

Inhibition of Fatty Acid Synthesis Induces Programmed Cell Death in Human Breast Cancer Cells¹

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Abstract

One of the key limiting factors in the treatment of advanced stage human epithelial malignancies is the lack of new, selective molecular targets for antineoplastic therapy. A substantial subset of human breast, ovarian, endometrial, colorectal, and prostatic cancers express elevated levels of fatty acid synthase, the major enzyme required for endogenous fatty acid biosynthesis, and carcinoma lines are growth inhibited by cerulenin, a noncompetitive inhibitor of fatty acid synthase. We have shown previously that the difference in fatty acid biosynthesis between cancer and normal cells is an exploitable target for metabolic inhibitors in the *in vitro* setting and *in vivo* in a human ovarian carcinoma xenograft in nude mice. Here, we report that cerulenin treatment of human breast cancer cells inhibits fatty acid synthesis within 6 h after exposure, that loss of clonogenic capacity occurs within the same interval, and that DNA fragmentation and morphological changes characteristic of apoptosis ensue.

Introduction

The treatment of human epithelial malignancies is limited by drug resistance and toxic side effects of therapy, which contribute to ultimate treatment failure for the majority of advanced stage cancer victims. Identification of new, selective molecular targets for antineoplastic therapy provides an opportunity for therapeutic advancement. Although disordered intermediary metabolism in cancer cells has been known for the better part of this century (1), little attention has been paid to fatty acid metabolism. One early study showed elevated levels of fatty acid synthesis in tumor tissues, although the significance of the observations was not appreciated (2). We and others (3-10) have recently shown that some clinical human ovarian, endometrial, breast, colorectal, and prostatic cancers overexpress FAS.³ Tumor cells which express high levels of fatty acid synthesizing enzymes use endogenously synthesized fatty acids for membrane biosynthesis and also appear to export large amounts of lipid.⁴ In marked contrast, normal cells preferentially utilize dietary lipids.

We have shown recently that these biochemical differences provide a selective target for metabolic inhibitors both *in vitro* and *in vivo*. Key observations are that: (a) inhibition of the β -ketoacyl synthase site of FAS is selectively cytotoxic to cancer cells with increased fatty

acid biosynthesis but not to normal skin fibroblasts *in vitro*; (b) addition of palmitate, the major direct product of FAS, reverses the cytotoxic effects of its inhibition, demonstrating mechanistic specificity (11-14); (c) elevations in fatty acid synthase expression in carcinoma cell lines are comparable to levels in primary human tumors assessed by immunohistochemistry; (d) fatty acid synthetic activities of a carcinoma line grown *in vitro* or as a murine xenograft are similar, and are 4- to >20-fold higher than normal murine tissues; and (e) treatment with the specific FAS inhibitor, cerulenin, produces regression of established ascites tumor, reduction in ascites incidence, delay in onset of ascites, and significantly increased survival in a nude mouse xenograft model of human ovarian carcinoma (15).

The mechanism by which inhibition of fatty acid synthesis produces its antitumor effect remains unexplained. The evidence presented here indicates that exposure of breast carcinoma cells to cerulenin produces inhibition of fatty acid synthesis within 6 h and produces irreversible lethal injury measured as a reduction of clonogenic capacity in the same interval. Treated breast carcinoma cells subsequently undergo DNA fragmentation and morphological changes characteristic of apoptosis.

Materials and Methods

Cell Lines and Culture Conditions. ZR-75-1, SKBR3, and MCF-7 cells were maintained in RPMI 1640 with 10% fetal bovine serum except as otherwise indicated. Cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere. A 5 mg/ml stock solution of cerulenin (Sigma Chemical Co.) in DMSO was diluted into experimental cultures to final concentrations of 5 or 10 μ g/ml.

Measurement of Endogenous Lipid Synthesis. ZR-75-1 cells were plated at 2×10^5 cells/well in 24-well plates and incubated overnight prior to use. Cerulenin (Sigma Chemical Co.) in DMSO was added for indicated incubation times. Each well was pulse labeled with 0.1 μ Ci [U-¹⁴C]acetate during the final 2 hours of drug treatment and washed, and cellular lipids were extracted (13) and assayed for ¹⁴C by scintillation counting. Controls consisted of cells incubated with DMSO alone. All determinations were performed in triplicate with error bars representing the SE.

Clonogenic Assay. After overnight incubation, 1×10^6 ZR-75-1, SKBR3, or MCF-7 cells were exposed to cerulenin (Sigma Chemical Co.) in DMSO (0.1-0.2%) for 6 h, washed, detached by trypsin digestion, counted, and replated at 1000 or 500 cells/60-mm plate in triplicate. Colonies were stained with crystal violet and counted 3-5 days after plating. Controls consisted of cells incubated with DMSO without cerulenin. Error bars represent the SE.

DNA Fragmentation Assay and Morphological Evaluation. Exponentially growing ZR-75-1 cells were plated at $1-5 \times 10^4$ cells/cm² in DMEM with 5% fetal bovine serum and 2 mM glutamine. After attachment, the medium was changed, and cells were incubated with or without cerulenin continuously until harvesting. At harvest, medium and trypsinized cells were combined, and DNA was extracted from pelleted cells and subjected to pulse field gel electrophoresis as described previously (16). Cells treated in parallel were spun onto glass slides, fixed in methanol, and stained with Hoechst 33342 for evaluation of morphological changes of apoptosis by fluorescence microscopy at 480 nm.

Received 2/12/96; accepted 4/25/96.

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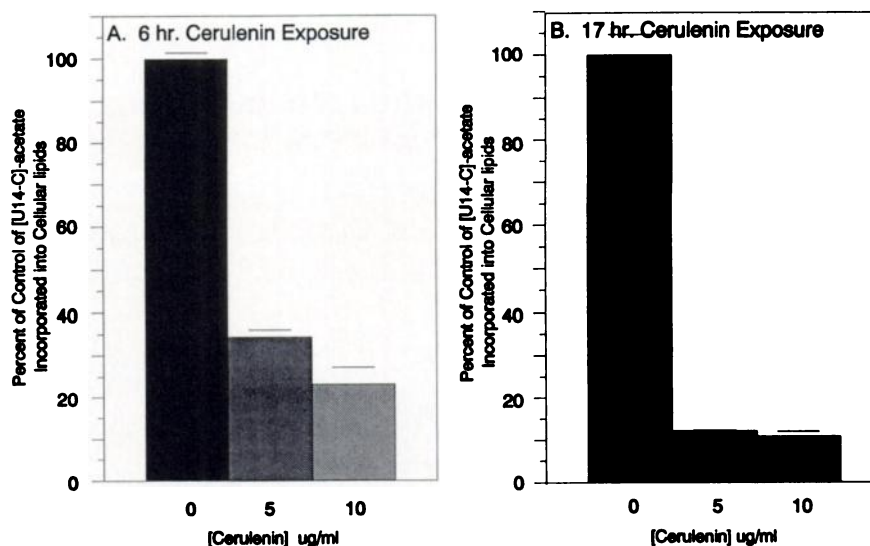
¹ Supported by grants from the National Cancer Institute (CA57545), the American Institute for Cancer Research, the W. W. Smith Charitable Trust, and the Susan G. Komen Foundation. E. P. was supported by USPHS Grant T32 AI 07247 and by a grant from the Stetler Research Fund. C. J. was supported by a fellowship from the Deutsche Forschungsgemeinschaft.

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³ The abbreviation used is: FAS, fatty acid synthase.

⁴ H. S. Heine, F. P. Kuhajda, F. D. Wood, A. Kayler, and G. R. Pasternack, Increased rates of fatty acid synthesis in breast cancer and identification of unique products, manuscript in preparation.

Fig. 1. Inhibition of fatty acid synthesis in breast carcinoma cells by cerulenin. ZR-75-1 cells were pulse labeled with [^{14}C]acetate after exposure to cerulenin (5 or 10 $\mu\text{g/ml}$) for 6 (A) or 17 h (B). There is a dose-dependent inhibition of incorporation of acetate into cellular lipids within 6 h of exposure to cerulenin. Error bars, SE.



Results

Inhibition of Fatty Acid Synthesis in Human Breast Cancer Cells by Cerulenin. Cerulenin, a fungal metabolite, is a potent, specific, noncompetitive inhibitor of FAS. The effect of cerulenin on fatty acid synthesis in breast carcinoma cells was assessed directly by quantitation of [^{14}C]acetic acid incorporation into cellular lipids via the fatty acid synthetic pathway without or with exposure to cerulenin (Fig. 1). ZR-75-1 cells exhibited a dose-dependent reduction of fatty acid synthesis 6 h after cerulenin exposure, with 77% reduction from control levels of fatty acid synthesis in cerulenin (10 $\mu\text{g/ml}$). The magnitude and rapid onset of this effect were similar to cerulenin inhibition of fatty acid synthesis in other cell lines and systems (13, 15). The magnitude of cerulenin inhibition of fatty acid synthesis increased further at 17 h; however, changes in viable cell number between treated and control cultures may have contributed to this increase.

Reduction of Clonogenic Potential of Human Breast Cancer Cells after Cerulenin Treatment. Studies of cellular injury support the notion that irreversible biochemical events that will inevitably lead to cell death occur well in advance of recognizable structural changes; in short, there is apparently a biochemical point of no return. To assess the cellular consequences of fatty acid synthesis inhibition, the clonogenicity of three human breast carcinoma cell lines was determined after a 6-h exposure to cerulenin (Fig. 2). All three cell lines exhibited dose-dependent reductions in clonogenic potential, with cytotoxicity ranging from 61 to 97% in cerulenin (10 $\mu\text{g/ml}$) after 6 h. When cerulenin treatment of MCF7 cells was increased to 12 h, there was an additional reduction in clonogenic potential by one log (data not shown).

Induction of Programmed Cell Death by Cerulenin. The cytotoxic effects of cerulenin must occur within the context of a cellular response. A wide variety of cellular injuries results in cell death by initiating a cellular program for autodestruction, or apoptosis. Fragmentation of genomic DNA to high molecular weight (>50 kb) fragments is a characteristic early event in apoptosis and may represent the committed step of the process. Field inversion gel electrophoresis was used to evaluate whether high molecular weight DNA fragmentation was a feature of the cellular response to cerulenin (Fig. 3A). ZR-75-1 cells generated detectable high molecular weight DNA fragments within 24 h after exposure to cerulenin, with increasing abundance of high molecular weight fragmented DNA through 72 h. DNA fragmentation to nucleosome-sized fragments was not observed.

To confirm that the cytotoxic effects of cerulenin were mediated via apoptosis, treated cells were examined for morphological changes of programmed cell death. Fig. 3B shows the chromatin condensation and nuclear fragmentation characteristic of cells undergoing apoptosis after cerulenin treatment of ZR-75-1 cells.

Discussion

The strategy of cancer chemotherapy by fatty acid synthesis inhibition is based on the therapeutic index provided by elevated fatty acid synthesis in tumor cells and the apparent tumor preference for the endogenous pathway. Treatment of the OVCAR-3 nude mouse xenograft model of human ovarian carcinoma has demonstrated potential clinical efficacy for this approach (15). The data reported here demonstrate that inhibition of fatty acid synthesis inflicts rapid, lethal injury to carcinoma cells via activation of the cell death program.

A wide range of influences, from radiation injury to hormonal stimuli, may trigger apoptosis. These results are novel in that perturbation of an intermediary metabolic pathway has been shown to

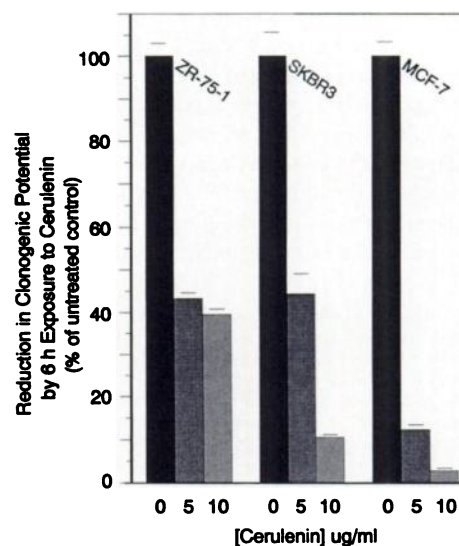


Fig. 2. Abrogation of clonogenicity of breast carcinoma cells after cerulenin exposure. Breast carcinoma cells were incubated in cerulenin (5 or 10 $\mu\text{g/ml}$) for 6 h, washed, and replated as described in "Materials and Methods." Cerulenin [10 $\mu\text{g/ml}$] produced 61–97% reduction in viable cells within 6 h of exposure. Error bars, SE.

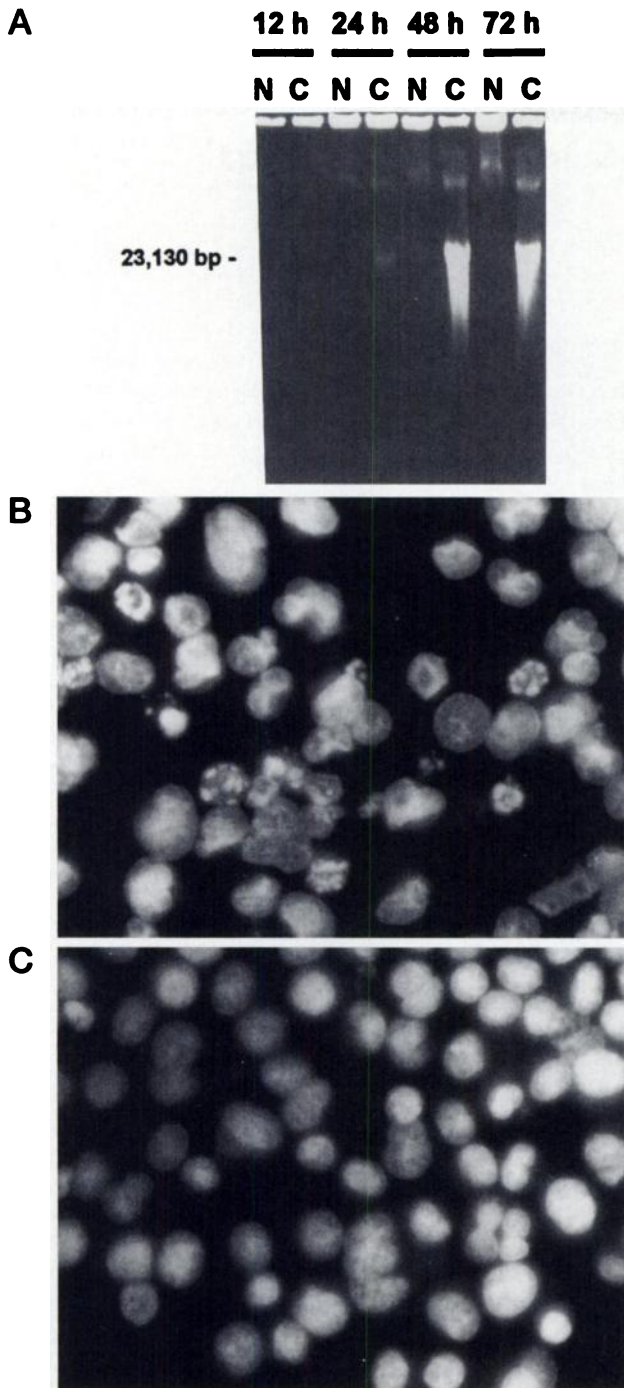


Fig. 3. Induction of programmed cell death by cerulenin. A, high molecular weight DNA fragmentation after exposure of ZR-75-1 cells to cerulenin (10 mg/ml) at 12, 24, 48, and 72 h. Cells were incubated in the absence (N) or presence (C) of cerulenin and then analyzed by field inversion gel electrophoresis to assess high molecular weight (≥ 50 kb) DNA fragmentation. B and C, morphological changes characteristic of programmed cell death, including chromatin condensation and nuclear fragmentation in ZR-75-1 cells exposed to cerulenin (10 mg/ml) for 72 h (B) versus controls (C). Cells are stained with Hoechst 33342.

trigger apoptosis. A detailed understanding of the linkage between endogenous fatty acid synthesis and regulation of apoptosis is not yet established; however, several observations support the notion that tumor cell fatty acid synthesis may be functionally linked to prolifer-

ation. Our studies have shown closely parallel expression of FAS and Ki-67, a proliferation antigen, in human tissues with rapid growth rates (3, 17).⁵ HL60 promyelocytic leukemia cells have elevated fatty acid synthetic activity, which is immediately down regulated along with proliferative activity after chemical induction of differentiation (14). Other investigators have shown induction of expression of metabolic enzymes, including FAS, immediately after serum stimulation of growth-arrested fibroblasts (18). Possible functional roles for endogenously synthesized fatty acids in cell growth include synthesis of structural lipids for membrane biosynthesis and lipid mediators of intracellular, autocrine or paracrine signals, as well as acylation of other macromolecules. We hypothesize that it is through inhibition of the growth-supportive functions of endogenous fatty acid synthesis that apoptosis is triggered, and that therapeutic selectivity may depend partially on this, because tissues that synthesize fat for purposes unrelated to proliferation (e.g., liver) appear resistant to cerulenin.

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