Cetuximab Reverses the Warburg Effect by Inhibiting HIF-1–Regulated LDH-A

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Abstract

Hypoxia-inducible factor-1 (HIF-1) plays a critical role in reprogramming cancer metabolism toward aerobic glycolysis (i.e., the Warburg effect), which is critical to supplying cancer cells with the biomass needed for proliferation. Previous studies have shown that cetuximab, an EGF receptor–blocking monoclonal antibody, downregulates the alpha subunit of HIF-1 (HIF-1α) through the inhibition of EGF receptor downstream cell signaling and that downregulation of HIF-1α is required for cetuximab-induced antiproliferative effects. However, the mechanism underlying these actions has yet to be identified. In this study, we used the Seahorse XF96 extracellular flux analyzer to assess the effect of cetuximab treatment on changes in glycolysis and mitochondrial respiration, the two major energy-producing pathways, in live cells. We found that cetuximab downregulated lactate dehydrogenase A (LDH-A) and inhibited glycolysis in cetuximab-sensitive head and neck squamous cell carcinoma (HNSCC) cells in an HIF-1α downregulation–dependent manner. HNSCC cells with acquired cetuximab resistance expressed a high level of HIF-1α and were highly glycolytic. Overexpression of a HIF-1α mutant (HIF-1αΔO6D) conferred resistance to cetuximab-induced G1 phase cell-cycle arrest, which could be overcome by knockdown of LDH-A expression. Inhibition of LDH-A activity with oxamate enhanced the response of cetuximab-resistant cells to cetuximab. Cetuximab had no noticeable inhibitory effect on glycolysis in nontransformed cells. These findings provide novel mechanistic insights into cetuximab-induced cell-cycle arrest from the perspective of cancer metabolism and suggest novel strategies for enhancing cetuximab response. Mol Cancer Ther; 12(10); 2187–99. ©2013 AACR.

Introduction

Glucose is an important source of energy and carbon for both normal and cancer cells. Unlike most normal cells—which metabolize glucose by a low rate of glycolysis followed by oxidative phosphorylation in the mitochondria through the tricarboxylic acid cycle (also known as the citric acid cycle or Krebs cycle)—cancer cells metabolize glucose by a high rate of glycolysis followed by lactate production in the cytosol even in the presence of abundant oxygen, a phenomenon known as aerobic glycolysis or the Warburg effect (1, 2). The Warburg effect is important for cancer cell proliferation because this process generates building blocks and reducing power, both of which are needed for the biosynthesis that fuels cell growth and proliferation (3, 4). This altered metabolism in cancer cells is a direct result of the aberrant cell signaling caused by overexpression of growth factor receptors, activation of oncogenes, and/or inactivation of tumor suppressor genes that permits unlimited cancer cell proliferation (5–7).

The transcription factor hypoxia-inducible factor-1 (HIF-1) plays a key role in reprogramming cell metabolism from oxidative phosphorylation to aerobic glycolysis. HIF-1 regulates the expression of the genes coding for proteins involved in various steps of cancer metabolism, from glucose uptake and subsequent glycolytic reactions to the generation of lactate and its secretion by lactate transporters (8). HIF-1 is a heterodimer consisting of a highly regulated HIF-1α subunit and a constitutively expressed HIF-1β subunit (9–12). A high level of HIF-1α protein is common in many types of solid tumors including tumors of the colon, lung, breast, stomach, ovary, pancreas, prostate, kidney, and head and neck (13–15). The high level of HIF-1α in cancer cells is caused not only by the decreased ubiquitination and degradation of HIF-1α protein via a posttranslational mechanism associated with tumor hypoxia (16, 17) but also by aberrant cell signaling, which increases HIF-1α protein expression via a translational mechanism (18–22).

Cetuximab is an EGF receptor (EGFR)-blocking monoclonal antibody approved for treating patients with head and neck cancers and colorectal cancers in combination...
with radiotherapy and/or chemotherapty (23, 24). We and others have previously shown that cetuximab binds to EGFR and blocks the ligand-induced activation of EGFR downstream cell signaling, which leads to G1 phase arrest of cell-cycle traversal and even apoptosis in certain circumstances (25–40). Our previous work showed that cetuximab can downregulate HIF-1α protein by inhibiting the PI3K/Akt and MEK/Erk pathways, and this down-regulation of HIF-1α is required, although may not be sufficient, for cetuximab to induce antiproliferative effects (41–45). Knockdown of HIF-1α by siRNA partially overcame the resistance caused by overexpression of constitutively active Ras mutant to cetuximab-induced antiproliferative effects (43–45).

These previous studies established the importance of HIF-1α downregulation in mediating cetuximab-induced antitumor effects; however, to our knowledge, no studies have carefully examined the mechanism that leads to growth inhibition after downregulation of HIF-1α by cetuximab. We hypothesized that cetuximab inhibits cancer cell proliferation through inhibition of glycolysis by downregulating HIF-1α, thereby reversing the Warburg effect that is critically important for cancer cell survival and proliferation. To test this hypothesis, we generated and characterized 2 pairs of genetically matched cetuximab-sensitive and -resistant head and neck cancer cell lines. We used the Seahorse XP96 extracellular flux analyzer to assess the effect of cetuximab treatment on glycolysis and mitochondrial respiration, the 2 major energy-producing pathways, in live cells. We further investigated the effect of cetuximab on the expression and enzymatic activity of lactate dehydrogenase-A (LDH-A), which regulates the conversion of pyruvate to lactate, and on the levels of glucose consumption, lactate production, and intracellular ATP in cetuximab-sensitive and -resistant cells. Our findings provide novel insights into the mechanisms underlying cetuximab-induced antiproliferative and apoptotic effects in cancer cells and suggest a novel therapeutic strategy for improving cetuximab response.

Materials and Methods

Reagents

Cetuximab was provided by ImClone Systems, an Eli Lilly company. All other reagents, including oxamate, were purchased from Sigma-Aldrich unless otherwise specified.

Cell lines and cell cultures

HN5, FaDu, SqCC/Y1, and TU167 head and neck squamous cell carcinoma (HNSCC) cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F12 medium supplemented with 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO2 atmosphere at 37°C as described previously (45, 46). Cetuximab-resistant HN5-R and FaDu-R cells were generated by exposing parental HN5 and FaDu cells to stepwise dose increases of cetuximab (up to 20 nmol/L) for 6 months or more. HN5-HIF-1α/ΔODD and FaDu-HIF-1α/ΔODD cells were established by transfecting parental HN5 and FaDu cells with pcDNA3.1 construct containing an HIF-1α oxygen-dependent degradation (ODD) domain deletion mutant (referred to as HIF-1α/ΔODD) using Lipofectamine 2000 (Life Technologies). The HN5-HIF-1α/ΔODD and FaDu-HIF-1α/ΔODD cells were maintained in medium containing 500 μg/mL neomycin. Immortalized nontransformed NOM9-TK human head and neck epithelial cells were maintained in serum-free keratinocyte growth medium supplemented with components of a keratinocyte growth medium SingleQuots kit including bovine pituitary extract, recombinant human EGF, insulin, hydrocortisone, and gentamicin sulfate (Lonza, Inc.; refs. 46, 47). The authenticity of the cell lines used in the current study has not been confirmed during the past 6 months.

Cell proliferation assay

Cells were cultured in 24-well plates with low-glucose (1 g/L), low-serum (0.5% FBS) medium (0.5 mL/well) at 37°C. Following the indicated treatments, 10 mg/mL MTT was added (50 μL/well), and the cells were incubated for an additional 2 hours. The cells were then lysed with a lysis buffer (500 μL/well) containing 20% SDS in dimethyl formamide/H2O (1:1, v/v; pH 4.7) at 37°C for at least 6 hours. The relative number of surviving cells in each group was determined by measuring the optical density (OD) of the cell lysates at an absorbance wavelength of 570 nm. The OD value of each treatment group was expressed as a percentage of the OD value of the untreated control cells.

Knockdown of gene expression by siRNA

LDH-A–targeted siRNA (target DNA sequence #1, GAGAAAGCCGTCTTAATT; #2, GATTAAGGCTTATCCGA; #3, CAGATTTAGGGACTGATAA), HIF-1α–targeted siRNA (target DNA sequence #1, CAAAGTTTACCTGACCTA; #2, GATTAATCTAGTTTGAAAT), and control siRNA were purchased from Sigma-Aldrich. The siRNA (200 pmol) and Lipofectamine 2000 (5 μL) were mixed in 100 μL of minimal essential medium (Opti-MEM, Life Technologies) for 15 minutes, and the siRNA/Lipofectamine 2000 mixture was added into the culture medium for 6 hours. After transfection, the medium was replaced with regular medium, and the cells were cultured for an additional 48 hours before the detection of knockdown of LDH-A and HIF-1α expression by Western blotting.

Western blot analysis

After the indicated treatments, cells were lysed in a lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 0.5% NP-40, 50 mmol/L NaF, 1 mmol/L Na2VO4, 1 mmol/L phenylmethylsulfonyl fluoride, 25 μg/mL aprotinin, and 25 μg/mL leupeptin and kept on ice for 15 minutes. The lysates were cleared by centrifugation, and the supernatants were collected. Equal amounts of protein lysate, as determined using the Pierce
Cetuximab Inhibits Aerobic Glycolysis

Glucose consumption assay

Cells were seeded in 6-well plates at $5 \times 10^5$ cells per well in 3 mL of phenol red–free, low-glucose, low-serum culture medium as described above. At the indicated times after treatment, an aliquot of 50 µL of the conditioned medium was collected from each well and diluted with 950 µL of distilled water (1:20). The glucose concentration in the diluted medium was measured using the Glucose (GO) Assay Kit (Sigma-Aldrich) according to the manufacturer’s instructions. Briefly, samples were mixed with the provided glucose assay reagent and incubated at 37 °C for 30 minutes before OD was measured at 540 nm. The measured ODs of the samples were compared with that of the standard glucose control. Glucose consumption was expressed as mg/10^6 cells at the indicated times.

Lactate production assay

Cells were seeded in 6-well plates at $5 \times 10^5$ cells per well in 3 mL of serum-free cell culture medium containing 1 g/L glucose. At the indicated times after treatment, an aliquot of 50 µL of the conditioned medium was collected from each well and diluted with 950 µL of distilled water (1:20). The lactate concentration in the diluted medium was measured using the Lactate Assay Kit from BioVision, Inc. Briefly, the sample was mixed with the provided reaction reagent and incubated at 37 °C for 30 minutes before the OD was measured at 570 nm. The measured ODs of the samples were compared with that of the standard lactate control. The levels of lactate were expressed as nmol/10^6 cells at the indicated times.

LDH-A activity assay

LDH-A activity was measured using a colorimetric LDH Activity Assay Kit (BioVision, Inc.), whose detection of LDH-A activity is based on a reaction in which LDH-A converts NAD to NADH in a specific time. Briefly, after various treatments, cell pellets were homogenized on ice in 0.5 mL of cold assay buffer. Supernatants were collected by centrifugation. Protein concentrations were determined using the Pierce Coomassie Plus colorimetric protein assay. Fifty-microliter aliquots (volume adjusted using assay buffer) containing equal amounts of protein and 50 µL of reaction reagent (48 µL of assay buffer and 2 µL of LDH-A substrate mix solution) were added to each well of a 96-well plate, and the plates were read under a microreader at 450 nm immediately ($T_0$) and 10 minutes after the reaction ($T_{10}$). LDH-A activity was expressed as an increase in OD values ($T_{10} - T_0$). The relative LDH-A activities in treated and untreated cells were expressed as percentages of the LDH-A activity in untreated cells after 4 hours in culture, which was arbitrarily set as 100.

Intracellular ATP assay

Intracellular ATP levels were determined using the ATP Bioluminescent Assay Kit (Sigma-Aldrich). Briefly, cells were seeded in 6-well plates at $5 \times 10^5$ cells per well and treated with 20 nmol/L cetuximab or left untreated in low-glucose, low-serum medium for 4 hours. The cells were then harvested and resuspended in 1 mL of PBS. An aliquot of 50 µL of the cell suspension was mixed with 100 µL of ATP-releasing reagent and 50 µL of distilled water in each well of a 96-well plate. The samples (100 µL) in each well were then transferred to a white opaque 96-well plate whose wells were each pre-filled with 100 µL of ATP assay mix. The amount of light emitted in each well was immediately measured using a FLUOstar Omega luminometer.

Flow cytometric analysis

After the indicated treatments, cells were harvested by trypsin and fixed in 70% ethanol overnight. After centrifugation and washing with PBS, the cells were stained with...
propidium iodide (50 μg/mL) and RNaseA (20 μg/mL) on ice for 30 minutes and then subjected to flow cytometric analysis with an LSRFortessa cell analyzer (BD Biosciences). Cell-cycle distribution data were analyzed using the FlowJo v10 data processing program.

**Apoptosis assays**

Apoptosis was measured using a colorimetric ELISA kit (Cell Death Detection ELISA, Roche Diagnostics Corp.) that quantitatively measures cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes). The procedure was conducted exactly according to the manufacturer’s instructions. Apoptosis was also detected by Western blotting with an antibody that recognizes both cleaved and uncleaved PARP (Cell Signaling Technology) as described previously (43, 44).

**Statistical analysis**

The Student t test was used for all statistical analyses. The data are presented as means ± SD. All experiments were repeated at least once with reproducible findings.

**Results**

**Cetuximab inhibits aerobic glycolysis, and resistance to cetuximab is linked to increased glycolytic flux**

To assess the effect of cetuximab treatment on cancer cell metabolism and determine whether this effect correlates with cetuximab-induced antiproliferative effects in cancer cells, we generated 2 pairs of isogenic cetuximab-sensitive and cetuximab-resistant HNSCC cell lines by subjecting cetuximab-sensitive HN5 and FaDu cells to stepwise dose increases of cetuximab (up to 20 nmol/L) in continuous culture for 6 months or more. The resultant sublines, HN5-R and FaDu-R, were considerably less sensitive to cetuximab-induced growth inhibition than the parental HN5 and FaDu cells treated under identical experimental conditions (Fig. 1A). HN5 cells had higher expression levels of EGFR, phosphorylated EGFR (represented by phosphorylation of EGFR at Y1068), and HIF-1α than FaDu cells in normoxic culture. Both HN5 and FaDu cells had lower levels of HIF-1α after cetuximab treatment (Fig. 1B). In contrast, HN5-R and FaDu-R cells had higher levels of HIF-1α than their parental cells in normoxic culture, and the levels of HIF-1α in HN5-R and FaDu-R cells were only minimally affected by cetuximab treatment. The level of LDH-A was decreased by cetuximab treatment in parental HN5 and FaDu cells but not in cetuximab-resistant HN5-R and FaDu-R cells. Interestingly, HN5-R and FaDu-R cells remained sensitive or partially sensitive to cetuximab-induced inhibition of cell signaling, as revealed by the inhibition of EGFR autophosphorylation (in FaDu-R cells, but not apparent in HN5-R cells) and reduced phosphorylation of Akt-S473 and Erk T202/Y204 (with slight difference in the extents between FaDu-R and HN5-R cells), 2 important signaling molecules downstream of EGFR (Fig. 1B). These interesting findings challenge the conventional concept that cetuximab resistance is linked to the failure for cetuximab to effectively inhibit EGFR downstream cell signaling and suggest that the resistance could occur through changes in the pattern of cell metabolism without significant changes in cell signaling pathways.

We further analyzed the metabolic profiles of the parental cell lines and the cetuximab-resistant sublines with and without cetuximab treatment using the Seahorse XF96 extracellular flux analyzer, which simultaneously records the metabolic patterns of aerobic respiration and glycolysis, the 2 major energy-yielding pathways in cells. Compared with the parental HN5 and FaDu cells, the cetuximab-resistant HN5-R and FaDu-R cells had higher ECARs, indicating that these cells had higher glycolysis rates than the parental cells (Fig. 1C). Cetuximab treatment lowered the ECAR and increased the OCR (an indicator of mitochondrial respiration rate) in HN5 and FaDu cells but not in HN5-R and FaDu-R cells. Mitochondrial stress challenge with oligomycin, an ATP synthase inhibitor, decreased the OCR in parental cells (although modestly) but not in the cetuximab-resistant cells, suggesting that the cetuximab-resistant cells were more glycolytic. Challenge with carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), an uncoupler of oxidative phosphorylation and ATP synthesis, resulted in the cetuximab-resistant cell lines having a higher capacity than the parental cells to increase ECAR, which was consistent with the finding that HIF-1α and LDH-A levels in the cetuximab-resistant sublines were higher than those in the parental cells.

To determine a direct role of high level of HIF-1α in promoting aerobic glycolysis via regulating LDH-A in HN5-R and FaDu-R cells, we knocked down HIF-1α with 2 different HIF-1α siRNA in parental and cetuximab-resistant cells and confirmed that the level of LDH-A in the cetuximab-resistant cells was markedly lowered after HIF-1α knockdown as did the level of LDH-A in parental cells after HIF-1α knockdown (Fig. 1D). Knockdown of HIF-1α in HN5-R and FaDu-R cells with the same 2 different HIF-1α siRNA significantly decreased ECAR and increased OCR in these cetuximab-resistant cells, indicating that knockdown of HIF-1α can reverse the Warburg effect in cetuximab-resistant cells, just as cetuximab reversed the Warburg effect in cetuximab-sensitive cells (Fig. 1E).

Taken together, these novel findings indicate that LDH-A was downregulated and glycolysis was inhibited via downregulation of HIF-1α by cetuximab in cetuximab-sensitive HN5 and FaDu cells but not in the cetuximab-resistant sublines. The resistant sublines exhibited increased glycolytic potential that could be tempered by knockdown of HIF-1α.

**Cetuximab reduces glucose consumption, lactate production, and intracellular ATP level in an HIF-1α downregulation-dependent manner**

To confirm the effect of cetuximab on inhibiting aerobic glycolysis, we directly measured glucose consumption

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Cetuximab downregulates LDH-A and inhibits glycolytic flux in HNSCC cells. A, HN5 and FaDu cells and their cetuximab-resistant sublines (HN5-R and FaDu-R) were treated with the indicated doses of cetuximab for 5 days in low-glucose (1 g/L), low-serum (0.5% FBS) medium. Cell growth responses to cetuximab were measured using an MTT assay. B, the indicated cells were treated with 20 nmol/L cetuximab or left untreated for 24 hours. Cell lysates were prepared, and equal amounts of cell lysates were subjected to Western blot analysis with the indicated antibodies. C, HN5, HN5-R, FaDu, and FaDu-R cells were treated with 20 nmol/L cetuximab or left untreated for 10 hours. Metabolic flux analysis was conducted to measure changes in ECAR and OCR and was followed by a mitochondrial stress test as indicated. The final concentrations of oligomycin, FCCP, antimycin, and rotenone were all 1 μmol/L. Error bars, SD. D, the indicated cell lines were subjected to knockdown of HIF-1α with each of 2 different HIF-1α siRNAs or control siRNA for 48 hours, followed by Western blotting with the indicated antibodies. Ctr, control. E, HN5-R and FaDu-R cells were subjected to knockdown of HIF-1α as described in D. Metabolic flux analysis was then conducted to measure ECAR and OCR.

Figure 1. Cetuximab downregulates LDH-A and inhibits glycolytic flux in HNSCC cells. A, HN5 and FaDu cells and their cetuximab-resistant sublines (HN5-R and FaDu-R) were treated with the indicated doses of cetuximab for 5 days in low-glucose (1 g/L), low-serum (0.5% FBS) medium. Cell growth responses to cetuximab were measured using an MTT assay. B, the indicated cells were treated with 20 nmol/L cetuximab or left untreated for 24 hours. Cell lysates were prepared, and equal amounts of cell lysates were subjected to Western blot analysis with the indicated antibodies. C, HN5, HN5-R, FaDu, and FaDu-R cells were treated with 20 nmol/L cetuximab or left untreated for 10 hours. Metabolic flux analysis was conducted to measure changes in ECAR and OCR and was followed by a mitochondrial stress test as indicated. The final concentrations of oligomycin, FCCP, antimycin, and rotenone were all 1 μmol/L. Error bars, SD. D, the indicated cell lines were subjected to knockdown of HIF-1α with each of 2 different HIF-1α siRNAs or control siRNA for 48 hours, followed by Western blotting with the indicated antibodies. Ctr, control. E, HN5-R and FaDu-R cells were subjected to knockdown of HIF-1α as described in D. Metabolic flux analysis was then conducted to measure ECAR and OCR.

and lactate production after cetuximab treatment in HN5 and FaDu cells that did or did not overexpress an experimentally created HIF-1α mutant (HIF-1α/ΔODD). This mutant retains the majority of the transcriptional activity of full-length HIF-1α and can be stably overexpressed in normoxic culture because of experimental deletion of the ODD domain in HIF-1α that renders HIF-1α highly unstable in normoxic culture (16, 17). In HN5 and FaDu
cells transfected with a control vector (HN5-neo and FaDu-neo cells), cetuximab inhibited glucose consumption and lactate production in a time-dependent manner (Fig. 2A and B). Consistent with the knowledge that HIF-1 upregulates glycolysis, overexpression of HIF-1α/ΔODD in HN5 and FaDu cells (HN5-HIF-1α/ΔODD and FaDu-HIF-1α/ΔODD cells) significantly increased the levels of glucose consumption and lactate production and conferred resistance to cetuximab-induced inhibition of glucose consumption and lactate production.

We also assessed the differences in the intracellular ATP levels of cells untreated and treated with cetuximab for 4 hours when no changes in cell numbers were detected between the 2 groups. The ATP levels of the HN5-neo and FaDu-neo cells treated with cetuximab were substantially lower than that of the untreated cells (down 39% and 31%, respectively). In contrast, the ATP levels of the HN5-HIF-1α/ΔODD and FaDu-HIF-1α/ΔODD cells treated with cetuximab were only modestly lower than that of untreated cells (down 15% and 11%, respectively; Fig. 2C). These findings are consistent with the knowledge that ATP generated during glycolysis is a major source of energy in cancer cells.

Cetuximab inhibits LDH-A expression and enzymatic activity by downregulating HIF-1α

To further elucidate the biochemical mechanisms underlying cetuximab-mediated inhibition of glycolysis via HIF-1α downregulation, we compared the effects of cetuximab on downregulation of LDH-A level and inhibition of EGFR downstream signaling pathways in HN5-neo cells with those in HN5-HIF-1α/ΔODD cells and in FaDu-neo cells with those in FaDu-HIF-1α/ΔODD cells. We found that cetuximab treatment downregulated the level of LDH-A in both HN5-neo and FaDu-neo cells but not in the cell lines overexpressing HIF-1α/ΔODD cells (Fig. 3A). Interestingly, similar to our finding that the phosphorylation levels of Akt and Erk in HN5-R and FaDu-R cells were sensitive or partially sensitive to cetuximab (Fig. 1B), the phosphorylation levels of these molecules in HN5-HIF-1α/ΔODD and FaDu-HIF-1α/ΔODD cells were also sensitive to cetuximab. The levels of HIF-1α increased steadily after the regular culture medium was changed for low-glucose, low-serum medium, which likely caused metabolic changes that can upregulate the HIF-1α level, such as mTOR activation. However, both HN5-neo and FaDu-neo cells treated with cetuximab had lower levels of HIF-1α than untreated cells at each time of

Figure 2. Cetuximab inhibits glucose consumption, lactate production, and intracellular ATP levels in a HIF-1α inhibition–dependent manner. A, HN5-neo, HN5-HIF-1α/ΔODD, FaDu-neo, and FaDu-HIF-1α/ΔODD cells were seeded in 6-well plates with 3 mL of low-glucose (1 g/L), low-serum (0.5% FBS) medium. The cells were treated with 20 nmol/L cetuximab or left untreated. At the indicated times after treatment, the level of glucose remaining in the conditioned medium was determined as described in Materials and Methods. B, the indicated cells were seeded and treated similarly as described in A. At the indicated times after treatment, the level of lactate produced in the conditioned medium was determined as described in Materials and Methods. C, the indicated cells were treated with 20 nmol/L cetuximab or left untreated in low-glucose (1 g/L), low-serum (0.5% FBS) medium for 4 hours. Cell pellets were harvested, and the intracellular level of ATP was measured using a luciferase-based ATP determination assay. The relative values of ATP in the treated groups were expressed as percentages of the value of ATP in the corresponding untreated groups. The data are presented as means ± SD.
measurement. The kinetics of cetuximab-mediated inhibition of EGFR downstream pathways was different: Erk phosphorylation was inhibited earlier than Akt phosphorylation; however, Erk phosphorylation recovered substantially 24 hours after treatment whereas Akt phosphorylation did not. In cells overexpressing HIF-1α/ΔODD, even the endogenous HIF-1α became resistant to cetuximab treatment, an interesting phenomenon that we reported previously (42). These findings provide further support that HIF-1α has a fundamental role in regulating cell response to cetuximab-induced growth inhibition.

Consistent with the decrease in LDH-A protein levels in HNS-neo and FaDu-neo cells after cetuximab treatment, the activity of LDH-A in catalyzing the production of lactate was also decreased in these cells as early as 4 hours after cetuximab treatment (Fig. 3B). In contrast, HNS-HIF-1α/ΔODD, and FaDu-HIF-1α/ΔODD cells had much higher LDH-A enzymatic activity than HNS-neo and FaDu-neo cells, and the elevated LDH-A activity was not inhibited by cetuximab (Fig. 3B). Together, these findings indicate that cetuximab downregulates HIF-1α, thereby downregulating LDH-A expression and inhibiting LDH-A activity to inhibit glycolysis.

**Knockdown of LDH-A overcomes resistance to cetuximab-induced G1 arrest**

Aerobic glycolysis has been suggested to be a means of fueling cancer cells with the biomass needed for proliferation (3, 48). To test our hypothesis that cetuximab inhibits cancer cell proliferation by downregulating HIF-1α and subsequently LDH-A to inhibit glycolysis, we conducted cell-cycle analysis after subjecting cells to treatment with cetuximab and/or knockdown of LDH-A by siRNA. We used 3 different LDH-A–specific siRNAs, all of which knocked down the level of LDH-A in all parental and sublines tested (Fig. 4A, C and E). LDH-A siRNA #1 and #2 were used for knockdown of LDH-A in the cells subjected to cell-cycle analysis.

As shown in Fig. 4B, in HN5-neo and FaDu-neo cells, the percentages of cetuximab-treated cells in G1 phase (85.0% and 83.2%, respectively) were higher than those of the untreated cells in G1 phase (67.9% and 64.9%, respectively). LDH-A knockdown alone by siRNA #1 increased the percentages of G1 phase cells modestly (73.0% and 70.2%, respectively) but the combination of cetuximab and LDH-A knockdown had an additive effect on G1 arrest (90.3% and 87.1%, respectively). Similar results were obtained using LDH-A siRNA #2 in HN5-neo and FaDu-neo cells (supplementary Fig. S1).

Both the cetuximab-resistant sublines (HN5-R and FaDu-R) and the HIF-1α/ΔODD-overexpressing cells (HN5-HIF-1α/ΔODD and FaDu-HIF-1α/ΔODD cells) were resistant to cetuximab-induced G1 arrest. As shown in Fig. 4D, compared with the percentages of untreated HNS-R cells and FaDu-R cells in G1 phase (53.3% and 60.9%, respectively), the percentages of cetuximab-treated HNS-R cells and FaDu-R cells in G1 phase were 54.0%...
and 60.7%, respectively. As shown in Fig. 4F, compared with the percentages of untreated HN5-HIF-1α/ΔODD cells and FaDu-HIF-1α/ΔODD cells in G1 phase (51.7% and 64.4%, respectively), the percentages of cetuximab-treated HN5-HIF-1α/ΔODD cells and FaDu-HIF-1α/ΔODD cells in G1 phase were modestly higher (59.6% and 71.0%, respectively). These findings are in agreement with the role of HIF-1α/ΔODD in counteracting cetuximab-mediated inhibition of glycolysis and downregulation of LDH-A (Figs. 2 and 3).

Knockdown of LDH-A alone had modest effects on increasing the percentage of G1 phase cells in the cetuximab-resistant cells. As shown in Fig. 4D, compared with the percentages of untreated HN5-R cells and FaDu-R cells in G1 phase (53.3% and 60.9%, respectively), the percentages of LDH-A knockdown HN5-R cells and FaDu-R cells in G1 phase were modestly higher (59.6% and 71.0%, respectively). As shown in Fig. 4F, compared with the percentages of untreated HN5-HIF-1α/ΔODD cells and FaDu-HIF-1α/ΔODD cells in G1 phase (51.7% and 64.4%, respectively), the percentages of LDH-A knockdown HN5-HIF-1α/ΔODD cells and FaDu-HIF-1α/ΔODD cells in G1 phase were modestly also higher (64.3% and 70.3%, respectively).
Importantly, knockdown of LDH-A by siRNA #1 largely restored the activity of cetuximab in inducing G1 cell-cycle arrest in cetuximab-resistant cells. Compared with the percentage of cells in G1 phase after cetuximab or LDH-A knockdown alone (Fig. 4D and F), the percentage of G1 phase cells after the combination of cetuximab and LDH-A knockdown increased to 72.3% and 76.5%, respectively, in HN5-R and FaDu-R cells, and increased to 84.7% and 83.0%, respectively, in HN5-HIF-1α/ΔODD and FaDu-HIF-1α/ΔODD cells. Similar results were obtained using LDH-A siRNA #2 (Supplementary Fig. S1).

Together, these findings confirm the role of HIF-1α in cetuximab-induced cell-cycle arrest and suggest that targeting LDH-A is an effective strategy for improving cetuximab response.

**Knockdown of LDH-A or inhibition of LDH-A with oxamate enhances the therapeutic effect of cetuximab in cancer cells**

To determine whether knockdown of LDH-A can also potentiate cetuximab-induced apoptosis, we used 2 independent apoptosis assays (one detecting PARP cleavage and the other detecting DNA fragmentation) to assess the induction of apoptosis in HN5 cells (parental HN5, HN5-R, and HN5-HIF-1α/ΔODD cells) and FaDu cells (parental FaDu, FaDu-R, and FaDu-HIF-1α/ΔODD cells) after subjecting the cells to treatment with cetuximab and/or knockdown of LDH-A. Treatment with cetuximab or knockdown of LDH-A alone had little effect on inducing apoptosis in parental cells or cetuximab-resistant cells. Cetuximab plus LDH-A knockdown caused an increase in the levels of PARP cleavage and DNA fragmentation in HN5 and FaDu cells but had only minimal or no effect in the cetuximab-resistant cells (Fig. 5A and B).

Next, we assessed the effects of oxamate, a small-molecule inhibitor of LDH-A (49), alone and in combination with cetuximab on the growth and survival of HNSCC cells after 5-day extended culture (Fig. 5C). MTT assays, which measure the cytostatic and cytotoxic effects of the treatments, showed that oxamate alone only modestly inhibited cell growth and survival in HN5 cells but had a stronger effect in HN5-R and HN5-HIF-1α/ΔODD cells, which are more glycolytic than their parental cells. Oxamate alone had a similarly modest effect on inhibiting cell growth and survival in FaDu, FaDu-R, and FaDu-HIF-1α/ΔODD cells; however, the combination of cetuximab and oxamate significantly enhanced the inhibition of cell growth and survival compared with either single treatment alone, particularly in the cetuximab-resistant sublines. The combination treatment yielded similar results in another 2 HNSCC cell lines, SqCC/Y1 and TU167, which are much less sensitive to cetuximab-induced growth inhibition than HN5 and FaDu cells (Supplementary Fig. S2). Together, these data suggest that co-targeting LDH-A with oxamate and cetuximab improves the therapeutic effect of cetuximab in cancer cells.

**Cetuximab has no significant effect on inhibiting glycolysis in nontransformed cells in normoxia**

To determine whether cetuximab affects glycolysis in nontransformed cells, we assessed changes in cell proliferation, glucose consumption, lactate production, LDH-A activity, and ATP level in NOM9-TK cells, an immortalized human head and neck epithelial cell line, treated with cetuximab or left untreated in normoxic culture. In the absence of EGF, NOM9-TK proliferation was slow and barely inhibited by cetuximab (Fig. 6A). Compared with cells treated under hypoxic conditions, cells treated under normoxic conditions expressed a very low level of HIF-1α. Twenty-four–hour cetuximab treatment inhibited EGFR autophosphorylation and Erk phosphorylation but not Akt phosphorylation (Fig. 6B). Although cetuximab inhibited LDH-A expression and aerobic glycolysis in HN5 and FaDu cells in normoxic culture (Fig. 1), it did not inhibit LDH-A expression in NOM9-TK cells (Fig. 6B) and did not significantly inhibit glucose consumption (Fig. 6C), lactate production (Fig. 6D), LDH-A activity (Fig. 6E), or ATP production (Fig. 6F) in the cells in normoxic culture. The absolute amount of lactate production in the NOM9-TK cells (Fig. 6D) was markedly less than that in the HN5 and FaDu cells during the same period (Fig. 2B). These findings indicate that cetuximab inhibits glycolysis selectively in cancer cells through the downregulation of LDH-A, which causes cancer cells that rely heavily on aerobic glycolysis for biosynthetic metabolism to arrest at G1 phase.

**Discussion**

In the current work, we showed that HNSCC cells with acquired resistance to cetuximab have higher levels of HIF-1α and are more glycolytic than their parental cells. Overexpression of a degradation-resistant HIF-1α (HIF-1α/ΔODD) elevated the glycolytic potential of the cells and conferred resistance to cetuximab-induced glycolysis inhibition and cell-cycle arrest. We also showed that cetuximab-induced HIF-1α downregulation led to LDH-A downregulation and that downregulation of LDH-A expression by siRNA or inhibition of LDH-A activity improved cancer cell response to cetuximab. Our findings provide strong evidence suggesting that glycolysis inhibition plays a major role in the cetuximab-induced inhibition of cell proliferation.

A fundamental difference in metabolism between cancer cells and normal cells is that cancer cells rely on an uninterrupted supply of biomass to fuel their unlimited proliferation. Cancer cells thus have evolved to adapt to the energy-less-efficient type of metabolism, that is, aerobic glycolysis or the Warburg effect, by directing the pyruvate toward production of lactate in the cytosol under the action of LDH-A, through which an adequate supply of biomass is produced via an elevated glycolytic flux. Cetuximab reverses the Warburg effect by inhibiting HIF-1α-regulated LDH-A in cancer cells, thereby decreasing the supply of biomass needed for proliferation; the cells are thus arrested at G1 phase. Unlike the metabolism...
Figure 5. LDH-A knockdown or inhibition enhances the therapeutic effect of cetuximab. The indicated cells were subjected to knockdown of LDH-A with specific siRNA or control siRNA for 48 hours and then treated with 20 nmol/L cetuximab or left untreated for 24 hours in low-glucose (1 g/L), low-serum (0.5% FBS) medium. A, after treatment, cell lysates were prepared, and equal amounts of cell lysates were subjected to Western blot analysis with the indicated antibodies. The quantification of the LDH-A and cleaved PARP bands was conducted using ImageJ software, and the data were expressed as fold increases in reference to the leftmost lane of the blots, the value of which was arbitrarily set as 1. B, the cell lysates in A were analyzed for the level of DNA fragmentation with an apoptosis ELISA kit as described in Materials and Methods. The data in the treated groups were expressed as fold increases in the optical density value compared with the value in the corresponding untreated cells. C, the indicated cells were treated with 20 nmol/L cetuximab and/or 5 mmol/L oxamate or left untreated for 5 days. Cell growth responses to cetuximab were measured using an MTT assay. The growth and survival of the treated groups were expressed as percentages of the OD value in the corresponding untreated groups. The data are presented as means ± SD.
Cetuximab Inhibits Aerobic Glycolysis

in cancer cells, the pyruvate in normal cells enters the mitochondria and is fully oxidized through the tricarboxylic acid cycle, thereby generating large amounts of ATP. This difference may explain why cetuximab inhibits glycolysis mainly in cancer cells, but not normal cells, after LDH-A downregulation.

We showed that knockdown of LDH-A expression or inhibition of LDH-A activity inhibited cell proliferation in both cetuximab-sensitive and cetuximab-resistant cells. Depending on the extent to which glucose-derived pyruvate is redirected to the tricarboxylic acid cycle for oxidative phosphorylation in cancer cells, inhibition of LDH-A could induce apoptosis in addition to cell-cycle arrest. This is because excess oxidative phosphorylation of the high level of pyruvate in cancer cells can cause overproduction of reactive oxygen species that are cytotoxic to cells, leading to apoptosis. However, in our models, the effects of knockdown of LDH-A or inhibition of LDH-A on inducing apoptosis seem to be limited. It was observed only when the targeting of LDH-A was combined with cetuximab in cetuximab-sensitive cells.

Another interesting finding of the current work is that the cetuximab-resistant cells (HN5-R, FaDu-R, HN5-HIF-1α/ΔODD, and FaDu-HIF-1α/ΔODD cells) remained at least partially sensitive to cetuximab-induced inhibition of major downstream cell signaling pathways of EGFR—that is, Akt phosphorylation and Erk phosphorylation—despite their resistance to cetuximab-induced growth inhibition. These findings suggest that, compared with these downstream signaling molecules, LDH-A is a better biomarker for predicting cancer cell responsiveness to cetuximab treatment. It is noteworthy that, despite the high level of HIF-1α or HIF-1α/ΔODD, the basal level of LDH-A was only modestly elevated in these cetuximab-resistant cells. This is most likely because the basal level of LDH-A was sufficient to drive glycolysis toward lactate production. It was when the cells were treated with cetuximab, an elevated HIF-1α or HIF-1α/ΔODD was needed to protect against downregulation of LDH-A by cetuximab.

In the current study, we did not explore the mechanisms underlying the increase in the basal HIF-1α levels.
that makes HN5-R and FaDu-R cells highly glycolytic. We found inconsistent changes in the levels of EGFR downstream cell signaling in these cells; for example, the level of activation-specific Akt phosphorylation was higher in HN5-R cells than in HN5 cells, whereas the level of activation-specific Erk phosphorylation was higher in FaDu-R cells than in FaDu cells. These findings suggest that either the underlying mechanisms are cell context-specific or additional mechanisms, such as Src activation, also play a role in upregulating HIF-1α in cetuximab-resistant cells. We are currently investigating these possibilities.

An important caveat to our findings is that downregulation of LDH-A is required but may not be sufficient for cetuximab-induced growth inhibition. The inhibition of other glycolytic pathway enzymes, which are also subject to regulation by HIF-1, may also be necessary for cetuximab-mediated inhibition of cancer cell proliferation.

In summary, we showed that cetuximab inhibits cancer cell proliferation by inhibiting glycolysis. Our findings suggest that LDH-A is a novel biomarker for predicting cetuximab response and a candidate target for sensitizing cetuximab-resistant cells to cetuximab treatment. Our work fills a major knowledge gap regarding the link between EGFR-targeted therapy and cancer cell metabolism and advances our understanding of how cetuximab inhibits the proliferation of cancer cells.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H. Lu, X. Li, Z. Fan Development of methodology: H. Lu, X. Li, Z. Luo Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Lu, X. Li, Z. Luo Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Lu, X. Li, Z. Luo, J. Liu, Z. Fan Writing, review, and/or revision of the manuscript: H. Lu, Z. Fan

Access to study materials or patients: Z. Fan Study supervision: Z. Fan

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