Cancer Therapy: Preclinical

Increased TGF-α as a Mechanism of Acquired Resistance to the Anti-EGFR Inhibitor Cetuximab through EGFR–MET Interaction and Activation of MET Signaling in Colon Cancer Cells

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Abstract

Purpose: Although cetuximab, an anti-EGF receptor (EGFR) monoclonal antibody, is an effective treatment for patients with KRAS wild-type metastatic colorectal cancer (mCRC), its clinical use is limited by onset of resistance.

Experimental Design: We characterized two colorectal cancer models to study the mechanisms of acquired resistance to cetuximab.

Results: Following chronic treatment of nude mice bearing cetuximab-sensitive human GEO colon xenografts, cetuximab-resistant GEO (GEO-CR) cells were obtained. In GEO-CR cells, proliferation and survival signals were constitutively active despite EGFR inhibition by cetuximab treatment. Whole gene expression profiling identified a series of genes involved in the hepatocyte growth factor (HGF)-MET–dependent pathways, which were upregulated in GEO-CR cells. Furthermore, activated, phosphorylated MET was detected in GEO-CR cells. A second colorectal cancer cell line with acquired resistance to cetuximab was obtained (SW48-CR). Inhibition of MET expression by siRNA restored cetuximab sensitivity in GEO-CR and SW48-CR cells, whereas exogenous activation of MET by HGF stimulation in cetuximab-sensitive GEO and SW48 cells induced resistance to cetuximab. Treatment of GEO-CR and SW48-CR cells with PHA665752, a selective MET inhibitor, inhibited cell growth, proliferation, and survival signals and impaired cancer cell migration. Overexpression of TGF-α, a specific EGFR ligand, was involved in the acquisition of cetuximab resistance in GEO-CR and SW48-CR cells. In fact, TGF-α overexpression induced the EGFR–MET interaction, with subsequent MET phosphorylation and activation of MET downstream effectors in GEO-CR and SW48-CR cells.

Conclusions: These results suggest that overexpression of TGF-α through induction of EGFR–MET interaction contributes to cetuximab resistance in colorectal cancer cells. The combined inhibition of EGFR and MET receptor could represent a strategy for preventing and/or overcoming cetuximab resistance in patients with colorectal cancer. 

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Introduction

Colorectal cancer is one of the most frequently diagnosed malignant diseases in Europe and one of the leading causes of cancer-related death worldwide (1). Recent therapeutic strategies for metastatic colorectal cancer (mCRC) have been focused on developing molecularly targeted therapies. The EGFR is expressed in 60% to 80% colorectal cancer and plays a key role in the development and progression of human cancers and for this reason it has been proposed as a target for anticancer therapies (2, 3). Cetuximab, an anti-EGF receptor (EGFR) blocking monoclonal antibody (mAb), is an effective treatment as single agent and in combination with standard chemotherapy regimens for patients with KRAS wild-type mCRC (4).

Cetuximab treatment is effective in a subgroup of patients with mCRC because resistance to anti-EGFR therapies could be due to the constitutive activation of signaling pathways acting downstream of the EGFR. Several retrospective clinical studies have demonstrated that patients with mCRC with tumors harboring a KRAS mutation have no clinical benefit following cetuximab treatment, whereas, in patients...
Translational Relevance

Cetuximab, an anti-EGFR monoclonal antibody (mAb), has proven to be effective in combination with chemotherapy or as a single agent for treatment of patients with KRAS wild-type metastatic colorectal cancer (mCRC). Clinical data indicate that even the best responses are transient and eventually all patients develop acquired resistance. This evidence triggered a series of studies on the molecular mechanisms of primary and acquired resistance to cetuximab. In the present study, we have demonstrated that resistance to cetuximab in colorectal cancer cells is mediated by TGF-α overexpression, which induced the EGFR–MET interaction with subsequent MET pathway activation. Blockade of both EGFR and MET receptor tyrosine kinases could represent a strategy for preventing and/or overcoming cetuximab resistance in patients with colorectal cancer. These results could be of relevant clinical interest for the design of translational research-based clinical studies with cetuximab in combination with MET inhibitors.

with wild-type KRAS tumors, a significant clinical benefit in terms of progression-free survival and overall survival has been observed (5, 6). The evidence that KRAS mutations were associated with the lack of response to cetuximab or panitumumab, another anti-EGFR blocking mAb, in patients with mCRC has led the U.S. Food and Drug Administration and the European Medicines Agency to restrict the use of anti-EGFR mAbs to patients with KRAS wild-type tumors. In addition to KRAS gene mutations, several retrospective studies in patients with chemorefractory mCRC have provided evidence that primary resistance to cetuximab could be correlated with mutations in other intracellular downstream effectors of EGFR activation, such as BRAF, NRAS, and PIK3CA exon 20 genes (7, 8). However, even among the molecularly enriched subset of patients with colorectal cancer with KRAS, BRAF, NRAS, and PIK3CA exon 20 wild-type genes, cetuximab is not uniformly clinically effective, suggesting that there are other undefined mechanisms of cetuximab resistance (7–10). Therefore, both intrinsic and acquired resistance mechanisms significantly limit the efficacy of anti-EGFR mAbs in the medical management of patients with mCRC. In this scenario, HER2 gene amplification has recently been identified as a potential mechanism of resistance to cetuximab in a subset of patients with mCRC with wild-type KRAS/NRAS/BRAF/PIK3CA genes (11–13). A proof-of-concept, a multiarm preclinical study in mice bearing human colorectal cancer tumors with HER2 amplification (‘xenopatients’), revealed that the combined inhibition of HER2 and EGFR induced long-lasting tumor regression (12). Other mechanisms of resistance have recently been reported, such as MET and KRAS amplification (14, 15). Therefore, an understanding of these mechanisms is necessary to design effective therapies for patients to prevent or to overcome clinical resistance to cetuximab treatment.

MET is a cell membrane tyrosine kinase receptor for the hepatocyte growth factor (HGF). Deregulation and consequent aberrant MET signaling may occur by different mechanisms and it has been reported that MET is overexpressed in a variety of cancers including colorectal cancer (16, 17). High expression of MET in colorectal is associated with development of distant metastases (18). Increased and deregulated MET signaling has been identified as one of the key pathways to bypass growth inhibition caused by drugs targeting the EGFR in lung adenocarcinoma (19, 20). Several clinical studies have evaluated the combination of MET and EGFR inhibitors in patients with non–small cell lung cancer (NSCLC; refs. 21–23).

Furthermore, a functional link between EGFR and MET has been suggested (24). In this respect, expression of EGFR and MET correlate in multiple malignancies such as choroidoma, prostate and ovarian carcinomas, and gastrinoma (25). EGFR has been implicated in HGF-induced hepatocyte proliferation and is required for MET-mediated colon cancer cell invasiveness (26). Despite these studies, the underlying mechanisms of EGFR-induced MET phosphorylation are not yet well understood.

In the present study, we have generated and characterized two human colon cancer cell models of acquired resistance to cetuximab to elucidate the molecular mechanisms of resistance. We have found that in cetuximab-resistant colorectal cancer cells, cell proliferation, and survival pathways are activated by MET. Interestingly, enhanced expression of the selective EGFR ligand TGF-α in cetuximab-resistant colorectal cancer cells is responsible for EGFR–MET interaction and subsequent EGFR-induced MET phosphorylation and activation. Finally, treatment of these cells with a selective MET inhibitor restores cetuximab sensitivity, suggesting that the combined inhibition of both EGFR and MET receptor tyrosine kinases (RTK) could represent a rational therapeutic strategy for preventing and/or overcoming cetuximab resistance in patients with mCRC.

Materials and Methods

Drugs

Cetuximab, an anti-EGFR human–mouse chimeric mAb was kindly provided by Merck Serono. PHA665752, a selective MET tyrosine kinase inhibitor (TKI), was purchased from Santa Cruz Biotecnology. PHA665752 was dissolved in sterile dimethyl sulfoxide and a 10 mmol/L working solution was prepared and stored in aliquots at −20°C. Working concentrations were diluted in culture medium just before each experiment.

Generation of cetuximab-resistant GEO and SW48 cells

Four- to six-week-old female balb/c athymic (nu−/nu−) mice were purchased from Charles River Laboratories. The research protocol was approved and mice were maintained in accordance with the institutional guidelines of the Second University of Naples Animal Care and Use Committee.
(Naples, Italy). Mice were acclimatized at the Second University of Naples Medical School Animal Facility for 1 week before being injected with cancer cells. Mice were injected subcutaneously with 2.5 × 10⁶ GEO cells that had been resuspended in 200 μL of Matrigel (BD Biosciences). When established tumors of approximately 200 to 300 mm³ in diameter were detected, mice were treated continuously by intraperitoneal (i.p.) injection with cetuximab (1 mg twice weekly) for the indicated time periods. Each treatment group consisted of 8 mice. Tumor size was evaluated twice per week by calliper measurements using the following formula: \( \pi/6 \times \text{larger diameter} \times (\text{smaller diameter})^2 \). The animals were sacrificed when the tumor diameter exceeded 1,500 mm³. Tumor from the treated group, were removed, digested, and suspended as a single cell, which were propagated in quadruplicate. The starting dose was the dose causing the inhibition of 50% of cancer cell growth (IC50). The drug concentrations of cetuximab. The starting dose was the dose causing the inhibition of 50% of cancer cell growth (IC50). The drug dose was progressively increased to 1 μg/mL in approximately 2 months, to 5 μg/mL after other 2 months, and finally, to 10 μg/mL after additional 2 months. The established cetuximab-resistant SW48 cancer cell line (SW48-CR) was then maintained in continuous culture with this maximally achieved dose of cetuximab that allowed cellular proliferation.

**Cell lines**

The human GEO colon cancer cell was a gift of Dr. N. Normanno (National Cancer Institute, Naples, Italy). The human SW48 colon cancer cell line was obtained from the American Type Culture Collection. Four GEO cetuximab-resistant clones were established as in vitro cell lines after cancer cell recovery and enzymatic treatment (27) from in vivo GEO tumor xenografts in mice, as previously described (27). GEO and GEO cetuximab-resistant clones were grown in Dulbecco’s Modified Eagle Medium (Lonza) supplemented with 20% FBS (Lonza), 1% penicillin/streptomycin (Lonza) and maintained in a humidified atmosphere of 95% air and 5% carbon dioxide (CO₂) at 37°C. SW48 and SW48-CR cells were grown in RPMI-1640 (Lonza) supplemented with 10% FBS, 1% penicillin/streptomycin. All cell lines were routinely screened for the presence of mycoplasma (Mycoplasma Detection Kit, Roche Diagnostics).

**Proliferation assay**

Cancer cells were seeded in 24-well plates and were treated with different concentrations of PHA665752 (range, 0.01–10 nmol/L), cetuximab (range, 0.01–20 μg/mL) alone or in combination for 96 hours. Cell proliferation was measured with MTT. The IC₅₀ was determined by interpolation from the dose-response curves. Results represent the median of three separate experiments, each performed in quadruplicate.

**Apoptosis assay**

GEO, GEO-CR, and SW48-CR cells were seeded in 6-well plates, treated for 72 hours, and stained with Annexin V–fluorescein isothiocyanate (FITC). Apoptotic cell death was assessed by counting the numbers of cells that stained positive for Annexin V–FITC and negative for propidium iodide using the Apoptosis Annexin V-FITC Kit (Invitrogen), coupled with fluorescence-activated cell sorting analysis.

**Western blotting and immunoprecipitation**

GEO, SW48, GEO-CR, and SW48-CR cells were seeded into 100 mm³ dishes and treated for 24 hours. Protein lysates containing comparable amounts of proteins, estimated by a modified Bradford assay (Bio-Rad), were subjected to immunoprecipitation or direct Western blot analysis. Immunocomplexes were detected with the enhanced Chemiluminescence Kit (Pierce Biotechnology Inc.). Anti-EGFR, phospho-EGFR (Tyr1068), HER2, HER3, HER4, p44/42 MAPK, phospho-p44/42MAPK, AKT, phospho-AKT (Ser473), MET, phospho-MET (Tyr 1234/1235), STAT3, phospho-STAT3, FAK, and phospho-FAK were purchased from Cell Signaling Technology. Monoclonal anti-α-tubulin antibody was purchased from Sigma Chemical Co. Secondary antibodies coupled to horseradish peroxidase were from GE Healthcare. Each experiment was done in triplicate. Two milligrams of protein lysates were immunoprecipitated with the required antibodies; immunocomplexes were recovered with protein G Sepharose (Roche Diagnostics) and detected by Western blotting.

**Microarray gene expression analysis**

Agilent (Agilent Technologies) microarray analyses were performed to assess baseline gene expression profile for GEO and GEO-CR cells using a one color labeling microarray system as previously described (28). The absolute amount and purity (A260/280 nm ratio) of total RNA samples were determined by spectrophotometry (Nanodrop, Thermofisher) and the size distribution was assessed by Agilent Bioanalyzer. Eight hundred nanograms of total RNA were converted into labeled cRNA with nucleotides coupled to a fluorescent dye (either Cy3 or Cy5) following the manufacturer’s protocol (Quick Amp Kit, Agilent). Yield and purity (A260/280 nm ratio) of cRNAs were determined by spectrophotometry (Nanodrop, Thermofisher). Of note, 825 ng of cRNA labeled from GEO colon cancer cell lines were hybridized to Agilent Human Whole Genome 4 × 44 k Microarrays. Data were extracted from slide image using Agilent Feature Extraction software (v.10.5). The raw data and associated sample information were loaded and processed by Gene Spring 11.5X (Agilent Technologies). For identification of genes significantly altered in resistant cells, total detected entities were filtered by signal intensity value (upper cutoff 100th and lower cutoff 20th percentile) and flag to remove very low signal entities. Experiments were carried out in triplicate and data were analyzed using Student t test (\( P < 0.05 \)) with a Benjamani–Hochberg multiple test correction to minimize selection of false positives. Of the significantly differentially expressed RNA, only those with greater than 2-fold increase or 2-fold decrease as compared with the controls were used for further analysis. Functional and network analyses of statistically significant
gene expression changes were performed using Ingenuity Pathways Analysis (IPA) 8.0 (Ingenuity Systems; http://www.ingenuity.com). Analysis considered all genes from the data set that met the 2-fold ($P < 0.05$) change cutoff and that were associated with biologic functions in the Ingenuity Pathways Knowledge Base. The significance of the association between the data set and the canonical pathway was measured in two ways: (i) ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway is displayed; (ii) Fisher exact test was used to calculate a $P$ value determining the probability that the association between the genes in the data set and the canonical pathway is explained by chance alone.

RNA interference and PCR analysis

The small inhibitor duplex RNAs (siRNA) (ON-target plus SMARTpool) siMET (human: #L-003156-00), siTGF-$\alpha$ (human: #L019737), and siHBEGF (human: #L019624) were from Dharmacon. The siCONTROL Non-targeting a (human: #L019737), and siHBEGF (human: #L019624) plus SMARTpool) siMET (human: #L-003156-00), siTGF-$\alpha$, and HBEGF in the con-

ant and HBEGF with a fluorimeter at 480/520 nm. Assays were performed in triplicate.

Gene mutation analysis

GEO, SW48, GEO-CR, and SW48-CR cells were seeded into 100 mm$^2$ dishes for 72 hours. DNA extraction was performed by the QIAeasy Kit (Qiagen) following the manufacturer’s instructions. The DNA was analyzed by next generation sequencing using the Ion AmpliSeq colon and lung panel as previously described. DNA was quantified by Nanodrop (Thermo Scientific). Ten nanograms of GEO, SW48, GEO-CR, and SW48-CR genomic DNA was analyzed by next generation sequencing using the Ion AmpliSeq colon and lung panel as previously described (30).

Statistical analysis

The statistical analyses of in $\text{vitro}$ and in $\text{vivo}$ data were carried out using Prism version 4.02 (GraphPad Software, Inc). The Student $t$ test was used to evaluate the statistical significance of the results. All $P$ values represent two-sided tests of statistical significance with $P$ value $<0.05$.

Results

In $\text{vivo}$ generation and characterization of a preclinical model of colon cancer cells with acquired resistance to cetuximab

To study the mechanisms of cetuximab-induced resistance, we generated cetuximab-resistant cells starting from cetuximab-sensitive GEO colon cancer cells, which were grown as tumor xenografts in $\text{vivo}$ in immunodeficient mice that were exposed to continuous treatment with an optimal
therapeutic dose of the drug (Fig. 1A). Continuous treatment caused tumor growth suppression within the first weeks of treatment. However, after approximately 12 weeks, in most mice GEO tumors resumed growth despite cetuximab treatment, reaching a growth rate comparable to untreated control GEO tumors within 18 to 20 weeks (Fig. 1A). Four cetuximab-resistant tumors were surgically removed and homogenized into single-cell suspensions used to generate in vitro GEO-CR cell lines, which displayed resistance to the growth inhibitory effects of cetuximab treatment (Fig. 1B). Only one of these cell lines (named GEO-CR cells) was further characterized and used for the subsequent experiments. As illustrated in Fig. 1B and C, GEO-CR cells were not sensitive to cetuximab treatment at doses up to 20 μg/mL, as compared with parental GEO cells.

The investigation of EGFR-dependent intracellular pathways revealed that treatment of GEO cells with cetuximab completely blocked EGFR phosphorylation and consequently the activation of downstream mitogen-activated protein kinase (MAPK) and AKT signals (Fig. 1D). Although cetuximab treatment markedly reduced EGFR phosphorylation in GEO cells, GEO-CR cells remained insensitive to cetuximab treatment. These findings suggest that the mechanisms of resistance to cetuximab in GEO-CR cells involve alterations in the EGFR signaling pathway.

Figure 1. Establishment and characterization of cetuximab-resistant colorectal cancer cells. A, mice bearing GEO cells were treated continuously by intraperitoneal injection with vehicle or cetuximab (1 mg daily twice weekly; n = 8/group). Treatments started when tumor reached volumes of 200 to 300 mm³. Animals were sacrificed when tumor reached 1,500 mm³ in size. Tumors from the cetuximab-treated group were removed, digested, and suspended as a single cell, which was propagated in in vitro culture. B, parental and cetuximab-resistant GEO clones were treated with increasing concentrations of cetuximab (0.01–20 μg/mL) for 96 hours and evaluated for proliferation by MTT staining, as described in Materials and Methods. The results are the average ± SD of three independent experiments each done in quadruplicate. C, apoptosis was evaluated with Annexin V staining, as described in Materials and Methods. GEO and GEO-CR were treated with cetuximab for 72 hours. The rate of apoptosis was expressed as a percentage of the total cells counted. Columns: means of three independent experiments. D, GEO and GEO-CR were treated with cetuximab (5 μg/mL) for 24 hours. Cell extracts were assayed by Western blotting to detect the indicated proteins. Experiments were repeated three times with similar results.
phosphorylation in GEO-CR cells, the downstream MAPK and AKT pathways were not completely blocked (Fig. 1D).

Identification of gene expression profiles that correlate with acquired resistance to cetuximab in GEO-CR cells

To investigate the potential molecular pathways involved in cetuximab resistance, mRNAs from GEO and GEO-CR cells were extracted and assessed for global gene expression changes by microarray analysis. In GEO-CR cells, 39 and 54 genes were identified whose expression was upregulated or downregulated, respectively, as compared with GEO cells \((P < 0.05)\). Among the 39 upregulated genes in GEO-CR cells, we identified 17 genes involved in the HGF–MET-dependent pathways, as described in Materials and Methods. Moreover, the mRNAs of two EGFR selective ligands were upregulated in GEO-CR cells as compared with GEO cells: TGF-α and heparin-binding EGF–like growth factor (HB-EGF). Among the 54 downregulated genes in GEO-CR cells, 7 genes were involved in DNA repair and 4 genes were involved in cell-cycle...
regulation (Supplementary Table S2A and S2B). By Western blot analysis, we found a moderate increase in total MET protein expression, which was not accompanied by a significant increase in MET mRNA in GEO-CR cells as compared with parental GEO cells. However, higher levels of activated phospho-MET protein were depicted (Fig. 2B).

Inhibition of MET expression by siRNA restores sensitivity to cetuximab

To determine whether MET activation could be involved in the acquisition of cetuximab resistance in GEO-CR cells, we investigated whether reduction of MET expression could restore cetuximab sensitivity. Transfection with a specific MET siRNA for 48 hours significantly reduced MET protein expression in GEO-CR cells, as shown in Fig. 3A. As illustrated in Fig. 3B and C, MET siRNA treatment slightly reduced cell growth with a little increase in apoptosis in GEO-CR cells. Although single-agent cetuximab treatment did not affect GEO-CR proliferation, cetuximab treatment in combination with MET silencing determined a statistical significant cell growth inhibition and proapoptotic effects in GEO-CR cells. MET silencing also restored the ability of cetuximab to inhibit MAPK and AKT activation in GEO-CR cells, as shown by downregulation of both phospho-MAPK and phospho-AKT levels (about 50% of inhibition, respectively; Fig. 3D).

To further evaluate whether MET could confer resistance in a different model of cetuximab-sensitive colorectal cancer cells, we used the SW48 colorectal cancer cell line, harboring KRAS, NRAS, BRAF, and PIK3CA wild-type genes, known to be cetuximab sensitive. Following continuous exposure to increasing doses of cetuximab for up to 6 months of SW48 cells, SW48-CR cells were obtained. As depicted in Supplementary Fig. S1A, SW48-CR cells were not sensitive to the growth inhibitory effect of cetuximab treatment at doses up to 20 μg/mL. The EGFR-dependent intracellular signaling pathways were not inhibited by cetuximab treatment in SW48-CR cells as compared with parental SW48 cells (Supplementary Fig. S1B). It has recently suggested that a mechanism of acquired resistance to cetuximab continuous treatment in human colorectal cancer cell lines that are initially sensitive, is the appearance of cancer clones with a mutated RAS gene (9, 10). To test this hypothesis, we have evaluated the presence of mutations in both the KRAS and the NRAS genes in GEO-CR and SW48-CR cells as compared with parental GEO and SW48 cells by next generation sequencing using the Ion AmpliSeq colon and lung cancer panel that evaluates the presence of mutations in 22 genes including exon 2, 3, and 4 KRAS and exon 2 and 3 NRAS (30). No additional mutations were observed in KRAS and NRAS genes in GEO-CR and SW48-CR cells (data not shown). Moreover, increased MET protein phosphorylation was observed in SW48-CR but not in parental SW48 cells, (Supplementary Fig. S1B). Similarly to GEO-CR cells, the inhibition of MET expression by siRNA treatment in combination with cetuximab determined a statistical significant cell growth inhibition and proapoptotic effects in SW48-CR (Supplementary Fig. S2A–S2C). Moreover, MET protein downregulation by siRNA treatment also restored the ability of cetuximab to inhibit MAPK and AKT activation in SW48-CR cells (Supplementary Fig. S2D).

Activation of MET by HGF treatment can rescue cetuximab-sensitive GEO and SW48 cells from cetuximab-induced cell growth inhibition

We next evaluated whether MET activation could confer resistance in two cetuximab-sensitive colorectal cancer cell lines (GEO and SW48). These colorectal cancer cell lines were extremely sensitive to cetuximab cell growth inhibition with IC50 of approximately 0.1 and 0.2 μg/mL, respectively (Fig. 3E). Treatment with the MET ligand HGF, significantly reduced cetuximab-induced cell growth inhibition in both cancer cells lines (Fig. 3F). In addition, despite treatment with cetuximab, HGF treatment caused MET phosphorylation and partial reactivation of MAPK and AKT in both GEO (Fig. 3G) and SW48 cells (Fig. 3H) compared with cetuximab treatment alone.

Treatment with a selective MET TKI sensitizes GEO-CR and SW48-CR cells to cetuximab

To further evaluate the role of MET activation in cetuximab resistance, treatment of GEO-CR and SW48-CR cells with PHA665752, a selective MET TKI, was performed. PHA665752 treatment of GEO-CR and SW48-CR cells induced a dose-dependent inhibition of cell growth with an IC50 of approximately 0.5 and 5 nmol/L, respectively (Fig. 4A and C). Moreover, although cetuximab treatment had no effect on cell growth in GEO-CR and SW48-CR cells, the combined treatment with PHA665752 restored the sensitivity of GEO-CR and SW48-CR cells to cetuximab in a dose-dependent manner (Fig. 4B and D). In particular, in GEO-CR cells the IC50s for treatment with cetuximab in combination with PHA665752 (0.01 or 0.05 nmol/L) were 0.1 and 0.01 μg/mL, respectively (Fig. 4B). We next assessed by Western blot analysis the effects of treatment of GEO-CR and SW48-CR cells with cetuximab and/or the selective MET inhibitor on the expression and activation of key proteins, which act downstream to EGFR and MET. As shown in Fig. 4E and F, single-agent cetuximab or single-agent PHA665752 treatment were able to completely inhibit EGFR and MET phosphorylation, respectively, while inducing a modest decrease of MAPK and AKT phosphorylation. However, the combined treatment with both drugs was able to fully abrogate phosphorylation of both MAPK and AKT in GEO-CR and SW48-CR cells. (Fig. 4E and F).

GEO and SW48 cells are differentiated colon carcinoma cells with little or no ability to migrate or to invade in vitro (35). GEO-CR and SW48-CR cells acquired the ability to migrate in an in vitro migration assay (Fig. 4G and H). To investigate whether the migration properties of GEO-CR and SW48-CR cells could be due to MET activation, we performed a migration assay on GEO-CR and SW48-CR cells in the presence of cetuximab, PHA665752 or their combination. Although single-agent cetuximab treatment had no effect on GEO-CR and SW48-CR cancer cell migration, PHA665752 significantly reduced the number of
Figure 3. Inhibition of MET expression restores cetuximab sensitivity in GEO-CR cells, and HGF-dependent MET activation rescues GEO and SW48 cells from cetuximab inhibition. A, GEO-CR cells were transfected with either a specific siRNA targeting MET or with a control (scrambled) RNA sequence and harvested 96 hours after transfection. Western blot analysis for MET expression was done as described in Materials and Methods. B, GEO-CR cells were transfected with a specific siRNA targeting MET. Twenty-four hours after transfection, cells were treated with cetuximab, 5 μg/mL. Viable cells were counted after 24 hours of treatment and plotted relative to untreated control. The results are average ± SD of three independent experiments each done in duplicate. Cetuximab plus si-MET versus si-MET (**, P < 0.005), cetuximab plus si-MET versus cetuximab (***, P < 0.0005). (Continued on the following page.)
cancer cells that were able to invade through the migration chambers. Moreover, this effect was markedly potentiated by the combined inhibition of EGFR and MET (Fig. 4G and H).

**TGF-α overexpression mediates acquired resistance to cetuximab by causing active EGFR–MET interaction in GEO-CR and SW48-CR cells**

Once demonstrated the MET role in the acquired cetuximab resistance of GEO-CR and SW48-CR cells, we further evaluated the potential mechanism(s) responsible for MET activation. One major mechanism of cell membrane growth factor receptor activation in cancer cells is the specific ligand stimulation. In this respect, HGF-driven autocrine or paracrine activation of MET has been observed in several cancers (16). However, little or no HGF secretion was detected in GEO, SW48, GEO-CR, and SW48-CR cells, suggesting that autocrine MET activation was not occurring in these cancer cell models (Fig. 5A and E). Moreover, it has been shown that several members of the EGFR family can transactivate MET by inducing the formation of heterodimers even in the absence of HGF (36). Therefore, a Western blot analysis was performed to evaluate the expression of the four members of the EGFR family. Although EGFR was expressed in GEO, SW48, GEO-CR, and SW48-CR cells, no expression of ERBB2, ERBB3, and ERBB4 was found in these cancer cell lines (data not shown). According to these results, we hypothesized that MET activation could be caused by interaction with the EGFR in GEO-CR and SW48-CR cells. By microarray gene expression analysis, the two specific EGFR ligands TGF-α and HB-EGF, were upregulated in GEO-CR cells as compared with GEO cells (Fig. 2A and Supplementary Table S1). ELISA assays confirmed that both growth factors were significantly secreted in the conditioned medium derived from GEO-CR cells as compared with parental GEO cells (Fig. 5A). Furthermore TGF-α levels were also significantly higher in the conditioned medium derived from SW48-CR cells (Fig. 5E). To examine whether TGF-α and/or HB-EGF enhanced expression could be causally involved in cetuximab resistance, GEO-CR cells were transfected with specific siRNAs for TGF-α or HB-EGF (Fig. 5B and C). TGF-α silencing, but not HB-EGF, was functionally relevant because it sensitized GEO-CR cells to the antiproliferative effects of cetuximab (Fig. 5B). Similarly, TGF-α downregulation by siRNA sensitized SW48-CR cells to cetuximab (Fig. 5F and G). Furthermore, inhibiting TGF-α expression by siRNA together with cetuximab treatment of GEO-CR and SW48-CR cells caused a significant suppression of MET phosphorylation as well as of downstream effectors MAPK, AKT, STAT3, and FAK (Fig. 5D and H).

Taken together, these results suggest a role for TGF-α overexpression in acquired cetuximab resistance in GEO-CR and SW48-CR cells. To test this hypothesis, further experiments were carried out in GEO and SW48 parental cells to evaluate whether exogenous TGF-α treatment by itself could lead to the acquisition of resistance to cetuximab. TGF-α stimulation of both cell lines resulted in decreased cetuximab sensitivity, because TGF-α could partially overcome the cetuximab induced growth inhibition (Fig. 6A). Moreover, TGF-α treatment of GEO and SW48 cells led to both EGFR and MET phosphorylation with an increase in MAPK activation and did not show evident effects on AKT (Fig. 6B). However, combined treatment with cetuximab partially rescues the cetuximab-mediated effects on MAPK and AKT phosphorylation. (Fig. 6B).

We next analyzed whether a functional cross talk between EGFR and MET in GEO-CR cells could be due to the interaction of the two growth factor receptors. For this purpose, GEO and GEO-CR protein extracts were immunoprecipitated with a specific anti-MET antibody and assayed by Western blotting with a specific anti-EGFR antibody. As shown in Fig. 6C, EGFR immunoprecipitated together with MET in GEO-CR cells, but not in GEO cells. Moreover, to elucidate the potential role of TGF-α in inducing EGFR–MET interaction, GEO, SW48, GEO-CR, and SW48-CR cells were treated with TGF-α in the presence or in the absence of cetuximab and lysates were immunoprecipitated with the anti-MET antibody and then assayed by Western blotting with the anti-EGFR antibody. As reported in Fig. 6D, TGF-α treatment induced EGFR–MET interaction in GEO and SW48 cells and increased the interaction GEO-CR and SW48-CR cells. The EGFR–MET interaction was also observed following combined treatment of cells with both TGF-α and cetuximab, suggesting that, even in the presence of cetuximab, TGF-α could induce a cross-interaction between EGFR and MET.

**Discussion**

Elucidating the mechanisms of cancer cell resistance to anticancer drugs is critical for the development of effective therapies. In this respect, mechanistic insights gained from

(Continued.) C, apoptosis was evaluated with Annexin V staining, as described in Materials and Methods. GEO-CR cells were transfected with a specific siRNA targeting MET. Ninety-six hours after transfection, cells received 5 μg/mL of cetuximab and apoptosis was measured after 24 hours of treatment. The rate of apoptosis was expressed as a percentage of the total cells counted. Columns: means of three independent experiments. Cetuximab plus si-MET versus si-MET or cetuximab (**P < 0.005). **D, Western blot analysis of cell signaling proteins in GEO-CR cells transfected with a specific siRNA targeting MET or with a scrambled, control siRNA for 96 hours and treated with the indicated dose of cetuximab for 24 hours. Total cell protein extracts were subjected to immunoblotting with the indicated antibodies, as described in Materials and Methods. E, GEO and SW48 cells were treated with increasing concentrations of cetuximab (0.01–10 μg/mL) for 96 hours and evaluated for proliferation by MTT staining, as described in Materials and Methods. The results are the average of three independent experiments, each done in quadruplicate. F, GEO and SW48 cells were treated with cetuximab (1 μg/mL), HGF (100 ng/mL), or their combination. Cell proliferation was determined after 96 hours. The results are the average ± SD of three independent experiments, each done in quadruplicate. G and H, analysis by Western blotting of protein expression in GEO and SW48 cancer cells, treated with cetuximab (1 μg/mL), HGF (100 ng/mL), or with their combination. Total cell protein extracts were subjected to immunoblotting with the indicated antibodies, as described in Materials and Methods.
Figure 4. PHA655752 restores sensitivity of GEO-CR and SW48-CR cells to cetuximab. A and C, GEO, GEO-CR (A), SW48, and SW48-CR (C) cells were treated with increased concentrations of PHA655752 (0.01–10 nmol/L) for 96 hours and evaluated for cell proliferation by MTT staining, as described in Materials and Methods. The results are the average ± SD of three independent experiments, each done in quadruplicate. B and D, GEO-CR (B) and SW48-CR (D) cells were treated with 2 doses of PHA655752 (0.01 or 0.05 nmol/L for GEO-CR and 5 or 10 nmol/L for SW48-CR), with increasing concentrations of cetuximab (0.01–10 μg/mL) or with a combination of both drugs for 96 hours and evaluated for cell proliferation by MTT staining, as described in Materials and Methods. The results are the average ± SD of three independent experiments, each done in quadruplicate. E and F, GEO-CR (E) and SW48-CR (F) cells were treated with PHA655752, cetuximab, or their combination at the indicated concentrations for 24 hours. The cell lysates were assayed by Western blotting with the indicated antibodies, as described in Materials and Methods. G and H, GEO-CR (G) and SW48-CR (H) cells were treated with PHA655752, cetuximab, or their combination at the indicated concentrations and analysis of in vitro cell migration was performed as described in Materials and Methods.
Figure 5. Inhibition of TGF-α expression reverts cetuximab resistance in GEO-CR and SW48-CR cells. A and E, TGF-α, HB-EGF, and HGF protein levels were measured in cell culture media by using Luminex technology, as described in Materials and Methods. TGF-α in GEO versus GEO-CR (\(^*\), \(P < 0.05\)), HBEGF in GEO versus GEO-CR (\(^\star\star\), \(P < 0.005\)), TGF-α in SW48 versus SW48-CR (\(^*\), \(P < 0.005\)). B and F, GEO-CR (B) and SW48-CR (F) cells were transfected with a specific TGF-α–targeting siRNA, or with a specific HB-EGF–targeting siRNA (only GEO-CR), or with a scrambled, control siRNA. TGF-α and HBEGF mRNA levels were measured by PCR, as described in Materials and Methods. C and G, GEO-CR (C) and SW48-CR (G) cells were transfected with a specific TGF-α–targeting siRNA, or with a specific HB-EGF–targeting siRNA (only GEO-CR), or with a scrambled, control siRNA. Twenty-four hours after transfection, cells were treated with cetuximab, 5 μg/mL. Viable cells were counted after 24 hours of treatment and plotted as a percentage untreated control cells. The results are the average ± SD of three independent experiments, each done in quadruplicate. D and H, Western blot analysis of protein expression in GEO-CR (D) and in SW48-CR (H) cells transfected with a specific TGF-α–targeting siRNA or with a scrambled, control siRNA and subsequently treated with the indicated dose of cetuximab for 24 hours. Total cell protein extracts were subjected to immunoblotting with the indicated antibodies, as described in Materials and Methods.
preclinical models can be used to design novel treatment strategies. In the past few years, an extensive effort has been made to understand the resistance mechanisms that cancer cells develop to overcome the anticancer efficacy of EGFR inhibitors (3, 4, 7).

The aim of this study was to examine the signaling mechanisms operating in human colorectal cancer cells that are sensitive to the antitumor activity of the anti-EGFR mAb cetuximab and that become resistant following continuous exposure to the drug. In fact, although cetuximab is an effective anticancer agent in the treatment of patients with mCRC, the occurrence of acquired resistance is a major clinical limitation. It has been shown that intrinsic resistance to cetuximab as well as other anti-EGFR mAbs panti-
tumumab can be the result of constitutive activation of KRAS signaling, due to the presence of KRAS gene mutations (4). For this reason, cetuximab therapy is limited to patients with mCRC with a tumor harboring wild-type KRAS gene. Moreover, the constitutive activation of other proteins by specific gene mutations are responsible of intrinsic resistance to anti-EGFR therapies in patients with colorectal cancer, including BRAF, NRAS, PI3KCA (exon 20) genes, or inactivation of the PTEN phosphatase (7, 37). It has recently been shown that in initially responding patients with colorectal cancer with a KRAS wild-type tumor, the resistance to anti-EGFR antibodies may occur by selection of cancer cell clones harboring a KRAS gene mutation (9, 10). However, approximately 25% of patients with colorectal cancer not responding to EGFR...
inhibitors are wild-type for KRAS, BRAF, NRAS, PIK3CA, and PTEN genes and the mechanism of resistance in these patients is still unknown (38). Therefore, both primary and acquired resistance mechanisms significantly limit the efficacy of anti-EGFR mAbs in the medical management of patients with colorectal cancer.

In the present study, we have generated and characterized two models of cetuximab resistance in human colon cancer cells. In the parental, cetuximab-sensitive, GEO, and SW48 colorectal cancer cells, cetuximab treatment was able to induce cell growth inhibition and apoptosis with MAPK and AKT phosphorylation reduction. On the contrary, this was not observed in the derived GEO-CR and SW48-CR cells, in which activation of MAPK and AKT, was not blocked, despite EGFR inhibition by cetuximab treatment. MAPK activation that could bypass EGFR inhibition is one of the potential mechanisms of acquired resistance to anti-EGFR therapies (39). In this respect, it has recently been shown that one mechanism of acquired resistance to cetuximab treatment could be the HER2 (ERBB2) activation (11). Similarly, Bertotti and colleagues identified HER2 gene amplification as a mechanism of resistance to cetuximab in mCRC that harbor wild-type KRAS, NRAS, BRAF, and PIK3CA genes (12). In this respect, although HER2 gene amplification was found to occur in only 2% to 3% of unselected mCRC specimens, a significantly higher frequency was found in metastatic KRAS wild-type patients with colorectal cancer that did not benefit from treatment with anti-EGFR mAbs (12). For this reason, we decided to examine the expression and the activation of HER2 and of other EGFR family members in GEO-CR and SW48-CR cells. However, no gene amplification and/or enhanced expression of HERB2, HERB3, or ERBB4 was found.

Our results suggest that MET activation may play a relevant role in determining acquired resistance to cetuximab. In particular, gene expression profiling of GEO and GEO-CR cells identified a series of genes involved in HGF–MET-dependent pathway, that were upregulated in GEO-CR cells as compared with GEO cells. Intriguingly, inhibition of MET correlated with a partially restored sensitivity to cetuximab in GEO-CR and SW48-CR cancer cells. In fact, MET silencing restores cetuximab ability to inhibit MAPK and AKT and cell proliferation. In contrast, HGF-mediated activation of MET in parental GEO and SW48 cells reduced the sensitivity cetuximab. The role of MET in EGFR resistance was also confirmed by using the selective MET inhibitor PHA665752 in combination with cetuximab that overcome the resistance in GEO-CR and SW48-CR cells.

Several studies have demonstrated that in lung adenocarcinoma–derived cells the EGF inhibition can be overcome by MET (19, 22). Moreover, HGF-dependent MET activation also proved to be a mechanism of intrinsic resistance to gefitinib in NSCLC cells with EGFR-activating mutations and no MET gene amplification (22). MET amplification is associated with acquired resistance to anti-EGFR treatment in patients with mCRC who have not selected a KRAS mutation during the therapy (14). In agreement with these results, it has been recently shown that HGF stimulation rescues cetuximab-sensitive colorectal cancer cells from EGFR inhibition by preventing cell-cycle arrest and inducing cell proliferations (40). Similarly, in HER2-overexpressing breast cancer cells, MET contributes to trastuzumab resistance (12). Conversely, MET-amplified gastric cancer cells were shown to be resistant to a MET-specific TKI when stimulated with EGF or heregulin-β (11). In these studies treatment of cancer cells with both MET and EGFR inhibitors could overcome resistance to a single inhibitor.

We have investigated the potential mechanisms by which MET can be activated. Although HGF-driven autocrine loop as a mechanism of MET activation was not demonstrated in GEO-CR and SW48-CR cells, here we provide evidence of a cross talk between EGFR and MET. In fact, we found that EGFR immunoprecipitated together with MET in GEO-CR and SW48-CR cells, but not in GEO and SW48 cells. This interaction was observed in parental cells following TGF-α treatment. In agreement with these results, several studies have shown an interaction between EGFR and MET that allows activation of MET following stimulation of different cell lines (A431, AKN-1, HepG2, AKN-1, HuH 6, and MRC5) with the EGFR selective ligands EGF or TGF-α (24). Stimulation of cells expressing both MET and EGFR with EGF resulted in phosphorylation of MET, and stimulation with ligands for both receptors resulted in synergistic activation of downstream modulators, indicating mutual activation of these two pathways (41).

EGFR ligands have been studied in several cancers as potential biomarkers for EGFR-targeted therapy. However, the results have been controversial depending on different tumor types and different clinical specimens used for testing (42, 43). In a study by Cohen and colleagues, changes in serum TGF-α levels in patients with head and neck cancer treated with gefitinib, were not associated with clinical response to gefitinib (43). In a study by Mutsaers and colleagues, TGF-α levels were increased in the plasma of patients with colorectal cancer during cetuximab treatment (44). However, increased TGF-α levels did not associate with cetuximab response (45). In contrast, more consistent results support the view that increased mRNA levels of amphiregulin (AREG) and epiregulin (EREG) in the tumors of patients with colorectal cancer are associated with cetuximab sensitivity. Khambata-Ford and colleagues were the first to demonstrate that AREG and EREG were significantly upregulated in chemotherapy refractory patients with colorectal cancer that obtained a clinical benefit from cetuximab treatment (42). The predictive value of EREG and AREG expression for cetuximab sensitivity was confirmed by Jacobs and colleagues, which analyzed primary tumors from refractory patients with mCRC treated with cetuximab-based therapies (46). Furthermore, in the 045 phase I study of cetuximab monotherapy as first-line treatment of patients with mCRC, Tabernero and colleagues found that AREG and EREG mRNA levels were significantly higher in tumor samples from patients with clinical response to cetuximab as compared with patients with disease progression (47). Although high level of expression of AREG and
EGFR ligands may predict a better response to treatment with cetuximab in patients with mCRC, HB-EGF and TGF-α overexpression could confer lack of sensitivity to EGFR inhibitors. In fact, in 045 clinical trial, TGF-α mRNA levels were significantly elevated in the tumors from patients with mCRC with disease progression after cetuximab therapy as compared with patient with clinical response (47). Furthermore, increased HB-EGF may be correlated with cetuximab resistance in head and neck squamous cell carcinoma (48).

Taken together, these results suggest that enhanced expression of different EGFR-specific ligands could have a different effect on the clinical activity of cetuximab. In this respect, the observation of TGF-α overexpression with activation of MET signaling in human colorectal cancer cells with acquired resistance to cetuximab treatment, as described in the present study, is in agreement with the above clinical findings.

The results of the present study suggest that overexpression of TGF-α, which induces EGFR–MET interaction, plays a relevant role in determining the development of acquired resistance in cetuximab-treated colorectal cancer cells. Therefore, the combined inhibition of both EGFR and MET RTKs could represent a rational therapeutic strategy for preventing and/or overcoming cetuximab resistance in patients with colorectal cancer.

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