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Epidermal Growth Factor Receptor (EGFR) Antibody Down-regulates Mutant Receptors and Inhibits Tumors Expressing EGFR Mutations*[S]

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Activating mutations in the kinase domain of the EGF receptor have been reported in non-small cell lung cancer. The majority of tumors expressing these mutants are sensitive to ATP mimetics that inhibit the EGFR tyrosine kinase. The effect of antibodies that bind to the ectodomain of the receptor is less clear. We report herein the effects and mechanisms of action of the antibody cetuximab in lung cancer cells that naturally express receptor mutations and in ErbB-null 32D hematopoietic cells transfected with mutant EGFR. Treatment with cetuximab down-regulated EGFR levels and inhibited cell growth both *in vitro* and *in vivo*. This was associated with inhibition of ligand-independent EGFR signaling. These effects were seen in 32D cells arguing the growth inhibitory action was not because of the blockade of autocrine ligand action. Both antibody-induced EGFR down-regulation and inhibition of growth required receptor dimerization as monovalent Fab fragments only eliminated receptor levels or reduced cell proliferation in the presence of anti-human IgG. Finally, cetuximab inhibited growth of H1975 lung cancer cells and xenografts, which expressed L858R/T790M EGFR and were resistant to EGFR tyrosine kinase inhibitors. These data suggest that cetuximab is an effective therapy against mutant EGFR-expressing cancer cells and thus can be considered in combination with other anti-EGFR molecules.

The epidermal growth factor receptor (EGFR)[3] is a tyrosine kinase receptor that is abnormally amplified and/or activated in a variety of tumors (1, 2). Therefore, EGFR has been identified as an important target in cancer (3). Two main strategies have been developed to target EGFR: low molecular weight tyrosine kinase inhibitors (TKIs) and monoclonal antibodies against the extracellular domain of EGFR (3). TKIs compete with ATP for binding to the intracellular kinase domain, thus preventing receptor activation and engagement of downstream signaling transducers (3). The best characterized TKIs against EGFR are gefitinib (Iressa) and erlotinib (Tarceva). Several monoclonal antibodies have been developed; among them is the chimeric antibody cetuximab (Erbitux), which competes with receptor ligands for binding to EGFR (4–6). In addition to blocking ligand binding, the monoclonal antibody 225, equivalent to cetuximab, undergoes internalization and, in some cells, induces receptor degradation and down-regulation (7–9). Combination treatment of gefitinib and cetuximab has been shown to be synergistic both *in vivo* and *in vitro* against EGFR-dependent tumor cells (10, 11).

EGFR overexpression is found in 43–89% of non-small cell lung cancers (NSCLC; reviewed in Ref. 12); however, receptor levels are not effective predictors of response to EGFR TKIs (13–15). Three groups recently reported somatic mutations in the *EGFR* gene in NSCLC (16–18). The mutations are either short in-frame deletions or insertions or substitutions clustered around the region encoding the ATP-binding pocket of the tyrosine kinase domain of the receptor in exons 18–21. The two most common mutations are an in-frame deletion in exon 19, which eliminates a conserved LREA motif and an L858R substitution in exon 21 (19). *In vitro* studies with cells expressing most of these mutants have shown that they are exquisitely sensitive to either gefitinib or erlotinib (17, 20, 21). Further, the majority of patients with these tumors exhibit durable clinical responses to gefitinib and erlotinib suggesting that they are "gain-of-function" mutations, which represent a functional marker of EGFR dependence in NSCLC.

Although the effects of gefitinib and erlotinib against cells expressing these receptor mutants have been dramatic, less is known about the effects of cetuximab. Mokohara *et al.* (22) reported weaker inhibitory action of cetuximab compared with gefitinib in cells harboring EGFR kinase domain mutations. This was based on short-term assays using tumor cells in culture. Similar results were reported by Amann *et al.* (20) against HCC827 lung cancer cells. In this report, we have studied the effects and mechanisms of action of cetuximab in lung cancer cells that display the two most common mutations in EGFR:

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; sEGFR, soluble extracellular domain of EGFR; WT, wild-type; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; LMP, low melting point; BrdUrd, bromodeoxyuridine; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; NSCLC, non-small cell lung cancer; TGF-α, transforming growth factor-α; TKI, tyrosine kinase inhibitor; FITC, fluorescein isothiocyanate.

deletion of amino acids Glu-746 to Ala-750 in PC9 cells (23) and the L858R point mutation in H3255 cells (21) as well as in 32D cells stably transfected with an exon 19 deletion mutant. Treatment with cetuximab down-regulated EGFR levels and inhibited cell growth both *in vitro* and *in vivo*. This was associated with inhibition of ligand-independent EGFR signaling. These effects were also seen in ErbB-null 32D cells. When transfected with wild-type EGFR, these cells still require addition of receptor ligands to grow (24–26), suggesting that in our study the growth inhibitory action of cetuximab was not because of blockade of autocrine ligands. Finally, cetuximab also inhibited growth of H1975 lung cancer cells, which express L858R/T790M EGFR and are resistant to EGFR TKIs (27). Taken together, these data suggest that cetuximab is an effective therapy against mutant EGFR-expressing cancer cells and, thus, can be considered in combination with other anti-EGFR molecules to maximize the efficacy of receptor-targeted therapy and/or the emergence of therapeutic resistance.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—PC9 cells were a gift from Kazuto Nishio (Japan). H3255 cells were a gift from Bruce Johnson (Dana-Farber Cancer Institute), and H1975 cells were from Adi Gazdar (University of Texas Southwestern Medical Center). A431 and 32D cells were obtained from American Tissue Culture Collection (Manassas, VA). PC9 and H3255 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS); H1975 were grown in RPMI 1640 medium with 5% FBS. 32D cells were grown in RPMI 1640 medium supplemented with 15% FBS and 5% conditioned media from WEHI-3B cells (as a source of interleukin-3). A431 cells were grown in improved minimum Eagle's medium Zn^{2+} option (Richter's modification) (Invitrogen) supplemented with 10% FBS and 0.2% phenol red.

Kinase Inhibitors and Antibodies—Gefitinib and erlotinib were provided by Alan Wakeling (AstraZeneca Pharmaceuticals) and Mark Sliwkowski (Genentech), respectively. Cetuximab as well as its monovalent (Fab) and divalent $F(ab')_2$ fragments were provided by Dan Hicklin (Imclone Systems, Inc.). We utilized the following antibodies: Y1068 P-EGFR, Y1173 P-EGFR, Akt, Ser-473 P-Akt, MAPK, and caspase-3 (Cell Signaling, Beverly, MA); total EGFR Ab-12 (Neomarkers, Fremont, CA); and P-MAPK (Promega, Madison, WI). Horseradish peroxidase-linked rabbit IgG and horseradish peroxidase-linked mouse IgG secondary antibodies were from Amersham Biosciences. Unconjugated anti-human IgG (H + L) was from Vector Laboratories (Burlingame, CA).

Plasmids and Mutagenesis—A wild-type EGFR cDNA sequence (GenBank™ accession no. NM_005228) was introduced into the pcDNA3.1 vector (Invitrogen) to add myc and His tags at the C terminus. To generate the deletion747–753insS EGFR mutant (del747–753insS), nucleotides 2240–2257 were removed from the EGFR cDNA using a PCR-based approach. Mutation was confirmed by sequencing; then the WT EGFR and the del747–753insS mutant were cut from pcDNA3.1 with PmeI and inserted in the pMSCVpuro. IRES.EGFP retrovirus vector as described (28).

Retroviral Infection—Retroviral supernatant for each vector was produced as described previously (29). 32D cells were resuspended in 1 ml of corresponding viral supernatant and 4 μg/ml Polybrene followed by overnight incubation. Viral supernatant was replaced with fresh medium, and cells were cultured for 48 h before sorting and pooling all green fluorescent protein-positive cells. EGFR levels were confirmed by Western blot and also by using a FACSCalibur flow cytometer (BD Biosciences).

Soft Agar Colony Formation Assay—3×10^4 cells were mixed with LMP agarose and overlaid on top of a 0.8% LMP agarose layer (in 35-mm dishes) containing corresponding EGFR inhibitors. Dishes were incubated in 5% CO_2 at 37 °C for 10–14 days. After treatment, pictures of representative fields were taken and colonies measuring $\geq$50 μm in diameter counted using the OMNICON tumor colony analyzer (BioLogics Inc., Manassas, VA).

Cell Proliferation and Apoptosis—Cells were seeded in complete medium in 12-well plates at a density of 3×10^4 cells/well in triplicate and then treated with gefitinib, erloninib, or cetuximab for 72 h. Cell numbers were measured with a Zeiss Coulter counter (Beckman Coulter, Miami, FL). To measure apoptosis, cells were treated with different concentrations of gefitinib, erloninib, or cetuximab. Both adherent and floating cells were harvested 72 h later and subjected to Western blot analysis to detect caspase-3 cleavage using an antibody from Cell Signaling.

Western Blot Analysis—Cells were treated with different concentrations of gefitinib, erloninib, or cetuximab for 24 h, lysed, and samples were analyzed by Western blot procedure as described previously (30). Immunoreactive bands were detected using horseradish peroxidase-conjugated secondary antibodies followed by SuperSignal Pico chemiluminescent substrate (Pierce).

Flow Cytometry Analysis of Cetuximab Binding to EGFR in PC9, H3255, and A431 Cells—To determine cetuximab binding to the EGFR mutants, we followed the same procedure described by Molder *et al.* (31). Instead of monoclonal antibody 528, we used 10 μg/ml cetuximab and 10 μg/ml nonspecific human IgG (Vector Labs, Burlingame, CA).

Competitive Binding Assay—Cells were grown to confluence, then washed twice with cold serum-free medium, and subsequently incubated with 1 ng/ml ^{125}I-TGF-α ± increasing concentrations of cetuximab for 3 h at 4 °C. Cells were solubilized with 0.5 N NaOH, and cpm were measured. For standardization purposes we obtained cell counts from unlabeled wells that were handled similarly to wells containing ^{125}I-TGF-α-labeled cells. Percent binding = (cpm sample/cpm control (no cetuximab)) x 100; a best curve fit was generated with GraphPad Prism 4 software, and the EC_{50} was calculated using a one-site competition equation: Y = bottom + (top-bottom)/1 + 10^(X-logEC_{50}), where X = log(concentration) and Y = binding.

Studies with PC9 and H1975 Xenografts—PC9 and H1975 cells (10^7) were injected subcutaneously in the right flank of six-week-old female athymic nude mice (Harlan Sprague-Dawley, Indianapolis, IN). Tumors were measured twice a week with calipers, and tumor volume in mm^3 was calculated according to the formula: $((\text{width})^2 \times (\text{height}))/2$. Treatment was

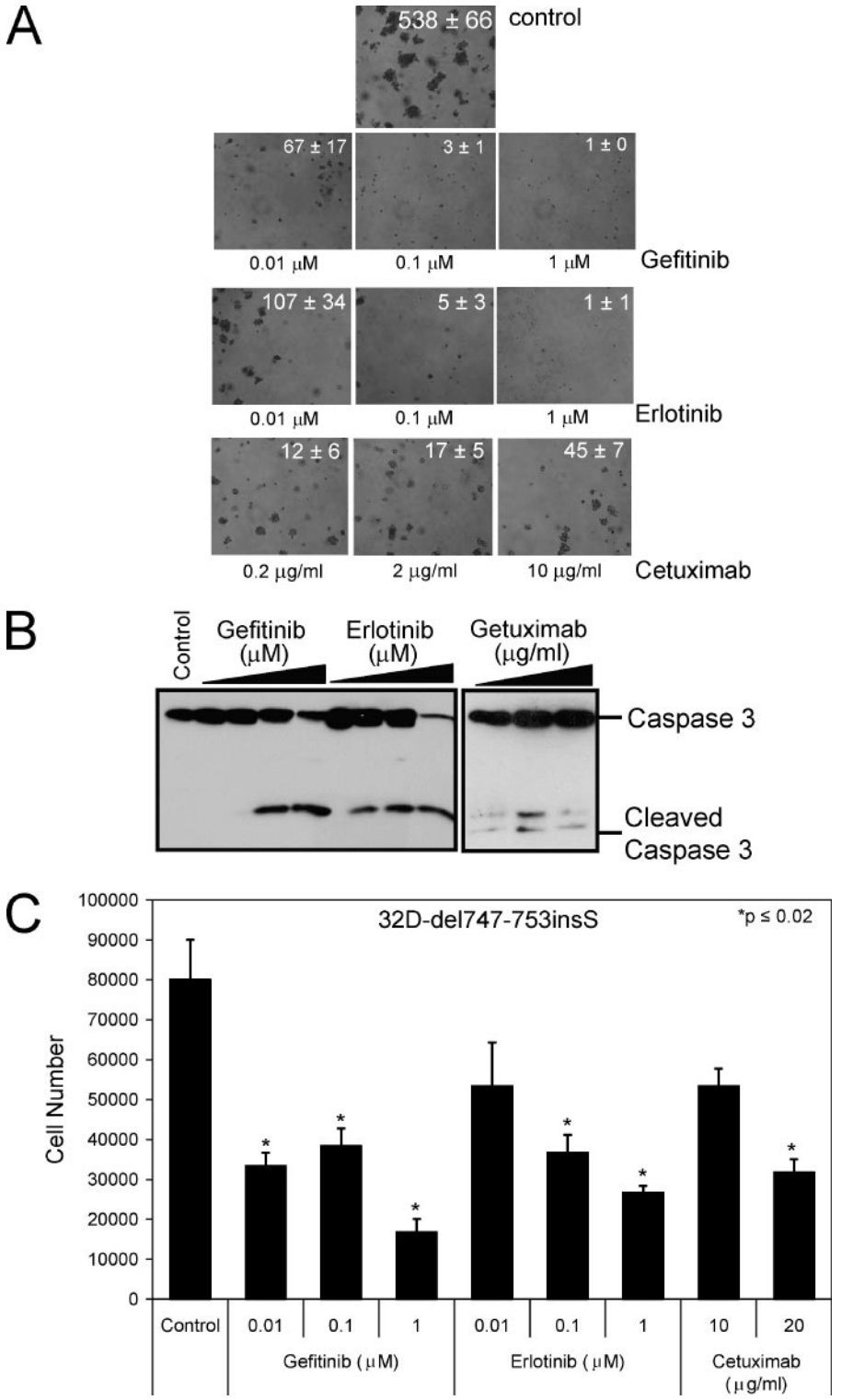


FIGURE 1. **EGFR antibody inhibits viability of EGFR mutant NSCLC.** *A*, PC9 cells (3×10^4 cells/plate) were mixed with LMP agarose, overlaid on top of a 0.8% LMP agarose layer, and then treated with gefitinib, erloninib, or cetuximab. 10 days later, pictures of representative fields (4×) were taken, and colonies measuring ≥50 μm in diameter were counted. Numbers in the right upper corners represent the average colony number ± S.E. of triplicate dishes. *B*, PC9 cells were treated with increasing concentrations of gefitinib, erlotinib, or cetuximab. After 72 h, floating and adherent cells were harvested and lysed. Cell lysates were subjected to immunoblot analysis for cleaved caspase-3. *C*, 32D-del747–753insS cells were seeded at a cell density of 3×10^4 cells/well in 12-well plates in the presence of 1% FBS and treated for 72 h with the indicated inhibitors. Cells were harvested, diluted (1:2) in trypan blue, and counted in a hemocytometer. Numbers represent average ± S.E. ($n = 3$). Student's *t* test was used for statistical comparisons.

started when tumors reached an average ≥200 mm^3 (day 27 and 15 post-injection of PC9 and H1975 cells, respectively). Mice with PC9 tumors were randomly assigned to one of the following treatment groups: gefitinib 200 mg/kg daily by oral gavage 5 days a week, 1 mg of cetuximab intraperitoneally twice a week, gefitinib + cetuximab at the same concentrations as above, and no treatment. On the third day of treatment, three mice per group were injected intraperitoneally with 0.3 ml BrdUrd (10 mg/ml in phosphate-buffered saline) 2.5 h before sacrifice. Tumors were collected, fixed in 10% formalin, and embedded in paraffin. Five μm sections from the paraffin blocks were stained for: 1) BrdUrd, according to the instructions in the BrdUrd staining kit of the Zymed Laboratories Inc.; and 2) TUNEL, according to instructions in the ApopTag peroxidase *in situ* apoptosis detection kit (Chemicon International, Temecula, CA). The percent of BrdUrd- and TUNEL-positive cells was determined by counting 10 random high power (400×) fields. Tumor lysates were prepared by grinding the tumors in TNE buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 1× protease inhibitor mixture), then a final concentration of 1% Nonidet P-40 was added, total protein was determined, and detection of EGFR levels was assessed by Western blot. Treatment was stopped on day 28. Tumor recurrences were retreated with the same original treatment. Mice with H1975 tumors were randomized to cetuximab *versus* phosphate-buffered saline only.

RESULTS

EGFR Antibody Inhibits Viability of EGFR Mutant NSCLC—We initially examined the effect of the EGFR antibody cetuximab and the TKIs gefitinib and erlotinib on anchorage-independent growth of PC9 cells. PC9 cells contain a del746–750 in exon 19 of the EGFR (32). Treatment with each inhibitor

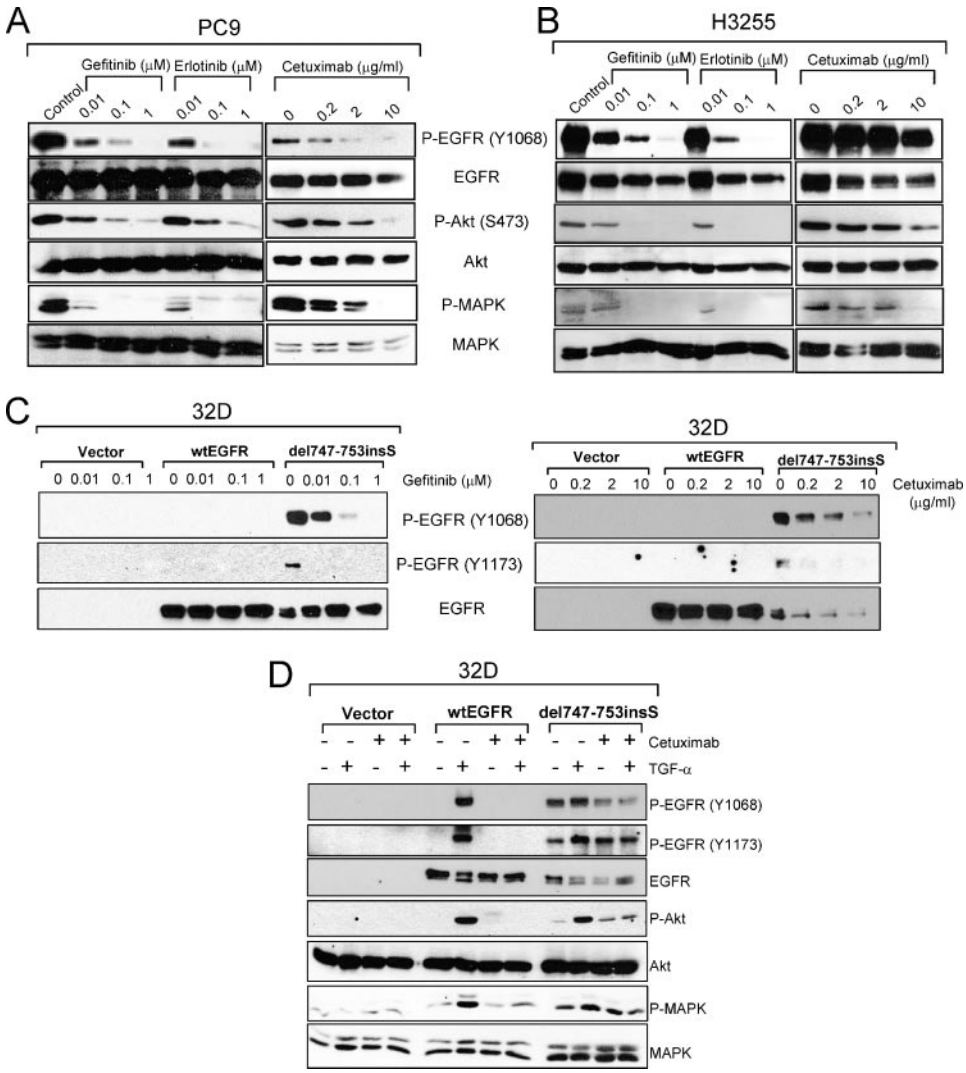


FIGURE 2. **EGFR antibody blocks receptor signaling.** PC9 (*A*) and H3255 (*B*) cells were treated with gefitinib, erlotinib, or cetuximab for 24 h. After treatment, cells were lysed; proteins were resolved by 8% SDS-PAGE followed by immunoblot analysis to detect phosphorylated and total EGFR, Akt, and MAPK. *C*, 32D cells expressing vector, WT EGFR, or the del747–753insS mutant were treated with gefitinib or cetuximab for 24 h; afterwards, cells were lysed and phosphorylated, and total EGFR levels were determined by Western blot. *D*, the indicated 32D cells were serum starved and then treated with cetuximab for 4 h followed by stimulation with TGF-α for 15 min. Phosphorylated and total EGFR, Akt, and MAPK levels were assessed by Western blot.

markedly inhibited PC9 colony formation in soft agar with an IC_{50} lower than 0.01 μM for the small molecules and <0.2 μg/ml for cetuximab (Fig. 1*A*). In addition, treatment with gefitinib and erlotinib induced apoptosis in PC9 cells, as shown by caspase-3 cleavage (Fig. 1*B*). Cetuximab also induced caspase-3 cleavage in PC9 cells, albeit with lower efficacy. It is worth noting that we detected both p20 and p17 caspase-3 cleaved species in the cetuximab treated cells but only the p20 form in the TKI treated cells, the basis for this apparent difference is unknown.

To confirm our results, we stably transfected wild-type EGFR or the mutant del747–753insS into murine hematopoietic 32D cells, which do not express EGFR or any other member of the ErbB receptor family, are dependent on interleukin-3 to grow, and are unresponsive to EGFR ligands (24–26). 32D cells expressing the del747–753insS EGFR mutant survived under low serum conditions (1% FBS), whereas cells transfected with WT EGFR did not (supplemental Fig. 1). Cells expressing the mutation proliferated in the absence of interleukin-3 or EGFR ligands in medium containing 15% FBS. However, WT EGFR-expressing cells required TGF-α to survive and grow under both low and high serum conditions (supplemental Fig. 1). Treatment of 32D-del747–753 cells with gefitinib, erlotinib, or cetuximab in low serum (1% FBS) also inhibited cell survival (Fig. 1*C*).

EGFR Antibody Blocks Receptor Signaling—We next determined whether cetuximab and the EGFR TKIs inhibited receptor signal transduction. H3255 cells contain an L858R activating mutation in the *EGFR* gene (16) as well as *EGFR* gene amplification and are exquisitely sensitive to gefitinib (21). EGFR, MAPK, and Akt were constitutively active in the absence of serum or EGFR ligands in both PC9 and H3255 cells. Cetuximab strongly inhibited P-EGFR in PC9 but modestly in H3255 cells (Fig. 2). In both cells, the antibody also inhibited P-MAPK and P-Akt in dose-dependent manner with complete inhibition at a receptor-saturating concentration of 10 μg/ml. In general, the anti-signaling effects of cetuximab were less potent than those observed with both gefitinib and erlotinib (Fig. 2, *A* and *B*). Treatment with cetuximab resulted in EGFR down-regulation in both cancer lines (Fig. 2, *A* and *B*). Gefitinib and erlotinib also down-regulated EGFR levels in H3255 cells (Fig. 2*B*). In a previous report from our group (28), this down-regulation in response to TKIs was not observed in 32D cells stably expressing EGFR-L858R, suggesting that the effect of gefitinib and erlotinib is specific to H3255 cells.

32D cells expressing del747–753insS-EGFR exhibited ligand-independent receptor activation, as measured by Y1068 and Y1173 P-EGFR immunoblot, which was blocked by both cetuximab and gefitinib (Fig. 2*C*). In contrast, 32D-EGFR (WT) cells did not show ligand-independent receptor activation (Fig. 2*C* and supplemental Fig. 2). We next serum-starved cells and pre-treated them with cetuximab for 4 h followed by stimulation with TGF-α. In 32D-EGFR (WT), the receptor was phosphorylated upon addition of ligand; this was blocked in cells pre-treated with cetuximab (Fig. 2*D*). In contrast, 32D-del747–753insS cells showed ligand-independent EGFR phosphorylation, which was slightly increased by TGF-α; both basal and

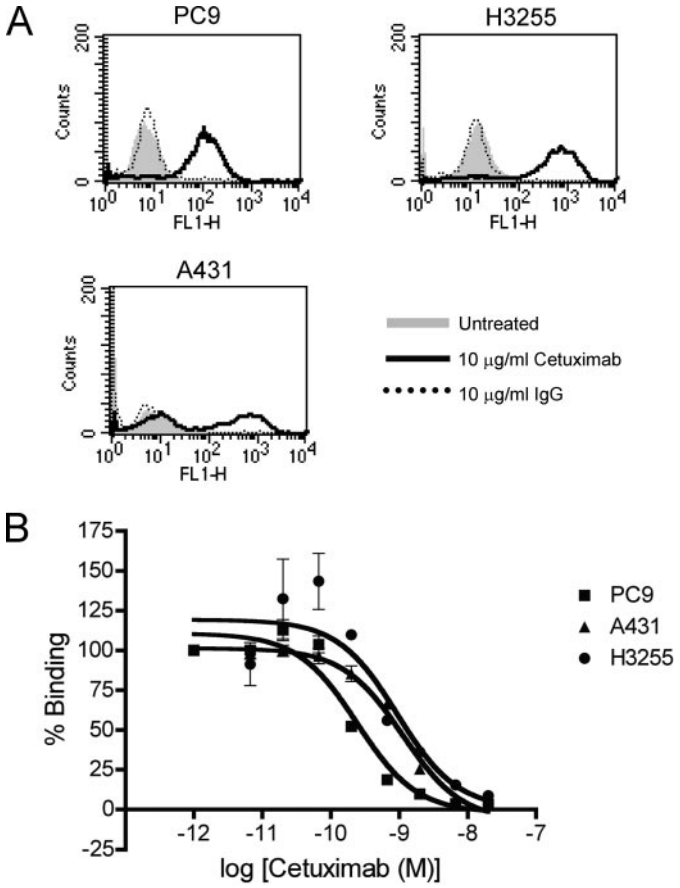


FIGURE 3. **Cetuximab binds to cells and blocks ligand binding.** *A*, cells were incubated with 10 μg/ml cetuximab or a nonspecific human IgG on ice for 1 h, after which they were resuspended in FITC-labeled anti-human IgG and incubated for 30 min. Flow cytometric analysis of FITC-positive cells was performed as described under "Experimental Procedures." *B*, cells were incubated with 1 ng/ml ^{125}I-TGF-α with or without increasing concentrations of the antibody for 3 h at 4 °C with gentle incubation. Cells were washed, solubilized with NaOH, and cpm counted in a gamma counter. Percent binding was calculated as follows: (cpm sample/cpm control (no cetuximab)) × 100.

ligand-induced mutant EGFR phosphorylation were inhibited by cetuximab (Fig. 2*D*). In addition, treatment with the antibody down-regulated mutant but not wild-type EGFR levels (Fig. 2, *C* and *D* and supplemental Fig. 2).

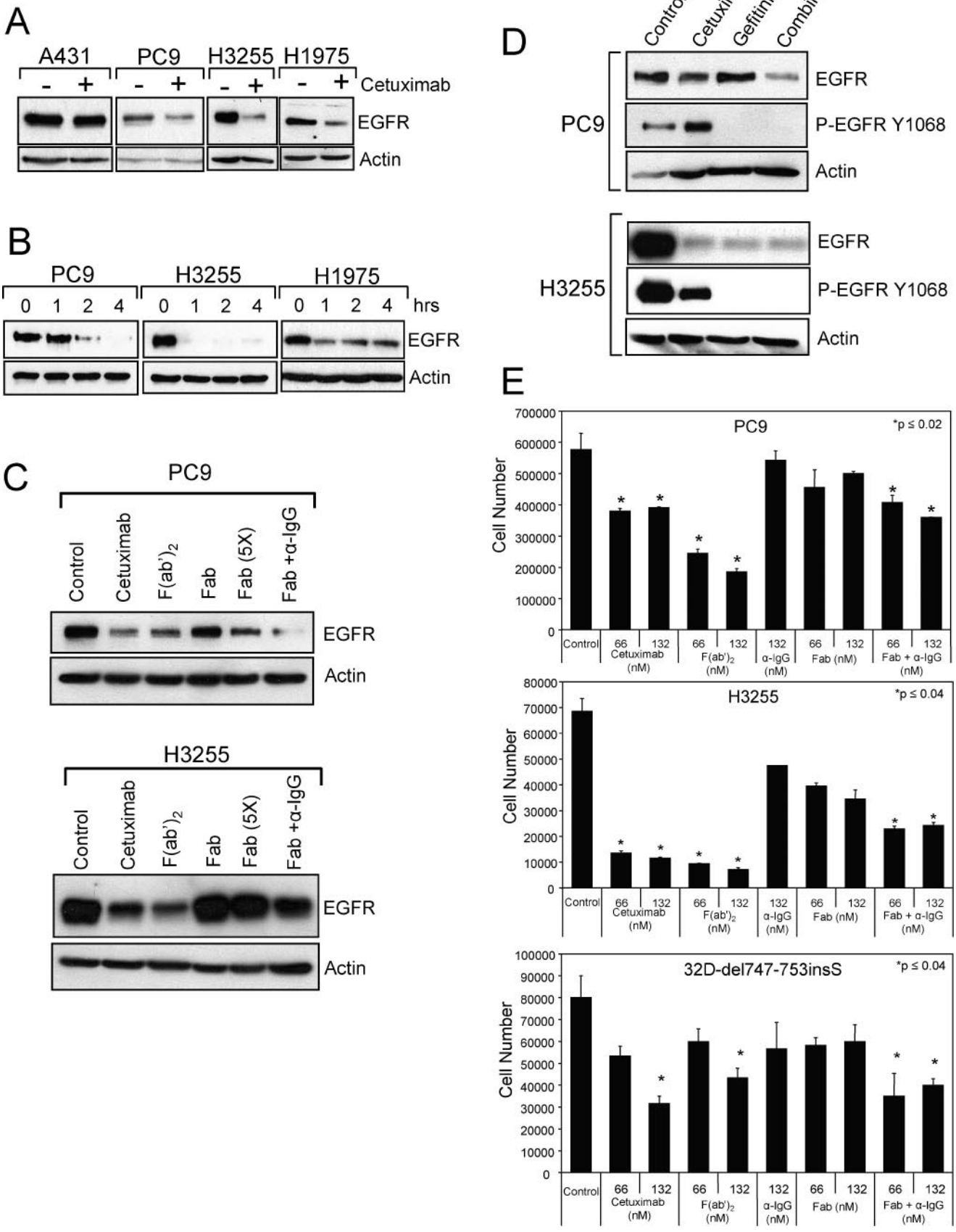
Cetuximab Binds to Mutant EGFR and Blocks Ligand Binding—We incubated A431, PC9, and H3255 cells with 10 μg/ml cetuximab or a nonspecific human IgG followed by an anti-human IgG FITC-labeled secondary antibody. A431 are squamous carcinoma cells with wild-type *EGFR* gene amplification (33). FITC-positive cells, indicative of cetuximab binding, were detected in all three cell lines with A431 and H3255 expressing roughly equivalent EGFR levels (Fig. 3*A*). To determine ligand binding affinity to mutant EGFRs, we incubated PC9, H3255, and A431 cells (as a control expressing wild-type EGFR) with ^{125}I-TGF-α and increasing concentrations of cetuximab. Cetuximab competed with labeled TGF-α binding in all three lines, with an EC_{50} of 2.29×10^{-10} M, 1.13×10^{-9} M, and 9.85×10^{-10} M for PC9, A431, and H3255 cells, respectively (Fig. 3*B*), suggesting similar binding affinity for wild-type (in A431) and mutant EGFR.

Cetuximab-induced Down-regulation Requires EGFR Dimerization—Exposure to a receptor-saturating concentration of cetuximab (20 μg/ml) for 24 h down-regulated EGFR in PC9, H3255, and H1975 cells but not appreciably in A431 cells (Fig. 4*A*). H1975 cells harbor the L858R mutation and a secondary mutation in its kinase domain (T790M) that confers resistance to gefitinib (27, 34). A time course experiment revealed that full down-regulation in the mutant EGFR expressing lung cancer cells was reached after 1–2 h of incubation with cetuximab (Fig. 4*B*). Fan *et al.* (36) reported that the EGFR down-regulation mediated by the antibody mAb225, the mouse counterpart of cetuximab, required receptor dimerization. To test this in mutant EGFR-expressing cells, we incubated PC9 and H3255 cells with cetuximab, a divalent fragment of cetuximab $F(ab')_2$, or a 5-fold higher concentration of a monovalent fragment (Fab). The binding affinity of Fab is 5-fold less than that of cetuximab or $F(ab')_2$ (36)[4]. Both cetuximab and $F(ab')_2$ but not the monovalent fragments induced EGFR down-regulation in PC9 and H3255 cells. An anti-human IgG antibody was added to cells incubated with Fab to artificially create a bivalent molecule containing two Fab fragments. This resulted in similar EGFR down-regulation as that induced by cetuximab or $F(ab')_2$ (Fig. 4*C*). Treatment with gefitinib did not interfere with cetuximab-induced receptor down-regulation (Fig. 4*D*), suggesting that receptor tyrosine kinase activity was not required for this effect of the antibody.

We next determined whether receptor down-regulation was required for the growth inhibitory effect of cetuximab. Growth of PC9 and H3255 cells was significantly inhibited by cetuximab (66 and 132 nM) and equimolar concentrations of $F(ab')_2$ but not by Fab fragments. However, when cells were co-incubated with Fab and anti-human IgG, PC9 and H3255 cell growth was inhibited (Fig. 4*E*, *top panels*). In 32D-del747–753insS cells, the higher (132 nM) concentration of cetuximab and $F(ab')_2$ fragments was required to inhibit proliferation. As in the NSCLC lines, monovalent Fabs did not inhibit proliferation of 32D-del747–753insS cells, but addition of anti-human IgG resulted in Fab-mediated reduction of growth (Fig. 4*E*, *lower panel*).

Antibody Treatment Inhibits EGFR-Mutant Tumor Growth in Vivo—We next examined whether the effects of cetuximab on EGFR mutant NSCLC also occurred *in vivo*. Athymic mice were injected with PC9 cells in the subcutaneous space; once xenografts reached a volume of at least 200 mm^3, they were randomized to no treatment, cetuximab, gefitinib, or the combination of both drugs for 4 weeks. Seventy-two hours after initiation of treatment, some tumors were harvested to assess inhibition of proliferation and/or induction of apoptosis. Tumor cell proliferation, as measured by BrdUrd incorporation, was significantly lower in all treatment groups relative to untreated controls, but this reduction was larger in both gefitinib-treated groups (Fig. 5*A*). Tumor cell death assessed by the proportion of TUNEL-positive cells in stained xenograft sections was higher than controls in all treatment groups. A single dose of cetuximab delivered on day 1 of therapy resulted in a 5-fold increase in the proportion of tumor cells undergoing

[4] Dan Hicklin, personal communication.



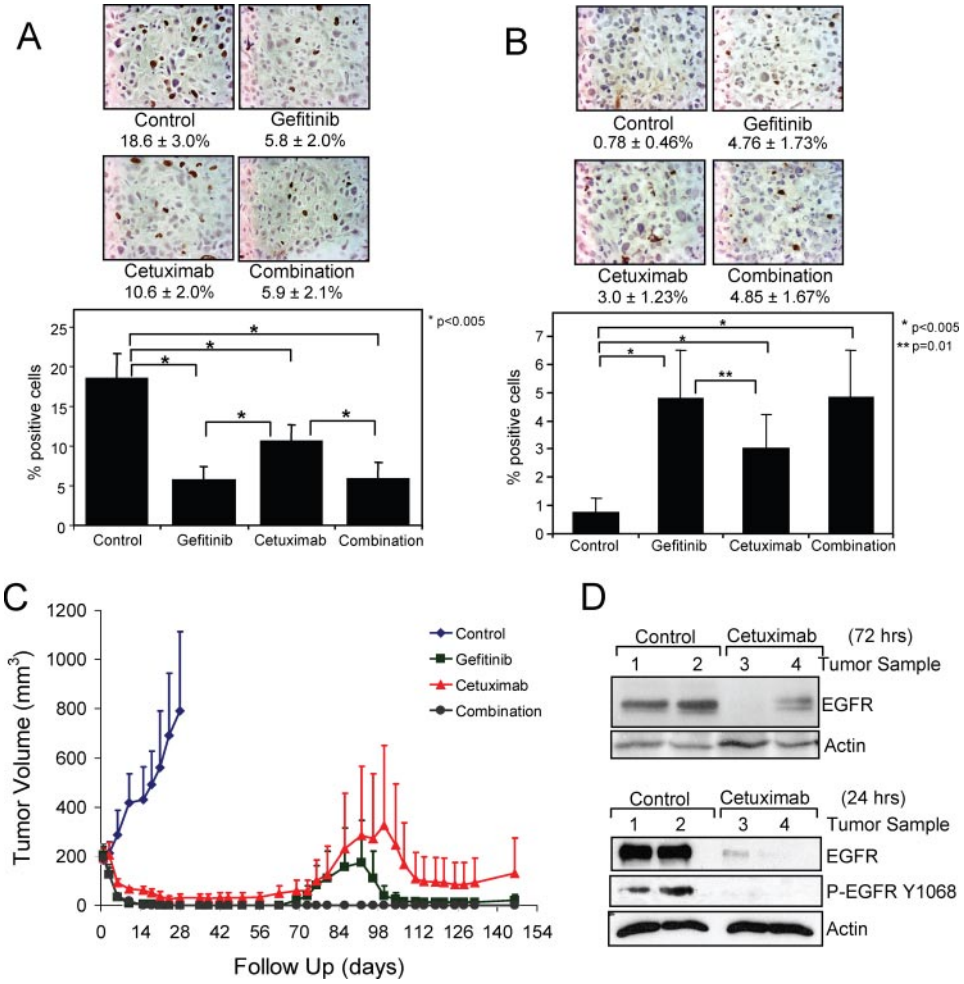


FIGURE 5. **Treatment with cetuximab alone eliminates established PC9 xenografts.** Detection by immunohistochemistry of (*A*) BrdUrd and (*B*) TUNEL-positive cells in PC9 xenografts harvested on day 3 after 1st dose of cetuximab and 2.5 h after the administration of 3 mg of BrdUrd intraperitoneally. The number of proliferating cells was determined by counting 10 random high-power fields (400×) and expressed as percentage of BrdUrd-positive cells ± S.E. ($n = 3$ mice/group). Student's *t* test was used for statistical comparisons. *C*, effect on tumor growth. Once tumors reached a volume ≥200 mm^3, mice were randomly assigned to a) no treatment, b) 200 mg/kg gefitinib by oral gavage daily, c) 1 mg of cetuximab intraperitoneally twice a week, or d) both the drugs. Tumor volumes were determined serially as described under "Experimental Procedures." After 4 weeks of therapy, all except one tumor in the cetuximab-treated group had been completely eliminated. Two tumors in the cetuximab group and one in the gefitinib group recurred after stopping the treatment. No recurrences were observed in the combination arm ($n = 5$) after 6 months of follow-up. Retreatment of the tumors that regrew with the same original drug induced tumor regression. *D*, two tumor samples treated with cetuximab or not were collected 24 or 72 h after 1st dose of the antibody and homogenized. Tumor lysates were separated by 7.5% SDS-PAGE followed by immunoblot analysis with an EGFR antibody. Actin was used as loading control.

apoptosis. Histopathological examination of antibody-treated tumors confirmed extensive evidence of nuclear condensation and chromatin fragmentation without evidence of a mononuclear or inflammatory infiltrate (supplemental Fig. 3). A higher induction of apoptosis was observed in mice treated with gefitinib either alone or in combination with the antibody compared with cetuximab alone (Fig. 5*B*). At 72 h, cetuximab did not enhance gefitinib-induced inhibition of BrdUrd staining or the percentage of cells undergoing programmed cell death (Fig. 5, *A* and *B*). After 4 weeks of therapy, all but one tumor in the cetuximab-only group were completely eliminated (Fig. 5*C*). Immunoblot of tumor homogenates harvested at 24 and 72 h after a single dose of cetuximab indicated almost complete down-regulation of EGFR (Fig. 5*D*). Two tumors in the cetuximab group and one in the gefitinib group recurred 47 days after discontinuation of single-drug treatment, whereas no recurrences were observed in the combination arm ($n = 5$) after 6 months of follow-up. Upon retreatment with the same drug, the tumor in the gefitinib group responded partially. Of those retreated with cetuximab, one had a complete and the other one a partial response.

Finally, we tested the effects of cetuximab on gefitinib-resistant H1975 xenografts, expressing L858R/T790M mutant EGFR (27). In culture, gefitinib or erlotinib had no effect, whereas the addition of cetuximab resulted in dose-dependent inhibition of H1975 cell growth in monolayer (Fig. 6*A*). Treatment with cetuximab was started once tumors were ≥200 mm^3 in volume. Tumors in mice treated with cetuximab for 3 weeks did not grow, whereas control tumors did (Fig. 6*B*). These results suggest that cetuximab is effective not only against tumors expressing gefitinib-sensitive activating mutations in the EGFR kinase domain, but also in those tumors that developed resistance to EGFR TKIs as a result of a secondary kinase domain mutation.

DISCUSSION

Activating mutations in the kinase domain of the EGFR were reported recently in NSCLC (16–18). Most of these mutants are exquisitely sensitive to ATP mimetics that inhibit ATP

FIGURE 4. **Cetuximab-induced EGFR down-regulation requires receptor dimerization.** *A*, A431, PC9, H3255, and H1975 cells were incubated with 20 μg/ml cetuximab for 24 h. EGFR levels in cell lysates were determined by Western blot; actin was used as loading control. *B*, PC9, H3255, and H1975 cells were incubated with 20 μg/ml cetuximab for the indicated times and then lysed and subjected to SDS-PAGE. EGFR levels were detected by Western blot. *C*, PC9 and H3255 cells were incubated for 24 h with equimolar (66 nM) concentrations of cetuximab, divalent $F(ab')_2$, monovalent fragments (Fab) at equimolar (66 nM) as well as a 5-fold higher concentration (396 nM), or Fab with anti-human IgG. EGFR levels in cell lysates were determined by Western blot. *D*, PC9 and H3255 cells were incubated for 24 h with 10 μg/ml cetuximab, 1 μM gefitinib, or both. Total EGFR and P-EGFR Y1068 levels were determined by Western blot. *E*, PC9, H3255, and 32D-del747–753insS cells were treated with cetuximab or equimolar concentrations of monovalent or divalent antibody fragments ± anti-human IgG. After 72 h, the monolayers were trypsinized and cell number measured in a Coulter counter. Each bar represents the mean cell number ± S.E. of three wells. Statistical analysis was done using Student's *t* test.

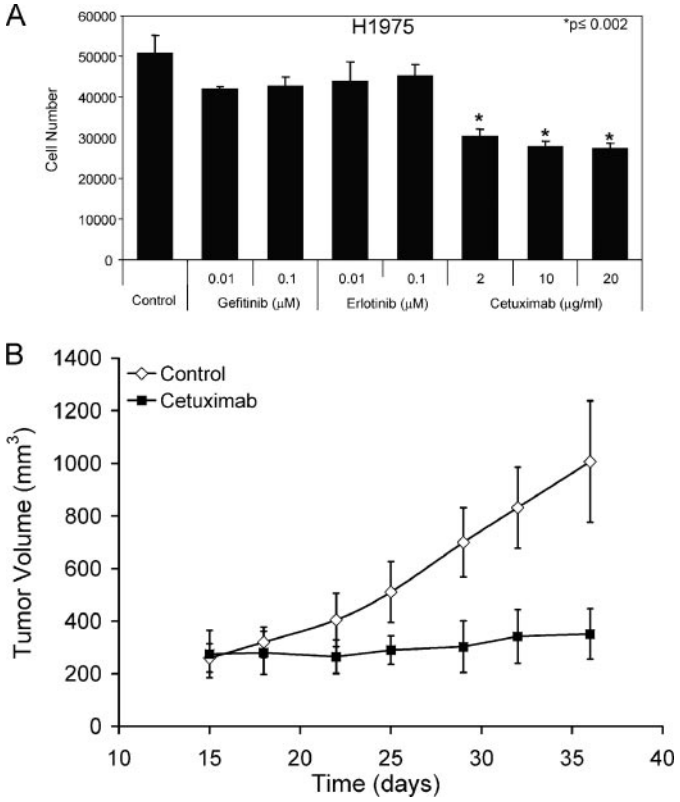


FIGURE 6. **Cetuximab inhibits growth of gefitinib- and erlotinib-resistant H1975 xenografts.** *A*, H1975 cells were treated with gefitinib, erlotinib, or cetuximab. After 72 h, the monolayers were trypsinized and cell numbers measured in a Coulter counter. Each *bar* represents the mean cell number ± S.E. of three wells. Statistical analysis was done using Student's *t* test. *B*, H1975 cells were injected subcutaneously in athymic mice. Treatment with 1 mg of cetuximab, intraperitoneally twice a week, started on day 15 once all tumors had reached an average volume ≥250 mm^3. Tumor diameters were monitored serially with calipers and calculated as described under "Experimental Procedures." Each data point represents the mean volume ± S.E. of eight mice/group.

binding to the kinase pocket of the receptor and thus block its catalytic activity. In this study, we show that cetuximab, a chimeric monoclonal antibody that binds to the extracellular domain of EGFR, induces down-regulation of mutant receptors and inhibits growth of cells expressing these mutations both *in vitro* and *in vivo*.

Treatment with cetuximab inhibited growth of PC9 and H3255 lung cancer cells, which contained del746_750 and L858R EGFR mutations, respectively, as well as 32D cells stably transfected with del747–753insS EGFR (Fig. 4*E*). Although EGFR phosphorylation was variably inhibited by cetuximab in the NSCLC lines, P-MAPK and P-Akt were markedly inhibited in both cells by concentrations of the antibody lower than those achieved at steady state in the serum of patients treated with conventional therapeutic doses of cetuximab (37). Despite the lack of a tight correlation between inhibition of P-EGFR with that of MAPK and Akt, we speculate that the inhibition of the latter two is EGFR-specific. First, the antibody binds a conserved region in EGFR and has not been shown to bind other molecules. Second, EGFR phosphorylation in antibody-treated cells can be misleading as it has been shown to occur after cell lysis but not lead to a mitogenic response (38), entirely consistent with the simultaneous inhibition of MAPK and Akt in this experiment.

In general, the effects of cetuximab on growth and basal EGFR signaling *ex vivo* were less dramatic than those induced by gefitinib and erlotinib. This difference could be explained by the different mechanisms of action of these two drugs. The small molecules directly compete with ATP for binding to the kinase domain of the receptor, and cetuximab competes with ligand for binding to the EGFR ectodomain and/or induces receptor down-regulation (9, 39). The ability of the small molecules TKI to easily diffuse into cells and thus block intracellular EGFR phosphorylation and signaling could explain their more robust inhibitory activity. Despite this limitation, cetuximab inhibited ligand-independent EGFR phosphorylation in PC9 and H3255 cells (Fig. 2, *A* and *B*) and in 32D cells transfected with the deletion mutant (Fig. 2, *C* and *D* and supplemental Fig. 2). Because the 32D-EGFR (WT) cells do not proliferate in the absence of added ligand (supplemental Fig. 1), we deduce that they do not express EGFR ligands, and therefore, the inhibitory effect of cetuximab against 32D expressing the deletion mutant cannot be explained by blockade of autocrine ligand action. These results agree in part with those of Mukohara *et al.* (22), in which in short term assays, the effects of cetuximab are less robust than those induced by gefitinib. However, this same group has just reported a dramatic inhibitory effect of >2 weeks of cetuximab therapy against temporally regulated mouse transgenic tumors expressing L858R EGFR and Del (exon 19) EGFR (40).

A short treatment with cetuximab down-regulated EGFR levels in PC9, H3255, and H1975 (Fig. 4, *A* and *B*) as well as in 32D cells expressing the deletion mutant. Receptor down-regulation was not observed in A431 cells (Fig. 4*A*) or in 32D cells transfected with wild-type EGFR (Fig. 2, *C* and *D* and supplemental Fig. 2). The equivalent receptor levels between A431 and H3255 cells and between 32D cells expressing wild-type or mutant EGFR suggest that these differences cannot be explained by differences in receptor content. Antibody-mediated down-regulation required receptor dimerization as treatment with monovalent (Fab) fragments of cetuximab did not decrease receptor levels or inhibited cell growth unless they were co-incubated with anti-human IgG (Fig. 4, *C* and *E*). The kinetics of down-regulation was variable but in general slower than that reported for ligand-induced down-regulation of wild-type EGFR. These results are interesting in face of the observation that mutant EGFRs are resistant to ligand-induced down-regulation (18, 28). The mechanisms to explain this resistance are unclear but two recent reports show constitutive association of EGFR mutants with the Hsp90 chaperone (28, 41). In one of these studies, treatment with the Hsp90 inhibitor geldanamycin restored TGF-α-induced mutant receptor ubiquitination and down-regulation in H3255 and H1975 cells and in 32D cells expressing L861Q and L858R EGFR (28). In the study herein though, treatment with cetuximab did not dissociate the constitutive EGFR·Hsp90 complex in PC9, H3255, and H1975 cells (supplemental Fig. 4), thus arguing against uncoupling of the EGFR·Hsp90 complex as a mechanism of receptor down-regulation.

One possible mechanism of receptor down-regulation is

inferred from structural studies with the soluble extracellular domain of EGFR (sEGFR). Fab fragments of cetuximab interact only with subdomain III of sEGFR, and this interaction is 50-fold stronger than the EGF-sEGFR interaction (35). Li *et al.* (35) reported that binding of cetuximab to sEGFR does not change when the pH is reduced from 7.0 to 5.0, suggesting it is not likely to dissociate from the receptor in the low pH environment of the endosome, thus potentially targeting the receptor for lysosomal degradation. Whether this explains the effect of cetuximab on mutant EGFR stability requires further investigation.

The ubiquitin ligase Cbl has been shown to ubiquitinate activated EGFR. This modification carries internalization and degradation signals that control endocytosis and sorting of receptors for destruction in the lysosomes (42, 43). Further, recruitment of Cbl to Tyr-1112 in the EGFR homologous ErbB2 receptor is associated with antibody-induced degradation of ErbB2 (44). In the case of cetuximab, several arguments suggest Cbl is not involved in antibody-mediated mutant receptor down-regulation. First, even though the EGFR mutants are constitutively phosphorylated in Tyr-1045, the Cbl binding site, and/or associated with Cbl in the absence of added ligands, they appear protected from ubiquitination and degradation (28, 45). Second, a Tyr-1045 mutant of EGFR, which cannot bind Cbl, is down-regulated upon treatment with EGFR monoclonal antibodies (46). Third, ErbB2 mutants lacking the entire cytoplasmic domain but still anchored in the plasma membrane undergo down-regulation upon treatment with ErbB2 antibody (46). In addition to supporting lack of involvement of Cbl in antibody-mediated receptor down-regulation, these data further imply that cytoplasmic motifs are not necessary for this effect of receptor antibodies. This is consistent with the effect of cetuximab against PC9, H3255, and H1975 cells, each exhibiting a different EGFR kinase domain mutation, which likely results in a different intracellular receptor conformation.

Finally, cetuximab inhibited growth of established xenografts expressing EGFR mutations. In PC9 tumors, this was associated with evidence of receptor down-regulation *in vivo*. Although the effect of cetuximab on established PC9 tumors was more delayed than that of gefitinib, the combination cetuximab and gefitinib was markedly effective and did not allow tumor recurrences after discontinuation therapy and long follow-up. In addition, cetuximab inhibited gefitinib-resistant H1975 tumors. Although we cannot rule out a mechanism of anti-tumor action involving the host, such as antibody-dependent, cell-mediated cytotoxicity (36, 47, 48), the inhibitory effect of $F(ab')_2$ fragments (Fig. 4*E*), which lack the antibody constant region required for engagement of the Fc receptor in immune cells, would argue against it being an obligatory mechanism of antibody-mediated antitumor action. Other arguments support a direct anti-oncogene effect of the antibody when given alone. These include the potent inhibition of proliferation and induction of apoptosis at 72 h, the prompt reduction of tumor EGFR content *in vivo* preceding full tumor shrinkage, and the lack of a mononuclear inflammatory infiltrate in tumors already showing evidence of treatment-induced toxicity (supplemental Fig. 3).

The effect of cetuximab alone against PC9 tumors and the inhibitory effect against L858R/T790M EGFR-expressing H1975 xenografts also raise the possibility that a combined approach may reduce the duration of anti-EGFR therapy and potentially abrogate the emergence of acquired resistance to TKIs. In addition, if mutant receptor down-regulation is causal to antibody-induced antitumor action, this provides a mechanism that can be used as experimental readout for the selection of preclinical combinations with other agents that work by reducing mutant receptor levels. These may include other EGFR antibodies as suggested by Friedman *et al.* (46), Hsp90 inhibitors (28, 41), and irreversible inhibitors of the EGFR kinase (49, 50).

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