Dose-Dependent Increases in Circulating TGF-α and Other EGFR Ligands Act As Pharmacodynamic Markers for Optimal Biological Dosing of Cetuximab and Are Tumor Independent

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Abstract

Purpose: The objective of this study was to characterize treatment-induced circulating ligand changes during therapy with epidermal growth factor receptor (EGFR) inhibitors and evaluate their potential as surrogate indicators of the optimal biological dose.

Experimental Design: Conditioned medium from human tumor cell lines, ascites fluid from tumor xenografts, and plasma samples from normal mice, as well as colorectal cancer patients, were assessed for ligand elevations using ELISA, following treatment with cetuximab (Erbilux), an anti–mouse EGFR neutralizing antibody, or a small-molecule EGFR tyrosine kinase inhibitor.

Results: A rapid elevation in human transforming growth factor α (TGF-α) was observed in all cell lines after treatment with cetuximab, but not with small-molecule inhibitors. The elevation showed a dose-response effect and plateau that corresponded to the maximal decrease in A431 proliferation in vitro and HT29 tumor growth in vivo. The TGF-α increase was exacerbated by ongoing ligand production and cleavage from the plasma membrane but did not involve transcriptional up-regulation of TGF-α or the matrix metalloproteinase tumor necrosis factor-α converting enzyme/ADAM17. Elevations in plasma TGF-α, amphiregulin, and epiregulin were also detected in normal mice treated with an anti–mouse EGFR monoclonal antibody, illustrating a host tissue–dependent component of this effect in vivo. Finally, circulating TGF-α increased in the plasma of six patients with EGFR-negative colorectal tumors during cetuximab treatment.

Conclusions: Treatment-induced increases in circulating ligands, particularly TGF-α, should be serially assessed in clinical trials of anti-EGFR therapeutic antibodies as potential biomarkers to aid in determination of the optimal biological dose.

With increasing knowledge of the molecular mechanisms that govern carcinogenesis and tumor progression came the discovery and development of numerous new drugs designed to specifically target the relevant molecular pathways involved. The epidermal growth factor receptor (EGFR) represents one such target (1, 2). Indeed, a number of drugs targeting this receptor have been approved for cancer treatment, and others are being evaluated in late phase clinical trials (3–6). Cetuximab (C225, Erbitux) is a chimeric anti–human EGFR monoclonal antibody (7) that has recently been approved for use in metastatic colorectal carcinoma (8, 9), as well as head and neck squamous cell carcinoma (10). However, despite the recent success of targeted therapy in certain clinical oncology trials, including those using cetuximab, there still exists an urgent need for strategies to improve efficacy, as well as reduce toxicity.

One difficulty has been establishing the dosage that will effectively inhibit the intended molecular target in the tumor and/or patient. Many targeted drugs do not elicit the same type of responses, such as immediate and substantial tumor regressions and/or potent toxic side effects, such as myelosuppression, characteristic of conventional cytotoxic chemotherapeutics (11). The successful application of many of the new molecularly targeted anticancer drugs requires determination of the optimal biological dose because, unlike traditional chemotherapy, the “maximum tolerated dose” is frequently not used and sometimes cannot be applied because of the absence of dose-limiting toxicities (12). Even if dose-limiting toxicities and a maximum tolerated dose can be defined, the optimal therapeutic activity may be seen at a dose lower than the maximum tolerated dose, the so-called optimal biological dose. Thus, determining the optimal biological dose is, by definition, highly empirical, which increases the odds of selecting...
Translational Relevance

This article deals with biomarkers that are potentially clinically useful for assessing activity and optimal dosing of anti–epidermal growth factor receptor (EGFR) antibodies. It is a follow-up to one published in Cancer Research from the Kerbel laboratory, where it was shown that a single injection of an antibody to vascular endothelial growth factor VEGF receptor-2 causes an acute and marked increase of circulating plasma VEGF and, moreover, did so in a dose-dependent fashion, which plateaued at what was known to be the optimal biological (antitumor) dose of this drug in mice. It was speculated that this could be a very simple clinical biomarker to help establish the optimal biological dose for antibodies to VEGF receptor-2 and, possibly, other antibodies that target other receptor tyrosine kinases. This hypothesis has now been partially validated in some recent clinical trials. Here, we report similar data, preclinical and clinical, using antibodies to the mouse EGFR or cetuximab, that is, rapid dose-dependent increases in several EGFR-binding circulating ligands in normal mice, such as transforming growth factor α, epiregulin, and amphiregulin. However, correlate with the optimal concentration of cetuximab required to inhibit A431 viable cell proliferation in vitro. In addition, TGF-α elevations in the ascites fluid from i.p. HT29 xenografts correlated with optimal tumor growth delay. When administered to normal mice, an anti–mouse EGFR neutralizing monoclonal antibody produced increases in plasma TGF-α, amphiregulin, and epiregulin, reflecting the contribution of host tissues to this effect in vivo. Again, these increases were not observed with the use of an EGFR-specific receptor tyrosine kinase inhibitor. Finally, in a clinical pilot study, significant increases in plasma TGF-α during cetuximab treatment were shown in EGFR-negative colorectal cancer patients and therefore could represent a useful aid to optimal dose determination for this agent and possibly other anti-EGFR targeting antibodies in clinical trials.

Materials and Methods

Tumor cell lines and culture conditions. HT29 human colorectal and A431 epidermoid carcinoma cell lines were obtained from American Tissue Culture Collection (Rockville, MD). The GEO colon carcinoma cell line was a gift from Dr. Lee Ellis. All tumor cell lines were maintained in tissue culture in DMEM supplemented with 5% fetal bovine serum, except HT29, which was grown in RPMI 1640 supplemented with 5% fetal bovine serum. All cell lines were grown in monolayer culture to 75% to 90% confluence, detached, made into single cell suspensions using 0.05% trypsin-EDTA, and washed once with complete medium for in vitro experiments or followed by two washes in fetal bovine serum-free medium before injection in vivo. Cells were counted using trypan blue and adjusted to the desired concentration for plating or injection.

Reagents and drugs. Cetuximab (C225, Erbitux) and an anti–mouse-specific EGFR neutralizing monoclonal antibody were provided by Imclone Systems, Inc. and were diluted in media for in vitro experiments and 0.9% sterile normal saline before i.p. injection in vivo. The activity of the anti–murine EGFR neutralizing antibody has been described previously (19). Erlotinib (Tarceva) was generously provided by Genentech. Vandetanib (Zactima) was generously provided by Alan Barge at AstraZeneca. Gefitinib (Iressa) was a gift from Dr. Ian Tannock. Each receptor tyrosine kinase inhibitor was diluted in DMSO for use in vitro (20). Final DMSO concentration was ≤1%, and vehicle controls were included. Erlotinib and vandetanib were given by oral gavage of homogeneous suspension in 1% Tween 80 in saline. Cycloheximide (Sigma) and TAPI-1 (Pepptides International, Inc.) were also reconstituted in DMSO for use in vitro.

In vitro dose-response and time-course experiments. Cells were plated in 24-well plates at 1 × 10⁵ cells/well and incubated until they reached 75% to 90% confluence. Fresh medium containing drug at the specified concentrations was then added to each well in triplicate. Wells containing drug and media, but not cells, were also included to assess potential cross-reactivity with the ELISA. Cells were incubated in the presence of drug or vehicle for 0.25 to 48 h. Conditioned medium was centrifuged at 16,000 g for 5 mins, and supernatant was frozen at -80°C until assayed. For ligand determination in lysates, medium was removed and cells were rinsed with cold PBS, scraped from plates in cold lysis buffer [20 mmol/L Tris (pH 7.5), 137 mmol/L NaCl, 100 mmol/L NaF, 10% glycerol, 1% NP40, 1 mmol/L Na₂VO₄], centrifuged, and resuspended in cold lysis buffer. Protein content of lysates was quantified using Bradford reagent (Bio-Rad) and standardized using known amounts of bovine serum albumin (Gibco). Human TGF-α, amphiregulin, epidermal growth factor (EGF), hepatin-binding EGF, betacellulin, and VEGF were assessed using commercially available sandwich ELISA assays or DuoSets (R&D Systems, Inc.), following manufacturer’s instructions with the following exception. In experiments with low signal detection, conditioned media was added with less or suboptimal or inadequate doses for clinical trials, increasing the risk that such trials will show lesser or minimal, if any, treatment benefit.

A drug-induced increase in circulating vascular endothelial growth factor (VEGF) has emerged as a “class effect” for patients treated with a variety of drugs that target VEGF receptor pathways (13–17). We previously reported an optimal biological dose marker for a neutralizing anti–mouse VEGF receptor-2 monoclonal antibody called DC101 (13). Administration of DC101 to normal and tumor-bearing mice resulted in a rapid and remarkable increase in the plasma levels of circulating VEGF, which was tumor-independent in origin, yet paralleled antitumor activity of the drug. The VEGF results, which have been observed clinically with VEGF receptor-2 antibodies and small-molecule receptor tyrosine kinase inhibitors, prompted us to examine EGFR-targeted agents to determine whether this surrogate marker paradigm may extend to this biological system. Given that therapeutic monoclonal antibodies such as cetuximab compete for the ligand-binding site on EGFR (18), the specific objective of this study was to determine and characterize changes in circulating EGFR ligands in response to the administration of cetuximab. The relevant small-molecule receptor tyrosine kinase inhibitors erlotinib, gefitinib, and vandetanib were also evaluated because previous studies with VEGF inhibitors have revealed ligand elevations following treatment with receptor tyrosine kinase inhibitors, as well as antibodies. Using a number of EGFR-positive tumor cell lines in vitro, we observed an increase in transforming growth factor α (TGF-α) in a dose-dependent manner, following treatment with cetuximab, but not with any of the receptor tyrosine kinase inhibitors. Neither transcriptional up-regulation of TGF-α mRNA nor that of the metalloproteinase tumor necrosis factor-α–converting enzyme (TACE), which is responsible for pro–TGF-α cleavage at the plasma membrane, was associated with the increases observed. The ligand plateau did, however, correlate with the optimal concentration of cetuximab required to inhibit A431 viable cell proliferation in vitro. In addition, TGF-α elevations in the ascites fluid from i.p. HT29 xenografts correlated with optimal tumor growth delay. When administered to normal mice, an anti–mouse EGFR neutralizing monoclonal antibody produced increases in plasma TGF-α, amphiregulin, and epiregulin, reflecting the contribution of host tissues to this effect in vivo. Again, these increases were not observed with the use of an EGFR-specific receptor tyrosine kinase inhibitor. Finally, in a clinical pilot study, significant increases in plasma TGF-α during cetuximab treatment were shown in EGFR-negative colorectal cancer patients and therefore could represent a useful aid to optimal dose determination for this agent and possibly other anti-EGFR targeting antibodies in clinical trials.
no dilution to the wells to maximize the signal. All other samples were diluted as per manufacturer's instructions. Human epiregulin was assessed by ELISA, according to the method of Khambata-Ford et al (21), using antibodies obtained from R&D Systems, Inc. Optical density was determined using the microplate reader Benchmark Plus (Bio-Rad Laboratories) set to 450 nm with a wavelength correction set to 540 nm. Mouse amphiregulin, epiregulin, and EGF were assessed similarly, using commercially available sandwich ELISA assays or DuoSets (R&D Systems, Inc.). Multiple mouse cytokines, including TGF-α and EGF, were also assessed using an angiogenesis array (Panomics, Inc.), and results were reported as a comparison to positive (set to 100) and negative controls (set to 0) obtained via densitometry.

**Cell proliferation studies.** A431 cells (3 × 10⁴) were plated in 96-well plates with 200-μL media and grown to 75% confluence, then incubated with cetuximab in quadruplicate over a dose range of 0.01 to 100 μg/mL for 90 h, and viable cell count was then assessed using the MTS method. Medium (100 μL) was removed from each of the four wells and pooled for TGF-α analysis by ELISA immediately before addition of 100 μL MTS reagent/well.

**RNA extraction and Northern blots.** Cells were trypsinized, washed with PBS, and centrifuged; pellets were resuspended in Trizol (Life Technologies, Inc.). Trizol extraction was carried out according to manufacturer's instruction. Human TGF-α and TACE/ADAM17 cDNA fragments were generated by reverse transcription-PCR, confirmed by sequencing, and used as a probe. Northern blotting was carried out as previously described (22), using 32P-dCTP labeled probes. Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control.

**In vivo experiments.** Female 6-8 wk old BALB/c mice (Jackson laboratories) were treated with 3 doses of murine EGFR monoclonal antibody or saline every 3 d by the i.p. route. Plasma samples were obtained 24 h after the third dose in Microtainer plasma separating tubes (Becton Dickinson), centrifuged at 7,500 × g for 15 mins at 4°C, and frozen at -80°C until analysis for mouse-specific EGF ligands, as outlined previously. Similar experiments were done with erlotinib (100 mg/kg), vandetanib (50 mg/kg), or vehicle control given daily by oral gavage for 7 d, with plasma samples obtained 24 h after the final treatment. For HT29 experiments, 2 × 10⁶ cells were injected into the peritoneum of 6- to 8-wk-old female athymic (nu/nu) mice (Harlan laboratories) and allowed to grow to a large tumor burden with ascites evident. Mice were then injected with a single dose of cetuximab, erlotinib, or saline, as outlined previously. Animals were sacrificed 24 h after injection, and ascites samples were collected in Microtainer plasma separating tubes and processed as for plasma samples. Growth delay experiments used HT29 cells that had been transfected with human choriionic gonadotropin β-subunit (β-hCG), as previously described (23), to allow for noninvasive tracking of tumor burden in the urine of treated mice. For the growth delay trial, 2 × 10⁶ HT29(β-hCG) cells were injected into the peritoneum, and cetuximab treatments were given at the doses indicated twice weekly, beginning 6 d after tumor injection. Urine was collected from mice weekly using metabolic cages, pooled together within each treatment group (n = 5), and frozen at -80°C until analysis. β-hCG was measured using a commercially available ELISA (Omega Diagnostics Ltd.) and standardized for urine creatinine measured by colorimetric assay (QuantiChrom, Bioassay Systems). This model for tracking tumor burden has been previously validated in our laboratory (23).

**Patient samples.** Following institutional research ethics board approval and informed consent, heparinized plasma samples were obtained from patients enrolled in a single-agent cetuximab clinical trial.

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**Fig. 1.** Dose-response and time-course analysis of TGF-α in conditioned media of EGRF-positive cell lines incubated with cetuximab. A, cells were plated in monolayer culture at 1 × 10⁵/well, grown to 75% to 90% confluence, and treated with cetuximab for 24 h. Conditioned medium was removed and assayed for TGF-α by ELISA, as outlined in Materials and Methods. B, identical experiment assessing amphiregulin in conditioned media of A431 cells with cetuximab treatment. Experiments conducted twice in triplicate. Symbols and bars, mean ± SD.
for metastatic EGFR-negative colorectal cancer. Cetuximab was administered at the standard dose regimen of 400 mg/m² for week 1, followed by weekly doses of 250 mg/m². Blood samples were obtained 2 h before, then 2 and 24 h following the first, second, and fourth treatment. Samples were centrifuged at 1,000 × g for 15 mins at 4°C, within 30 mins of collection, and aliquots were stored at -80°C until analysis.

**Statistical analysis.** Results of in vitro assays are reported as the mean ± SD and in vivo assays and patient samples as mean ± SEM. Statistical significance was assessed by one-way ANOVA, followed by repeated measures ANOVA followed by Newman-Keuls test using GraphPad Prism software package v.4.0 (GraphPad Software, Inc.). The level of significance was set at *P* < 0.05 (in figures, *** < 0.001, 0.001 < ** < 0.01, and 0.01 < * < 0.05).

**Results**

Dose-dependent ligand elevations are rapidly observed with anti-EGFR antibody but not receptor tyrosine kinase inhibitor treatment in vitro. To assess changes in ligand levels after drug treatment, human cancer cell lines that express EGFR were chosen from a wide variety of tumor types. Upon incubation with increasing doses of cetuximab for 24 hours, a dose-response effect on the level of TGF-α in conditioned media was observed in each cell line, which reached a plateau after a certain drug concentration had been reached (Fig. 1A). The cell lines reported in Fig. 1 include A431 epidermoid, HT29 colorectal, and GEO colorectal carcinoma; however, TGF-α elevations were also observed in U87 glioma, MDA MB231 breast, SW839 renal, BxPC3 pancreatic, DU145 prostate, and A549 lung carcinoma cell lines (data not shown). The level of TGF-α attained and the concentration of cetuximab at ligand plateau varied between cell lines. TGF-α was not the only ligand to show this elevation because a dose response was also noted for amphiregulin with the A431 cell line (Fig. 1B). To test how quickly the ligand elevation is observed, time-course studies were done using HT29. The level of TGF-α differed between

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**Fig. 2.** TGF-α increases are not the result of transcriptional up-regulation and are dependent on ligand cleavage by TACE. A, large increases in TGF-α in conditioned media are observed after incubation with cetuximab for 24 h in the HT29, A431, and GEO cell lines (top), but there is no detectable difference in TGF-α mRNA between treated and untreated samples in the cell lysates, as assessed by Northern blot (bottom). Lanes of Northern blot correspond to the cell lines represented in the bars of graph directly above. B, cycloheximide (100 μg/mL) suppresses the cetuximab-induced increase in TGF-α to basal level in HT29 cells after 6 h. Cells were preincubated with cycloheximide for 30 mins before addition of cetuximab. Similar results were obtained with A431 (not shown). C, large increases in TGF-α in conditioned media (A, top) correspond to decreases in TGF-α protein in lysates, as assessed by ELISA, and expressed at picogram/gram total protein (bottom). Bars in bottom correspond to lanes and bars directly above in A. D, TAPI-1 treatment (100 μg/mL) suppresses the cetuximab-induced increase in TGF-α to near basal level in HT29 cells after 6 h. Cells were not preincubated with TAPI-1 before addition of cetuximab. Similar results were obtained with A431 (not shown). Cetuximab dose is 100 μg/mL in all experiments. Symbols and bars, mean ± SD.
cetuximab-treated and untreated cultures at 15 minutes after drug addition, and accumulation of TGF-α in conditioned media became much more pronounced after 2 hours (data not shown). Similar experiments were done with the receptor tyrosine kinase inhibitors erlotinib, gefitinib, and vandetanib. Upon incubation with receptor tyrosine kinase inhibitor at concentrations up to 100 μmol/L for 24 hours, no elevations in TGF-α were observed compared with untreated cell cultures (data not shown).

Increased TGF-α is not the result of transcriptional up-regulation and is impacted by ligand cleavage. To investigate whether the TGF-α increase could be the result of a feedback response to EGFR signaling blockade resulting in increased TGF-α production, Northern blots were done on lysates from the HT29, A431, and GEO cell lines incubated with cetuximab for 24 hours. No differences in TGF-α mRNA after treatment were apparent, indicating that the increase in TGF-α likely does not occur as a compensatory response to treatment at the transcriptional level (Fig. 2A) during this period. However, ongoing TGF-α production is required to appreciate the maximum ligand effect during EGFR blockade because experiments using cycloheximide in HT29 showed abrogation of the cetuximab-induced TGF-α elevation in conditioned media (Fig. 2B). The cycloheximide experiments were also done in A431 cells, with similar results (data not shown). Next, to examine the effects of cetuximab treatment on cell-associated TGF-α protein, ELISA of cell lysates was done. TGF-α levels were decreased in lysates following treatment (Fig. 2C), which prompted examination of the possibility that up-regulation of pro–TGF-α cleavage was occurring. TGF-α, like many receptor tyrosine kinase growth factors, exists as a pro-ligand attached to the plasma membrane is cleaved by proteases for release of its active fragment (24). The enzyme implicated in TGF-α cleavage is TACE/ADAM-17 (25). Inhibition of TACE activity using the MMP inhibitor TAPI-1 revealed near complete, but not total, abrogation of the TGF-α increase induced by cetuximab (Fig. 2D), showing the important contribution of TACE to TGF-α elevation during cetuximab treatment. In addition, to rule out a compensatory increase in TACE production, Northern blots were done and did not reveal a difference in TACE mRNA levels with and without exposure to cetuximab (Fig. 2A).

TGF-α plateau predicts the optimal cetuximab concentration to inhibit tumor proliferation/viability in vitro. To assess whether the increase in ligands such as TGF-α may have potential as a pharmacodynamic marker of optimal EGFR blockade, A431 and GEO cells, previously showed to be sensitive to EGFR inhibition in vitro (26, 27), were incubated with cetuximab in a dose-response study for 90 hours. When an aliquot of conditioned media was removed for TGF-α analysis, followed by MTS assay, the maximum decrease in viable cell number corresponded to a steep increase in TGF-α in conditioned media (Fig. 3). Thymidine proliferation assays done with A431 showed a similar result (data not shown). Erlotinib caused a large reduction in viable cell number with this assay (~50% at 90 hours) without a corresponding change in ligand levels in conditioned media compared with vehicle control.

Tyrosine kinase inhibitor studies were also done in HT29 cells transfected with h-hCG show a TGF-α dose-response relationship 24 h after a single dose of cetuximab. No elevation was seen following erlotinib treatment (50 mg/kg).

Surrogate Markers for Optimal Cetuximab Dosing
Ligand elevations show a dose-response effect and parallel tumor suppression in a xenograft model. Given that the target of cetuximab is human EGFR, assessment of ligand changes in vivo necessitated the implantation of a human tumor xenograft into immunodeficient mice. In an attempt to obtain sufficient growth factor levels for assay by ELISA, HT29 tumors were implanted into the peritoneal cavity. To track growth delay, HT29 cells were used that had been transfected with β-hCG. This secreted artificial tumor marker was previously developed and validated by Shih et al. (28), and used successfully in the HT29 i.p. ascites tumor model in our laboratory (23). Plasma levels of TGF-α were not detectable using this model; however, ascites fluid samples provided sufficient growth factor for detection. This tumor showed a TGF-α dose-response and plateau at the ≥500-μg cetuximab dose 24 hours after a single treatment (Fig. 4A). This effect was not observed after treatment with erlotinib (50 mg/kg i.p.). With this information, a growth delay trial was done in this model with cetuximab dosing twice weekly i.p. beginning 6 days after inoculation. Tumor burden assessment via urinary β-hCG measurements was obtained weekly. β-hCG began to increase between weeks 4 and 5, and by week 6, the superior performance of cetuximab at the 500- and 1,000-μg doses (as predicted by TGF-α elevation in previous studies) became evident (Fig. 4B).

Plasma ligand elevations occur following anti-EGFR antibody, but not receptor tyrosine kinase inhibitor, treatment of non tumor-bearing mice. To assess the potential contribution of host EGFR-positive tissue blockade, non-tumor-bearing BALB/c mice were treated with the anti–mouse EGFR antibody for 1 week. Plasma samples taken from these mice revealed increases in plasma amphiregulin (Fig. 5A), as well as epiregulin, but not EGF (data not shown). When mice were treated with high doses of erlotinib by daily gavage for 1 week, there were no significant increases in any of these ligands (Fig. 5A; similar results obtained for vandetanib; data not shown). For confirmation of the effect of species specificity, cetuximab (which only binds human EGFR) was included as a negative control and did not produce an increase in any of the ligands tested (Fig. 5A). Assessment of mouse TGF-α was carried out using a mouse antibody array, which showed a 3.2-fold increase in antibody-treated versus control (Fig. 5B). Although less quantitative, this assay confirmed the TGF-α results that were shown using in vitro models. This array also confirmed the low levels of EGF in mouse plasma before and

![Graph A](image1.png)

**Fig. 5.** Anti–mouse EGFR neutralizing antibody but not the receptor tyrosine kinase inhibitor erlotinib causes an increase in EGFR ligands in non-tumor-bearing mice. A, mice were treated with daily oral gavage of erlotinib (100 mg/kg) or vehicle for 7 d or i.p. injection on days 1, 4, and 7 of cetuximab (1 mg), anti–mouse EGFR antibody (1 mg), or saline (5 mice per group). Plasma samples were obtained 24 h after the final treatment and assayed for amphiregulin by ELISA, as outlined in Materials and Methods. B, angiogenesis array showing elevated TGF-α in plasma along with apparent elevations in fibroblast growth factor-α, leptin, tumor necrosis factor-α, and decreased VEGF. EGF was not detectable by this assay (pooled plasma from 5 mice per group). EGFR antibody dose and schedule as in (A). Symbols and bars, mean ± SEM. C, ligand increases observed in normal mice are dose dependent. Mice were treated with 3 doses of anti–mouse EGFR antibody or saline i.p. (days 1, 4, and 7). Plasma samples were obtained 24 h after the final treatment and assayed for amphiregulin and epiregulin by ELISA. Symbols and bars, mean ± SEM.
binding EGF, and betacellulin levels were not consistently detectable in all six patients but increased upon cetuximab treatment in some cases (data not shown). Interestingly, circulating VEGF, which is not a direct ligand for EGFR but is impacted by EGFR signaling, seemed to mildly decrease in patients treated with cetuximab (Fig. 6B), as may be expected from previous studies (28).

**Discussion**

To date, the study of potential biomarkers for application to EGFR targeted drugs has focused mainly on predicting which patients will respond favorably to treatment, and the use of sequential biopsies with assessment of signaling proteins histologically has shown some promise in this regard (26, 31), as has the presence or absence of ras mutations in colorectal carcinoma (21). In addition, EGFR ligands have been studied in tumor tissue as prognostic markers for treatment response and/or survival in experimental models and clinical trials. Interestingly, high levels of tumor amphiregulin and epiregulin gene expression were associated with a positive response to cetuximab in a recent colorectal cancer trial (21). Although it is currently unknown whether or not the serial assessment of ligand changes would be useful for individual patient prognosis, this test may have utility as a pharmacodynamic marker indicative of adequate drug exposure. Attractive features of ligand monitoring in the circulation, when compared to other biomarkers, include the ease of application of ELISA technology and the noninvasive nature of repeated sample acquisition.

EGFR signaling is prevalent in many tissues, and the side effect profile of EGFR inhibitors reflects this fact (1, 4, 32, 33). Increases in serum or plasma TGF-α in patients may therefore reflect the degree of EGFR blockade throughout the entire body, that is, normal and tumor tissue, which is why it is not surprising to observe this increase in non-tumor-bearing mice, as well as patients with EGFR-negative tumors, the difficulties inherent in proper tumor EGFR classification notwithstanding (34, 35). Although cutaneous rash is a host tissue response that has shown potential as a predictive factor for response to EGFR inhibitors, as well as survival in some studies (32, 36), assessing the effectiveness of EGFR blockade using the severity of cutaneous side effects reflects only one tissue type, whereas it is possible that circulating ligand changes may be more reflective of total body EGFR. It would be of interest to undertake parallel studies comparing the extent of TGF-α and other ligand increases with the severity of rash.

TGF-α was the only ligand in this study that became consistently elevated upon EGFR antibody treatment and would therefore seem to be the most promising for potential application as a biomarker, although the number of patients assessed in this pilot study was small. Potential reasons for the observed variability between ligands include differences in receptor affinity and/or intracellular trafficking (37–39). Further studies are needed to investigate the role such factors may have in treatment-induced ligand changes.

In contrast to cetuximab, none of the three EGFR-targeted receptor tyrosine kinase inhibitors evaluated produced an elevation in EGFR ligands in conditioned media or plasma; however, the treatment time was relatively short, and the

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**Fig. 6.** Cetuximab treatment increases plasma TGF-α in patients with EGFR-negative colorectal carcinoma. A. Plasma samples from six patients obtained before the first, second, and fourth cycles of treatment, and assayed for TGF-α by ELISA, as outlined in Materials and Methods. B. Plasma VEGF levels from six patients over the same time points as mentioned previously.
ATP mimetics, which bind inside the cell, continue to allow accumulation, regardless of whether or not the growth factor disruption of this clearance by cetuximab results in free TGF-\(\beta\). Because receptor binding and internalization represent a example, would be quickly internalized after binding EGFR (24). Before receptor blockade. This conclusion is also consistent with the fact that neutralizing antibodies such as cetuximab have been used as tools to study autocrine EGFR signaling (44). Before the use of a neutralizing antibody, it was difficult to study growth factor levels in conditioned media because TGF-\(\alpha\), for example, would be quickly internalized after binding EGFR (24). Because receptor binding and internalization represent a major clearance mechanism for this growth factor (45, 46), disruption of this clearance by cetuximab results in free TGF-\(\alpha\) accumulation, regardless of whether or not the growth factor source is autocrine in nature. Conversely, the small-molecule ATP mimetics, which bind inside the cell, continue to allow ligand to engage surface EGFR. Therefore, in addition to their vast differences in pharmacokinetics, the discrepancy between EGFR antibodies and receptor tyrosine kinase inhibitors could also be related to these differing sites of drug binding.

The inability of TGF-\(\alpha\) to be cleared after blockade of its normal route of internalization does not address the question of whether or not production or cleavage becomes up-regulated as a result of EGFR blockade. Our Northern blot results would seem to refute this possibility. In fact, a positive-feedback autocrine loop has previously been described for the A431 cell line, among others (21, 27, 47). Treatment with cetuximab actually resulted in a reduction of TGF-\(\alpha\) mRNA at 4 hours (27), so it is perhaps not surprising that there was not a compensatory increase at the transcriptional level in our experiments. Similar results were also obtained about ligand cleavage activity becoming down-regulated upon disruption of EGFR signaling in that same study (27). In addition, the very rapid onset of differences in TGF-\(\alpha\) between treated and untreated cell cultures may also be significant mechanistically because it decreases the likelihood that up-regulated growth factor production is exclusively responsible for these results. However, the resulting increase in circulation is perhaps exacerbated by other factors such as ongoing constitutive tissue TGF-\(\alpha\) production and/or decreased cell surface EGFR expression that has been shown with cetuximab treatment (48).

Finally, another possible implication of these findings is the fact that certain EGFR ligands may bind more than the receptor being targeted by monoclonal antibody; epiregulin, heparin-binding EGF, and betacellulin can all bind EGFR and HER4, for example (24). The biological implications of these ligand increases secondary to antibody treatment that targets only EGFR remain a priority for investigation. In addition, the possible increase in ligands considered "off target," such as fibroblast growth factor \(\alpha\), leptin, and tumor necrosis factor-\(\alpha\) is also of interest. A similar finding, but with differing cytokines, was recently shown with sunitinib (49). The consequences of such host changes are currently unknown but worthy of further study. In the meantime, the clinical utility of elevated circulating TGF-\(\alpha\), as well as other EGFR ligands, as potential pharmacodynamic biomarkers during EGFR-directed antibody treatment is indicated, including the incorporation of serial ligand assessment into the design of future clinical trials.

Disclosure of Potential Conflicts of Interest

Y. Wu and L. Witte are employed by Imclone Systems, Inc., and R.S. Kerbel is a Canada Research Chair.

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References

Surrogate Markers for Optimal Cetuximab Dosing


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