Malonyl-Coenzyme-A Is a Potential Mediator of Cytotoxicity Induced by Fatty-Acid Synthase Inhibition in Human Breast Cancer Cells and Xenografts

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Abstract

A biologically aggressive subset of human breast cancers and other malignancies is characterized by elevated fatty-acid synthase (FAS) enzyme expression, elevated fatty acid (FA) synthesis, and selective sensitivity to pharmacological inhibition of FAS activity by cerulenin or the novel compound C75. In this study, inhibition of FA synthesis at the physiologically regulated step of carboxylation of acetyl-CoA to malonyl-CoA by 5-(tetradecyl)-2-furoic acid (TOFA) was not cytotoxic to breast cancer cells in clonogenic assays. FAS inhibitors induced a rapid increase in intracellular malonyl-CoA to several fold above control levels, whereas TOFA reduced intracellular malonyl-CoA by 60%. Simultaneous exposure of breast cancer cells to TOFA and an FAS inhibitor resulted in significantly reduced cytotoxicity and apoptosis. Subcutaneous xenografts of MCF7 breast cancer cells in nude mice treated with C75 showed FA synthesis inhibition, apoptosis, and inhibition of tumor growth to less than 1/8 of control volumes, with comparable toxicity in normal tissues. The data suggest that differences in intermediary metabolism render tumor cells susceptible to toxic fluxes in malonyl-CoA, both in vitro and in vivo.

Introduction

A number of studies have demonstrated surprisingly high levels of FAS expression (EC 2.3.1.85) in virulent human breast cancer (1,2), as well as other cancers (3,4). FAS expression has also been identified in intraductal and lobular in situ breast carcinoma, lesions associated with increased risk for the development of infiltrating breast cancer (5). FAS is the principal synthetic enzyme of FA synthesis, which catalyzes the NADPH-dependent condensation of malonyl-CoA and acetyl-CoA to produce predominantly the 16-carbon saturated free FA palmitate (6). Ex vivo measurements in tumor tissue have revealed high levels of both FAS and FA synthesis, indicating that the entire genetic program is highly active consisting of some 25 enzymes from hexokinase to FAS (3). Cultured human cancer cells treated with inhibitors of FAS, including the fungal product cerulenin and the novel compound C75, demonstrated a rapid decline in FA synthesis, with subsequent reduction of DNA synthesis and cell cycle arrest, culminating in apoptosis (7,8). These findings suggested a vital biochemical link between FA synthesis and cancer cell growth. Importantly, these effects occurred despite the presence of exogenous FAs in the culture medium derived from fetal bovine serum. Although it has been possible to rescue the cytotoxic effect of cerulenin on certain cells in FA-free culture conditions by the addition of exogenous palmitate, most cancer cells were not rescued from FA synthesis inhibition by the pathway end product (data not shown; Ref. 9). Thus, it has been unresolved whether the cytotoxic effect of FA synthesis inhibition on most cancer cells resulted from end product starvation or from some other biochemical mechanism. If FA starvation mediated the cytotoxic effects of cerulenin and C75, then any other FA synthesis inhibitor of similar potency should produce similar effects. To test this idea, we compared the effects on cancer cells of inhibition of ACC (EC 6.4.1.2), the rate-limiting enzyme of FA synthesis, with the effects of FAS inhibitors.

Fig. 1A outlines the portion of the FA synthesis pathway containing the target enzymes of the inhibitors used in this study. TOFA is an allosteric inhibitor of ACC, blocking the carboxylation of acetyl-CoA to malonyl-CoA. Once esterified to CoA, TOFA-CoA allosterically inhibits ACC with a mechanism similar to long chain acyl-CoAs, the physiological end-product inhibitors of ACC (10). Both cerulenin (11) and C75 (8) are inhibitors of FAS, preventing the condensation of malonyl-CoA and acetyl-CoA into FAs. Cerulenin is a suicide inhibitor, forming a covalent adduct with FAS (12), whereas C75 is likely a slow-binding inhibitor (13). We now report that using TOFA, we achieved FA synthesis inhibition in human breast cancer cell lines comparable to inhibition by cerulenin or C75. Surprisingly, however, TOFA was essentially nontoxic to human breast cancer cells. These data suggest that FA starvation is not a major source of cytotoxicity to cancer cells in serum supplemented culture. Rather, high levels of the substrate, malonyl-CoA, resulting specifically from inhibition of FAS, may mediate cytotoxicity of cerulenin and C75.

Materials and Methods

FA Synthesis Inhibitors. Cerulenin was obtained from Sigma. C75 and TOFA were synthesized in the laboratory of C. A. Townsend in the Department of Chemistry, Johns Hopkins University.

Cell Lines, Culture Conditions, Metabolic Labeling, and Clonogenic Assays. The human breast cancer cell lines, SKBR3 and MCF7 were maintained in RPMI with 10% fetal bovine serum. Cells were screened periodically for Mycoplasma contamination (Gen-probe). All inhibitors were added as stock 5 mg/ml solutions in DMSO. For FA synthesis activity determinations, 5 x 10^4 cells/well in 24-well plates were pulse labeled with [U-14C]acetate after exposure to drug, and lipids were extracted and quantified as described previously (8). For MCF7 cells, pathway activity was determined after 2 h of inhibitor exposure. SKBR3 cells demonstrated slower response to FAS inhibitors, possibly because of their extremely high FAS content, so pathway activity was determined after 6 h of inhibitor exposure. For clonogenic assays, 4 x 10^3 cells were plated in 25-cm^2 flasks with inhibitors added for 6 h in concentrations listed. To rescue MCF7 cells with TOFA (see Fig. 3C), the TOFA was added 1 h prior to the FAS inhibitors. Equal numbers of treated cells and controls were plated in 60-mm dishes. Clones were stained and counted after 7–10 days.
Flow-cytometric Quantitation of Apoptosis. Apoptosis was measured by multiparameter flow cytometry using a FACStar® Plus flow cytometer equipped with argon and krypton lasers (Becton Dickinson). Apoptosis was quantified using merocyanine 540 staining (Sigma), which detects altered plasma membrane phospholipid packing that occurs early in apoptosis, added directly to cells from culture (8, 14). In some experiments, chromatin conformational changes of apoptosis were simultaneously measured as decreased staining with LDS-751 (Exciton; Ref. 15). Merocyanine 540 (10 μg/ml) was added as a 1 mg/ml stock in water. Cells were stained with LDS-751 at a final concentration of 100 nM from a 1 mM stock in DMSO. The merocyanine 540-positive cells were marked by an increase in red fluorescence, collected at 575 ± 20 nm, 0.5–2 logs over merocyanine 540-negative cells. Similarly, the LDS-751 dim cells demonstrated a reduction in fluorescence of 0.5–1.5 logs relative to normal cells, collected at 660 nm with a DF20 band pass filter. Data were collected and analyzed using CellQuest software (Becton Dickinson). In these experiments, all LDS-751 dim cells were merocyanine 540 bright; however, a population of merocyanine 540 bright cells were detected that were not yet LDS-751 dim. All merocyanine 540 bright cells were classified as apoptotic.

Measurement of Malonyl-CoA. Malonyl-CoA levels were measured in MCF-7 cells using the HPLC method of Corkey (16). Briefly, 2.5 × 10^5 cells/well in 24-well plates were subjected to 1.2 ml of 10% trichloroacetic acid at 4°C after various drug treatments. The pellet mass was recorded, and

Fig. 1. Inhibitors of the FA synthesis pathway. A, a schematic representation of the FA synthesis pathway showing the specificity of cerulenin and C75 for FAS and of TOFA for ACC. The three FA synthesis inhibitors reduced FA synthesis activity (incorporation of [U-14C]acetate into extractable lipids) by comparable amounts in SKBR3 breast carcinoma cells (B) and in MCF7 breast carcinoma cells (D). The cytotoxic activity of the three FA synthesis inhibitors was determined by clonogenic assay in the dose range for FA synthesis inhibition. A 6-h exposure to cerulenin or C75 reduced the clonogenic fraction of SKBR3 breast carcinoma cells (Student’s t test, P = 0.0002 for C75, P < 0.0001 for cerulenin; C) and MCF7 breast carcinoma cells (P = 0.0004 for C75, P < 0.0001 for cerulenin; E), whereas TOFA did not.
the supernatant was washed six times with 1.2 ml of ether and reduced to dryness using vacuum centrifugation at 25°C. CoA esters were separated and quantitated using reversed-phase HPLC on a 5-μm Supelco C18 column with a Waters HPLC system running Millenium32 software, monitoring 254 nm as the maximum absorbance for CoA. The following gradients and buffers were used: buffer A, 0.1 M potassium phosphate, pH 5.0; buffer B, 0.1 M potassium phosphate, pH 5.0, with 40% acetonitrile. Following a 20-min isocratic run with 92% buffer A, 8% buffer B at 0.4 ml/min, flow was increased to 0.8 ml/min over 1 min, whereupon a linear gradient to 10% buffer B was run until 24 min and then held at 10% buffer B until 50 min, at which point a linear gradient was run to 100% buffer B at 53 min, completing at 60 min. The following CoA esters (Sigma) were run as standards: malonyl-CoA, acetyl-CoA, glutathione-CoA, succinyl-CoA, HMG-CoA, and free CoA. Samples and standards were dissolved in 50 μl of buffer A. CoA esters eluted sequentially as follows: malonyl-CoA, glutathione-CoA, free CoA, succinyl-CoA, HMG-CoA, and acetyl-CoA. Quantitation of CoA esters was performed by the Millenium32 software.

**Xenograft Studies.** s.c. flank xenografts of the human breast cancer cell line, MCF-7 in nude female mice (Harlan) were used to study the antitumor effects of C75 in vivo. All animal experiments complied with institutional animal care guidelines. All mice received a 90-day slow-release s.c. estrogen pellet (Innovative Research) in the anterior flank 7 days before tumor inoculation. MCF7 cells (10^7 cells) were xenografted from culture in DMEM supplemented with 10% FBS and 10 μg/ml insulin. Treatment began when measurable tumors developed about 10 days after inoculation. Eleven mice (divided between two separate experiments of five and six mice) were treated i.p. with weekly doses of C75 at 30 mg/kg in 0.1 ml of RPMI. Dosing was based on a single dose LD_{10} determination of 40 mg/kg in BALB/c mice; 30 mg/kg has been well tolerated in outbred nude mice. Eleven control mice (divided in the same way as the treatment groups) received RPMI alone. Tumor volume was measured with calipers in three dimensions. Experiment was terminated when controls reached the surrogate end point. In a parallel experiment to determine FA synthesis activity in treated and control tumors, a group of MCF-7 xenografted mice were treated with C75 or vehicle at above doses and sacrificed after 3 h. Tumor and liver tissue were ex vivo labeled with [U^{14}C]acetate. Malonyl-CoA is the first of these to elute, with a column retention time of 19–22 min. The overlay of chromatographs in Fig. 2 is a representative chromatograph demonstrating the separation of malonyl-CoA resulting from exposure to cerulenin (17).

**FAS Immunohistochemistry.** Immunohistochemistry for FAS was performed on the MCF-7 xenografts using a mouse monoclonal anti-FAS antibody (1) at 1:2000 on the DAKO Immunostainer using the LSAB2 detection kit.

**Results and Discussion**

**TOFA, Cerulenin, and C75 All Inhibited FA Synthesis in Human Breast Cancer Cells but Showed Differential Cytotoxicity.** In standard pulse labeling experiments in which breast cancer cell lines SKBR3 and MCF7 were labeled for 2 h after exposure to FA synthesis inhibitors, TOFA, Cerulenin, and cerulenin all produced dose-dependent inhibition of [U^{14}C]acetate incorporation into lipids (Fig. 1, A and D). The maximal pathway inhibition achieved with each drug was somewhat variable among cell lines. In numerous similar experiments (not shown), TOFA maximally inhibited FA synthesis in the 1–5 μg/ml dose range in all cell lines tested, and cerulenin and C75 maximally inhibited FA synthesis at about 10 μg/ml. Although all inhibitors reduced FA synthesis to comparable degrees, TOFA was nontoxic or stimulatory to the cancer cell growth in the dose range for ACC inhibition, as measured by clonogenic assays, whereas cerulenin and C75 were significantly cytotoxic in the dose range for FAS inhibition (Fig. 1, C and E). The profound difference between the cytotoxic effects of ACC and FAS inhibition demonstrated that the acute reduction of FA production per se was not the major source of cell injury after FAS inhibition. Alternatively, these data suggested that cytotoxicity resulted from a biochemical effect of FAS inhibition that was not shared by ACC inhibition.

**Malonyl-CoA Levels Were Markedly Increased with FAS Inhibition and Reduced by TOFA.** The most obvious difference in the expected results of inhibiting these two enzymes was that malonyl-CoA levels should fall after ACC inhibition but increase after FAS inhibition. Although not previously investigated in eukaryotes, recent data in Escherichia coli have demonstrated elevated levels of malonyl-CoA resulting from exposure to cerulenin (17).

Direct measurement of CoA esters in MCF7 cells by reversed-phase HPLC of acid soluble extracts from drug-treated cells confirmed that both cerulenin and C75 caused a rapid increase in malonyl-CoA levels, whereas TOFA reduced malonyl-CoA levels. Fig. 2A is a representative chromatograph demonstrating the separation and identification of CoA derivatives important in cellular metabolism. Malonyl-CoA is the first of these to elute, with a column retention time of 19–22 min. The overlay of chromatographs in Fig. 2B shows that cerulenin treatment led to a marked increase in malonyl-CoA over the control, whereas TOFA caused a significant reduction. The chemical identity of the malonyl-CoA was independently confirmed by spiking samples with standards (not shown). The anal-
ysis of multiple experiments shown in Fig. 2C demonstrated that following a 1-h exposure to cerulenin or C75 at 10 μg/ml, malonyl-CoA levels increased by 930 and 370%, respectively, over controls, whereas TOFA treatment (20 μg/ml) led to a 60% reduction of malonyl-CoA levels. The concentration of TOFA required for maximal reduction of malonyl-CoA levels was 4-fold higher than the dose for pathway inhibition shown in Fig. 1, B and D. However, optimal cultures for extraction of CoA derivatives had 5-fold higher cell density than the cultures used in the other biochemical and viability assays presented. The remarkable increase in malonyl-CoA after FAS inhibition can be attributed in part to the release of long-chain fatty acyl-CoA inhibition of ACC, leading to an increase in ACC activity (Fig. 1A). Moreover, the cerulenin-induced increase in malonyl-CoA levels occurred within 30 min of treatment (930 ± 15% increase over control, data not shown), within the time frame of FA synthesis inhibition and well before the onset of DNA synthesis inhibition or early apoptotic events (8). Thus, high levels of malonyl-CoA were a characteristic effect of FAS inhibitors and temporally preceded the other cellular responses, including apoptosis.

Inhibition of ACC Rescued Breast Cancer Cells from FAS Inhibition. If the elevated levels of malonyl-CoA resulting from FAS inhibition were responsible for cytotoxicity, then it should be possible to rescue cells from FAS inhibition by reducing malonyl-CoA accumulation with TOFA. Co-administration of TOFA and cerulenin to SKBR3 cells (Fig. 3A) abrogated the cytotoxic effect of cerulenin alone in clonogenic assays. In MCF7 cells (Fig. 3C), TOFA rescued both cerulenin and C75 when cells were exposed to TOFA for 1 h prior to the FAS inhibitors. Representative flow cytometric analyses of SKBR3 cells (Fig. 3B) and MCF7 (Fig. 3D) substantiated these findings, because TOFA rescued cells from cerulenin induced apoptosis. These experiments also confirmed the differential cytotoxicity between TOFA (<5% increase in apoptosis; no reduction in clonogenicity) compared to cerulenin (>85% apoptosis; 70% reduction in clonogenicity). Taken together, these studies suggest that high malonyl-CoA levels may play a role in the cytotoxic effect of FAS inhibitors on cancer cells.

In Vivo Inhibition of FAS Led to Reduced Tumor Growth. Previous studies have demonstrated local efficacy of cerulenin against a human cancer xenograft (18) but were limited by the failure of cerulenin to act systemically. The similar responses of breast cancer cells to cerulenin and C75 in vitro suggested that C75 might be effective in vivo against xenografted breast cancer cells. To determine whether the effects of FAS inhibition seen in vitro would translate to an in vivo setting requiring systemic activity, we tested C75 against s.c. MCF-7 xenografts in athymic nude mice, to quantitate effects on FA synthesis and the growth of established solid tumor.

FA synthesis pathway activity in tissues of xenografted mice was determined by ex vivo pulse labeling with [U14C]acetate. The tumor xenografts had 10-fold higher FA synthesis activity than liver, highlighting the difference in pathway activity between benign and malignant tissues (Fig. 4A). FAS expression in the MCF-7 xenograft paralleled the high level of FA synthesis activity (Fig. 4B). i.p.

Fig. 3. ACC inhibition rescued the cytotoxic effects of FAS inhibition in breast cancer cells. A, pretreatment with TOFA rescued SKBR3 cells from cerulenin cytotoxicity as determined by clonogenic assays (Student’s t test, P = 0.001). C, similarly, TOFA reduced both cerulenin and C75 cytotoxicity in MCF7 cells (P = 0.0016 for C75, P < 0.0001 for cerulenin). Using merocyanine 540 staining as an indicator of apoptosis, TOFA rescued both SKBR3 cells (B) and MCF7 cells (D) from cerulenin cytotoxicity.

A.

B.

C.

D.
injections of C75 at 30 mg/kg reduced FA synthesis in ex vivo labeled liver by 76% and in the MCF-7 xenografts by 70% within 3 h (Fig. 4A). These changes in FA synthesis preceded histological evidence of cytotoxicity in the xenograft, which became evident 6 h after treatment (Fig. 4, C and D). The C75-treated xenografts showed numerous apoptotic bodies throughout the tumor tissue, which were not seen in vehicle-treated tumors. Histological analysis of liver and other host tissues following C75 treatment showed no evidence of any short or long term toxicity (not shown).

Weekly i.p. C75 treatment retarded the growth of established s.c. MCF-7 tumors compared to vehicle controls, demonstrating a systemic antitumor effect (Fig. 4E). After 32 days of weekly treatments, there was a greater than 8-fold difference in tumor growth in the treatment group compared to vehicle controls. Sim-
ilar to cerulenin, transient reversible weight loss was the only toxicity noted (18).

The systemic pharmacological activity of C75 provided the first analysis of the outcome of systemic FAS inhibitor treatment. The significant antitumor effect of C75 on a human breast cancer xenograft in the setting of physiological levels of ambient FAs was similar to the in vitro result in serum supplemented culture and was consistent with a cytotoxic mechanism independent of FA starvation. Furthermore, the result suggested that malonyl-CoA accumulation may not be a significant problem in normal tissues, possibly because FA synthesis pathway activity is normally low, even in lipogenic organs, such as the liver. It is of further interest that whereas malonyl-CoA was the predominant low molecular weight CoA conjugate detected in breast cancer cells in these experiments, other studies have reported predominantly succinyl-CoA and acetyl-CoA in cultured hepatocytes (16). Differences in CoA derivative profiles may be indicative of larger differences in energy metabolism between cancer cells and hepatocytes.

The identification of malonyl-CoA as a potential mediator of cytotoxicity, possibly via induction of apoptosis in cancer cells, although unanticipated, was not surprising given its pivotal role in cellular metabolism. In addition to its function as a substrate for FA synthesis, malonyl-CoA regulates FA oxidation by inhibiting carnitine palmitoyltransferase I at the outer mitochondrial membrane (19). Physiologically, the elevated levels of malonyl-CoA occurring during FA synthesis reduce FA oxidation to prevent a futile cycle of simultaneous FA synthesis and degradation. During starvation or feeding with high-fat diets, fat synthesis ceases, malonyl-CoA levels fall, and FAs enter the mitochondrion for energy production. Malonyl-CoA is thus a crucial regulatory metabolic intermediate in cellular energy metabolism. How superphysiological levels of malonyl-CoA may lead to apoptosis is not yet known; however, carnitine palmitoyltransferase I, which is regulated by malonyl-CoA, has been shown to interact directly with Bcl-2 at the mitochondrial membrane (20). This convergence suggests that high levels of malonyl-CoA may either induce apoptosis directly or alter mitochondrial metabolism to increase susceptibility to apoptosis from other signals. Thus, further investigation of malonyl-CoA and CoA metabolism in cancer cells may yield new insights into cancer cell metabolism and selective susceptibility to anti-metabolite therapy.

References


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