Published in final edited form as:

Mol Cancer Ther. 2013 October ; 12(10): . doi:10.1158/1535-7163.MCT-12-1245.

Cetuximab reverses the Warburg effect by inhibiting HIF-1–regulated LDH-A

Haiquan Lu^{1,2}, Xinqun Li¹, Zhongguang Luo^{1,3}, Jie Liu³, and Zhen Fan^{1,2,*}

¹Department of Experimental Therapeutics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

²Graduate School of Biomedical Sciences, The University of Texas Health Sciences Center at Houston, Houston, TX, USA

³Department of Digestive Diseases, Huashan Hospital, Fudan University, Shanghai, China

Abstract

Hypoxia-inducible factor-1 (HIF-1) plays a critical role in reprogramming cancer metabolism towards 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 epidermal growth factor receptor-blocking monoclonal antibody, downregulates the alpha subunit of HIF-1 (HIF-1 α) through the inhibition of epidermal growth factor receptor downstream cell signaling and that downregulation of HIF-1 α 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 a HIF-1 α downregulation–dependent manner. HNSCC cells with acquired cetuximab resistance expressed a high level of HIF-1 α and were highly glycolytic. Overexpression of a HIF-1 α mutant (HIF-1 α / ODD) 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.

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

*Corresponding author: Zhen Fan, Department of Experimental Therapeutics, Unit 1950, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA. Tel.: +1 713 745 3560; Fax: +1 713 745 3562; zfan@mdanderson.org.

Conflicts of Interest: The authors declare no conflicts of interest.

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-1 α subunit and a constitutively expressed HIF-1 β subunit (9–12). A high level of HIF-1 α 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-1 α in cancer cells is caused not only by the decreased ubiquitination and degradation of HIF-1 α protein via a posttranslational mechanism associated with tumor hypoxia (16, 17), but also by aberrant cell signaling, which increases HIF-1 α protein expression via a translational mechanism (18–22).

Cetuximab is an epidermal growth factor (EGF) receptor (EGFR)-blocking monoclonal antibody approved for treating patients with head and neck cancers and colorectal cancers in combination with radiotherapy and/or chemotherapy (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-1 α protein by inhibiting the PI3K/Akt and MEK/Erk pathways, and this downregulation of HIF-1 α is required, although may not be sufficient, for cetuximab to induce antiproliferative effects (41–45). Knockdown of HIF-1 α by small interfering RNA (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-1 α 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-1 α by cetuximab. We hypothesized that cetuximab inhibits cancer cell proliferation through inhibition of glycolysis by downregulating HIF-1 α , thereby reversing the Warburg effect that is critically important for cancer cell survival and proliferation. To test this hypothesis, we generated and characterized two pairs of genetically matched cetuximab-sensitive and cetuximab-resistant head and neck cancer cell lines. We used the Seahorse XF96 extracellular flux analyzer to assess the effect of cetuximab treatment on glycolysis and mitochondrial respiration, the two 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 adenosine triphosphate (ATP) in cetuximab-sensitive and cetuximab-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 (New York, NY). All other reagents, including oxamate, were purchased from Sigma-Aldrich (St. Louis, MO) 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% fetal bovine serum (FBS), 2 mM 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 nM) for 6 months or more. HN5-HIF-1 / ODD and FaDu-HIF-1 / ODD cells were established by transfecting parental HN5 and FaDu cells with pcDNA3.1 construct containing a HIF-1 oxygen-dependent degradation (ODD) domain deletion mutant (referred to as HIF-1 / ODD) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). The HN5-HIF-1 / ODD and FaDu-HIF-1 / ODD 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 basal 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., Walkersville, MD) (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 methylthiazolyldiphenyl-tetrazolium bromide (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% sodium dodecyl sulfate (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, GGAGAAAGCCGTCTTAATT; #2, GATTAAGGGTCTTTACGGA; #3, CAGATTTAGGGACTGATAA), HIF-1 -targeted siRNA (target DNA sequence #1, CAAAGTTCACCTGAGCCTA; #2, GATTAAGGGTCTTTACGGA; #3, CAGATTTAGGGACTGATAA), 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 prior to the detection of knockdown of LDH-A and HIF-1 expression by Western blotting.

Western blot analysis

After the indicated treatments, cells were lysed in a lysis buffer containing 50 mM TrisHCl (pH 7.4), 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, 25 $\mu\text{g}/\text{ml}$ aprotinin, and 25 $\mu\text{g}/\text{ml}$ 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 Coomassie Plus colorimetric protein assay (Thermo Fisher Scientific), were separated by SDS–polyacrylamide gel electrophoresis, blotted onto nitrocellulose, and probed with primary antibodies against HIF-1 (BD Biosciences); Akt, S473-phosphorylated Akt, T202/Y204-phosphorylated Erk, Y1068-phosphorylated EGFR, and LDH-A (Cell Signaling Technology); MAPK (Santa Cruz Biotechnology); and EGFR and β -actin (Sigma-Aldrich). The signals were visualized using the enhanced chemiluminescence detection kit (GE Healthcare). Western blotting results were quantified using the ImageJ v1.46 image processing program (National Institutes of Health, Bethesda, MD).

Metabolic flux assay

The bioenergetic flux of cells in response to cetuximab or HIF-1 silencing was assessed using the Seahorse XF96 extracellular flux analyzer (Seahorse Biosciences). For cetuximab treatment, the indicated cells were plated at 2×10^4 cells/well in XF96 plates and incubated with DMEM/F12 regular cell culture medium in a 5% CO_2 atmosphere at 37°C for 24 hours. The medium was then changed to low-glucose, low-serum medium, and the cells were incubated for another 24 hours and then treated with 20 nM cetuximab or left untreated for 10 hours. The medium was then replaced with unbuffered DMEM XF assay medium (pH adjusted to 7.4 using 1 N sodium hydroxide) supplemented with 2 mM glutamine and 1 g/L glucose with or without 20 nM cetuximab. For HIF-1 silencing, the indicated cells were treated with HIF-1 siRNA or control siRNA for 48 hours. The cells were then plated at 3×10^4 cells/well in XF96 plates and incubated with DMEM/F12 regular cell culture medium in a 5% CO_2 atmosphere at 37°C for another 24 hours. The medium was then replaced with unbuffered DMEM XF assay medium as described above. After media changes, the cells were placed in a 37°C CO_2 -free incubator for 1 hour. The basal oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were then determined using the XF96 plate reader with the standard program as recommended by the manufacturer.

Glucose consumption assay

Cells were seeded in 6-well plates at 5×10^5 cells/well in 3 mL of phenol red–free, lowglucose, low-serum cell 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 calculated by subtracting the concentration of glucose remaining in the medium at the indicated time from the concentration of glucose present in fresh cell culture medium. Glucose consumption was expressed as $\text{mg}/10^6$ cells at the indicated times.

Lactate production assay

Cells were seeded in 6-well plates at 5×10^5 cells/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. (Milpitas, CA). 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/10⁶ 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 (T₀) and 10 minutes after the reaction (T₁₀). LDH-A activity was expressed as an increase in OD values (T₁₀-T₀). 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 5×10⁵ cells/well and treated with 20 nM cetuximab or left untreated in low-glucose, low-serum medium for 4 hours. The cells were then harvested and resuspended in 1 mL of phosphate-buffered saline (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 cytometry 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 enzyme-linked immunosorbent assay (ELISA) kit (Cell Death Detection ELISA, Roche Diagnostics Corp., Indianapolis, IN) that quantitatively measures cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes). The procedure was performed exactly according to the manufacturer's instructions. Apoptosis was also detected by Western blotting with an antibody that recognizes both cleaved and uncleaved poly(adenosine diphosphate ribose) polymerase (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 ± standard deviations. 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 two 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 nM) 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 (Figure 1A). HN5 cells had higher expression levels of EGFR, phosphorylated EGFR (represented by phosphorylation of EGFR at Y1068), and HIF-1 than FaDu cells in normoxic culture. Both HN5 and FaDu cells had lower levels of HIF-1 after cetuximab treatment (Figure 1B). In contrast, HN5-R and FaDu-R cells had higher levels of HIF-1 than their parental cells in normoxic culture, and the levels of HIF-1 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), two important signaling molecules downstream of EGFR (Figure 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 two 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, suggesting that these cells had higher glycolysis rates than the parental cells (Figure 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, indicating 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-1 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-1 in promoting aerobic glycolysis via regulating LDH-A in HN5-R and FaDu-R cells, we knocked down HIF-1 with two different HIF-1 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-1 knockdown as did the level of LDH-A in parental cells after HIF-1 knockdown (Figure 1D). Knockdown of HIF-1 in HN5-R and FaDu-R cells with the same two different HIF-1 siRNA significantly decreased ECAR and increased OCR in these cetuximab-

resistant cells, indicating that knockdown of HIF-1 can reverse the Warburg effect in cetuximab-resistant cells, just as cetuximab reversed the Warburg effect in cetuximab-sensitive cells (Figure 1E).

Taken together, these novel findings indicate that LDH-A was downregulated and glycolysis was inhibited via downregulation of HIF-1 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-1.

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

To confirm the effect of cetuximab on inhibiting aerobic glycolysis, we directly measured glucose consumption and lactate production after cetuximab treatment in HN5 and FaDu cells that did or did not overexpress an experimentally created HIF-1 mutant (HIF-1 / ODD). This mutant retains the majority of the transcriptional activity of full-length HIF-1 and can be stably overexpressed in normoxic culture because of experimental deletion of the ODD domain in HIF-1 that renders HIF-1 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 (Figure 2A and 2B). Consistent with the knowledge that HIF-1 upregulates glycolysis, overexpression of HIF-1 / ODD in HN5 and FaDu cells (HN5-HIF-1 / ODD and FaDu-HIF-1 / ODD 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 two 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-1 / ODD and FaDu-HIF-1 / ODD cells treated with cetuximab cells were only modestly lower than that of untreated cells (down 15% and 11%, respectively) (Figure 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-1 α

To further elucidate the biochemical mechanisms underlying cetuximab-mediated inhibition of glycolysis via HIF-1 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-1 / ODD cells and in FaDu-neo cells with those in FaDu-HIF-1 / ODD 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-1 / ODD (Figure 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 (Figure 1B), the phosphorylation levels of these molecules in HN5-HIF-1 / ODD and FaDu-HIF-1 / ODD cells were also sensitive to cetuximab. The levels of HIF-1 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-1 level, such as mTOR activation. However, both HN5-neo and FaDu-neo cells treated with cetuximab had lower levels of HIF-1 than untreated cells at each time of measurement. The kinetics of cetuximab-mediated inhibition of EGFR downstream pathways were 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-1 / ODD, even the endogenous HIF-1 became resistant to cetuximab treatment, an interesting phenomenon that we reported previously (42). These findings provide further support that HIF-1 has a fundamental role in regulating cell response to cetuximab-induced growth inhibition.

Consistent with the decrease in LDH-A protein levels in HN5-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 (Figure 3B). In contrast, HN5-HIF-1 / ODD, and FaDu-HIF-1 / ODD cells had much higher LDH-A enzymatic activity than HN5-neo and FaDu-neo cells, and the elevated LDH-A activity was not inhibited by cetuximab (Figure 3B). Together, these findings indicate that cetuximab downregulates HIF-1, 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-1 and subsequently LDH-A to inhibit glycolysis, we performed cell cycle analysis after subjecting cells to treatment with cetuximab and/or knockdown of LDH-A by siRNA. We used three different LDH-A-specific siRNA, all of which knocked down the level of LDH-A in all parental and sublines tested (Figure 4A, 4C, and 4E). LDH-A siRNA #1 and #2 were used for knockdown of LDH-A in the cells subjected to cell cycle analysis.

As shown in Figure 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 Figure 1).

Both the cetuximab-resistant sublines (HN5-R and FaDu-R) and the HIF-1 / ODD-overexpressing cells (HN5-HIF-1 / ODD and FaDu-HIF-1 / ODD cells) were resistant to cetuximab-induced G1 arrest. As shown in Figure 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 cetuximab-treated HN5-R cells and FaDu-R cells in G1 phase were 54.0% and 60.7%, respectively. As shown in Figure 4F, compared with the percentages of untreated HN5-HIF-1 / ODD cells and FaDu-HIF-1 / ODD cells in G1 phase (51.7% and 64.4%, respectively), the percentages of cetuximab-treated HN5-HIF-1 / ODD cells and FaDu-HIF-1 / ODD cells in G1 phase were 52.5% and 67.9%, respectively. These findings are in agreement with the role of HIF-1 / ODD in counteracting cetuximab-mediated inhibition of glycolysis and downregulation of LDH-A (Figures 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 Figure 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 Figure 4F, compared with the percentages of untreated HN5-HIF-1 / ODD cells and FaDu-HIF-1 / ODD cells in G1 phase (51.7% and 64.4%, respectively), the percentages of LDH-A knockdown HN5-HIF-1 / ODD cells and FaDu-HIF-1 / ODD 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 (Figure 4D and 4F), 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-1 / ODD and FaDu-HIF-1 / ODD cells. Similar results were obtained using LDH-A siRNA #2 (supplementary Figure 1).

Together, these findings confirm the role of HIF-1 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 two 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-1 / ODD cells) and FaDu cells (parental FaDu, FaDu-R, and FaDu-HIF-1 / ODD 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 (Figure 5A and 5B).

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 (Figure 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-1 / ODD 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-1 / ODD 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 two HNSCC cell lines, SqCC/Y1 and TU167, which are much less sensitive to cetuximab-induced growth inhibition than HN5 and FaDu cells (supplementary Figure 2). 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 (Figure 6A). Compared with cells treated under hypoxic conditions, cells treated under normoxic conditions expressed a very low level of HIF-1. Twenty-four-hour cetuximab treatment inhibited EGFR autophosphorylation and Erk phosphorylation but not Akt phosphorylation (Figure 6B). Whereas cetuximab inhibited LDH-A expression and aerobic glycolysis in HN5 and FaDu cells in normoxic culture (Figure 1), it did not inhibit LDH-A expression in NOM9-TK cells (Figure 6B) and did not significantly inhibit glucose consumption (Figure 6C), lactate

production (Figure 6D), LDH-A activity (Figure 6E), or ATP production (Figure 6F) in the cells in normoxic culture. The absolute amount of lactate production in the NOM9-TK cells (Figure 6D) was markedly less than that in the HN5 and FaDu cells during the same period (Figure 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-1 α and are more glycolytic than their parental cells. Overexpression of a degradation-resistant HIF-1 α (HIF-1 α / ODD) 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-1 α 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, i.e., 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-1 α -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 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-1 α / ODD, and FaDu-HIF-1 α / ODD cells) remained at least partially sensitive to cetuximab-induced inhibition of major downstream cell signaling pathways of EGFR—i.e., 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-1 α or HIF-1 α / ODD, 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 towards lactate production. It was when the cells were treated

with cetuximab, an elevated HIF-1 or HIF-1 / ODD 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-1 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-1 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 demonstrated 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Jay Dunn of Seahorse Bioscience for technical assistance with the Seahorse XF96 extracellular flux analyzer.

Grant Support

This work was supported in part by US National Institutes of Health (NIH) R01 award (CA129036) and R21 award (DE021883) to Z. Fan. The work was also supported in part by the NIH through MD Anderson's Cancer Center Support Grant, CA016672.

References

1. Warburg O. On the origin of cancer cells. *Science*. 1956; 123:309–14. [PubMed: 13298683]
2. Hsu PP, Sabatini DM. Cancer cell metabolism: Warburg and beyond. *Cell*. 2008; 134:703–7. [PubMed: 18775299]
3. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 2009; 324:1029–33. [PubMed: 19460998]
4. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. *Cell Metab*. 2008; 7:11–20. [PubMed: 18177721]
5. Grander D. How do mutated oncogenes and tumor suppressor genes cause cancer? *Med Oncol*. 1998; 15:20–6. [PubMed: 9643526]
6. Dang CV, Semenza GL. Oncogenic alterations of metabolism. *Trends Biochem Sci*. 1999; 24:68–72. [PubMed: 10098401]

7. DeBerardinis RJ, Sayed N, Ditsworth D, Thompson CB. Brick by brick: metabolism and tumor cell growth. *Curr Opin Genet Dev.* 2008; 18:54–61. [PubMed: 18387799]
8. Semenza GL. HIF-1: upstream and downstream of cancer metabolism. *Curr Opin Genet Dev.* 2010; 20:51–6. [PubMed: 19942427]
9. Maxwell PH, Pugh CW, Ratcliffe PJ. Inducible operation of the erythropoietin 3' enhancer in multiple cell lines: evidence for a widespread oxygen-sensing mechanism. *Proc Natl Acad Sci U S A.* 1993; 90:2423–7. [PubMed: 8460154]
10. Firth JD, Ebert BL, Pugh CW, Ratcliffe PJ. Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3' enhancer. *Proc Natl Acad Sci U S A.* 1994; 91:6496–500. [PubMed: 8022811]
11. Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem.* 1994; 269:23757–63. [PubMed: 8089148]
12. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci U S A.* 1995; 92:5510–4. [PubMed: 7539918]
13. Semenza GL. Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. *Crit Rev Biochem Mol Biol.* 2000; 35:71–103. [PubMed: 10821478]
14. Semenza GL. Involvement of hypoxia-inducible factor 1 in human cancer. *Intern Med.* 2002; 41:79–83. [PubMed: 11868612]
15. Semenza GL. Defining the role of hypoxia-inducible factor 1 in cancer biology and therapeutics. *Oncogene.* 2010; 29:625–34. [PubMed: 19946328]
16. Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, et al. HIF α targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science.* 2001; 292:464–8. [PubMed: 11292862]
17. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, et al. Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science.* 2001; 292:468–72. [PubMed: 11292861]
18. Jiang BH, Agani F, Passaniti A, Semenza GL. V-SRC induces expression of hypoxia-inducible factor 1 (HIF-1) and transcription of genes encoding vascular endothelial growth factor and enolase 1: involvement of HIF-1 in tumor progression. *Cancer Res.* 1997; 57:5328–35. [PubMed: 9393757]
19. Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM, et al. Modulation of hypoxia-inducible factor 1 α expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res.* 2000; 60:1541–5. [PubMed: 10749120]
20. Treins C, Giorgetti-Peraldi S, Murdaca J, Semenza GL, Van Obberghen E. Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. *J Biol Chem.* 2002; 277:27975–81. [PubMed: 12032158]
21. Fukuda R, Hirota K, Fan F, Jung YD, Ellis LM, Semenza GL. Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. *J Biol Chem.* 2002; 277:38205–11. [PubMed: 12149254]
22. Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1 α (HIF-1 α) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. *Mol Cell Biol.* 2001; 21:3995–4004. [PubMed: 11359907]
23. Cunningham D, Humblet Y, Siena S, Khayat D, Bleiberg H, Santoro A, et al. Cetuximab monotherapy and cetuximab plus irinotecan in irinotecan-refractory metastatic colorectal cancer. *N Engl J Med.* 2004; 351:337–45. [PubMed: 15269313]
24. Bonner JA, Harari PM, Giralt J, Azarnia N, Shin DM, Cohen RB, et al. Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. *N Engl J Med.* 2006; 354:567–78. [PubMed: 16467544]

25. Fan Z, Masui H, Altas I, Mendelsohn J. Blockade of epidermal growth factor receptor function by bivalent and monovalent fragments of 225 anti-epidermal growth factor receptor monoclonal antibodies. *Cancer Res.* 1993; 53:4322–8. [PubMed: 8364927]
26. Fan Z, Baselga J, Masui H, Mendelsohn J. Antitumor effect of anti-epidermal growth factor receptor monoclonal antibodies plus cis-diamminedichloroplatinum on well established A431 cell xenografts. *Cancer Res.* 1993; 53:4637–42. [PubMed: 8402640]
27. Fan Z, Mendelsohn J, Masui H, Kumar R. Regulation of epidermal growth factor receptor in NIH3T3/HER14 cells by antireceptor monoclonal antibodies. *J Biol Chem.* 1993; 268:21073–9. [PubMed: 8407944]
28. Fan Z, Lu Y, Wu X, Mendelsohn J. Antibody-induced epidermal growth factor receptor dimerization mediates inhibition of autocrine proliferation of A431 squamous carcinoma cells. *J Biol Chem.* 1994; 269:27595–602. [PubMed: 7961676]
29. Wu X, Fan Z, Masui H, Rosen N, Mendelsohn J. Apoptosis induced by an anti-epidermal growth factor receptor monoclonal antibody in a human colorectal carcinoma cell line and its delay by insulin. *J Clin Invest.* 1995; 95:1897–905. [PubMed: 7706497]
30. Peng D, Fan Z, Lu Y, DeBlasio T, Scher H, Mendelsohn J. Anti-epidermal growth factor receptor monoclonal antibody 225 up-regulates p27^{KIP1} and induces G1 arrest in prostatic cancer cell line DU145. *Cancer Res.* 1996; 56:3666–9. [PubMed: 8706005]
31. Wu X, Rubin M, Fan Z, DeBlasio T, Soos T, Koff A, et al. Involvement of p27KIP1 in G1 arrest mediated by an anti-epidermal growth factor receptor monoclonal antibody. *Oncogene.* 1996; 12:1397–403. [PubMed: 8622855]
32. Fan Z, Shang BY, Lu Y, Chou JL, Mendelsohn J. Reciprocal changes in p27(Kip1) and p21(Cip1) in growth inhibition mediated by blockade or overstimulation of epidermal growth factor receptors. *Clin Cancer Res.* 1997; 3:1943–8. [PubMed: 9815583]
33. Ciardiello F, Bianco R, Damiano V, De Lorenzo S, Pepe S, De Placido S, et al. Antitumor activity of sequential treatment with topotecan and anti-epidermal growth factor receptor monoclonal antibody C225. *Clin Cancer Res.* 1999; 5:909–16. [PubMed: 10213228]
34. Ye D, Mendelsohn J, Fan Z. Androgen and epidermal growth factor down-regulate cyclin-dependent kinase inhibitor p27^{Kip1} and costimulate proliferation of MDA PCa 2a and MDA PCa 2b prostate cancer cells. *Clin Cancer Res.* 1999; 5:2171–7. [PubMed: 10473102]
35. Liu B, Fan Z. The monoclonal antibody 225 activates caspase-8 and induces apoptosis through a tumor necrosis factor receptor family-independent pathway. *Oncogene.* 2001; 20:3726–34. [PubMed: 11439335]
36. Liu B, Fang M, Lu Y, Mendelsohn J, Fan Z. Fibroblast growth factor and insulin-like growth factor differentially modulate the apoptosis and G1 arrest induced by anti-epidermal growth factor receptor monoclonal antibody. *Oncogene.* 2001; 20:1913–22. [PubMed: 11313939]
37. Nasu S, Ang KK, Fan Z, Milas L. C225 antiepidermal growth factor receptor antibody enhances tumor radiocurability. *Int J Radiat Oncol Biol Phys.* 2001; 51:474–7. [PubMed: 11567823]
38. Karashima T, Sweeney P, Slaton JW, Kim SJ, Kedar D, Izawa JI, et al. Inhibition of angiogenesis by the antiepidermal growth factor receptor antibody ImClone C225 in androgen-independent prostate cancer growing orthotopically in nude mice. *Clin Cancer Res.* 2002; 8:1253–64. [PubMed: 12006546]
39. Milas, L.; Fan, Z.; Mason, K.; Ang, KK. Role of epidermal growth factor receptor and its inhibition in radiotherapy. In: Nieder, C.; Milas, L.; Ang, KK., editors. *Modification of Radiation Response: Cytokines, growth factors, and other biological targets.* Springer; Berlin: 2003. p. 189-204.
40. Lu Y, Li X, Liang K, Luwor R, Siddik ZH, Mills GB, et al. Epidermal growth factor receptor (EGFR) ubiquitination as a mechanism of acquired resistance escaping treatment by the anti-EGFR monoclonal antibody cetuximab. *Cancer Res.* 2007; 67:8240–7. [PubMed: 17804738]
41. Luwor RB, Lu Y, Li X, Mendelsohn J, Fan Z. The antiepidermal growth factor receptor monoclonal antibody cetuximab/C225 reduces hypoxia-inducible factor-1 alpha, leading to transcriptional inhibition of vascular endothelial growth factor expression. *Oncogene.* 2005; 24:4433–41. [PubMed: 15806152]

42. Lu Y, Liang K, Li X, Fan Z. Responses of cancer cells with wild-type or tyrosine kinase domain-mutated epidermal growth factor receptor (EGFR) to EGFR-targeted therapy are linked to downregulation of hypoxia-inducible factor-1alpha. *Mol Cancer*. 2007; 6:63. [PubMed: 17931419]
43. Li X, Lu Y, Liang K, Pan T, Mendelsohn J, Fan Z. Requirement of hypoxia-inducible factor-1alpha down-regulation in mediating the antitumor activity of the anti-epidermal growth factor receptor monoclonal antibody cetuximab. *Mol Cancer Ther*. 2008; 7:1207–17. [PubMed: 18483308]
44. Lu Y, Li X, Lu H, Fan Z. 1, 9-Pyrazoloanthrones downregulate HIF-1alpha and sensitize cancer cells to cetuximab-mediated anti-EGFR therapy. *PLoS One*. 2010; 5:e15823. [PubMed: 21209911]
45. Lu H, Liang K, Lu Y, Fan Z. The anti-EGFR antibody cetuximab sensitizes human head and neck squamous cell carcinoma cells to radiation in part through inhibiting radiation-induced upregulation of HIF-1alpha. *Cancer Lett*. 2012; 322:78–85. [PubMed: 22348829]
46. Zhao M, Sano D, Pickering CR, Jasser SA, Henderson YC, Clayman GL, et al. Assembly and initial characterization of a panel of 85 genomically validated cell lines from diverse head and neck tumor sites. *Clin Cancer Res*. 2011; 17:7248–64. [PubMed: 21868764]
47. Chakravarti N, Kadara H, Yoon DJ, Shay JW, Myers JN, Lotan D, et al. Differential inhibition of protein translation machinery by curcumin in normal, immortalized, and malignant oral epithelial cells. *Cancer Prev Res (Phila)*. 2010; 3:331–8. [PubMed: 20145189]
48. Vander Heiden MG, Locasale JW, Swanson KD, Sharfi H, Heffron GJ, Amador-Noguez D, et al. Evidence for an alternative glycolytic pathway in rapidly proliferating cells. *Science*. 2010; 329:1492–9. [PubMed: 20847263]
49. Zhao Y, Liu H, Liu Z, Ding Y, Ledoux SP, Wilson GL, et al. Overcoming trastuzumab resistance in breast cancer by targeting dysregulated glucose metabolism. *Cancer Res*. 2011; 71:4585–97. [PubMed: 21498634]

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 nM 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 nM cetuximab or left untreated for 10 hours. Metabolic flux analysis was performed 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 μ M. Error bars indicate standard deviations. D. The indicated cell lines were subjected to knockdown of HIF-1 with each of two different HIF-1 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-1 as described in (D). Metabolic flux analysis was then performed to measure ECAR and OCR.

Figure 2. Cetuximab inhibits glucose consumption, lactate production, and intracellular ATP levels in a HIF-1 inhibition-dependent manner

A. HN5-neo, HN5-HIF-1 / ODD, FaDu-neo, and FaDu-HIF-1 / ODD 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 nM 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 nM 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 $\pm$ standard deviations.

Figure 3. Cetuximab downregulates LDH-A expression and reduces LDH-A activity in a HIF-1 inhibition-dependent manner

HN5 and FaDu cells expressing control vector (HN5-neo and FaDu-neo) or HIF-1 / ODD (HN5-HIF-1 / ODD and FaDu-HIF-1 / ODD) were treated with 20 nM cetuximab or left untreated in low-glucose (1 g/L), low-serum (0.5% FBS) medium for the indicated times. A. 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 band was performed 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 indicated cells were harvested and homogenized, and samples containing equal amounts of proteins were subjected to an LDH-A activity assay as described in Materials and Methods. The data are presented as means $\pm$ standard deviations.

Figure 4. Overexpression of HIF-1 / ODD confers resistance to cetuximab-induced cell cycle arrest, which can be overcome by knockdown of LDH-A

A, C, and E. The indicated cells were subjected to knockdown of LDH-A with each of three different LDH-A-specific siRNA or control siRNA in low-glucose (1 g/L), low-serum (0.5% FBS) medium for 48 hours. Cell lysates were prepared, and equal amounts of cell lysates were subjected to Western blot analysis with the indicated antibodies. B, D, and F. The indicated cells were subjected to knockdown of LDH-A with LDH-A siRNA #1 or control siRNA as described in (A, C, and E). The cells were then treated with 20 nM cetuximab or left untreated for 24 hours and then fixed and stained with propidium iodide for cell cycle distribution analysis by flow cytometry. The data are presented as means $\pm$ standard deviations.

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 nM 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 performed 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 nM cetuximab and/or 5 mM 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 optical density value in the corresponding untreated groups. The data are presented as means $\pm$ standard deviations.

Figure 6. Cetuximab does not affect glycolysis in nontransformed cells

A. NOM9-TK cells were treated with 20 nM cetuximab or left untreated for 5 days in chemically defined keratinocyte growth medium supplemented with components from the SingleQuots kit except EGF. Cell growth response to cetuximab was measured using an MTT assay. B. NOM9-TK cells were treated with 20 nM cetuximab or left untreated for 24 hours under normoxia. Cells treated the same way under hypoxia served as controls. Cell lysates were subjected to Western blot analysis with the indicated antibodies. C and D. NOM9-TK cells were seeded in 6-well plates with 3 mL of low-glucose (1 g/L) cell culture medium and treated with 20 nM cetuximab or left untreated for the indicated times. At the indicated times after treatment, the level of glucose remaining in the conditioned medium (C) and the level of lactate produced in the conditioned medium (D) were determined with assays described in Materials and Methods. E. NOM9-TK cells were treated with 20 nM cetuximab or left untreated for the indicated times. Cells were harvested and homogenized, and samples containing equal amounts of proteins were subjected to an LDH-A activity assay as described in Materials and Methods. F. NOM9-TK cells were treated with 20 nM cetuximab or left untreated in serum-free keratinocyte growth medium for 4 hours. Cell pellets were harvested, and the intracellular level of ATP was measured using a luciferase-based ATP determination assay. The data are presented as means $\pm$ standard deviations.