. The *EGFR* status for the cell lines was determined in our previous study (Okabe *et al*, 2007). Three cell lines (H460, H292, and H1299) possess wild-type *EGFR* alleles, whereas the other two cell lines (Ma-1 and H1975) harbour *EGFR* mutations (Table 1). Ma-1 cells have an in-frame deletion in

Table 1 Characteristics of NSCLC cell lines

Cell line	EGFR surface expression	*EGFR* status
H460	Low	Wild type
H292	High	Wild type
H1299	Low	Wild type
Ma-1	Moderate	del(E746–A750)
H1975	Low	L858R/T790M

EGFR = epidermal growth factor receptor; NSCLC = non-small cell lung cancer

exon 19 (E746–A750). H1975 cells harbour the L858R mutation in exon 21 and a secondary mutation in exon 20 (T790M). Activating mutations in exons 19 and 21 are associated with sensitivity to EGFR-TKIs (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004; Cappuzzo *et al*, 2005; Mitsudomi *et al*, 2005; Takano *et al*, 2005), whereas the T790M mutation contributes to the development of resistance to these drugs (Kobayashi *et al*, 2005; Pao *et al*, 2005). Our flow cytometric analysis demonstrated that H292 and Ma-1 cells express high and moderate levels of EGFR on the cell surface, respectively, whereas H460, H1299, and H1975 cells showed a low level of surface EGFR expression (Figure 1).

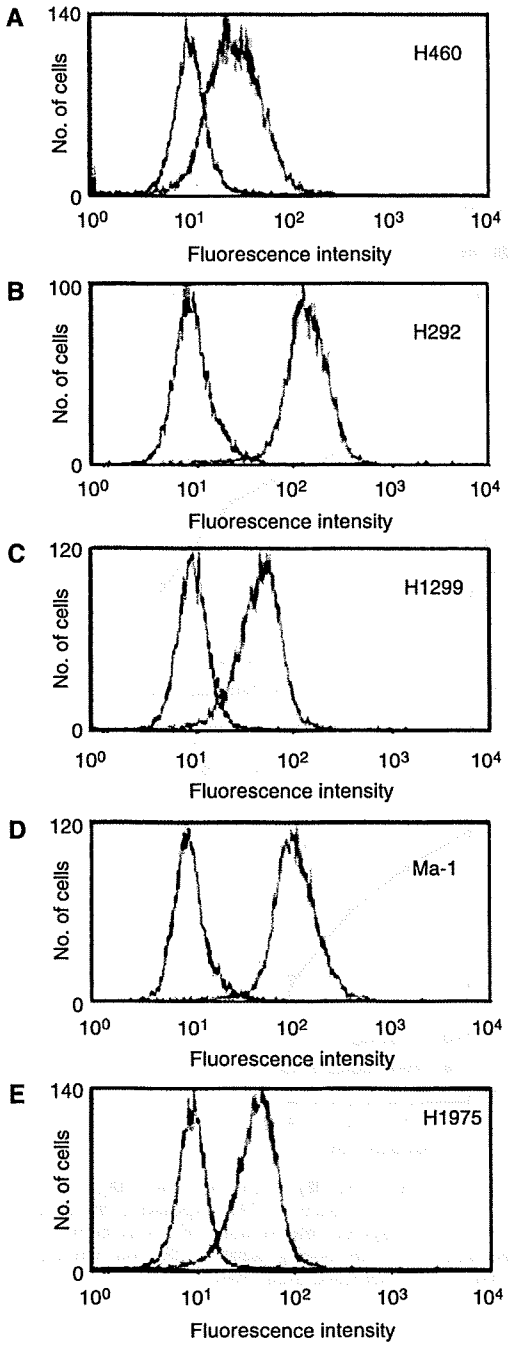

Figure 1 Expression of EGFR on the surface of NSCLC cells. Surface expression of EGFR on H460 (**A**), H292 (**B**), H1299 (**C**), Ma-1 (**D**), and H1975 (**E**) cells was determined by flow cytometry. Representative histograms of cells stained with an anti-EGFR mAb (red peak) or with an isotype-matched control mAb (black peak) are shown.

Effect of nimotuzumab on EGFR phosphorylation

Next, we determined whether nimotuzumab inhibits ligand-induced EGFR phosphorylation in the five NSCLC cell lines. The cells were deprived of serum overnight, exposed to various concentrations of nimotuzumab, or to gefitinib, for 15 min, and then stimulated with EGF for 15 min. In the NSCLC cells that harbour wild-type *EGFR* (H460, H292, and H1299), phosphorylation of EGFR was undetectable in the absence of EGF, but was markedly induced on exposure of the cells to this growth factor. The EGF-induced phosphorylation of EGFR in these cells was completely inhibited by the EGFR-TKI gefitinib. Nimotuzumab also inhibited the EGF-induced EGFR phosphorylation in a concentration-dependent manner in H292 cells (which have a high level of surface EGFR expression), whereas it did not substantially affect such phosphorylation in H460 or H1299 cells (both of which have a low level of surface EGFR expression) (Figure 2A–C). We previously showed that the basal level of EGFR phosphorylation was increased in the *EGFR* mutant NSCLC cell lines Ma-1 and H1975, indicative of constitutive activation of the EGFR tyrosine kinase (Okabe *et al*, 2007). The phosphorylation of EGFR in EGF-treated Ma-1 cells (which have a moderate level of surface EGFR expression) was inhibited by gefitinib as well as by nimotuzumab in a concentration-dependent manner (Figure 2D). In contrast, the constitutive activation of EGFR in H1975 cells (which have a low level of surface EGFR expression) was inhibited partially by gefitinib but was unaffected by nimotuzumab (Figure 2E). These results suggested that the inhibition of EGFR phosphorylation by nimotuzumab may be related to the surface expression level of EGFR rather than to the mutational status of *EGFR*.

Augmentation of the cytotoxic effect of radiation in NSCLC cells by nimotuzumab *in vitro*

We examined whether nimotuzumab might enhance the anticancer effect of γ-radiation in the five NSCLC cell lines with the use of a clonogenic assay. Tumour cells were incubated with or without nimotuzumab for 24 h, exposed to various doses of γ-radiation, and then allowed to form colonies in drug-free medium for 10–14 days. Survival curves revealed that, whereas nimotuzumab had no effect on the radiation sensitivity of H460, H1299, or H1975 cells, it enhanced the cytotoxic effect of radiation in H292 and Ma-1 cells, with a dose enhancement factor of 1.5 and 1.3, respectively (Figure 3). These results showed that nimotuzumab increased the radiosensitivity of the NSCLC cell lines with high or moderate levels of surface EGFR expression, consistent with the inhibitory effects of this antibody on EGFR signalling.

Augmentation of the antitumor effect of radiation in NSCLC cells by nimotuzumab *in vivo*

To determine whether the nimotuzumab-induced potentiation of the response of NSCLC cells to radiation observed *in vitro* might also be apparent *in vivo*, we injected three of the cell lines into nude mice to elicit the formation of solid tumours. The mice were then treated with nimotuzumab, radiation, or both modalities. In the H460 xenograft model, tumour growth was inhibited by radiation alone but not by nimotuzumab alone, and the effect of radiation was not promoted by nimotuzumab (Figure 4A). In contrast, radiation and nimotuzumab each inhibited the growth of tumours formed by H292 (Figure 4B) or Ma-1 (Figure 4C) cells during the first few weeks after treatment. Thereafter, the rate of

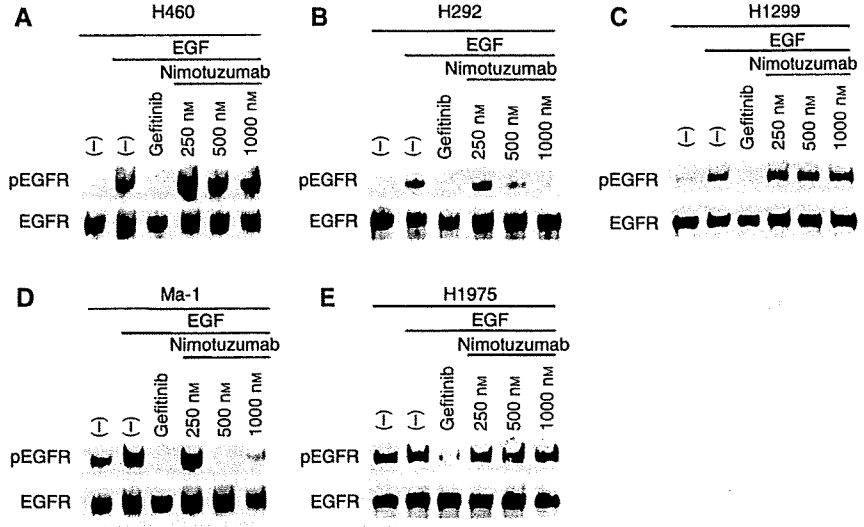

Figure 2 Effect of nimotuzumab on EGFR phosphorylation in NSCLC cells. H460 (**A**), H292 (**B**), H1299 (**C**), Ma-1 (**D**), and H1975 (**E**) cells were deprived of serum overnight and then incubated first for 15 min in the absence or presence of the indicated concentrations of nimotuzumab or gefitinib (10 μM) and then for an additional 15 min in the additional absence or presence of EGF (100 ng ml^{-1}). Cell lysates were then subjected to immunoblot analysis with antibodies to the Tyr1068-phosphorylated form of EGFR (pEGFR) as well as with those to total EGFR.

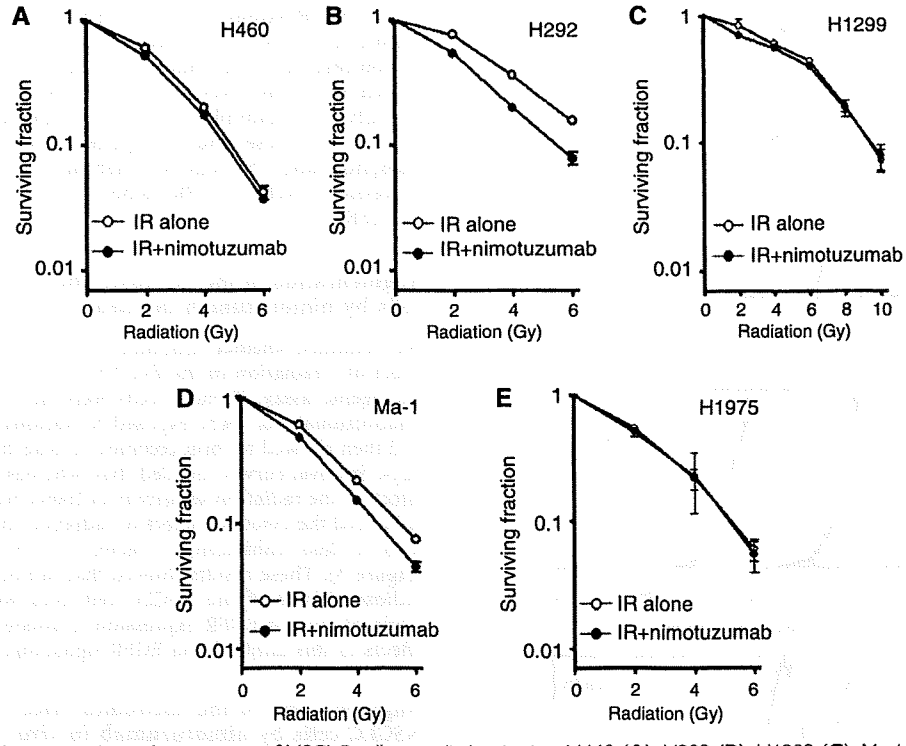

Figure 3 Effect of nimotuzumab on the response of NSCLC cells to radiation *in vitro*. H460 (**A**), H292 (**B**), H1299 (**C**), Ma-1 (**D**), and H1975 (**E**) cells were incubated with or without 700 nM nimotuzumab in medium supplemented with 1% fetal bovine serum for 24 h, exposed to the indicated doses of γ-radiation, and then incubated in drug-free medium supplemented with 10% serum for 10–14 days for determination of colony-forming ability. Survival curves were generated after correction of colony formation observed for combined treatment with ionising radiation (IR) and nimotuzumab by that apparent for treatment with nimotuzumab alone. Data are means ± s.d. of triplicates from a representative experiment.

tumour growth increased to a value similar to that seen in control animals. Combined treatment with radiation and nimotuzumab resulted in a substantial delay in tumour growth and subsequent inhibition of the growth rate of H292 and Ma-1 xenografts. The growth delay after treatment with nimotuzumab alone, radiation alone, or both nimotuzumab and radiation was thus 27.2, 19.6, and 53.6 days, respectively, for H292 cells and 26.7, 13.0, and 78.3 days, respectively, for Ma-1 cells (Table 2). The enhancement factor for the effect of nimotuzumab on the efficacy of radiation was 1.3 for H292 cells and 4.0 for Ma-1 cells, revealing the effect to be more

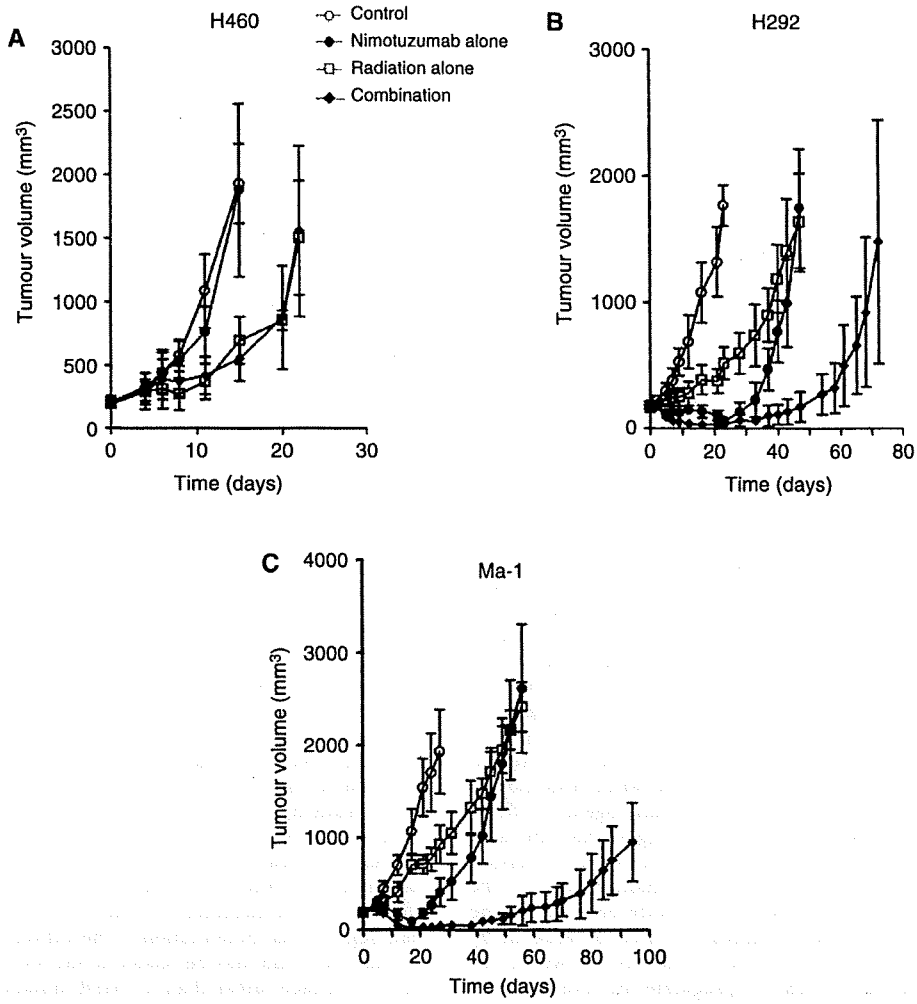

Figure 4 Effect of nimotuzumab on the response of NSCLC cells to radiation *in vivo*. H460 (**A**), H292 (**B**), or Ma-1 (**C**) cells were injected subcutaneously in athymic nude mice. Treatment was initiated when tumours in each group achieved an average volume of approximately 170–200 mm^3. Mice were treated with a single dose of nimotuzumab (1.0 mg per mouse) intraperitoneally, a single dose of γ-radiation (10 Gy), or neither (control) or both modalities, and tumour volume was determined at the indicated time points thereafter. Data are means ± s.d. for seven to eight mice per group.

Table 2 Tumour growth delay in nude mice treated with nimotuzumab, radiation, or both modalities

	H460		H292		Ma-1	
Treatment	**Days[a]**	**GD[b]**	**Days**	**GD**	**Days**	**GD**
Control	10.4		13.2		15.1	
Nimotuzumab alone	11.8	1.4	40.4	27.2	41.8	26.7
Radiation alone	20.4	10.0	32.8	19.6	28.1	13.0
Nimotuzumab+radiation	20.5	10.1	66.8	53.6	93.4	78.3
Enhancement factor	0.86		1.3		4.0	

GD = growth delay [a]Time required for xenografts in each group to achieve a fivefold increase in volume. [b]The additional time (days) required for xenografts in each treatment group to achieve a fivefold increase in volume relative to the corresponding time for xenografts in the control group.

than additive. No pronounced tissue damage or toxicities such as diarrhoea or a decrease in body weight of >10% were observed in mice in any of the four treatment groups. These results thus suggested that nimotuzumab potentiated the antitumor activity of radiation in H292 and Ma-1 cells *in vivo* as well as *in vitro*.

DISCUSSION

Somatic mutations in the EGFR kinase domain and *EGFR* amplification have been associated with a better response to EGFR-TKIs, such as gefitinib and erlotinib, in patients with NSCLC (Lynch *et al*, 2004; Paez *et al*, 2004; Pao *et al*, 2004; Cappuzzo *et al*, 2005; Mitsudomi *et al*, 2005; Takano *et al*, 2005). Given that little is known of the relation between such *EGFR* alterations and the response to treatment with anti-EGFR mAbs, we investigated the antitumor effect of combined treatment with the anti-EGFR mAb nimotuzumab and radiation in NSCLC cell lines of differing *EGFR* status.

The antitumor effect of EGFR-specific mAbs has been thought to result from inhibition of ligand binding to EGFR and consequent inhibition of EGFR activation (Li *et al*, 2005; Marshall, 2006). We, therefore, examined the effect of nimotuzumab on EGF-dependent EGFR signalling. Nimotuzumab inhibited the EGF-induced or constitutive phosphorylation of EGFR in H292 and Ma-1 cells (with high and moderate levels of surface EGFR expression, respectively), consistent with the mode of action of this antibody. However, nimotuzumab did not block EGF-induced or constitutive EGFR phosphorylation in H460, H1299, or H1975 cells (all with a

low level of surface EGFR expression). These observations suggest that the inhibitory effect of nimotuzumab on EGFR signalling depends on the expression level of EGFR on the cell surface. A clonogenic cell survival assay revealed that nimotuzumab enhanced the cytotoxic effect of radiation in H292 and Ma-1 cells, but not that in H460, H1299, or H1975 cells. These findings support the notion that the inhibition of EGFR signalling by nimotuzumab is responsible, at least in part, for the enhancement of the cytotoxic effect of radiation by this antibody. Irradiation of tumour cells has been shown to activate EGFR via ligand-independent and ligand-dependent mechanisms, possibly accounting for radiation-induced acceleration of tumour cell repopulation and the development of radioresistance (Schmidt-Ullrich *et al*, 1997, 2003; Dent *et al*, 2003). Such radiation-induced activation of EGFR-dependent processes may represent a rationale for combined treatment with radiation and EGFR inhibitors. It remains to be determined whether nimotuzumab is able to block radiation-induced activation of EGFR.

Consistent with our *in vitro* results, we found that nimotuzumab enhanced the antitumor effect of radiation on H292 or Ma-1 cells in nude mice. Such enhancement was not apparent for tumours formed by H460 cells. Nimotuzumab alone also manifested a substantial antitumor effect for xenografts formed by H292 or Ma-1 cells but not for those formed by H460 cells. Together these results suggest that the efficacy of nimotuzumab monotherapy is a prerequisite for augmentation of radioresponse by this mAb. Nimotuzumab was previously shown to induce the regression of A431 tumour xenografts *in vivo* as a result of inhibition of both tumour cell proliferation and tumour angiogenesis (Crombet-Ramos *et al*, 2002). Immunohistochemical analysis of tumour specimens from head and neck cancer patients treated with the combination of nimotuzumab and radiation also showed evidence of antiproliferative and antiangiogenic effects (Crombet *et al*, 2004). These observations suggest that effects of nimotuzumab on both NSCLC cell proliferation and tumour angiogenesis might contribute to the enhancement of the antitumor efficacy of radiation by this antibody observed in the present study. Enhancement of the anticancer effect of radiation by the anti-EGFR mAb cetuximab was previously shown to be increased by transfection of cells to upregulate the level of EGFR expression, suggesting that potentiation of the antitumor efficacy of radiation by anti-EGFR mAbs is related to the absolute level of EGFR expression (Liang *et al*, 2003; Bonner *et al*, 2004). This finding is consistent with our present results showing that potentiation of the antitumor activity of radiation by nimotuzumab was related to the level of surface EGFR expression. The nimotuzumab-resistant cell line H460 harbours a mutant form of *KRAS* (Balko *et al*, 2006) that has been associated with resistance to cetuximab (Lievre *et al*, 2006). However, we found that nimotuzumab also failed to inhibit EGF-induced EGFR phosphorylation and to enhance the cytotoxic effect of radiation in H1299 cells, which harbour wild-type *KRAS* (Coldren *et al*, 2006). These observations thus support the notion that a low level of EGFR expression at the cell surface is related to resistance to combined treatment with nimotuzumab and radiation, irrespective of *KRAS* status.

We demonstrated that nimotuzumab inhibited EGFR phosphorylation and enhanced the antitumor effect of radiation in *EGFR* mutant Ma-1 cells (with a moderate level of surface EGFR expression) but not in *EGFR*-mutant H1975 cells (with a low level of surface EGFR expression). Nimotuzumab also potentiated the cytotoxic effect of radiation in H292 cells, which harbour wild-type *EGFR* alleles and have a high level of surface EGFR expression. These findings support the notion that *EGFR* mutation is not the major determining factor for enhancement of the antitumor effect of radiation by nimotuzumab, consistent with previous observations with cetuximab (Barber *et al*, 2004; Tsuchihashi *et al*, 2005). However, the mechanisms underlying such enhancement of the antitumor effect of radiation may differ between NSCLC cells harbouring wild-type or mutant *EGFR* alleles. We and others have previously shown that mutations in the tyrosine kinase domain of EGFR are associated with increased ligand-independent tyrosine kinase activity of EGFR (Lynch *et al*, 2004) and aberrant EGFR signalling (Amann *et al*, 2005; Okabe *et al*, 2007). Given that cell-cycle checkpoints activated by ionising radiation are defective in *EGFR*-mutant NSCLC cell lines (Das *et al*, 2006), the constitutive activity of EGFR in such cells may result in unchecked DNA synthesis and in apoptosis on exposure to ionising radiation. It is possible that these defects in *EGFR*-mutant cells affect the enhancement of the antitumor efficacy of radiation by nimotuzumab.

In summary, we have shown that nimotuzumab enhanced the antitumor efficacy of radiation *in vitro* and *in vivo*, providing a rationale for future clinical investigations of the therapeutic efficacy of nimotuzumab in combination with radiotherapy. Our data suggest that potentiation of the antitumor activity of radiation by nimotuzumab may be related to the level of EGFR expression at the cell surface rather than to *EGFR* mutation. The preselection of patients on the basis of genetic factors that predict treatment sensitivity or resistance may thus be required for the combination therapy with nimotuzumab and radiation.

ACKNOWLEDGEMENTS

We thank S Ono for technical assistance.

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Int. J. Cancer: **122,** 1530–1538 (2008)

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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Received 7 June 2007; Accepted after revision 26 September 2007
DOI 10.1002/ijc.23253
Published online 21 November 2007 in Wiley InterScience (www.interscience.wiley.com).

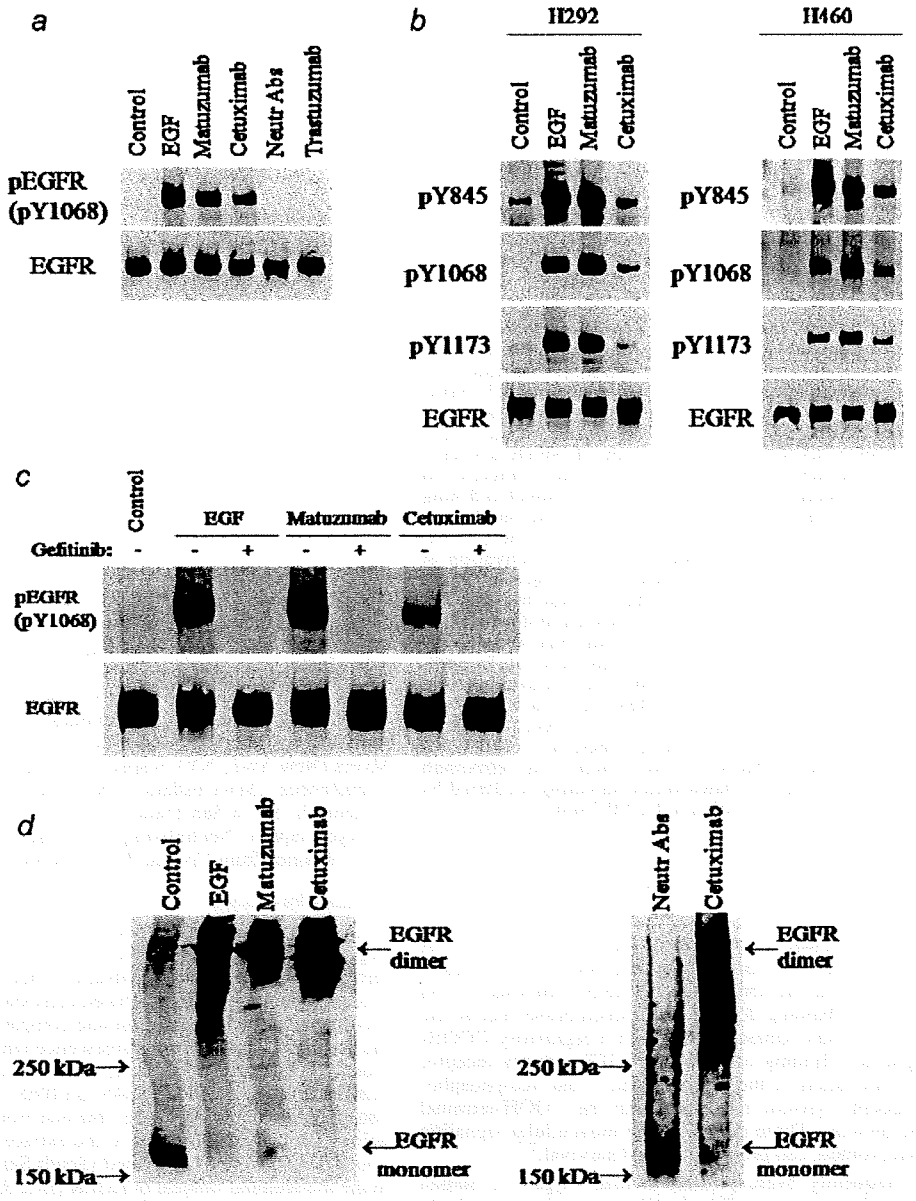

FIGURE 1 – EGFR phosphorylation induced by matuzumab or cetuximab as a result of receptor dimerization and activation of the receptor tyrosine kinase. (*a*) H292 cells were deprived of serum overnight and then incubated for 15 min in the absence (Control) or presence of matuzumab (200 nM), cetuximab (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.

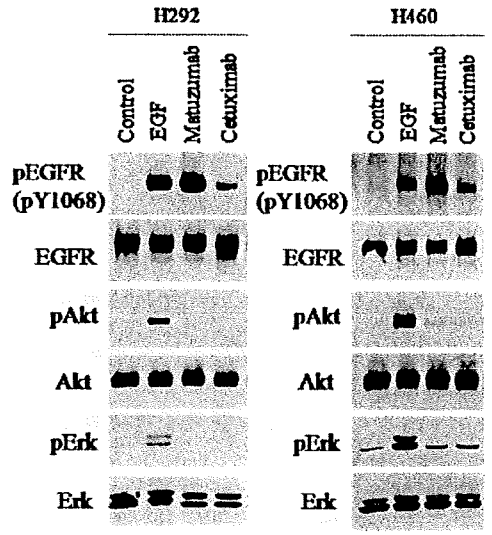

FIGURE 2 – Failure of matuzumab or cetuximab to activate Akt or Erk. H292 or H460 cells were deprived of serum overnight and then incubated for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or EGF (100 ng/ml). Cell lysates were subjected to immunoblot analysis with antibodies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

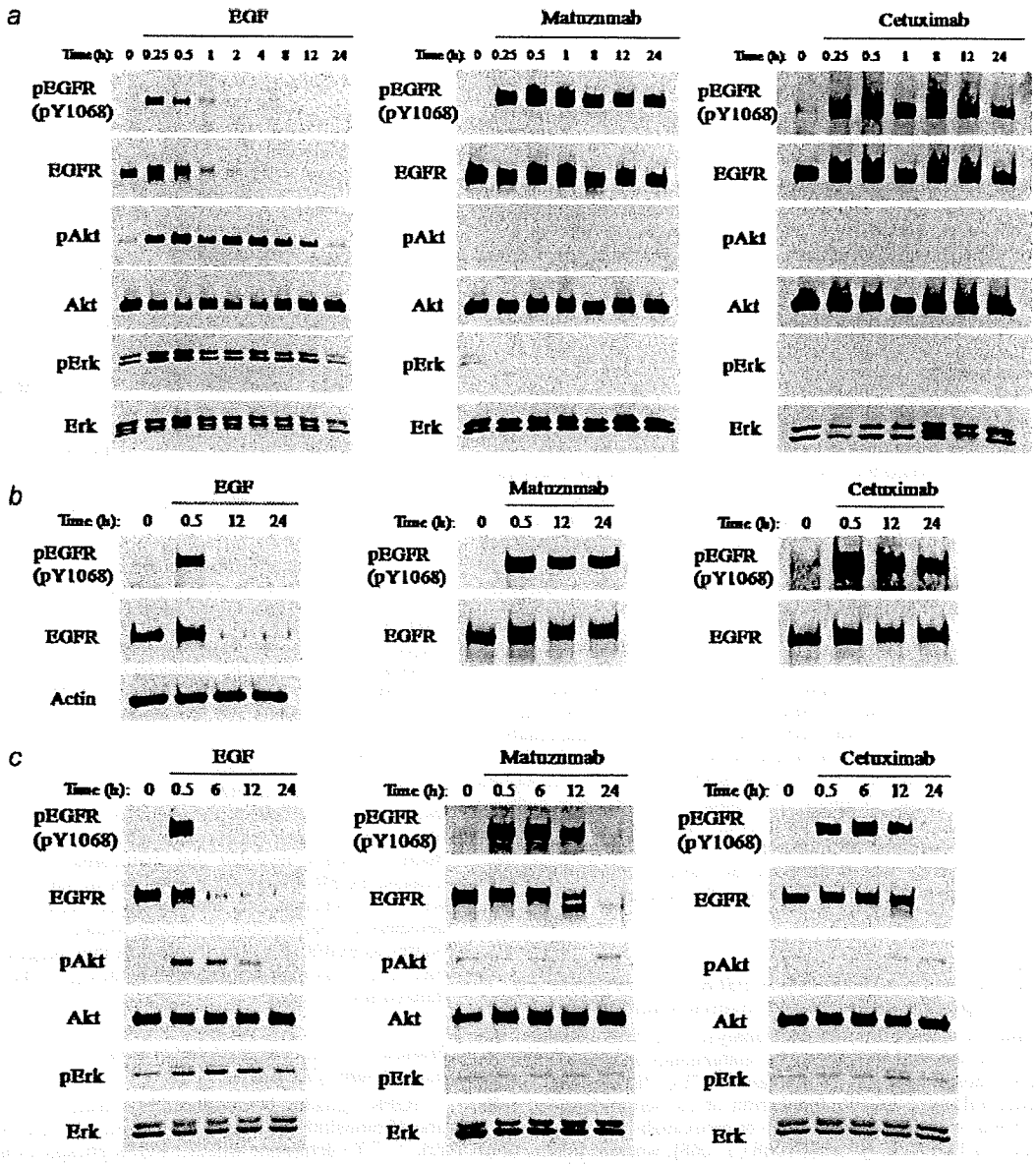

FIGURE 3 – Lack of EGFR turnover in cells treated with matuzumab or cetuximab. (*a*) H292 cells were deprived of serum overnight and then incubated for the indicated times in the presence of EGF (100 ng/ml), matuzumab (200 nM) or cetuximab (100 nM), respectively. Cell lysates were subjected to immunoblot 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

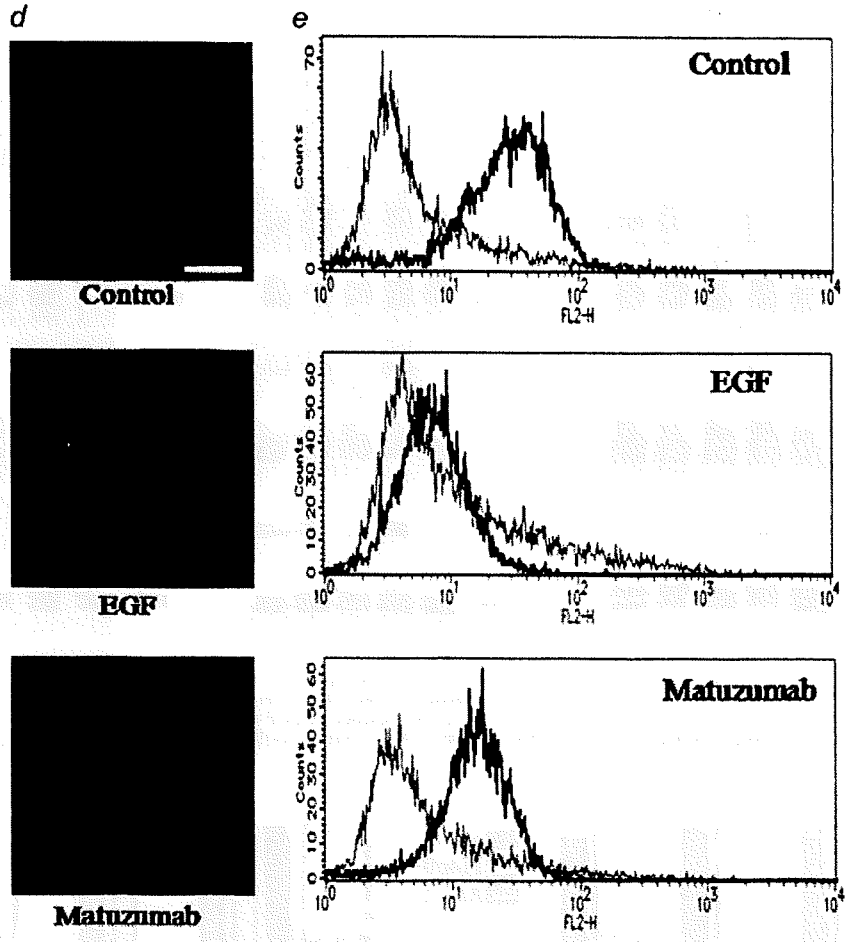

FIGURE 3 – CONTINUED

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

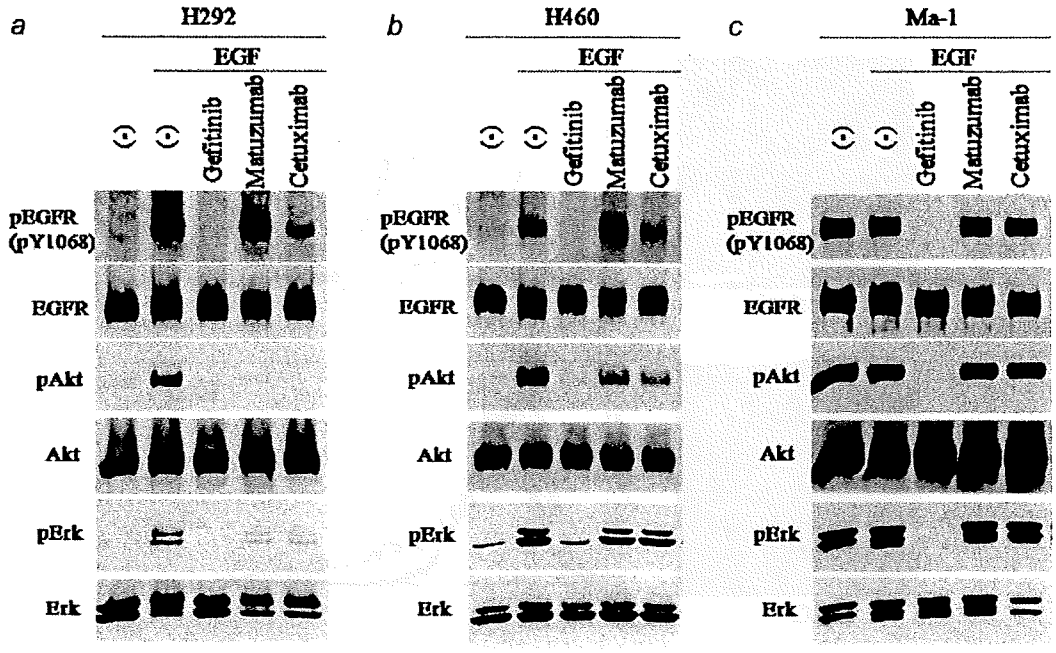

FIGURE 4 – Effects of matuzumab and cetuximab on EGF-induced EGFR signaling. H292 (*a*), H460 (*b*) and Ma-1 (*c*) cells were deprived of serum overnight and then incubated first for 15 min in the absence or presence of matuzumab (200 nM), cetuximab (100 nM) or gefitinib (10 μM) and then for an additional 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.

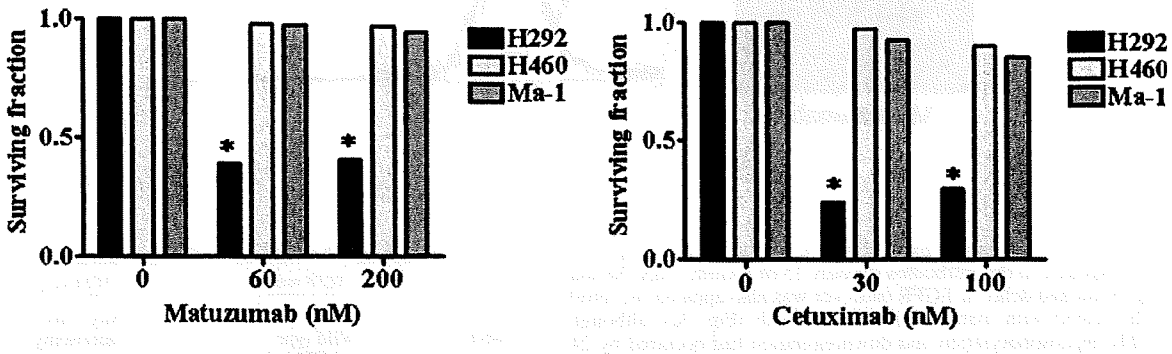

FIGURE 5 – Effects of matuzumab and cetuximab on cell survival. H292, H460 or Ma-1 cells were plated at a density of 200 cells per 25-cm^2 flask in triplicate and cultured for 7 days in the presence of the indicated concentrations of matuzumab or cetuximab. They were then incubated with medium alone 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.

Acknowledgements

The authors thank Ms. Erina Hatashita and Ms. Yuki Yamada for technical assistance.

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Differential Constitutive Activation of the Epidermal Growth Factor Receptor in Non–Small Cell Lung Cancer Cells Bearing *EGFR* Gene Mutation and Amplification

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Abstract

The identification of somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) in patients with non–small cell lung cancer (NSCLC) and the association of such mutations with the clinical response to EGFR tyrosine kinase inhibitors (TKI), such as gefitinib and erlotinib, have had a substantial effect on the treatment of this disease. *EGFR* gene amplification has also been associated with an increased therapeutic response to EGFR-TKIs. The effects of these two types of *EGFR* alteration on EGFR function have remained unclear, however. We have now examined 16 NSCLC cell lines, including eight newly established lines from Japanese NSCLC patients, for the presence of *EGFR* mutations and amplification. Four of the six cell lines that harbor *EGFR* mutations were found to be positive for *EGFR* amplification, whereas none of the 10 cell lines negative for *EGFR* mutation manifested *EGFR* amplification, suggesting that these two types of *EGFR* alteration are closely associated. Endogenous EGFRs expressed in NSCLC cell lines positive for both *EGFR* mutation and amplification were found to be constitutively activated as a result of ligand-independent dimerization. Furthermore, the patterns of both *EGFR* amplification and EGFR autophosphorylation were shown to differ between cell lines harboring the two most common types of *EGFR* mutation (exon 19 deletion and L858R point mutation in exon 21). These results reveal distinct biochemical properties of endogenous mutant forms of EGFR expressed in NSCLC cell lines and may have implications for treatment of this condition. [Cancer Res 2007;67(5):2046–53]

Introduction

The epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein with an extracellular ligand binding domain, a transmembrane region, and a cytoplasmic tyrosine kinase domain and is encoded by a gene (*EGFR*) located at human chromosomal region 7p12 (1–3). The binding of ligand to EGFR induces receptor dimerization and consequent conformational changes that result in activation of the intrinsic tyrosine kinase, receptor autophosphorylation, and activation of a signaling cascade (4, 5). Aberrant signaling by EGFR plays an important role in cancer development and progression (3).

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doi:10.1158/0008-5472.CAN-06-3339

EGFR is frequently overexpressed in non–small cell lung cancer (NSCLC) and has been implicated in the pathogenesis of this disease (6, 7). Given the biological importance of EGFR signaling in cancer, several agents have been synthesized that inhibit the receptor tyrosine kinase activity. Two such inhibitors of the tyrosine kinase activity of EGFR (EGFR-TKI), gefitinib and erlotinib, both of which compete with ATP for binding to the tyrosine kinase pocket of the receptor, have been extensively studied in patients with NSCLC (8, 9). We and others have shown that a clinical response to these agents is more common in women than in men, in Japanese than in individuals from Europe or the United States, in patients with adenocarcinoma than in those with other histologic subtypes of cancer, and in patients who have never smoked than in those with a history of smoking (10–14). Mutations in the tyrosine kinase domain of EGFR have also been detected in a subset of lung cancer patients and shown to predict sensitivity to EGFR-TKIs (15–17). Indeed, the clinical characteristics of patients with known *EGFR* mutations are similar to those of other individuals most likely to respond to treatment with EGFR-TKIs (18–22). These mutations arise in the first four exons (exons 18–21) corresponding to the tyrosine kinase domain of EGFR, and they affect key amino acids surrounding the ATP-binding cleft (23, 24). In-frame deletions that eliminate four highly conserved amino acids (LREA) encoded by exon 19 are the most common type of *EGFR* mutation, with missense point mutations in exon 21 that result in a specific amino acid substitution at position 858 (L858R) being the second most common. In addition to *EGFR* mutations, other molecular changes may play a role in determining sensitivity to EGFR-TKIs (22, 25–28). NSCLC patients with an increased *EGFR* copy number, as revealed by fluorescence *in situ* hybridization (FISH), have thus been found to show an increased response rate to and prolonged survival after gefitinib therapy (22, 25–27).

Given that *EGFR* is mutated or amplified (or both) in NSCLC, it is important to determine the biological effects of such *EGFR* alterations on EGFR function (15, 29–32). Transient transfection of various cell types with vectors encoding wild-type or mutant versions of EGFR showed that the activation of mutant receptors by EGF is more pronounced and sustained than is that of the wild-type receptor (15, 30). However, detailed biochemical analysis of NSCLC cell lines with endogenous *EGFR* mutations has been limited. We have now identified *EGFR* mutations in three NSCLC cell lines newly established from Japanese patients. Furthermore, we have characterized a panel of 16 NSCLC cell lines for *EGFR* mutations and amplification and evaluated the relation between the presence of these two types of *EGFR* alteration and sensitivity to gefitinib. The effects of *EGFR* alterations on activation status of EGFR and on downstream signaling were also evaluated.