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Acetyl-CoA Carboxylase- α Inhibitor TOFA Induces Human Cancer Cell Apoptosis

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Abstract

Acetyl-CoA carboxylase- α (ACCA) is a rate-limiting enzyme in long chain fatty acid synthesis, playing a critical role in cellular energy storage and lipid synthesis. ACCA is upregulated in multiple types of human cancers and small interfering RNA-mediated ACCA silencing in human breast and prostate cancer cells results in oxidative stress and apoptosis. This study reports for the first time that TOFA (5-tetradecyloxy-2-furoic acid), an allosteric inhibitor of ACCA, is cytotoxic to lung cancer cells NCI-H460 and colon carcinoma cells HCT-8 and HCT-15, with an IC₅₀ at approximately 5.0, 5.0, and 4.5 μ g/ml, respectively. TOFA at 1.0–20.0 μ g/ml effectively blocked fatty acid synthesis and induced cell death in a dose-dependent manner. The cell death was characterized with PARP cleavage, DNA fragmentation, and annexin-V staining, all of which are the features of the apoptosis. Supplementing simultaneously the cells with palmitic acids (100 μ M), the end-products of the fatty acid synthesis pathway, prevented the apoptosis induced by TOFA. Taken together, these data suggest that TOFA is a potent cytotoxic agent to lung and colon cancer cells, inducing apoptosis through disturbing their fatty acid synthesis.

Keywords

Acetyl-CoA carboxylase- α ; apoptosis; cancer; TOFA; and fatty acid synthesis

Introduction

Acetyl-CoA carboxylase (ACC) is a biotin-dependent, multi-domain enzyme with biotin carboxylase (BC) and carboxyltransferase (CT) activity in most eukaryotes. ACC catalyzes

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the irreversible carboxylation of acetyl-CoA via a two-step mechanism [1–3]. In sequence, BC catalyzes an ATP-dependent carboxylation of biotin with bicarbonate as a CO₂ donor, and CT promotes the carboxyl transfer from biotin to acetyl CoA to form malonyl-CoA. The malonyl-CoA is a dual functional compound that serves as a substrate of fatty acid synthase (FAS) for acyl chain elongation and as an inhibitor of carnitine palmitoyltransferase 1 (CPT-1), a key enzyme in long chain fatty acid β -oxidation. This feature of the malonyl-CoA ensures that the two processes, fatty acid synthesis and oxidation, do not occur simultaneously [3,4].

Two ACC isoforms, ACC- α (ACCA) and ACC- β (ACCB), have been identified in mammals, which are encoded by different genes [2,5]. In normal tissues, ACCA is enriched in lipogenic tissues, such as the liver and adipose, as well as the mammary gland during lactation, but ACCB is expressed abundantly in the heart, skeletal muscle and liver, the tissues with active fatty acid oxidation [2,6]. ACC activity is strictly controlled at the transcriptional and posttranslational levels, as well as by a metabolite-mediated allosteric mechanism [3,7, 8]. Reversible phosphorylation, characterized with deactivation by phosphorylation, is the major posttranslational regulatory mechanism; and insulin, glucagon, and many growth factors are involved in the regulation of the phosphorylation-dephosphorylation switch of the ACCA. In addition, breast cancer 1 (BRCA1), a tumor suppressor protein, blocks Ser⁷⁹ residue from dephosphorylation through direct association of its BRCT tandem domain at C-terminus with the ACCA; and aldo-keto reductase family 1 member B10 (AKR1B10) binds to the ACCA, blocking its degradation through the ubiquitination-proteasome pathway [9,10].

Interestingly, ACCA is upregulated in many types of human cancers, such as breast and liver carcinomas, and likely contributes to the growth and proliferation of cancer cells by stimulating lipogenesis [1,9,11,12]. In prostate and breast cancer cells, RNAi-mediated silencing of ACCA inhibits fatty acid synthesis, arrests cell cycle, and induces caspase-mediated apoptosis [13,14]. Therefore, the elevated expression and critical role of the ACCA in cell growth and survival lead to a notion that this protein may be a valuable target for anticancer therapy. TOFA (5-tetradecyloxy-2-furoic acid) is an allosteric inhibitor of the ACCA. Inside the cell, such as adipocytes and hepatocytes, TOFA is converted to TOFyl-CoA (5-tetradecyloxy-2-furoyl-CoA), exerting an allosteric inhibition on ACCA [15,16]. In this study, we evaluated the effects of TOFA on fatty acid synthesis and survival of human lung cancer cells NCI-H460 and colonic carcinoma cells HCT-8 and HCT-15, and found for the first time that TOFA induces the apoptosis of these cancer cells in a dose-dependent manner.

Materials and Methods

Cell culture

NCI-H460, human lung cancer cells, and HCT-8 and HCT-15, human colonic carcinoma cells, were purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 medium (Hyclone, UT) containing 10% fetal bovine serum and 2 mM glutamine at 37°C, 5% CO₂. For TOFA (Sigma, MO) exposures, cells were grown at 60% of confluence, and TOFA was applied at the indicated concentrations for 24 hours.

ACCA silencing

Scrambled and ACCA specific siRNAs [14] were chemically synthesized (Ambion, TX) and delivered into NCI-H460, HCT-8 and HCT-15 cells (3.5×10^4 in Opti-MEM I medium) as described previously [17].

Palmitic acid rescues

Palmitic acids were provided with a bovine serum albumin (BSA) complex [14]. Briefly, 4 volumes of 4% fatty acid-free BSA (Hyclone, UT) in 0.9% NaCl were mixed with 1 volume

of 5 mM palmitate (Sigma, MO) in ethanol and incubated at 37 °C for 1 hour to form 1 mM palmitate-BSA complex. Rescues were exerted by adding the palmitate-BSA complex (100 μ M) to the TOFA-treated cells at the indicated time.

MTT assay

TOFA cytotoxicity was assessed using a MTT [(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole] Cell Proliferation kit (Roche, IN) as described previously [18]. Briefly, cells (5,000/well) were seeded in 96-well plates overnight and then exposed to TOFA at indicated concentrations for 72 hours. Viable cells were detected.

Western blot

Cells were lysed in complete lysis buffer (Roche, IN), followed by centrifugation at 14,000 rpm for 15 min to collect the soluble proteins. Proteins (100 μ g) were separated on 8% SDS-PAGE and then subjected for immuno-blotting analysis as previous described [9].

Apoptosis

Apoptosis was evaluated by PARP cleavage, DNA fragmentation, and annexin-V staining and FACScan analysis. PARP cleavage was detected by Western blot as described above; DNA fragmentation was examined as addressed previously [19]; and annexin-V staining and FACScan was performed as previously described [17].

Fatty acid synthesis

Cells were pulsed with 1 μ Ci 2-¹⁴C-labeled acetate (53 mCi/mmol; Amersham Biosciences, NJ) per well of 12-well plates for 4 hours at 37°C, 5% CO₂, followed by lipid synthesis analysis as previously described [9].

Statistical analysis

Student's *t*-test was used for statistically significant analysis with INSTAT statistical analysis package (Graph Pad Software, CA). Significance was defined as $p < 0.05$.

Results

TOFA is cytotoxic to cancer cells

TOFA blocks fatty acid synthesis by inhibiting ACCA, the rate-limiting enzyme of the fatty acid synthesis pathway [15,16]. To evaluate the effects of this inhibition on cell growth and survival, we examined the viability of human NCI-H460, HCT-8, and HCT-15 cancer cells exposed to TOFA at 1.0 to 50.0 μ g/ml. As demonstrated in Figure 1, TOFA showed strong cytotoxicity to all three human cancer cell lines, with an IC₅₀ at approximately 5.0, 5.0, and 4.5 μ g/ml for NCI-H460, HCT-8, and HCT-15 cells, respectively.

TOFA induces apoptosis by inhibiting fatty acid synthesis

To understand the mechanisms of cytotoxicity of TOFA, we examined the expression of its targeting protein, ACCA, in the NCI-H460, HCT-8 and HCT-15 cancer cells and measured the lipid synthesis of these cells in response to TOFA at 1.0 to 20.0 μ g/ml. As shown in Figure 2, ACCA is highly expressed in all three cell lines and is sensitive to TOFA, reflected on the significant inhibition of the fatty acid synthesis by TOFA at 5.0 μ g/ml ($p < 0.05$) or higher ($p < 0.01$). Therefore, TOFA may affect the viability of these human cancer cells by inhibiting their fatty acid synthesis.

Fatty acids are the precursor of phospholipids, and reduced fatty acid synthesis leads to breast cancer cell apoptosis [13]. To understand the mechanisms of cell death induced by TOFA, we examined the apoptosis of the NCI-H460, HCT-8 and HCT-15 cells when exposed to TOFA at 1.0 to 20.0 $\mu\text{g/ml}$ by evaluating PARP cleavage, DNA fragmentation, and then annexin-V staining. In a dose-dependent manner, TOFA induced PARP cleavage and DNA fragmentation in the NCI-H460 and HCT-15 cancer cells (Figure 3A & B). PARP cleavage and DNA fragmentation also occurred in the HCT-8 cells to a similar extent (data not shown). Using annexin-V staining and FACScan analysis, we quantitatively assessed the apoptosis of these cancer cells in response to TOFA. Figure 3C demonstrates the dead cell number induced by TOFA. The data represent the total apoptotic cells, that is, the sum of annexin-V positive (early apoptotic) and annexin-V plus PI positive (late apoptotic) cells.

To confirm the specificity of ACCA inhibition and cell death induced by TOFA, we knocked down ACCA expression in NCI-H460, HCT-8 and HCT-15 cells using a specific small interfering RNA (siRNA) [14]. Cell death was assessed by annexin-V staining and FACScan analysis. As shown in Figure 3D, the transient delivery of chemically synthesized ACCA siRNA led to 80 ~ 90% knockdown of ACCA protein, concomitant with significant apoptosis. Exposing the ACCA silencing cells to TOFA at 10 $\mu\text{g/ml}$ slightly exacerbated apoptotic cell death, but no statistical significance was tested ($p > 0.05$). It may be due to the inhibition to the remaining ACCA in these cells.

Palmitic acids rescue cell death induced by TOFA

To further confirm that TOFA induces cell death by blocking fatty acid synthesis, we performed a palmitic acid rescue study. Palmitic acids (16:0) are the end-products of the fatty acid synthesis pathway and therefore, supplementing cells with palmitic acids could theoretically improve their survival. In this study, 100 μM palmitic acids were added into the cells that were exposed to 10 $\mu\text{g/ml}$ of TOFA and cell death was examined by annexin-V staining and FACScan analysis. As shown in Figure 4, palmitic acids significantly eradicated the apoptosis induced by TOFA, proving that TOFA induces the cell death by depleting the cellular fatty acids. Similar rescues were observed in the ACCA silencing cells (data not shown).

Discussion

Long chain fatty acids are the building blocks of biomembranes, playing a critical role in cell proliferation and survival [13,20]. Lipogenic alterations are early events during cancer development and the up-regulation of lipogenic enzymes, such as FAS and ACCA, has been documented in a variety of cancers, including breast, prostate, ovary, lung, colon, and endometrial cancers [1,11,12,21–23]. For instance, FAS is overexpressed in the earliest stages of prostate neoplastic transformation (PIN lesions) and expression levels are positively correlated to the grades of PIN lesions and invasive carcinomas [22,24]. In tumor cells, newly synthesized lipids are mainly phospholipids, the major components of biomembranes, meeting the need of rapid cell division. More importantly, the newly synthesized lipids are enriched with saturated or monounsaturated fatty acids, which tend to partition into detergent resistant membrane microdomains or rafts, mediating cell migration, signal transduction, and intracellular trafficking [21,25,26]. Therefore, the fatty acid synthesis pathway may be a potent therapeutic target of cancer. TOFA is an allosteric inhibitor of the ACCA. It has been reported that upon the cell types and/or organs, TOFA inhibits fatty acid synthesis, increases fatty acid oxidation and ketogenesis, and decreases triglyceride synthesis and very low density lipoprotein (VLDL) production [16,27]. In this study, we found for the first time that TOFA induces apoptosis of the human lung cancer cells NCI-H460 and colonic carcinoma cells HCT-8 and HCT-15 in a dose-dependent manner.

TOFA showed high toxicity to the NCI-H460, HCT-8, and HCT-15 cancer cells (Figure 1). Exposing the cells to TOFA at 1.0 to 20.0 $\mu\text{g/ml}$ effectively inhibited the fatty acid synthesis and induced apoptosis. In this study, the apoptotic cell death was assessed via PARP cleavage and DNA fragmentation, and then quantitatively confirmed by annexin-V staining and FACSscan analysis for the accuracy of data collection and interpretation. As seen in Figure 3, the cleaved PARP and DNA fragments were semi-quantitatively correlated with the dosages of the TOFA applied, consistent with the FACSscan data that measured the dead cell number. During the apoptosis, phospholipid phosphatidylserine (PS) translocates from the inner to outer leaflet of the plasma membrane. Annexin-V binds to PS and upon the stages of apoptosis, the dead cells may be stained by annexin-V alone (so called early apoptosis) or annexin-V plus PI that stains DNA in cells with disintegrating membranes (so called late apoptosis). Therefore, annexin-V staining and FACSscan analysis is able to quantitatively identify the stages of apoptosis. In this study, data were reported as a total of early and late apoptosis for the conciseness.

The specificity of TOFA-induced cell death through the ACCA inhibition and fatty acid depletion was confirmed by ACCA silencing and palmitic acid (16:0) rescue studies. As reported in breast cancer cells [13], ACCA knockdown resulted in apoptosis of the lung (NCI-H460) and colon (HCT-8 and HCT-15) cancer cells while palmitic acids (16:0), the end products of fatty acid synthesis pathway, could reverse the cell death induced by TOFA. Importantly, further exposing the cells with ACCA silencing to TOFA at 10 $\mu\text{g/ml}$ only slightly aggravated the cell death induced by ACCA silencing ($p > 0.05$), which is most likely due to the inhibition on the remaining ACCA in these cells. Taken together, we believe that in this study TOFA induced the death of the NCI-H460, HCT-8 and HCT-15 cancer cells by depleting cellular long chain fatty acids. This study result is consistent with that previously reported. ACCA inhibition by soraphen A, an inhibitor of the BC domain [28], or knockdown by siRNA [13,14] resulted in apoptotic cell death. Therefore, ACCA may be a potent target for cancer intervention; and identification of TOFA as a cytotoxic agent for the lung and colon cancer cells may facilitate the development of novel, sensitive ACCA inhibitors as antitumor agents.

It is noteworthy that controversial results were reported from other studies. For instance, ACCA inhibition by TOFA does not affect the survival of breast (MCF-7) and ovary (SKOV3) cancer cells [29–31] or even on opposite, prevents the cell death induced by C75, a FAS inhibitor via blocking malonyl-CoA accumulation [32]. A clear explanation on such contradictory observations is currently lacked. However, two facts need to be taken into consideration in data interpretation: 1) ACCA is mutated in some breast cancer tissues [33], which may alter the binding and/or inhibitory activity of TOFA and 2) there exist two ACC isoforms with distinct function in fatty acid synthesis or oxidation [2,6]. Therefore, it is merited to examine the ACCA mutations and define the ACC isoform in TOFA resistant cells.

Conclusion

This study demonstrates that through inhibiting fatty acid synthesis, TOFA induces apoptosis of the lung (NCI-H460) and colon (HCT-8 and HCT-15) carcinoma cells in a dose-dependent fashion. This study result facilitates the development of novel, more sensitive ACCA inhibitors as antitumor agents.

Abbreviations

ACCA	acetyl-CoA carboxylase- α
ACCB	acetyl-CoA carboxylase- β

AKR1B10	aldo-keto reductase family 1 B10
BC	biotin carboxylase
CPT-1	carnitine palmitoyltransferase 1
CT	carboxyltransferase
FAS	fatty acid synthetase
PARP	Poly (ADP-ribose) polymerase
TOFA	5-tetradecyloxy-2-furoic acid

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Figure 1. Cytotoxicity of TOFA

NCI-H460, HCT-8 and HCT-15 cells were seeded into 96-well plates at 5,000 cells/well. After incubation overnight, cells were fed with fresh medium containing TOFA at indicated concentrations, followed by continuing incubation for 72 hours. Thereafter, viable cells were assessed by MTT cell proliferation kit following the manufacture's instructions. Data indicates the mean $\pm$ SD from three independent measurements.

B) Fatty acid synthesis**A) ACCA expression** **Figure 2. Inhibition of fatty acid synthesis by TOFA**

A) Western blot. Soluble proteins (100 μg each) were used to detect the ACCA levels in the NCI-H460, HCT-8 and HCT-15 cells, as described in the Materials and Methods. Beta-actin was probed to indicate the relative amounts of loaded proteins. B) Fatty acid synthesis. Cells were exposed to TOFA at the indicated concentrations for 24 hours. At 4 hours before harvesting, cells were pulsed with 1 μCi 2-¹⁴C-labeled acetate per well of 12-well plates and subjected to fatty acid synthesis analysis as described in the Materials and Methods. Values represent mean ± SD from three independent measurements. * $p < 0.05$ and ** $p < 0.01$, compared to the corresponding control cells without TOFA. ACCA, acetyl-CoA carboxylase- α .

A) PARP cleavage **B) DNA fragmentation** **D) ACCA silencing and cell death****C) Annexin-V staining and FACSscan** **Figure 3. Apoptosis induced by TOFA**

Cells (2×10^6) were spread and incubated at regular conditions overnight, followed by exposure to TOFA at indicated concentrations for 24 hours. **A)** PARP cleavage. Cells were collected by rubber scrapers and soluble proteins (100 μg each) were used to detect the PARP cleavage by Western blot. Beta-actin was probed to indicate the relative amounts of loaded proteins. **B)** DNA fragmentation. Medium and PBS wash solutions were collected and pooled with the cells harvested by rubber scrapers. DNA ladders were prepared and visualized as described in the Materials and Methods. M, 100 bp DNA ladders. **C)** FACSscan analysis. PBS wash solutions and cell suspensions harvested by trypsinization were pooled. Annexin-V staining and FACSscan analysis were performed as described in the Materials and Methods. Data indicate

the total apoptotic cells (early plus later apoptotic cells). See text in the Results for more details. Values represent mean $\pm$ SD from three independent measurements. * $p < 0.05$, compared to the corresponding control cells without TOFA. *D*) Apoptosis induced by ACCA silencing. Chemically synthesized ACCA siRNA (50 nM) was delivered into the cells (1×10^5) and in the presence or not of TOFA (10 μ g/ml), apoptotic cells were assessed by annexin staining and FACSscan as described in the Materials and Methods. Scrambled siRNA was used as a control. Values represent mean $\pm$ SD from three independent measurements. * $p < 0.05$, compared to the scrambled control cells. ACCA, acetyl-CoA carboxylase- α ; PARP, Poly (ADP- ribose) polymerase; Scr, scrambled siRNA; and siR, ACCA siRNA.

Figure 4. Palmitate rescues of apoptosis induced by TOFA

Cells (2×10^6) were spread and incubated at regular conditions overnight, followed by exposure to TOFA at 10 $\mu\text{g/ml}$ and palmitic acids at 100 μM at the same time. After incubation for 24 hours, cells were subjected to annexin-V staining and FACScan analysis. Values represent mean $\pm$ SD from three independent measurements. * $p < 0.05$, compared to the corresponding control cells without TOFA; # $p < 0.05$, compared to the cells exposed to TOFA but not palmitic acids (PA).