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Original Article

Acetyl-CoA carboxylase rewires cancer metabolism to allow cancer cells to survive inhibition of the Warburg effect by cetuximab

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ABSTRACT

Cetuximab inhibits HIF-1-regulated glycolysis in cancer cells, thereby reversing the Warburg effect and leading to inhibition of cancer cell metabolism. AMP-activated protein kinase (AMPK) is activated after cetuximab treatment, and a sustained AMPK activity is a mechanism contributing to cetuximab resistance. Here, we investigated how acetyl-CoA carboxylase (ACC), a downstream target of AMPK, rewires cancer metabolism in response to cetuximab treatment. We found that introduction of experimental ACC mutants lacking the AMPK phosphorylation sites (ACC1_S79A and ACC2_S212A) into head and neck squamous cell carcinoma (HNSCC) cells protected HNSCC cells from cetuximab-induced growth inhibition. HNSCC cells with acquired cetuximab resistance contained not only high levels of T172-phosphorylated AMPK and S79-phosphorylated ACC1 but also an increased level of total ACC. These findings were corroborated in tumor specimens of HNSCC patients treated with cetuximab. Cetuximab plus TOFA (an allosteric inhibitor of ACC) achieved remarkable growth inhibition of cetuximab-resistant HNSCC xenografts. Our data suggest a novel paradigm in which cetuximab-mediated activation of AMPK and subsequent phosphorylation and inhibition of ACC is followed by a compensatory increase in total ACC, which rewires cancer metabolism from glycolysis-dependent to lipogenesis-dependent.

Introduction

The Warburg effect, also known as “aerobic glycolysis”, refers to a phenomenon first observed by Otto Warburg over 80 years ago in which cancer cells use glycolysis to generate lactate as the primary means for glucose metabolism, even when the cellular level of oxygen is sufficient for oxidation of pyruvate [1]. It is believed that cancer cells, by consuming large amounts of glucose via glycolysis, gain sufficient biomass-building materials for cell growth and proliferation. Targeting the Warburg effect, therefore, has been considered an attractive approach for cancer treatment [2–5]. We previously reported that cetuximab, a Food and Drug Administration-approved anti-epidermal growth factor receptor (EGFR) antibody, exerts its antitumor activity at least in part via inhibiting the Warburg effect through downregulating hypoxia-inducible factor-1 alpha (HIF-1α) [6–8], the regulatory subunit of HIF-1, which is a key transcription factor that regulates almost every biochemical step of glycolysis, as well as glucose uptake and lactate production and excretion [9,10].

More recently, we reported that inhibition of HIF-1 transcriptional activity by cetuximab does not always lead to successful inhibition of cell proliferation [11]. In human head and neck squamous cell carcinoma (HNSCC) cells, we observed that the response to cetuximab-mediated growth inhibition was linked to the activity status of the cell energy sensor AMP-activated protein kinase (AMPK). HNSCC cells with a low basal level of AMPK activity were more sensitive to cetuximab-induced growth inhibition and exhibited a transient activation of AMPK after cetuximab treatment. In contrast, HNSCC cells with a high basal level of AMPK activity were less sensitive to cetuximab-induced growth inhibition despite effective inhibition of EGFR downstream signaling by cetuximab [11].

An emerging paradigm is that cancer cells may rewire metabolic pathways from a glycolysis-dependent pattern to a lipogenesis-dependent pattern with fatty acid oxidation in response to

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treatments targeting the Warburg effect [12]. AMPK, through phosphorylation of acetyl-CoA carboxylase (ACC), plays an important role in maintaining cell energy homeostasis when cells are under stress [13–15]. AMPK-mediated phosphorylation of ACC1 at Ser79 [16] and ACC2 at Ser221 (Ser212 in mice) [17] is a well-described mechanism that leads to inhibition of fatty acid synthesis and stimulation of fatty acid β-oxidation, through which cells survive under energy stress. However, in vivo data supporting this paradigm, particularly data from patients, have been limited. Few studies have used clinical data to investigate the impact of the AMPK and ACC axis on cancer cell response to therapies targeting the Warburg effect.

In this study, by using ACC1 and ACC2 experimental mutants lacking the corresponding AMPK phosphorylation sites (ACC1_S79A and ACC2_S212A) [18], we further dissected the role of ACC in HNSCC cell response to cetuximab treatment. We first examined the role of the ACC mutants in an experimental Warburg effect model in which overexpression of HIF-1α in HEK293 cells renders the cells highly dependent on glucose supply in culture medium. We found that both ACC1 activity and ACC2 activity are indispensable for HEK293 cell survival in low glucose culture, which mimics the outcome of therapies targeting the Warburg effect. We next demonstrated that ACC rewires cancer metabolism to allow HNSCC cells to survive inhibition of the Warburg effect by cetuximab. We showed that co-targeting ACC with TOFA, an allosteric inhibitor of ACC, substantially improved the response of cetuximab-resistant HNSCC xenografts to cetuximab treatment. We further corroborated our observations in tumor specimens from patients with HNSCC treated with or without cetuximab.

Materials and methods

Patients

Tumor specimens were obtained from patients treated at the Department of Head and Neck Surgical Oncology, Tianjin Medical University Cancer Institute & Hospital, Tianjin, China, during 2007–2013. Tumor specimens from six patients who underwent post-cetuximab surgery and had complete medical records available were used for immunohistochemical evaluation of T172-phosphorylated AMPK, S79-phosphorylated ACC1, and total ACC. Surgical specimens from another 12 patients with complete medical records who were treated with the same chemotherapy regimen without cetuximab during the same period were used as controls. Informed consent was obtained for research use of these specimens.

Cell culture

293 Human kidney embryonic cells (HEK293) and human HNSCC cells (HN5, FaDu, Tu159, OSC19, MDA1986, UMSCC1, and Tu167) were maintained in high glucose (4.5 g/L) Dulbecco's modified Eagle's medium/F12 medium (50/50, v/v) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 atmosphere at 37 °C as previously described [19,20].

Plasmids

The complementary DNAs encoding HIF-1α_P402A/P564A and HIF-1α_ΔODD, originally provided by Dr. L. Eric Huang (University of Utah School of Medicine), were subcloned into a modified pLEX-MCS lentiviral vector (Thermo Fisher Scientific) via the *Bam*HI and *Not*I sites. ACC1_S79A and ACC2_S212A pLent6-D-TOPO vectors were provided by Dr. Nissim Hay (University of Illinois at Chicago).

Western blotting

The procedure for Western blotting was previously described [8,11]. The primary antibodies used for Western blotting and their sources were as follows: HIF-1α and SREBP-1c, BD Biosciences; T172-phosphorylated AMPK, AMPK, S79-phosphorylated ACC1, ACC1, ACC2, and FASN, Cell Signaling Technology; and β-actin, Sigma–Aldrich.

Live/dead cell viability assay and cell survival proportions

The LIVE/DEAD cell viability assay kit (Life Technologies) was used to distinguish live versus dead cells as we recently described [11,21]. For determination of the cell survival proportions after culture in low glucose medium (1 mM) supplemented with 0.5% FBS, cells were plated at a low density ($0.7–1 \times 10^5$ cells per well in a 12-well plate) in regular medium; the next day, the culture medium was switched to low glucose medium. At various time points after the switch to low glucose medium, the cells were washed once with PBS and incubated with 4 μM calcein acetoxymethyl ester. Five different areas were then randomly selected and imaged under a fluorescence microscope. The imaging data were analyzed using the ImageJ software program, and cell survival proportions were calculated by dividing the number of surviving cells at various time points after the switch to low glucose medium by the number of surviving cells before the switch [22–26].

Glucose consumption assay

Cells were seeded in six-well plates at 5×10^5 cells/well in 3 mL of phenol red-free 5 mM glucose, 0.5% FBS cell culture medium as described above. At various time points 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) as we previously described [8].

Apoptosis assays

Apoptosis was measured by detection of PARP cleavage using Western blotting with an antibody that recognizes both cleaved and uncleaved PARP (Cell Signaling Technology); by quantitative measurement of the levels of cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) using a Cell Death Detection ELISA kit (Roche Diagnostics Corp.); or by quantitative measurement of apoptotic cells using a flow cytometer after staining of cells with FITC-conjugated annexin V and propidium iodide (Life Technologies), according to the vendors' protocols [7,21,27].

Knockdown of ACC1 and ACC2 gene expression by siRNA

ACC1-targeted siRNA (target DNA sequence #1, CUAUGAGGGAGUCAAGUAU; #2, CUAUGAGGUGGAUCGGAGA), ACC2-targeted siRNA (#1, CCUACAAUGGGAACAGCUA; #2, GAACUUAACCGGAUGCGUA), and control siRNA were purchased from Sigma–Aldrich. The siRNA-mediated gene expression knockdown was performed as we previously described [8,11].

Quantitative real-time PCR

Total RNA was extracted from cells using a modified chloroform/phenol procedure (Trizol; Life Technologies). First-strand cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Invitrogen). The fluorescent real-time PCR reaction was performed in a thermal cycler (7500 Fast Real-Time PCR System, Applied Biosystems) for 40 cycles (denaturation at 95 °C for 15 s and annealing at 60 °C for 30 s) in iQ SYBR green supermix (Bio-Rad) with a final volume of 10 μL containing 0.5 μL of cDNA template with the specific primers targeting human ACC1 (ACACA) (forward: 5′-ATGTCTGGCTTGCACCTAGTA-3′; reverse: 5′-CCCCAAAGC-GAGTAACAAATTCT-3′) or ACC2 (ACACB) (forward: 5′-AGAAGACAAGAAGCAGG CAAAC-3′; reverse: 5′-GTAGACTCACGAGATGAGCCA-3′) or 0.5 μL of an internal control cDNA with specific primers targeting human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5′-CATGTTCGTCATGGGGTGAACCA-3′, reverse: 5′-AGTGATGGCATGGACTGTGGTCAT-3′). Fluorescent readings from real-time PCR reaction products were quantitatively analyzed by determining the difference in cycle number of crossing point (CP) between the target gene (ACC1 and ACC2) and GAPDH. Relative gene expression was calculated using the Pfaffl method [28], and changes of ACC1 and ACC2 mRNA expression in cetuximab-treated cells were obtained by comparison with the ACC1 and ACC2 mRNA expression in untreated cells.

Cell survival and proliferation assays

Cell survival and proliferation assays were performed using the MTT (methylthiazolyldiphenyl-tetrazolium bromide) method as we previously described [29,30]. 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 in each treatment group was then expressed as a percentage of the OD value in the untreated control cells, and the percentage values were plotted against treatments.

Animal studies and bioluminescence tumor imaging

Animal experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Swiss female nude mice (5–6 weeks old) were obtained from the colony facility maintained by the Department of Experimental Radiation Oncology at MD Anderson Cancer Center. HN5-R and UMSCC1 cells infected with a pLEX-based recombinant lentivirus containing a firefly luciferase cDNA were implanted subcutaneously on the right flanks of nude mice (6×10^6 cells/mouse in 100 μL of serum-free medium). Treatments were started on day 15, when xenograft volume reached approximately 120 mm^3. TOFA was first dissolved in DMF (10 mg/mL) and then diluted 1:50 with PBS. Tumor volume was calculated using the formula $\pi/6 \times ab^2$ (a: length; b: width, a > b). Bioluminescent imaging of xenografts was performed with the Xenogen in vitro imaging system in