Requirement of hypoxia-inducible factor-1α down-regulation in mediating the antitumor activity of the anti–epidermal growth factor receptor monoclonal antibody cetuximab

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Abstract

We tested our novel hypothesis that down-regulation of hypoxia-inducible factor-1α (HIF-1α), the regulated subunit of HIF-1 transcription factor that controls gene expression involved in key functional properties of cancer cells (including metabolism, survival, proliferation, invasion, angiogenesis, and metastasis), contributes to a major antitumor mechanism of cetuximab, an approved therapeutic monoclonal antibody that blocks activation of the epidermal growth factor receptor. We showed that cetuximab treatment down-regulates HIF-1α levels by inhibiting synthesis of HIF-1α rather than by enhancing degradation of the protein. Inhibition of HIF-1α protein synthesis was dependent on effective inhibition of the phosphoinositide-3 kinase (PI3K)/Akt pathway by cetuximab, because the inhibition was prevented in cells transfected with a constitutively active PI3K or a constitutively active Akt but not in cells with a constitutively active MEK. Overexpression of HIF-1α conferred cellular resistance to cetuximab-induced apoptosis and inhibition of vascular endothelial growth factor production in sensitive cancer cell models, and expression knockdown of HIF-1α by RNA interference substantially restored cellular sensitivity to the cetuximab-mediated antitumor activities in experimental resistant cell models created by transfection of an oncogenic Ras gene (G12V) or by concurrent treatment of the cells with insulin-like growth factor-I. In summary, our data show that cetuximab decreases HIF-1α protein synthesis through inhibition of a PI3K-dependent pathway and that an effective down-regulation of HIF-1α is required for maximal therapeutic effects of cetuximab in cancer cells. [Mol Cancer Ther 2008; 7(5):1207–17]

Introduction

The notion of targeting the epidermal growth factor receptor (EGFR) for cancer therapy, first proposed more than two decades ago (1–4), was based on several seminal findings, including observations that EGFR contains tyrosine kinase activity and can be cell transforming in a ligand-dependent manner (5–7) and that overexpression of EGFR is common in many solid tumors and is often accompanied by autocrine or paracrine production of transforming growth factor-α, one of the EGFR ligands (8). Results from recent clinical studies have shown antitumor activity of EGFR-targeted therapies, using receptor-blocking monoclonal antibodies or small-molecule receptor tyrosine kinase inhibitors (9–11). Unfortunately, however, most responses seem to occur only in a minority of patients whose tumors appear to depend on EGFR-mediated cell signaling. Many patients whose tumors express, or even highly express, EGFR do not achieve clinical responses. Accumulating evidence indicates that there are multiple intrinsic resistance mechanisms that can affect the overall response of tumor cells to EGFR-targeted therapies (12).

The major intrinsic resistant mechanisms found in various types of EGFR-expressing cancers appear to be bypass of the requirement for EGFR-activated cell signaling in these cells, as a result of activation of other growth factor receptors that have a spectrum of downstream pathways overlapping with EGFR, such as insulin-like growth factor-I receptor (IGF-IR), or constitutive activation of key signal-transducing molecules in the pathway due to genetic aberrations, such as mutational activation of the Ras oncogene (13) or mutational inactivation of the PTEN tumor suppressor gene (14). Thus, except for a small proportion of tumors in which survival and proliferation are exclusively dependent on EGFR-mediated cell signaling, many tumors are able to escape the treatment effect of EGFR inhibitors by activating EGFR downstream cell signaling in an EGFR-independent manner. With recognition that this is a major challenge to the therapeutic efficacy of EGFR-targeting agents, identification of key intermediate signal transducers or effector molecules in pathways downstream from EGFR, which also can be activated by EGFR-independent mechanisms, will provide guidance for designing novel combinatorial therapeutic approaches that

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may enhance therapeutic responses or overcome tumor resistance to EGFR-targeted therapy.

Originally discovered as a nuclear factor that binds to the human erythropoietin gene enhancer and that is elevated by hypoxic conditions (15), hypoxia-inducible factor-1α (HIF-1α) is now known to bind to specific *cis*-elements in the promoters of many genes after heterodimerization with its binding partner, HIF-1β subunit. The products of these genes can play important roles in the process of cancer development and progression, including tumor angiogenesis, cell survival and proliferation, invasion and metastasis, glucose metabolism, and drug resistance (16, 17). It is now well established that the induction of HIF-1α during hypoxia is due to inhibition of oxygen-dependent degradation of the HIF-1α protein (18). In contrast, oxygen-independent up-regulation of HIF-1α was first reported in cells transfected with the v-*Src* oncogene (19) or treated with insulin and IGF-I (20); such up-regulation occurs mainly at the protein synthesis level, requiring activation of a phosphoinositide-3 kinase (PI3K)–dependent pathway (21–25).

We hypothesize that HIF-1α is a major effector molecule of EGFR-activated cell signaling, which also can be regulated by EGFR-independent mechanisms. We and others recently found that treatment of responsive cancer cells with cetuximab or the small-molecule EGFR tyrosine kinase inhibitor gefitinib down-regulated the levels of HIF-1α, leading to decreased production of vascular endothelial growth factor (VEGF), and that the responses of cancer cells to EGFR-targeted therapy are linked to decreases in the HIF-1α level following treatment (26–28). In the present study, we have further elaborated on the mechanism by which cetuximab decreases HIF-1α. We found that treatment of cells with cetuximab down-regulated the level of HIF-1α through inhibition of the PI3K pathway under both normoxic and hypoxic conditions. This capability was impaired in cells following expression of a constitutively active Ras mutant or with concurrent exposure to IGF-I, both of which lead to EGFR-independent activation of PI3K. The failure of cetuximab to down-regulate HIF-1α was associated with a decreased activity of cetuximab in inducing apoptosis and inhibiting VEGF production in these cells. Expression knockdown of HIF-1α by RNA interference substantially restored cellular sensitivity to cetuximab treatment. Taken together, our results indicate that inhibition of HIF-1α is required for maximal therapeutic response of cancer cells to EGFR-targeted therapies. Therefore, resistance of cancer cells to cetuximab treatment conferred by EGFR-independent activation of EGFR downstream cell signaling might be overcome by combining novel therapeutic approaches targeting HIF-1α.

Materials and Methods

Cell Lines and Cell Culture

A431 human vulvar squamous carcinoma cells and DiFi colorectal adenocarcinoma cells were described previously (29–32). HCC827 and H3255 human non-small cell lung cancer cell lines were kindly provided by Dr. John Minna (The University of Texas Southwestern Medical Center) through Dr. Jonathan M. Kurie (The University of Texas M. D. Anderson Cancer Center). All cell lines were grown and maintained in DMEM or Ham's F12 supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin and incubated in a humidified atmosphere (95% air and 5% CO_2) at 37°C.

Western Blot Analysis and Blotting Antibodies

Cultured cells were harvested with a rubber scraper and washed twice with cold PBS. Cell pellets were lysed and kept on ice for at least 10 min with a buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 0.5% NP-40, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, 25 μg/mL leupeptin, and 25 μg/mL aprotinin. The lysates were cleared by centrifugation, and the supernatants were collected. Equal amounts of lysate protein were separated by SDS-PAGE, and Western blot analyses were done with various specific primary antibodies. Antibodies directed against HIF-1α and Ras were obtained from BD Biosciences PharMingen. The antibodies directed against total and S473-phosphorylated Akt, total and T202/Y204-phosphorylated ERK, and poly(ADP-ribose) polymerase (PARP) were obtained from Cell Signaling Technology. Specific signals were visualized using an enhanced chemiluminescence detection kit (Amersham).

Quantitative Real-time PCR

Total RNA was extracted from A431 cells without or with cetuximab treatment using a modified chloroform/phenol procedure (Trizol; Invitrogen/Life Technologies). First-strand cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad). The fluorescent real-time PCR was done in a Bio-Rad iCycler thermal cycler for 40 cycles (denaturation at 95°C for 10 s and annealing at 61°C for 60 s) in a final volume of 25 μL containing 1 μL cDNA template with the specific primers targeting human HIF-1α (forward 5′-TGCAACATGGAAGGTATTGC-3′ and reverse 5′-CCAAGCAGGTCATAGGTGGT-3′) or 1 μL of an internal control cDNA with specific primers targeting human glyceraldehyde-3-phosphate dehydrogenase (forward 5′-GAAGGTGAAGGTCGGAGTC-3′ and reverse 5′-GAAGATGGTGATGGGATTTC-3′) in iQ SYBR Green supermix (Bio-Rad). Fluorescent readings from real-time PCR products were quantitatively analyzed by determining the difference in Ct (threshold cycle) between HIF-1α and glyceraldehyde-3-phosphate dehydrogenase (ΔCt). Relative gene expression was calculated as $2^{-\Delta Ct}$, and changes of HIF-1α mRNA expression in cetuximab-treated cells were obtained by comparison with the HIF-1α mRNA expression in untreated cells.

cDNA Constructs and Transfection

The pcDNA3 expression constructs containing wild-type HIF-1α or its oxygen-dependent degradation domain (ODD) deletion mutant (HIF-1α/ΔODD) were kindly provided by Dr. L. Eric Huang (University of Utah School of Medicine). pEGFP-HIF-1α construct was prepared by

subcloning of HIF-1α from the pcDNA3 construct into pEGFP-C1 vector (Clontech-BD Biosciences). The myristoylated PI3K expression construct was kindly provided by Dr. Gordon Mills (The University of Texas M. D. Anderson Cancer Center). The myristoylated Akt expression construct was described previously (33). The constitutively active MEK1 (S217E/S221D) pcDNA3.1 construct was subcloned from pMCL vector containing the MEK1 mutant insert that was originally provided by Dr. Natalie Ahn (University of Colorado). Transfection of these constructs was done with LipofectAMINE 2000 (Invitrogen).

Small Interfering RNA and Transfection

A 21-mer oligonucleotide RNA duplex [small interfering RNA (siRNA)] targeting the ODD domain of HIF-1α (AACTGATGACCAGCAACTTGA) and control scramble oligonucleotide duplex were custom synthesized by Qiagen. In the pilot experiments, we tested different concentrations of siRNA oligonucleotides with different time intervals of transfection using LipofectAMINE 2000. The transfection efficiency was judged based on the uptake of FITC-conjugated oligonucleotides, and the efficiency of siRNA-mediated HIF-1α protein suppression was determined by Western blotting analysis.

Apoptosis Assay

Apoptosis was measured by an ELISA kit (Roche Diagnostics) that quantitatively measures cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) and by Western blot analysis with an antibody that recognizes both uncleaved and cleaved PARP after various treatments as we have reported previously (34, 35). In these experiments, the A431 cells were cultured with cetuximab for 16 h in serum-free medium, which enhances their susceptibility to apoptosis.

Measurement of VEGF Levels in Conditioned Medium

VEGF levels in the conditioned medium 24 h after transfection of siRNA or cetuximab treatment were measured by ELISA (R&D Systems). VEGF levels were normalized to the number of cells of each sample in the culture wells and expressed as picograms of VEGF per 10^6 cells. Experiments were repeated twice. Student's *t* test was used for statistical analysis using the combined data from each of the replicates.

Results

Cetuximab Down-regulates HIF-1α via Inhibition of New Protein Synthesis

We recently reported that treatment with cetuximab down-regulates HIF-1α levels in A431 cells, a representative cell line that responds well to the treatment (26). Experiments were designed to extend our observations by determining whether this down-regulation of steady-state levels of HIF-1α after cetuximab treatment reflected increased degradation, decreased synthesis of the protein, or both. We first determined the sensitivity of HIF-1α down-regulation by cetuximab in A431 cell culture to proteasomal inhibition with lactacystin, which allowed us to determine whether protein degradation played a role. If cetuximab down-regulates HIF-1α via enhanced degradation, proteasomal inhibition would cause a slower rate of HIF-1α decrease in response to cetuximab compared with HIF-1α degradation without proteasomal inhibition. However, we found that, except for a marked elevation in the basal level of HIF-1α due to an overall inhibition of HIF-1α degradation by lactacystin, there remained a cetuximab treatment time-dependent decrease in the HIF-1α level that was parallel with the time-dependent decrease of HIF-1α by cetuximab in the presence of DMSO, a vehicle control of lactacystin (Fig. 1A, *left*). This observation suggests that the decrease in HIF-1α on cetuximab treatment was not mediated through enhancing protein degradation. To further rule out involvement of protein degradation in cetuximab-mediated down-regulation of HIF-1α, we compared the degradation rate of HIF-1α protein in the presence or absence of cetuximab following cycloheximide treatment, which shuts down new protein synthesis globally, allowing us to track degradation of existing proteins, including HIF-1α. We found that, shortly after exposure of the cells to cycloheximide, the level of existing HIF-1α protein started to decline, with the half-life being <15 min in normoxic culture; by 60 min, ~100% of the HIF-1α was degraded (Fig. 1A, *right*). The presence of cetuximab in the culture medium yielded a completely overlapping degradation curve for HIF-1α, indicating that inhibition of EGFR with cetuximab has no effect on further facilitating HIF-1α degradation in normoxia.

We further assessed the efficacy of cetuximab in decreasing cellular HIF-1α levels during hypoxia when the normal degradation process of HIF-1α is generally inhibited due to reduced access to oxygen, which is required for HIF-1α ubiquitination and subsequent degradation. Figure 1B shows the changes in the levels of HIF-1α in hypoxic cells compared with normoxic cells after cetuximab treatment. As expected, hypoxic cultures of both A431 and DiFi cells in a 1% O_2 chamber yielded higher basal levels of HIF-1α than were found in normoxic cultures. Cetuximab decreased the levels of HIF-1α in these cells under both hypoxic and normoxic conditions. The effects of cetuximab on the inhibition of EGFR downstream signal transduction, as measured by the levels of phosphorylated ERK and Akt, were similar in normoxic and hypoxic cells. These results provide further evidence suggesting that cetuximab is unlikely to down-regulate HIF-1α via enhancing protein degradation.

To determine whether cetuximab decreases the HIF-1α protein level by translational or transcriptional mechanisms or both, we did real-time PCR to measure the levels of HIF-1α mRNA in A431 cells during cetuximab treatment. Compared with untreated cells, cells treated with cetuximab showed a marked decrease in HIF-1α mRNA in a time-dependent manner (Fig. 1C). Within 24 h of cetuximab treatment, HIF-1α mRNA synthesis was decreased by as much as 70% in the cells. To further determine whether cetuximab regulates HIF-1α via noncoding mRNA sequences that direct its translation and/or gene expression, we transfected A431 cells with a green fluorescent protein

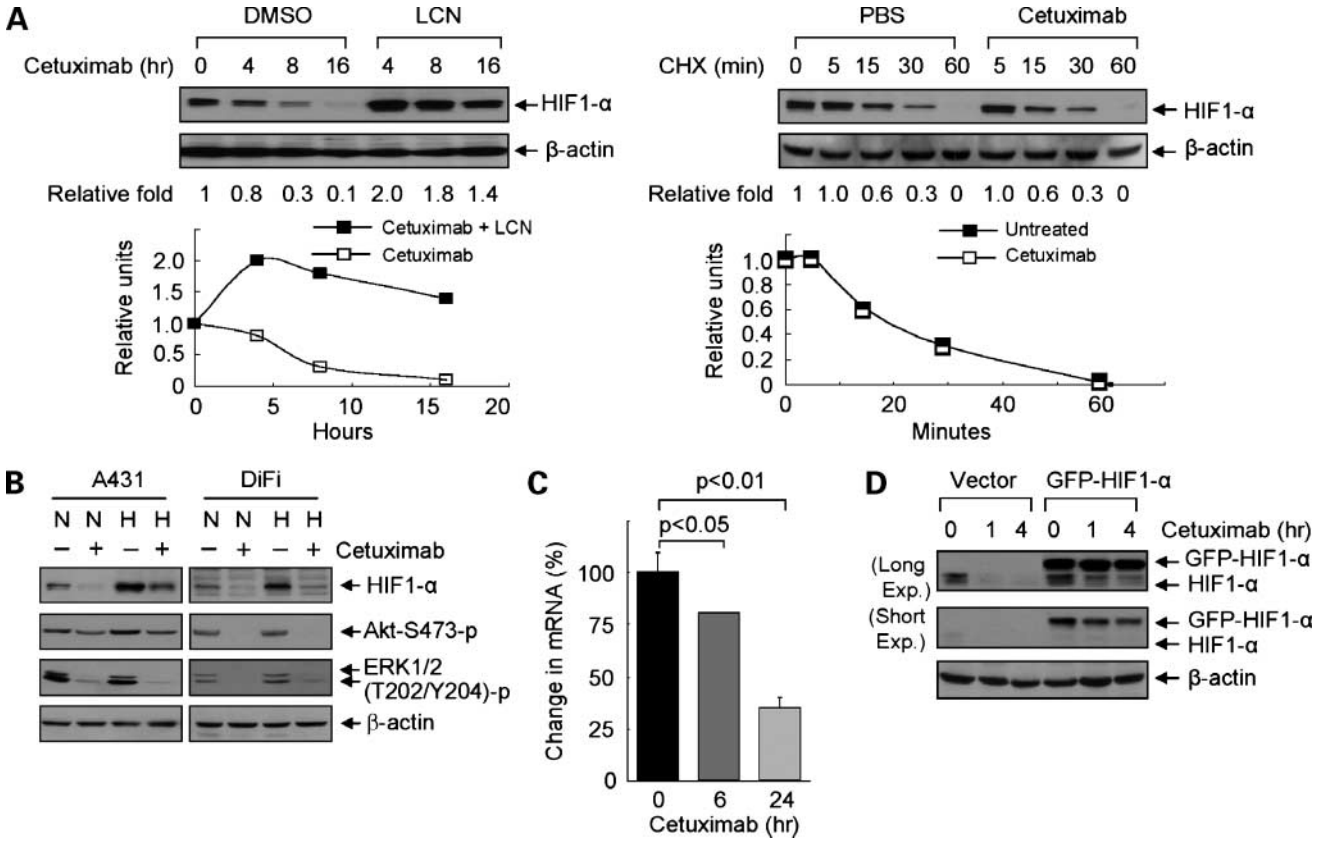

Figure 1. Cetuximab down-regulates HIF-1α level via inhibition of new HIF-1α protein expression. **A,** *left,* A431 cells were treated with 10 nmol/L cetuximab in the absence or presence of 10 μmol/L lactacystin (*LCN*) for up to 16 h; *right,* A431 cells were exposed to 10 μmol/L cycloheximide (*CHX*) without or with 10 nmol/L cetuximab for up to 60 min. Aliquots of cell samples at each indicated time point were prepared and subjected to Western blot analysis using antibody directed against a region near the COOH-terminal of HIF-1α (amino acid sequence from 610 to 727). The levels of β-actin served as an internal control for equal protein loading in each lane. Relative fold means the densitometric value of HIF-1α normalized by the corresponding densitometric value of β-actin expression under each treatment condition; the value for untreated cells was taken as unity. **B,** A431 and DiFi cells were left untreated or treated with 10 nmol/L cetuximab for 16 h under normoxic (*N*) or hypoxic (*H*; 1% O_2) conditions. After treatment, the cells were lysed, and equal amounts of cell lysate were subjected to Western blot analysis using the indicated antibodies. **C,** RNA samples from A431 cells that were treated with cetuximab for 0, 6, or 24 h were used for real-time reverse transcription-PCR. The absorbance in the treatment groups was expressed as a percentage of the value of untreated cells. Average of three independent experiments. Student's *t* test was used for statistical analysis of comparisons between the values of untreated and treated cell samples as shown. **D,** A431 cells were transiently transfected with pEGFP-C1 control vector or the pEGFP-HIF-1α construct by LipofectAMINE 2000 for 6 h. The cells were then subcultured overnight. On the next day, the cells were subjected to 10 nmol/L cetuximab treatment for 0, 1, or 4 h, and cell lysates were prepared and subjected to Western blot analysis using the indicated antibodies. *Exp.,* exposure time.

fusion construct containing a wild-type HIF-1α that lacks the natural noncoding sequences and then used Western blot analysis to examine the effect of cetuximab on the level of green fluorescent protein-HIF-1α fusion protein (Fig. 1D). We found the effect of cetuximab on decreasing the level of green fluorescent protein-HIF-1α fusion protein was much weaker than its effect on the endogenous HIF-1α, indicating cetuximab mainly regulates noncoding mRNA sequences of HIF-1α; however, cetuximab treatment also modestly reduced the level of green fluorescent protein-HIF-1α fusion protein (Fig. 1D, short exposure), which suggests that cetuximab may have a role in affecting HIF-1α synthesis at the translational level as well.

Cetuximab Down-regulates HIF-1α Levels through Inhibition of a PI3K/Akt-Dependent Pathway

The MEK- and PI3K-mediated signal pathways are two major downstream signal pathways inhibited on cetuximab treatment in responsive cells. To determine inhibition of which pathway (or both) mediates down-regulation of HIF-1α by cetuximab, we investigated the effects of a MEK-specific inhibitor (PD98059) and a PI3K-specific inhibitor (LY294002) on cellular levels of HIF-1α and the respective effects of expression of constitutively active MEK1 and PI3K on the cetuximab-induced decrease in HIF-1α levels.

We found that the basal level of HIF-1α was unchanged on PD98059 treatment, although PD98059 clearly inhibited MEK kinase activity at the doses tested, as shown by decreased phosphorylation of its substrate ERK (Fig. 2A, *top*). Further, expression of a constitutively active MEK (MEK1-S217E/S221D) in A431 cells conferred cellular resistance to cetuximab-induced inhibition of ERK phosphorylation; however, these cells remained sensitive to cetuximab-induced decrease in HIF-1α protein level (Fig. 2A, *bottom*). In contrast, overnight exposure of the

cells to LY294002 led to a dose-dependent decrease in the HIF-1α level, which was concurrent with the decrease in the activation-specific phosphorylation level of Akt and which was prevented upon expression of a myristoylated Akt known to be constitutively active (Fig. 2B, *top*). Expression of a myristoylated PI3K or a myristoylated Akt led to an overall increase in the basal level of HIF-1α and resistance to the cetuximab-mediated decrease of HIF-1α, indicating a critical role of PI3K/Akt inhibition in the cetuximab-mediated decrease of HIF-1α (Fig. 2B, *middle* and *bottom*). Taken together, these findings suggest that cetuximab decreases HIF-1α via inhibition of the PI3K/Akt pathway rather than the MEK/ERK pathway. It is noteworthy, however, that VEGF, as one of the major HIF-1α-targeted gene products, was sensitive to both PD98059 and LY294002 treatments when the VEGF levels were measured in the conditioned medium of treated cell cultures (Fig. 2C). This finding underscores the fact that, although the MEK/ERK pathway may not affect the HIF-1α level, MEK/ERK may participate in VEGF regulation via another mechanism, such as the phosphorylation of HIF-1α coactivator p300/CBP by MEK/ERK, as reported by others (36).

Down-regulation of HIF-1α Functions as a Major Antitumor Mechanism of Cetuximab

To confirm the critical role of HIF-1α down-regulation in mediating the antitumor activity of cetuximab, we examined whether experimental elevation of the HIF-1α level and expression knockdown of endogenous HIF-1α would affect responses of cancer cells to cetuximab-mediated antitumor activity correspondingly. Because of the susceptibility of wild-type HIF-1α to degradation in normoxia, we used a mutant HIF-1α construct (termed HIF-1α/ΔODD) in which the ODD domain of HIF-1α was deleted but in which its transcriptional activity was largely retained. This approach not only allowed us to keep the expressed HIF-1α (HIF-1α/ΔODD) stably high in normoxic culture but also provided an opportunity for us to selectively knock down endogenous (wild-type) HIF-1α by using a RNA interference approach specifically targeting the ODD domain, thereby sparing the transfected HIF-1α/ΔODD. Indeed, as

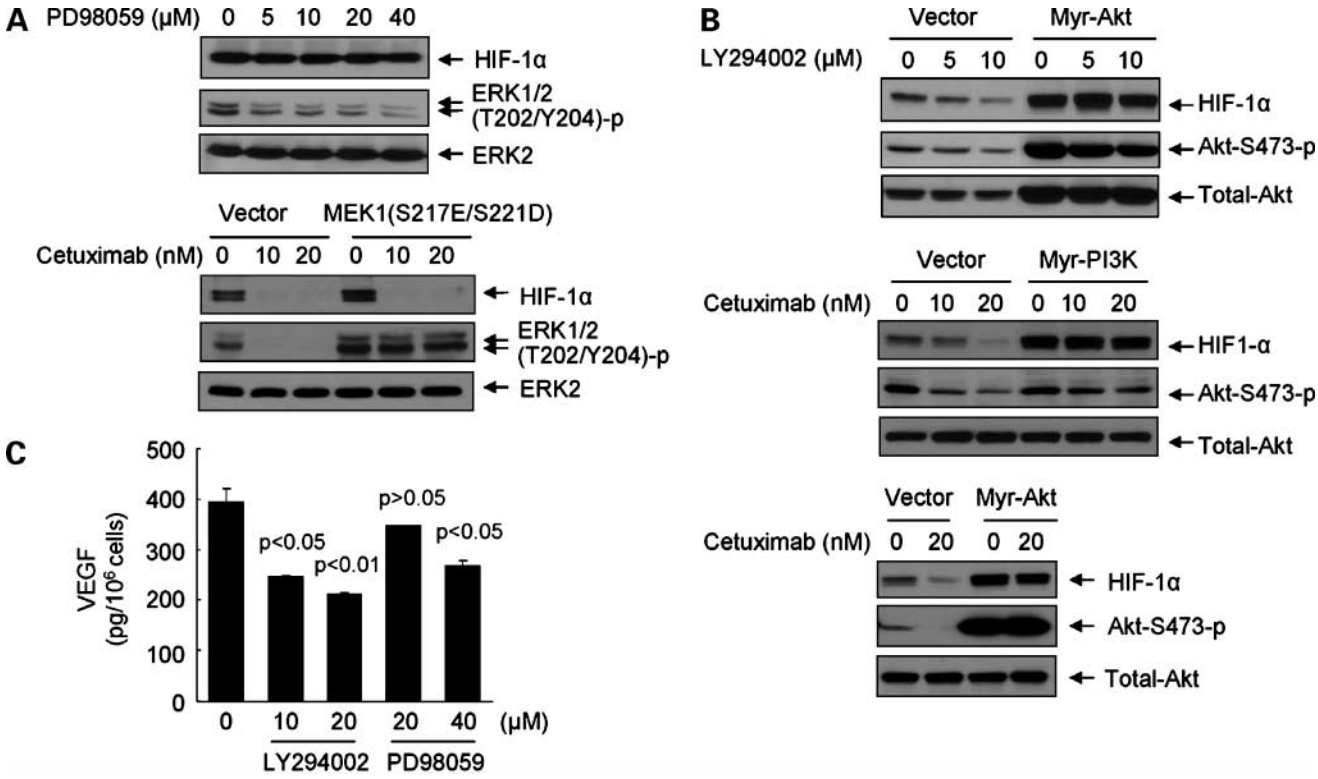

Figure 2. Cetuximab decreases the HIF-1α level through inhibition of a PI3K/Akt-dependent pathway. **A,** A431 cells were treated overnight (16 h) with the indicated concentrations of PD98059 (*top*) or were transiently transfected with a constitutively active MEK1 (S217E/S221D) construct or control vectors (pcDNA3.1) for 24 h followed by overnight (16 h) incubation without or with the indicated concentrations of cetuximab (*bottom*). Cell lysates were prepared and subjected to Western blot analysis with the indicated antibodies. **B,** A431 cells were transiently transfected with control vectors or myristoylated Akt for 24 h and then treated with various doses of LY204002 overnight (16 h; *top*) or were transiently transfected with control vector or a myristoylated PI3K construct (*middle*) or a myristoylated Akt construct (*bottom*) for 24 h followed by overnight (16 h) incubation without or with the indicated concentrations of cetuximab. The cell lysates were prepared and subjected to Western blot analysis with the indicated antibodies. **C,** A431 cells were exposed to the indicated concentrations of LY294002 and PD98059 for overnight (16 h). The conditioned media were used for detection of the VEGF level by ELISA. Mean ± SD. *P* values were determined by Student's *t* test for comparisons between untreated control and LY294002- or PD98059-treated cell groups.

we expected, compared with endogenous HIF-1α, which was sensitive to cetuximab treatment, the transfected HIF-1α/ΔODD (appearing as a truncated HIF-1α protein recognizable by the HIF-1α antibody) was maintained at high levels and was resistant to cetuximab treatment (Fig. 3A). A 21-mer HIF-1α siRNA oligonucleotide targeting the ODD domain effectively knocked down the expression level of endogenous HIF-1α but not the transfected HIF-1α/ΔODD level (Fig. 3B, *lane 3* versus *lane 4*).

We next compared the antitumor effects of cetuximab in A431neo (control vector cells) and A431HIF-1α/ΔODD cells. Figure 3C shows that an overnight exposure of A431neo cells and A431HIF-1α/ΔODD to cetuximab in the absence of serum reduced the levels of endogenous HIF-1α (Fig. 3C, *top*). In A431neo cells, incubation with cetuximab resulted in cleavage of PARP and an increase of histone-associated DNA fragmentation in the cytoplasm (Fig. 3C, *bottom*), both of which are characteristic of apoptosis. In contrast, expression of the cetuximab-resistant HIF-1α/ΔODD in A431 HIF-1α/ΔODD was sufficient to counteract the apoptotic effects of cetuximab under identical experimental conditions (Fig. 3C).

We further compared the effect of cetuximab on reducing the level of VEGF production in conditioned medium by A431neo and A431HIF-1α/ΔODD cells as another measurement of antitumor activity by the treatment (Fig. 3D). Expression of the cetuximab-resistant HIF-1α/ΔODD in A431 cells (A431HIF-1α/ΔODD) showed a notable increase in the basal level of VEGF compared with the control vector-transfected cells (*lane 5* versus *lane 1*); unexpectedly, however, although HIF-1α/ΔODD is cetuximab resistant, the VEGF level remained as sensitive to cetuximab in A431HIF-1α/ΔODD cells as in A431neo cells (a 62% decrease compared with a 64% decrease). We speculated that the high level of cetuximab-sensitive endogenous HIF-1α in A431 HIF-1α/ΔODD cells was the cause of unchanged cellular sensitivity to cetuximab-induced inhibition of VEGF production. Indeed, the effect of cetuximab on decreasing the VEGF level in A431HIF-1α/ΔODD cells became much weaker after the expression of endogenous HIF-1α was silenced compared with that in the control cells (a 26% inhibition versus a 71% inhibition) and also with the same cells in which the expression of endogenous HIF-1α was not silenced (a 26% inhibition versus a 62% inhibition; $P < 0.01$).

Interestingly, silencing the expression of endogenous HIF-1α alone only moderately decreased the VEGF level in both control cells (*lane 1* versus *lane 3*) and A431 HIF-1α/ΔODD cells (*lane 5* versus *lane 7*). These observations suggest that, although HIF-1α is important in regulating VEGF levels, its action alone may not have a major effect on the basal level of VEGF in normoxia compared with the results following inhibition of EGFR by cetuximab treatment, which can lead to inhibition of several components, such as HIF-1α, AP1, STAT3, and SP1, which are collectively involved in transcriptional regulation of VEGF (37). However, although HIF-1α silencing alone only moderately decreased the basal level of VEGF in normoxia, our data suggest that down-regulation of HIF-1α by cetuximab is important for its contribution to the overall effect of cetuximab-mediated inhibition of VEGF production, because failure of cetuximab to down-regulate HIF-1α/ΔODD was accompanied by a decreased effect of cetuximab on inhibiting VEGF production (*lane 4* versus *lane 8*).

These observations further support our hypothesis that down-regulation of HIF-1α is required for maximal activity of cetuximab. Our results also suggested that the effect of HIF-1α on conferring cellular resistance to apoptosis is more direct than its effect on regulating VEGF production, because the expression of VEGF is well known to be coregulated by several other factors as noted.

HIF-1α Silencing Overcomes the Cellular Resistance to Cetuximab Conferred by Constitutive or Alternative Activation of EGFR Downstream Signaling

Because of the critical role of HIF-1α down-regulation in maximizing the antitumor activity of cetuximab and because of the strong link between HIF-1α down-regulation and PI3K inhibition following cetuximab treatment, we hypothesized that cellular resistance to cetuximab due to the presence of EGFR-independent activation of PI3K would be reversed to a great extent by targeting HIF-1α. Validation of this hypothesis could lead to a novel therapeutic strategy involving a combination of inhibitory agents. We tested this hypothesis in principle using RNA interference in two clinically relevant experimental models in which PI3K was constitutively activated or alternatively activated.

We first tested resistance to cetuximab-induced down-regulation of HIF-1α caused by K-Ras mutation, which is one of the most common genetic aberrations in solid tumors (38). Compared with basal phosphorylation levels of PI3K/Akt and MEK/ERK in A431neo cells, the basal level of phosphorylated Akt was higher in A431 cells stably transfected with an oncogenic RasG12V mutant (A431RasG12V), although the increase in the basal level of phosphorylated ERK was modest (Fig. 4A). As expected, A431RasG12V cells were insensitive to cetuximab-mediated inhibition of these pathways. In concert with these differences, A431RasG12V cells contained a higher basal level of HIF-1α and were resistant to cetuximab-mediated decreases in the HIF-1α level compared with A431neo cells.

Figure 4B shows the effect of HIF-1α silencing on enhancing induction of PARP cleavage by combination use with cetuximab in A431RasG12V cells. It is interesting to note that, under comparable experimental conditions, the expression of HIF-1α was knocked down by siRNA much more efficiently in A431RasG12V cells than in A431neo cells (Fig. 4B, *lanes 1* and *3* versus *lanes 5* and *7*). Cetuximab treatment and HIF-1α silencing each induced only a moderate cleavage of PARP in A431neo cells, which was markedly enhanced by combination of the two (Fig. 4B, *lanes 1-4*). A431RasG12V cells were completely resistant to cetuximab-induced cleavage of PARP and partially resistant to HIF-1α silencing-induced cleavage of PARP. Importantly, combination of the two treatments resulted in a much greater cleavage of PARP, indicating a greater induction of apoptosis (Fig. 4B, *lanes 5-8*).

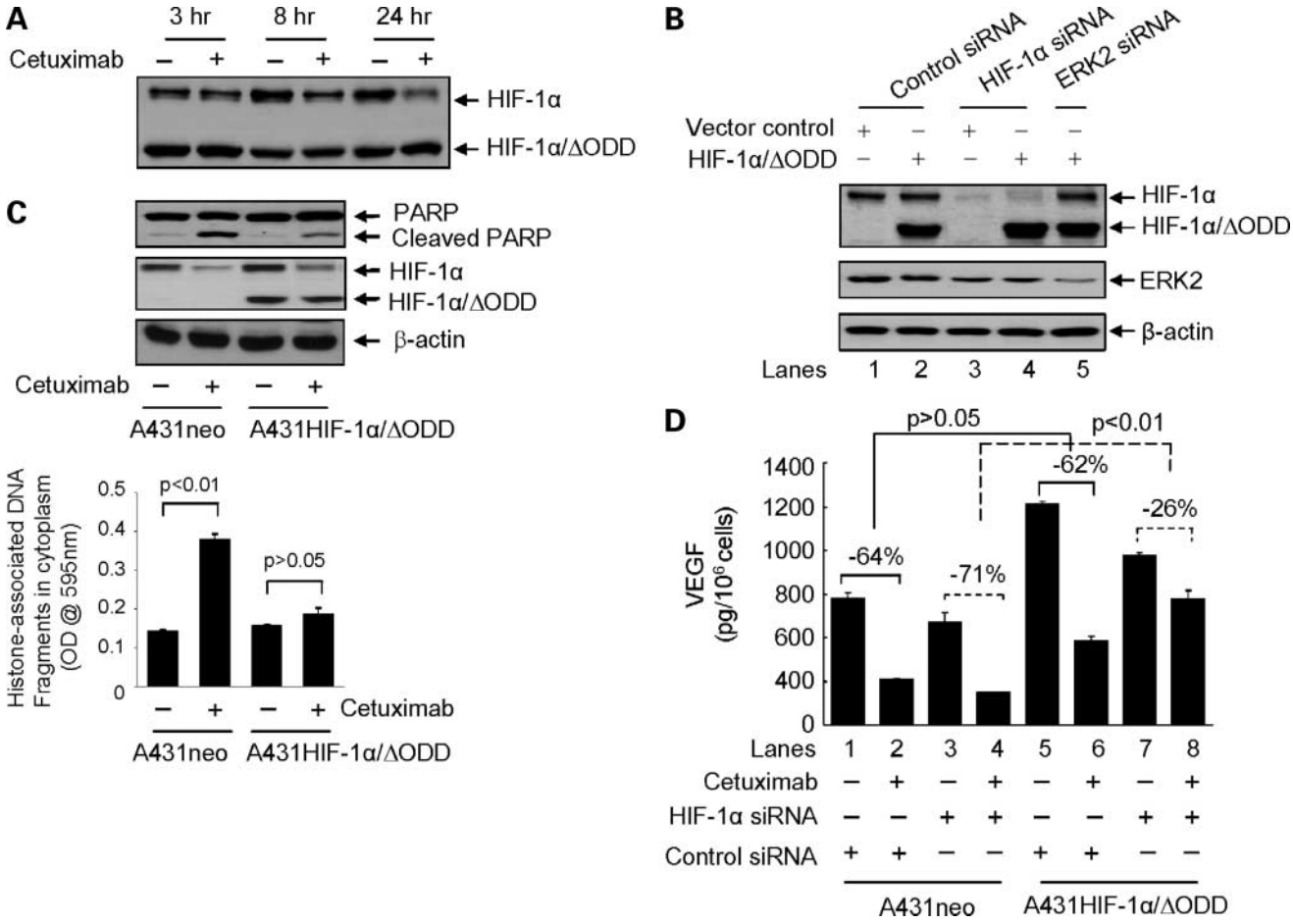

Figure 3. Down-regulation of HIF-1α is a major antitumor mechanism of cetuximab. **A,** A431 cells were transiently transfected with a HIF-1α/ΔODD construct for 24 h followed by exposure to 10 nmol/L cetuximab for the indicated periods. Cell lysates were prepared at each time point for Western blot analysis with a HIF-1α antibody as described in Fig. 1. **B,** A431 cells were transiently transfected with the HIF-1α/ΔODD or a control vector (pcDNA3.1) along with a scrambled siRNA (negative control siRNA), an ERK2-siRNA (positive control siRNA), and a HIF-1α ODD-targeting siRNA as indicated. Seventy-two hours after the transfection, the cell lysates were prepared and subjected to Western blot analysis with the indicated antibodies. **C,** A431 cells were transiently transfected with either the HIF-1α/ΔODD construct or control vector (pcDNA3.1) for 24 h followed by a 16-h culture without or with 10 nmol/L cetuximab. The cells were then harvested for measuring the cleavage of PARP by Western blot analysis (*top*) and for quantifying the levels of histone-associated DNA fragmentation in the cytoplasm on cetuximab treatment by ELISA (*bottom*). The expression of HIF-1α/ΔODD construct was measured as described in **A**. The levels of β-actin served as an internal control for equal protein loading in each lane. **D,** A431 cells were transiently transfected with the HIF-1α/ΔODD construct or control vector (pcDNA3.1) along with the HIF-1α ODD-targeting siRNA or scrambled siRNA for 72 h. The cells were cultured with or without 10 nmol/L cetuximab for 24 h, and the VEGF secreted into the conditioned media by the cells was measured by ELISA. *P* values for the comparisons shown were determined by Student's *t* test.

Figure 4C shows the result of HIF-1α silencing on overcoming oncogenic K-Ras-mediated resistance to cetuximab-induced inhibition of VEGF expression. Although expression of RasG12V in cells did not increase the basal level of VEGF production in A431 cells, which is likely due to a constitutively high basal level of VEGF in these cells, expression of RasG12V did confer a strong resistance to cetuximab-induced inhibition of VEGF production with a 62% inhibition in A431neo cells compared with only a 14% inhibition in A431RasG12V cells (Fig. 4C, *lanes 1* and *2* versus *lanes 5* and *6*). Interestingly, expression knockdown of HIF-1α seemed to decrease the VEGF level more effectively in A431RasG12V cells than in A431neo cells with a 39% inhibition in A431neo cells compared with a 65% inhibition in A431RasG12V cells (Fig. 4C, *lanes 1* and *3* versus *lanes 5* and *7*). This coincides with the finding of more effective silencing of HIF-1α by siRNA in A431RasG12V cells than in A431neo cells (Fig. 4B). Regardless of these differences, combination of the two treatments achieved comparable and enhanced inhibition of VEGF production in A431neo and A431RasG12V cells. Together, these results indicated that oncogenic Ras mutant-conferred resistance of cancer cells to the cetuximab-induced cleavage of PARP (an indicator of apoptosis) and inhibition of VEGF production (an indicator of tumor angiogenesis) may be overcome by expression knockdown of HIF-1α in the resistant cells.

We next investigated the scenario of cellular resistance to cetuximab-induced down-regulation of HIF-1α caused by

alternative activation of EGFR downstream signal pathways using concurrent activation of IGF-IR as an example. We conducted the study to include several cell lines that are highly dependent on EGFR signaling for survival and are thus extremely sensitive to cetuximab-induced apoptosis. Figure 5 shows the results from experiments showing the effects of HIF-1α silencing on overcoming cellular resistance to cetuximab-induced apoptosis produced by concurrent exposure of the sensitive cells to IGF-I stimulation. IGF-I stimulated PI3K/Akt, MEK/ERK, and several other pathways independently of EGFR and thereby conferred resistance to cetuximab-induced phosphorylation inhibition of Akt and ERK in these cells. Apoptosis was induced by cetuximab in H3255 non-small cell lung cancer cells and DiFi colorectal cancer cells as shown by the characteristic appearance of PARP cleavage (Fig. 5A and B). The extent of apoptosis caused by cetuximab was clearly reduced in both cell lines when the cells were incubated with IGF-I concurrently. We found that expression knockdown of HIF-1α by conventional liposome-based transient transfection of siRNA is not technically feasible in H3255 and DiFi cells (data not shown). To address whether HIF-1α silencing will overcome IGF-I-mediated resistance to cetuximab, we used another cell line (HCC827 non-small cell lung cancer cells) to study the effect of HIF-1α siRNA on counteracting the protection conferred by IGF-I against cetuximab-induced apoptosis (Fig. 5C). Treatment of HCC827 cells with cetuximab resulted in PARP cleavage, which was effectively prevented by concurrent exposure of the cells to IGF-I in the medium (Fig. 5C, *lane 2* versus *lane 4*); however, this IGF-I-mediated protection was eliminated when the expression of HIF-1α was silenced by HIF-1α siRNA (*lane 6* versus *lane 8*). This observation suggests that the resistance of cancer cells to cetuximab-mediated induction of apoptosis conferred by alternative activation of EGFR downstream signaling pathways can be overcome by approaches targeting HIF-1α.

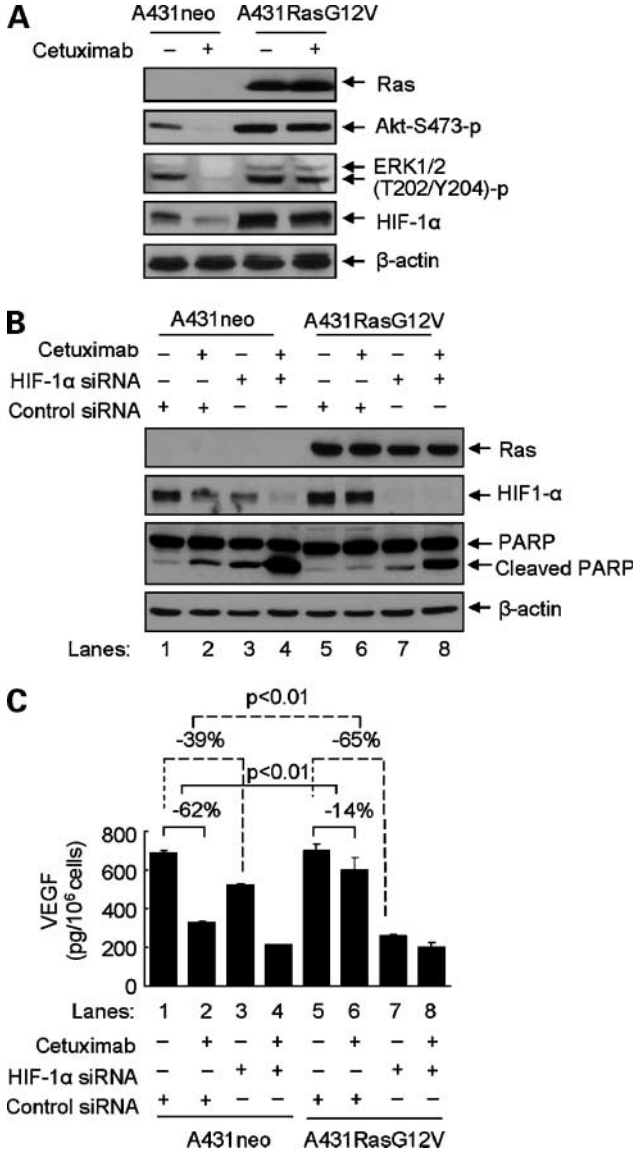

Figure 4. Effect of HIF-1α silencing on overcoming oncogenic Ras-mediated resistance to cetuximab-induced PARP cleavage and inhibition of VEGF production. **A,** A431neo and A431RasG12V cells were left untreated or treated with 10 nmol/L cetuximab for 16 h. The cells were then lysed, and cell lysates in equal amounts of protein were subjected to Western blot analysis using the antibodies as indicated. **B** and **C,** A431neo and A431RasG12V cells were subjected to HIF-1α expression silencing with scrambled or HIF-1α-specific siRNA for 72 h followed by cell culture with or without 10 nmol/L cetuximab for 16 h. Cell lysates were then prepared and subjected to Western blot analysis with the antibodies as indicated in **B**, and the VEGF secreted into the conditioned medium by the cells was measured by ELISA as shown in **C**. The significance of VEGF decreases in each individually treated group was compared with the value for the control group. *P* values (*top*) were determined by Student's *t* test.

Discussion

In this study, we tested our hypothesis that HIF-1α is a major downstream effector molecule of the EGFR pathway and that down-regulation of HIF-1α protein contributes a major antitumor mechanism of the EGFR-blocking antibody cetuximab. We found that cetuximab decreased the HIF-1α level through inhibition of HIF-1α protein synthesis in a PI3K inhibition-dependent manner, and we provided proof-in-principle using a HIF-1α RNA inference approach that cetuximab resistance caused by EGFR-independent activation of PI3K can be overcome by targeting HIF-1α. Our results are consistent with previous findings from several other laboratories showing that cell signal transduction leading to activation of the PI3K/Akt pathway stimulates HIF-1α protein synthesis (21, 22, 39–41). The demonstrated role of the PI3K/Akt/mTOR pathway in regulating synthesis of HIF-1α suggests a strong link between the levels of HIF-1α and inhibitability of PI3K in cancer cells on treatment with EGFR inhibitors (28).

A high level of HIF-1α is associated with increased tumor angiogenesis, invasion, and resistance to conventional treatments (42–44). These important roles of HIF-1 in cancer cell biology and its ubiquitous increase associated with hypoxia in solid tumors already make it a rational

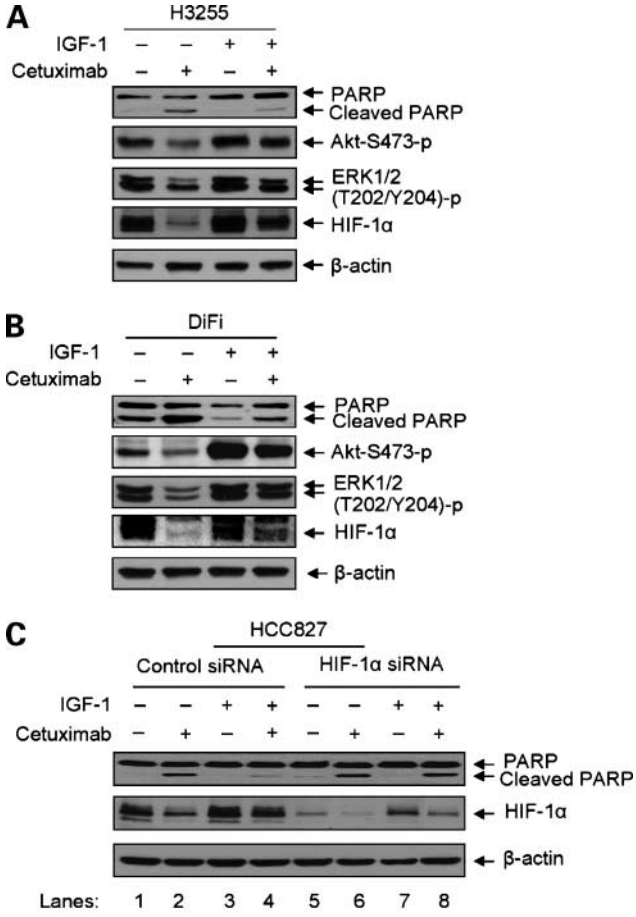

Figure 5. Effect of HIF-1α silencing on reversing IGF-I-mediated protection against cetuximab-induced PARP cleavage. **A** and **B,** H3255 and DiFi cells were left untreated or treated with 10 nmol/L cetuximab with or without concurrent treatment with 10 nmol/L IGF-I overnight (16 h) as indicated. The cells were lysed, and equal amounts of cell lysates were subjected to Western blot analysis using the antibodies indicated. **C,** HCC827 cells were first subjected to a 72-h culture period after transfection with a scrambled or HIF-1α-specific siRNA. The cells were then left untreated or treated with 10 nmol/L cetuximab with or without concurrent treatment with 10 nmol/L IGF-I overnight as in **A** and **B**. The cells were lysed, and equal amounts of cell lysates were subjected to Western blot analysis using the antibodies indicated. The levels of β-actin served as an internal control for equal protein loading in each lane.

target for therapeutic interventions (17, 45). We propose here that a combination of EGFR-targeting agents with HIF-1α-targeting approaches will be more effective than either one alone, because, as mentioned above, although targeting EGFR covers a broad range of downstream signaling networks activated by the receptor tyrosine kinase, some of its downstream signaling pathways can be bypassed due to alternative and/or constitutive activation of these pathways at various levels. Although targeting HIF-1α offers a narrower spectrum than does targeting of EGFR, it leads to a more specific inhibition of major cancer-related cell signaling, thereby reinforcing a major antitumor mechanism of EGFR-targeted therapy. This is particularly important when the EGFR inhibitors are unable to down-regulate HIF-1α levels due to the presence of EGFR-independent activation of PI3K mechanisms in resistant tumors, even when other downstream pathways such as MEK/ERK and STAT3 remain sensitive to EGFR inhibition.

This point of view is further strengthened by our findings showing that, although inhibition of MEK with PD98059 failed to decrease the level of HIF-1α compared with the effect of inhibition of PI3K with LY294002, the conditioned medium from cultures of the PD98059-treated cells showed a moderate but significant decrease in levels of VEGF compared with untreated cells. This result is consistent with the knowledge that MEK/ERK is involved in the transcriptional activity of HIF1 by phosphorylating the HIF-1 cofactor p300/CEB (36), which is required for the transcriptional activities of HIF-1 (46, 47). Additionally, MEK/ERK-mediated cell signaling is important for phosphorylation of c-fos, which is a component of the AP1 transcription factor, another important regulator of VEGF expression. Other VEGF transcription activators such as STAT3 and SP1 can be affected by targeting the EGFR. Thus, the outcomes of targeting HIF-1α and EGFR are extremely complementary.

Ras functions as a major molecular switch linking EGFR activation to downstream cytoplasmic and nuclear events. Oncogenic mutations of the Ras gene are present in ~30% of all human cancers (38). K-Ras mutations occur frequently in non-small cell lung cancer, colorectal cancers, and pancreatic carcinomas; H-Ras mutations are common in bladder, kidney, and thyroid carcinomas; N-Ras mutations are found in melanoma, hepatocellular carcinoma, and hematologic malignancies (38). In addition, high levels of H-Ras are found in >50% of human breast cancers (38). Thus, oncogenic mutation of Ras family oncogenes represents a clinically relevant mechanism that can confer resistance to EGFR-targeted therapy on cancer cells (13). We showed here that silencing of HIF-1α substantially restored the cellular response to cetuximab-induced inhibition of VEGF production in A431 cells following transfection with an oncogenic Ras mutant (RasG12V).

Our study showed that HIF-1α also plays a role in the IGF-I-mediated protection against apoptosis induced by cetuximab and that knockdown of HIF-1α expression resensitizes the cells to EGFR inhibition-induced apoptosis. It is important to note that there are a plethora of Ras and IGF-IR effectors in addition to PI3K and HIF-1α that may be coupled to cetuximab resistance in theory; however, our experimental data clearly showed that HIF-1α played a major role in the resistance, and silencing HIF-1α substantially improved therapeutic responses of cancer cells to cetuximab treatment in these clinically relevant scenarios.

Given that EGFR-independent activation of PI3K may prevent EGFR inhibitors from down-regulating HIF-1α, it might also be advantageous to combine PI3K inhibitors with EGFR inhibitors to overcome tumor resistance (35). It would be interesting to compare the effect of HIF-1α inhibition with that of PI3K inhibition on overcoming tumor resistance to EGFR inhibitors. Each strategy may have certain advantages and disadvantages. For example,

inhibition of PI3K may have a better therapeutic efficacy due to a wide scope and coverage of downstream signaling but may also be accompanied by increased toxicity. In addition, the efficiency of PI3K in inhibiting HIF-1α synthesis may be affected by intermediate factors that yet to be characterized. By direct targeting of HIF-1α, these potential concerns are eliminated.

In recent years, interest has grown in combining EGFR-targeting agents with compounds that inhibit the function of VEGF, and results from preclinical studies have shown enhanced antitumor activity for the combination treatment (48–50). Clinical trials are ongoing. Although the rationale for using such combinations was different from that of our current study, VEGF is one of major genes regulated by HIF-1, although HIF-1 is not the only VEGF regulator. Moreover, it should be noted that targeting HIF-1 is expected not only to inhibit VEGF expression but also to transcriptionally inhibit >60 known genes, many of which promote the cancer phenotypes.

In conclusion, our results have shown that down-regulation of HIF-1α is required for maximal therapeutic response of cancer cells to cetuximab. We believe that combinatorial therapies targeting EGFR and HIF-1α will increase the spectrum of patients who respond to EGFR-targeted therapy and propose to pursue this study in future preclinical settings and clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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