Epidermal Growth Factor Receptor Dynamics Influences Response to Epidermal Growth Factor Receptor Targeted Agents

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Abstract

Analysis of gene expression of cancer cell lines exposed to erlotinib, a small molecule inhibitor of the epidermal growth factor receptor (EGFR), showed a marked increase in EGFR mRNA in resistant cell lines but not in susceptible ones. Because cetuximab induces EGFR down-regulation, we explored the hypothesis that treatment with cetuximab would interfere with erlotinib-induced EGFR up-regulation and result in antitumor effects. Exposure of the resistant biliary tract cancer cell line HuCCT1 but not the susceptible A431 epidermoid cell line to erlotinib induced EGFR mRNA and protein expression. Combined treatment with cetuximab blunted the erlotinib-induced EGFR up-regulation and resulted in inhibition of cell proliferation and apoptosis in the HuCCT1 cells. Blockage of erlotinib-induced EGFR synthesis in HuCCT1 cells by small interfering RNA resulted in identical antitumor effects as cetuximab, providing mechanistic specificity. In mice xenografted with A431, HuCCT1, and the pancreatic cancer cell line Panc430, maximal growth arrest and decrease in Ki67 proliferation index were documented with combined therapy, and EGFR down-regulation was observed in cetuximab-treated tumors. These results may indicate that resistance to EGFR kinase inhibition may be, at least in part, mediated by a highly dynamic feedback loop consisting of up-regulation of the EGFR upon exposure to EGFR kinase inhibitors. Abrogation of this response by small interfering RNA-mediated EGFR mRNA down-regulation and/or by cetuximab-mediated protein clearance induced tumor arrest across several cancer models with different EGFR expression levels, suggesting that resistance and sensitivity are dynamic events where proportional decrease in the target rather than absolute content dictates outcome. (Cancer Res 2005; 65(8): 3003-10)

Introduction

The epidermal growth factor receptor (EGFR) is a membrane receptor with an extracellular domain, a single α-helix transmembrane domain, and an intracellular domain with tyrosine kinase activity. Ligand binding induces EGFR homodimerization and heterodimerization with other HER proteins, activation of tyrosine kinase activity, and autophosphorylation. EGFR signaling ultimately increases proliferation, angiogenesis, metastasis, and decreases apoptosis. Two major strategies have been developed to target the EGFR: the use of small molecules that compete with ATP for binding to the kinase pocket, and the use of monoclonal antibodies directed against the external domain of the receptor. Erlotinib (Tarceva, OSI Pharmaceuticals, Uniondale, New York, NY) is a quinazoline derivative that reversibly inhibits the tyrosine kinase of EGFR, showing *in vitro* and *in vivo* activity in human cancer cell lines (1, 2). Cetuximab (Erbitux, ImClone Systems, New York, NY) is a quimeric mouse-human monoclonal antibody that induces down-regulation of the EGFR (3). EGFR-directed therapies have shown a consistent but low level of clinical activity across tumor types, and factors determining their efficacy are largely unknown. In addition, little is known about the effect of EGFR-targeted agents at the molecular level, the response that these agents elicit in the cell machinery, and whether this response may be relevant in spontaneous and acquired resistance. Applying a broad-range gene expression evaluation strategy followed by sequential, increasingly specific investigational steps, this study was conducted to determine the mechanisms of resistance to tyrosine kinase inhibitors, and to devise rational ways of targeting the EGFR as an anticancer therapy.

Materials and Methods

Drugs. Erlotinib was provided by OSI Pharmaceuticals and cetuximab was provided by ImClone Systems.

***In vitro* treatment.** HuCCT1 and A431 cells were seeded in medium supplemented with 10% fetal bovine serum. When 50% to 60% confluence was reached, cells were serum-starved overnight, after which they were treated with growth media, erlotinib (5 μmol/L), cetuximab (20 nmol/L), or erlotinib (5 μmol/L) plus cetuximab (20 nmol/L).

Gene expression analysis. Microarray hybridization was done on the Affimetrix U133A gene array, containing ~22,000 unique human transcripts. Sample preparation and processing were done as described in the Affimetrix GeneChip Expression Analysis Manual (Affimetrix, Inc., Santa Clara, CA). The CEL files generated by the Affimetrix Microarray Suite (MAS) version 5.0 were converted into DCP files using dCHIP (http://www.dCHIP.org), as described previously (4). Genes that were differentially expressed 3-fold or greater in 0 versus 1 or 0 versus 24 hours were then identified by defining the appropriate filtering criteria in the dCHIP software (mean E / mean B > 3; mean E − mean B = 100; $P < 0.05$, t test).

Western blot analysis. Following treatment during 1, 6, and 24 hours, cells were harvested. Equal amounts of protein (50 μg) were resolved on 10% polyacrylamide gels. Gels were transferred onto nitrocellulose membranes that were incubated overnight at 4°C with antibodies against phospho-EGFR, phospho-MAPK, and phospho-Akt (#2232, #2234, #9271, and #9101, respectively, Cell Signaling Technology, Beverly, MA). The immunoreactive proteins were detected using the enhanced chemiluminescence method (Amersham, Piscataway, NJ).

Quantitative real-time reverse transcription-PCR analysis. Total RNA was extracted from cell pellets using the RNeasy Mini Kit (Qiagen, Valencia, CA). cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) following the manufacturer's instructions. Relative quantification of EGFR mRNA was achieved using an iCycler iQ real-time PCR detection system (Bio-Rad) with Sybr green as the fluorophore (Bio-Rad).

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Primer sequences used for EGFR were used as previously published (5). Accumulation of the specific PCR products was detected as an increase in fluorescence that was plotted against cycle number to determine the C_T values. Relative expression (RE) of the mRNA analyzed was estimated using the formula: $RE = 2^{-\Delta C_T}$, where $\Delta C_T = C_T$ (mRNA) $- C_T$ (glyceraldehyde-3-phosphate dehydrogenase).

ELISA assay. An immunoezymatic assay (ELISA) was used for quantification of EGFR (Oncogene Research Products, San Diego, CA) following the manufacturer's instructions.

Terminal deosynucleotidyl transferase nick-end labeling assay. Quantification of apoptosis was assessed in duplicate by the terminal deosynucleotidyl transferase nick-end labeling (TUNEL) technique, using a commercially available kit (Guava TUNEL Kit, Guava Technologies, Hayward, CA).

***Egfr* gene silencing by small interfering RNA.** Small interfering RNA (siRNA) specific for the *egfr* gene (Super Array, Frederick, MD) was used. Cells were plated in a 24-well plate at 5×10^4 per well, and after 24 hours were transfected with siRNA and LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Control consisted of HuCCT1 cells in the presence of the transfection reagent without siRNA, and a duplicate of the treatment arms without siRNA. After 24 hours, the cells were treated with growth media, erlotinib (5 μmol/L), cetuximab (20 nmol/L), or erlotinib (5 μmol/L) plus cetuximab (20 nmol/L). Cells were harvested 24 hours later to measure the amount of EGFR mRNA and protein, and apoptosis. The siRNA transfection was done in duplicate, and the experiment repeated twice.

***In vivo* growth inhibition studies.** Six-week-old female athymic nude mice (Harlan, IN) were used for this purpose. A431, HuCCT1, and Panc430 cells (5×10^6) were injected s.c. in each flank. Tumors were grown to a size of 0.2 cm^3, and mice were stratified by tumor volume into different groups (six mice, 12 tumors, per group) that were treated with vehicle, erlotinib 50 mg/kg i.p. once a day for 14 days, cetuximab 50 mg/kg i.p. every 3 days for 14 days, or erlotinib 50 mg/kg i.p. once a day + cetuximab 50 mg/kg i.p. every 3 days for 14 days.

Immunohistochemical analysis. Tumors were fixed and paraffin-embedded using standard procedures. Five-micron sections were stained after citrate-steam antigen retrieval with Ki67 (M7187, Dako, Carpinteria, CA) and EGFR (28-0005, Zymed, San Francisco, CA) primary antibodies. A biotinylated secondary antibody was used, followed by streptavidin-conjugated horseradish peroxidase and 3,3′-diaminobenzidine chromogen (K0690, Dako).

Results

Gene expression analysis of the response to erlotinib of sensitive and resistant cell lines. An exploratory, broad-range evaluation was done assessing the changes in gene expression occurring after treatment with erlotinib. After treating naturally resistant (HuCCT1, IC_{50} > 20 μmol/L) and naturally sensitive (SNU308, IC_{50} < 1 μmol/L) biliary cancer cell lines with erlotinib, 18 and 12 genes were dys-regulated after 1 hour of treatment (Table 1), and 41 and 61 after 24 hours, respectively. Among them, a significant increase in EGFR mRNA levels was observed in HuCCT1 cells compared with baseline at 1 and 24 hours (P = 0.028 and P = 0.017, respectively), whereas no change in EGFR mRNA was documented in SNU308 cells. Considering this differential reaction in terms of gene expression of a target in response to pharmacologic inhibition in resistant versus sensitive cell lines and its potential mechanistic implications, we conducted further experiments to better understand the molecular and translational implications of these findings.

Evaluation of epidermal growth factor receptor mRNA response to erlotinib, cetuximab and the combination of both by real-time reverse transcription-PCR. We next investigated the pattern of response to EGFR-targeted therapies using reverse transcription-PCR and ELISA analysis to evaluate EGFR mRNA and protein dynamics, respectively. Because cetuximab induces EGFR down-regulation, we explored the hypothesis that it would interfere with erlotinib-induced EGFR up-regulation. Treatment of HuCCT1 cells confirmed that erlotinib induced EGFR mRNA

Table 1. Genes with significant (P value < 0.05) up-regulation or down-regulation after 1 hour of treatment with erlotinib in HuCCT1 and SNU308 cells

	Gene	Fold	P value
HuCCT1	*Hypothetical protein FLJ20047*	24.3	0.040
	Consensus 217P22 on 6p21.1-21.31/dynein heavy	23.8	0.008
	EGFR	18.4	0.028
	Consensus AF052090.1/23950 mRNA/mRNA	13.7	0.028
	Consensus M78162/gi:273899/ EST01755	9.4	0.012
	Chymotrypsinogen B1	9.2	0.047
	Lipoprotein, Lp(a)	7.1	0.014
	Interleukin 6 (interferon, β2)	4.3	0.030
	Down syndrome critical region gene 1	3.7	0.032
	Interleukin 11	3.7	0.026
	Transcription factor 8 (represses interleukin 2 expression)	3.3	0.035
	Gastric intrinsic factor (vitamin B synthesis)	3.1	0.044
	Tenascin XB	−3.2	0.025
	Polymerase (RNA) II (DNA directed) polypeptide B (140 kDa)	−3.6	0.048
	Parvin, β	−4.0	0.039
	Bone morphogenetic protein 2	−4.1	0.023
	Glycoprotein M6B	−6.9	0.036
	Chimerin (chimaerin) 2	−7.0	0.039
SNU308	*Connective tissue growth factor*	13.9	0.019
	v-jun sarcoma virus 17 oncogene homologue	12.9	0.032
	Cysteine-rich, angiogenic inducer, 61	12.9	0.021
	Inhibitor of DNA binding 2, dominant-negative helix-loop-helix protein	6.8	0.019
	Hypothetical protein FLJ20972	5.4	0.044
	Prostaglandin E receptor 4 (subtype EP4)	5.2	0.045
	Activating transcription factor 3	4.6	0.029
	Tenascin XB	3.7	0.027
	Hypothetical protein FLJ20071	−3.0	0.020
	Consensus AK025247.1/ FLJ21594/10437718/ Hs.288571/FLJ21594	−4.6	0.041
	Solute carrier family 12 (sodium/potassium/chloride transporters)	−5.6	0.032
	Consensus AI952772/ 5745082/Hs.300865 immunoglobulin lambda	−8.3	0.017

synthesis (Fig. 1*A*). Furthermore, this up-regulation of mRNA levels was closely followed by a 2-fold increase in the transcription of the protein (Fig. 1*B*). A decrease in protein was observed both in cells treated with cetuximab alone, as well as in those treated with the combination, suggesting that cetuximab is able to abrogate the erlotinib-induced increase in EGFR transcription. In EGFR-dependent, erlotinib-sensitive (IC_{50} < 1 μmol/L) A431 squamous carcinoma cells, there was no differential increase in EGFR mRNA after treatment with erlotinib (Fig. 1*C*). Erlotinib did not induce an increase of EGFR, and cetuximab induced less significant changes in protein content compared with control cells than in HuCCT1 cells (Fig. 1*D*).

To examine whether the abovementioned differences in response to erlotinib were related to differential EGFR signaling in EGFR inhibition, we conducted Western blot analysis of both cell lines in the presence of the two drugs alone and in combination at different time points (Fig. 1*E*). The studies documented a significant (and identical) inhibition of EGFR activation in both cell lines by erlotinib, cetuximab, but especially with the combination. Inhibition of MAPK was more profound in A431; in HuCCT1, Akt activation was not significantly inhibited by any agent alone, and the effect of the combination was discrete.

Effect of transfecting HuCCT1 cells with small interfering RNA against the epidermal growth factor receptor. In order to

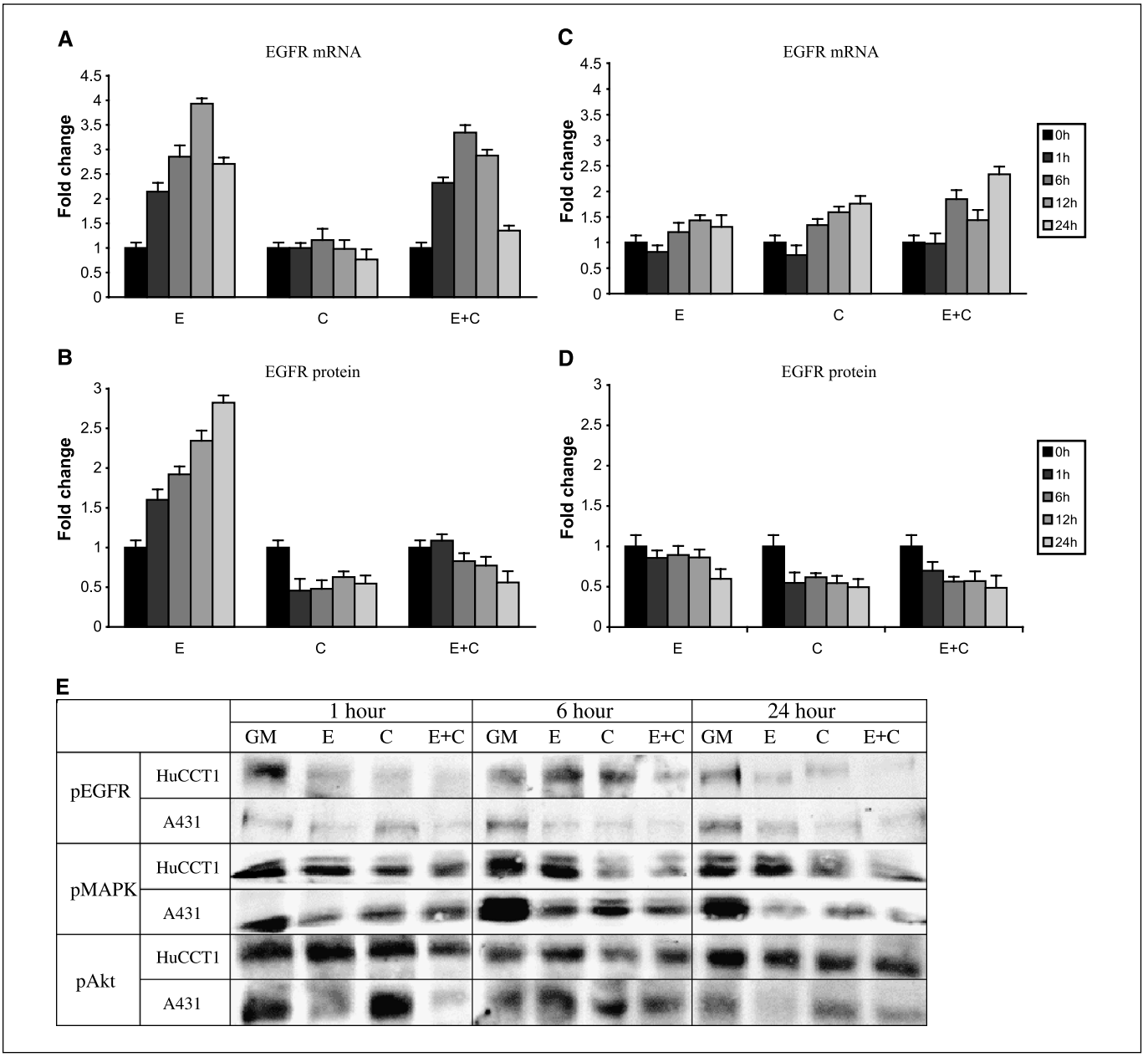

Figure 1. EGFR mRNA and protein expression *in vitro* in HuCCT1 and A431 cell lines. *A* and *B,* erlotinib treatment of HuCCT1 cells induces a steady increase in EGFR mRNA, which is followed by augmented protein expression; *C* and *D,* there is no increase in EGFR mRNA after treatment with erlotinib in A431 cells; *E,* EGFR pathway activity after treatment with erlotinib, cetuximab, or the combination of both agents in HuCCT1 and A431 cell lines. After overnight serum-starvation, cells were treated for 1, 6, and 24 hours; (*C*) cetuximab, (*E*) erlotinib, (*GM*) growth media. mRNA and protein values are ratios normalized to their correlative control values, and represent mean ± SD of four determinations.

fully assess the hypothesis that resistance to erlotinib in HuCCT1 cells may be in part mediated by the erlotinib-induced up-regulation of the target, we aimed at down-regulating EGFR transcription by means of interfering with EGFR mRNA content. HuCCT1 cells were transfected with siRNA against the EGFR, and treated during 24 hours with erlotinib, cetuximab, and the combination (Fig. 2). EGFR mRNA synthesis decreased in control cells by 50%, and was not affected by treatment with the transfection reagent alone (data not shown). The induction of EGFR mRNA by erlotinib was efficiently abrogated by EGFR siRNA. We confirmed that EGFR protein levels closely followed mRNA dynamics, as evidenced by the decrease in protein levels induced by EGFR siRNA; cetuximab and EGFR siRNA showed an additive effect in diminishing EGFR. The combination induced a nonsignificantly higher growth arrest compared with no treatment or single agent erlotinib/cetuximab (data not shown), and siRNA treatment enhanced this effect. In nontransfected cells, the combined treatment induced a 5.6- to 7.4-fold increase in cell apoptosis when compared with erlotinib or cetuximab treatment alone, respectively. In siRNA-transfected cells, apoptosis was higher in all treatment modalities compared with no transfection, but this difference was considerably superior in erlotinib-treated cells.

***In vivo* tumor growth modulation of erlotinib, cetuximab, and the combination of both.** To confirm the molecular events described previously, and to determine the effect of these drugs in a model closer to a clinical context, we tested our hypothesis in several *in vivo* models, including an EGFR-negative cell line added to widen the spectrum. Cancer cell lines with high (A431, vulvar), intermediate (HuCCT1, biliary), and low (Panc430, pancreatic) EGFR expression levels were xenografted in mice that were subsequently treated (Fig. 3). In A431-bearing mice, erlotinib induced short-lasting growth arrest, cetuximab prompted a more delayed tumor control, and the combination achieved both a rapid and a durable tumor growth arrest. Cetuximab significantly decreased EGFR levels by day 1 of treatment. In HuCCT1-bearing mice, cetuximab was equally effective in controlling growth when given alone or in combination with erlotinib, and EGFR levels equally decreased in both groups. To test whether cetuximab efficacy was dependent on prior EGFR-directed therapy, and to further assess the hypothesis that growth correlated with EGFR content, mice initially allocated to erlotinib received additional cetuximab (four doses in 14 days) after completion of erlotinib therapy (*arrow*); a growth interruption was observed, and EGFR levels decreased. In Panc430 xenografts, the combined treatment showed a modest, albeit significantly higher effect than the individual drugs, but only after 28 days. EGFR consistently decreased in cetuximab-treated tumors.

Immunohistochemical analysis of HuCCT1 and A431 tumors. In order to assess immunohistochemical efficacy end

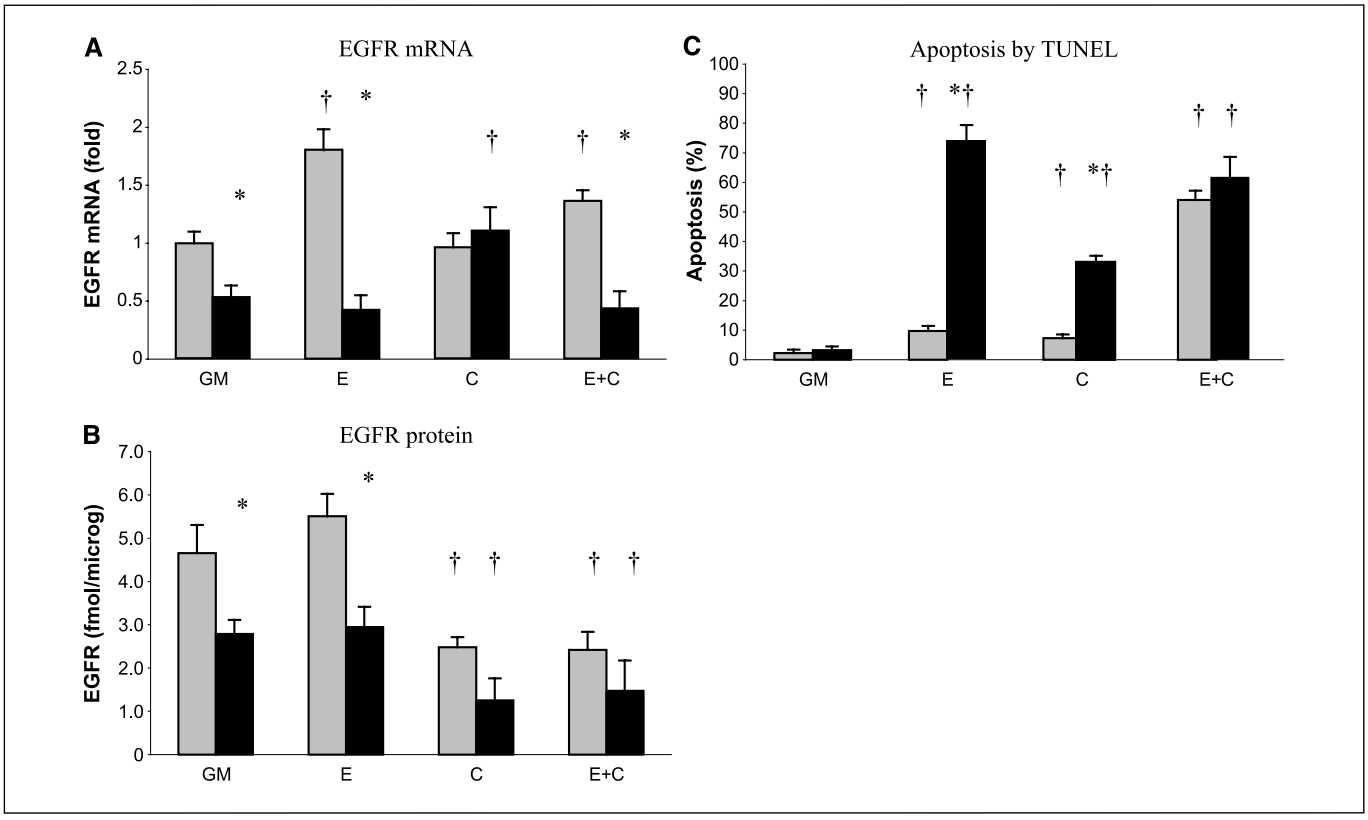

Figure 2. Effect of 24 hours of treatment of HuCCT1 cells after being transfected with siRNA directed against the EGFR (*gray columns,* not transfected; *black columns,* transfected). *A,* EGFR mRNA synthesis, as assessed by real-time reverse transcription-PCR. The induction of EGFR synthesis induced by erlotinib is efficiently abrogated by siRNA; *B,* EGFR protein levels closely follow mRNA dynamics, as evidenced by the fact that siRNA induces a significant decrease in protein levels; cetuximab and siRNA have an additive effect in down-regulating EGFR; *C,* TUNEL assay in untransfected cells showed that, whereas erlotinib or cetuximab alone modestly increased the proportion of cells undergoing apoptosis (baseline 2.3%, erlotinib 9.7%, cetuximab 7.3%), the combination achieved a dramatic increase (54.1%) in cells undergoing programmed cell death. In siRNA-treated cells, however, maximal apoptosis was observed in erlotinib-treated cells. siRNA nonsignificantly increased the proportion of apoptotic cells treated with growth media (from 2.3% to 3.2%); (*C*) cetuximab, (*E*) erlotinib, (*GM*) growth media. mRNA values are normalized to baseline, and represent mean ± SD of four determinations. Protein values represent mean ± SD of four determinations. Apoptosis values represents mean ± SD of two determinations. *, $P < 0.05$ (*t* test), comparing nontransfected versus transfected within treatment pairs; †, $P < 0.05$ (*t* test), comparing GM versus different treatments within transfection groups.

points, we then evaluated the variations in the proliferative index Ki67 in paraffin specimens of HuCCT1 and A431 tumors after 14 days of treatment (Fig. 3*D*). A significant decrease in Ki67 staining (from 3+ to 2+) was documented in HuCCT1 tumors treated with cetuximab, but especially in those treated with erlotinib and cetuximab (from 3+ to 1+), whereas no significant variation in proliferation was observed in erlotinib-treated tumors. In A431, where a baseline higher proliferation was documented (4+), erlotinib and cetuximab decreased Ki67 staining to 2+, whereas the combined treatment resulted in a 1+ score. Staining for EGFR was high in both types of xenografts and no differences were observed by a blinded pathologist.

Discussion

The aims of this study were to evaluate the sequence of events that follow EGFR inhibition, and to seek rational ways of overcoming drug resistance. It was hypothesized that a combined approach inhibiting the intracellular kinase activity with a small molecule, and blocking the receptor's functioning with an extracellular-acting monoclonal antibody would achieve this second goal. It was observed that resistance to EGFR kinase inhibition was in part mediated by a highly dynamic EGFR up-regulation, EGFR relative tumor content correlated with tumor growth, and decreasing EGFR tumor content by monoclonal antibody-mediated protein clearance induced tumor arrest across different *in vitro* and *in vivo* models. Subsequently, a third proof-of-principle strategy (short-circuiting this response with siRNA) was successfully applied to validate these findings.

The observation that a resistant cell line responded to the inhibition of a given enzymatic activity initiating a compensatory feedback loop that in a matter of hours incremented the total amount of receptor, is a rather classic pharmacologic paradigm, representing a homeostatic, adaptative mechanism to overcome target inhibition. Therefore, resistance and sensitivity may be redefined as the ability or inability of the cell to adapt to a changing environment, and the fact that decreasing the target while maintaining a constant drug concentration dramatically increases cell kill as assessed by apoptotic indexes suggests that the ultimate outcome is dictated by dynamic processes and quantitative ratios of drug/target rather than static, qualitative features. This compensatory effect may explain some apparently paradoxical findings observed in several clinical trials (6), where up-regulation of phosphorylated EGFR was observed after treatment with erlotinib in breast cancer patients. In another report, modifications of EGFR serum values during treatment for non–small cell lung cancer seemed to reflect gefitinib activity; responding patients had decreasing EGFR serum levels compared with refractory patients, where an increment from baseline was observed (7). The abrogation of the compensatory feedback loop with siRNA rendered the cell defenseless to the pharmacologic insult, reverting an innate resistance to erlotinib. However, at present, we are unable to define the cellular mechanism that senses and transduces EGFR functional status, and this leads us to the second implication of this report, namely the potential of dual targeting strategies.

Recent reports have shown the additive effect of a tyrosine kinase inhibitor in combination with cetuximab in head and neck cancer (8) and A431 (9) models. The mechanism responsible for this higher efficacy of dual targeting was not addressed in those reports, and to our knowledge this is the first insight into a potential mechanistic explanation of that observation. In our model, physically decreasing the amount of protein using an extracellular-acting monoclonal antibody increased sensitivity to the pharmacologic inhibition of the kinase activity, inducing a synergistic effect in terms of induction of apoptosis *in vitro*, and an additive effect in terms of tumor growth arrest *in vivo*. We can hypothesize that the cell may avoid entering apoptosis either with part of the receptor pharmacologically inhibited, or with a reduced total amount of receptor, but is unable to cope with the impact of both modulations. This threshold effect may be supported by recent reports suggesting that the presence of certain mutations in the catalytic domain of the EGFR augment the sensitivity of cells and tumors to gefitinib (10, 11). One of these reports shows that transfection of the mutated receptor to a naturally resistant, EGFR wild-type cell line induces sensitivity to a constant gefitinib concentration (10), indicating that these mutations might make the receptor susceptible to a clinically achievable drug concentration range, that is in turn unable to efficaciously inhibit the wild-type receptor in the majority of the patients. However, the incidence of EGFR mutations is considered to be relatively low, and this sole factor may not explain the preliminary reports of overall survival advantage found in a placebo-controlled trial of erlotinib in chemotherapy-refractory non–small cell lung cancer patients. It is relevant to note that in consonance with prior reports (12), siRNA-mediated EGFR down-regulation by itself had no effect on cell growth and/or apoptosis, and that the factor implicated in maximal apoptosis was erlotinib treatment. In contrast, other reports show that siRNA of the EGFR in A549 lung cancer cells inhibited cellular proliferation and motility and enhanced chemosensitivity to cisplatin by down-regulating the receptor (13). An even more provocative report documented that i.v. siRNA therapy targeting the EGFR prolonged survival in a glioma model (14).

The third significant aspect of this report is the observation that proportional decreases in EGFR rather than absolute baseline protein content dictated growth arrest. Both monoclonal antibody- or siRNA-mediated targeting of the EGFR provided evidence of a positive correlation between EGFR proportional protein content and growth, regardless of the method of evaluation used (*in vitro* apoptosis, *in vivo* tumor growth, or *in vivo* proliferation assessment by Ki67). This was especially true in HuCCT1 tumors, where second-line cetuximab therapy prompted a late decrease in EGFR paralleled by a modest, albeit significant growth arrest. It is noteworthy that Panc430 tumors presented EGFR levels 17- and 12-fold lower than A431 and HuCCT1 tumors, respectively. Notwithstanding, cetuximab decreased EGFR in the same proportion (40-50% from baseline after 14 days of treatment), and achieved a growth inhibitory effect that was significant across all three models (although more evident in the high-EGFR cell line). These results suggest that the relevant factor may be the proportional decrease in EGFR total activity/content achieved, and not the absolute baseline amount of EGFR present in a given cell line or tumor. The activity of gefitinib has been observed in cells that express high and low levels of EGFR (15), and synergistic effects along with chemotherapeutic agents was not dependent upon a high level of EGFR expression (16). The former observations have also been documented in a clinical setting: EGFR status correlates poorly with response to both monoclonal antibodies (17, 18) and tyrosine kinase inhibitors (19, 20). Another potential reason for this lack of correlation is that immunohistochemistry portrays a static, nonfunctional picture of the cellular scenario. In this report,

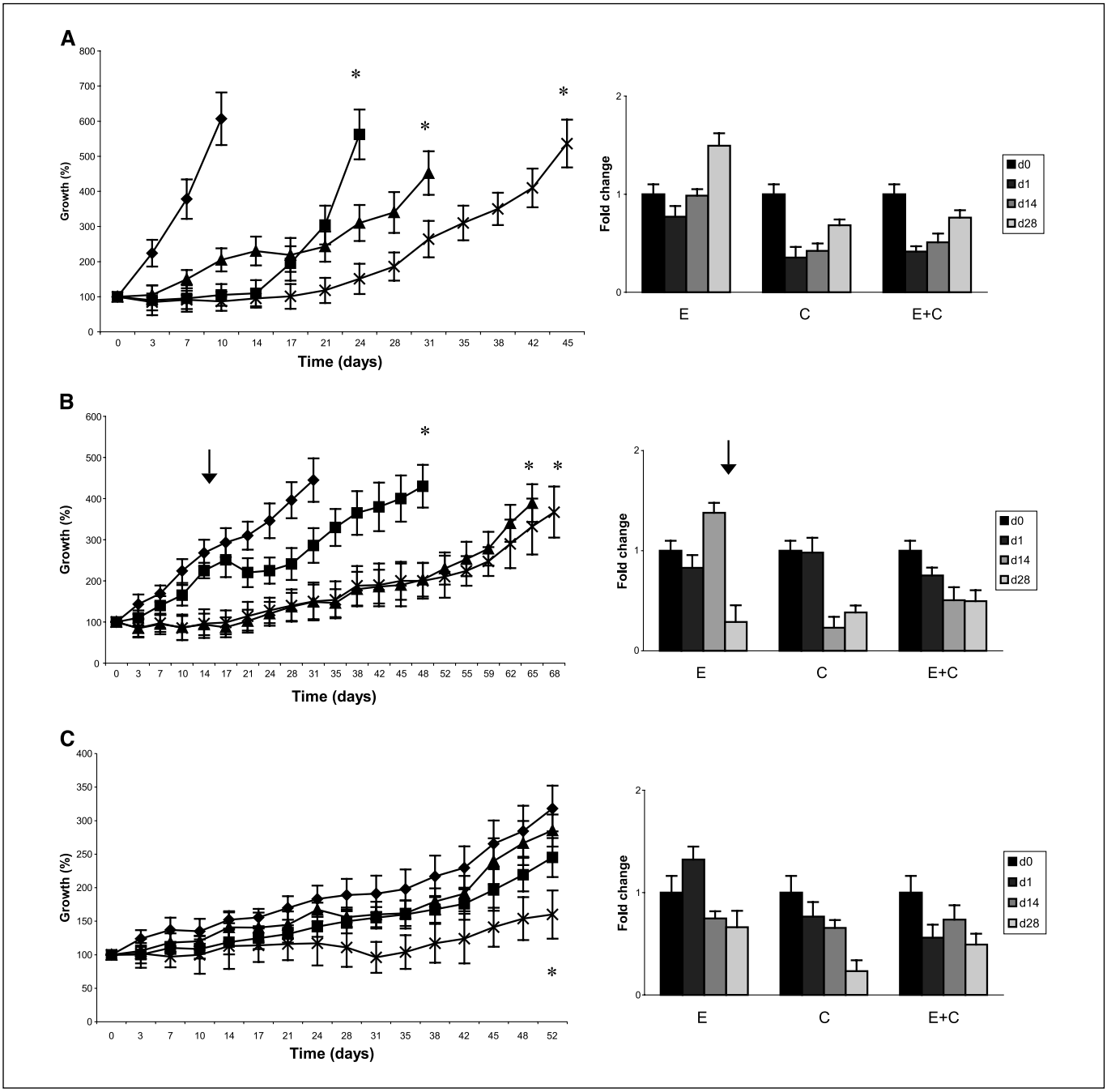

Figure 3. *A-C,* growth evaluation of A431 (*A*), HuCCT1 (*B*), and Panc430 (*C*) cell lines *in vivo*. Mice were treated with vehicle (♦), erlotinib (■), cetuximab (▲), or a combination of both (×) for 14 days. Survival surgery was done at baseline, and 1, 14, and 28 days after starting therapy. Tumor samples were snap-frozen, and EGFR levels at each time point from all treatment groups were determined by ELISA. Tumor growth plots (*left graphs*); relative EGFR levels as assessed by ELISA (*right graphs*); in HuCCT1, four doses of cetuximab were given after completion of erlotinib (*arrow*), and growth was significantly arrested. In Panc430, growth was significantly lower in the combination group only after 28 days of initiating therapy.

immunohistochemical assessment lacked the sensitivity to detect absolute differences up to 50% in EGFR content documented with more accurate, quantitative assays. The present findings indicate that EGFR regulation is highly sensitive and dynamic, significant changes can occur in short periods of time, and EGFR-directed therapy itself may induce such changes. Strategies consisting of seriated biopsies may be preferable to single, baseline evaluation to accurately evaluate EGFR dynamics in a clinical setting.

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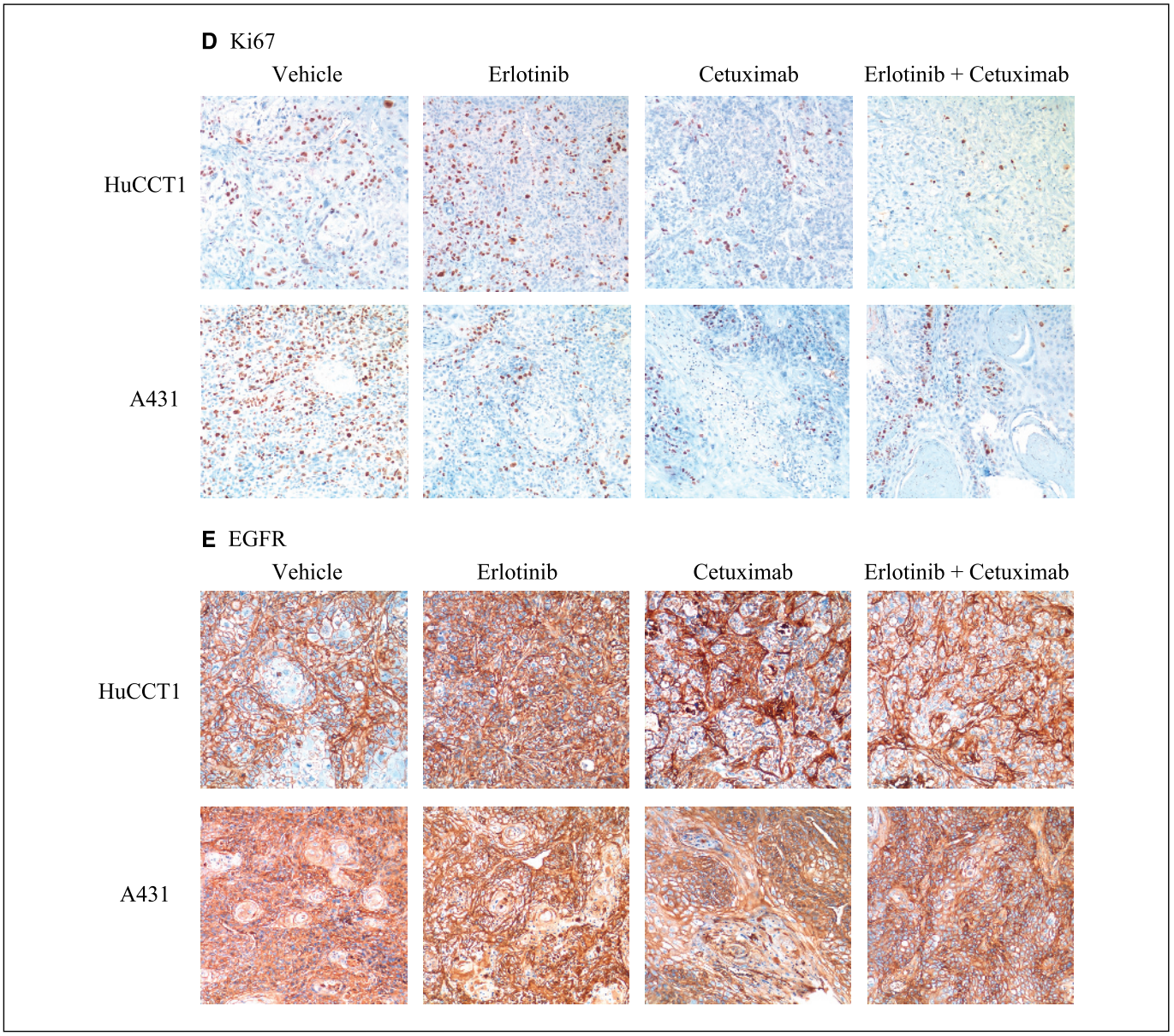

Figure 3. *Continued*. *D-E,* analysis of Ki67, and EGFR expression in HuCCT1 and A431 mice xenografts. Growth values are expressed as percentage relative to baseline ± SD (n = 12 tumors per group). Protein values are ratios normalized to their correlative control values, and represent mean ± SD of four determinations; (*C*) cetuximab, (*E*) erlotinib. *, $P < 0.05$ (t test), compared with control.

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