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Fatty acid synthesis: A potential selective target for antineoplastic therapy

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ABSTRACT **OA-519 is a prognostic molecule found in tumor cells from breast cancer patients with markedly worsened prognosis. We purified OA-519 from human breast carcinoma cells, obtained its peptide sequence, and unambiguously identified it as fatty acid synthase through sequence homology and enzymology. Tumor fatty acid synthase is an ≈270-kDa polypeptide which specifically abolished immunostaining of human breast cancers by anti-OA-519 antibodies. Tumor fatty acid synthase oxidized NADPH in a malonyl-CoA-dependent fashion and synthesized fatty acids composed of 80% palmitate, 10% myristate, and 10% stearate from acetyl-CoA, malonyl-CoA, and NADPH with a specific activity of 624 nmol of NADPH oxidized per min per mg. Tumor cell lines with elevated fatty acid synthase showed commensurate increases in incorporation of [U-^{14}C]acetate into acylglycerols demonstrating that fatty acid synthase increases occur in the context of overall increases in endogenous fatty acid synthesis. Cerulenin inhibited acylglycerol synthesis in tumor cells and fibroblast controls in a dose-dependent fashion and also caused a growth inhibition which generally paralleled the level of endogenous fatty acid synthesis. Supraphysiologic levels of palmitate, 14 μM in dimethyl sulfoxide, significantly reversed the growth inhibition caused by cerulenin at concentrations of up to 5 μg/ml, indicating that cerulenin-mediated growth inhibition was due to fatty acid synthase inhibition.**

In 1989, we reported the immunologic identification of a prognostic molecule in the tumor cells of breast cancer patients with a poor prognosis for recurrence and death due to their disease (1). This prognostic factor was statistically independent of important clinical parameters including tumor size, lymph node involvement, and assessment of estrogen and progesterone receptors; subsequent studies also showed it to be independent of other prognostic molecules including c-erb-B2 and cathepsin D (2). Tumors marked by this prognostic molecule were nearly 4 times more likely to recur and metastasize than tumors not so marked, representing a prognostic power as strong as the presence of cancer in the axillary lymph nodes of patients with T_1 or T_2 primaries (1–3).

We now report the unambiguous identification of this prognostic molecule, OA-519 [originally identified through Hpr epitopes (1, 4)], as a functional fatty acid synthase (FAS) through peptide sequencing and enzymologic studies. In tumor cells, FAS activity varies approximately in parallel with flux through the fatty acid synthetic pathway. Tumor cells displaying such unusually high levels of endogenous fatty acid synthesis appear to be dependent on this synthesis, since inhibition of FAS inhibits growth of human carcinoma cell lines commensurate with their FAS levels. Since FAS activity in the tissues of humans eating a diet containing normal amounts of fats is extremely low (5), the presence of markedly elevated FAS activity in aggressive tumors may provide a highly selective basis for anticancer therapy.

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METHODS

Purification of OA-519. A lysate of ZR-75-1 cells prepared by Dounce homogenization at 1.5×10^6 cells per ml in lysis buffer (20 mM Tris·HCl, pH 7.5 at 4°C/1 mM EDTA/0.1 mM diisopropyl fluorophosphate/0.1 mM phenylmethanesulfonyl fluoride) was centrifuged at 16,000 × *g* for 30 min at 4°C. After passing through a 0.45-μM filter, the lysate was applied to a Sephacryl S-200 (Pharmacia) gel filtration column (2.5 cm × 90 cm) equilibrated in lysis buffer at pH 8.0 at 4°C supplemented with 100 mM KCl and 1 mM 2-mercaptoethanol. Fractions containing protein immunoreactive with polyclonal anti-OA-519 peptide antibody as judged by Western blot analysis of a 4% SDS/polyacrylamide gel were pooled, diluted with an equal volume of lysis buffer without KCl, and loaded onto a Mono Q HR 5/5 anion-exchange column (Pharmacia). The column was washed for 15 min at 1 ml/min, and bound material was eluted with a linear 60-ml gradient over 60 min to 1 M KCl. Fractions containing the immunoreactive ≈270-kDa protein as shown by Western blot were pooled. This procedure results in substantially pure preparations (>95%) of FAS (OA-519) as judged by Coomassie-stained gels.

Protein Sequencing. Peptide sequence was obtained by removing residual contaminants from OA-519 by HPLC hydroxyapatite chromatography with a Bio-Rad MAPS analytical HPHT cartridge. With a 0–600 mM phosphate gradient, OA-519 was eluted at 200 mM phosphate and was >99% pure by Coomassie staining after SDS/PAGE. Purified OA-519 was digested with *Staphylococcus aureus* V8 protease, which cleaves C-terminally to glutamic residues, and resultant peptides were resolved by reversed-phase HPLC and subjected to microsequencing (6). Additional informative sequence was obtained when OA-519 underwent limited proteolysis by *S. aureus* V8 protease to produce two large peptides of 150 and 134 kDa. After digestion, the peptides were resolved by SDS/PAGE and transferred to poly(vinylidene difluoride) membrane for microsequencing (7, 8).

Enzyme Activity. FAS enzyme activity was demonstrated with purified protein by (*i*) incorporation of [2-^{14}C]malonyl-CoA into fatty acids with subsequent TLC analysis and (*ii*) incorporation of unlabeled precursors into fatty acids for GLC analysis. For analysis of enzyme activity by TLC, 4.2 μg of OA-519 (211 μg/ml, purified without protease inhibitors) in 20 μl of purification buffer (20 mM Tris·HCl/270 mM

Abbreviation: FAS, fatty acid synthase.

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KCl/1 mM EDTA/1 mM dithiothreitol, pH 7.5 at 25°C) was added to a reaction mixture to give 500 μM NADPH, 166.6 μM acetyl-CoA, and 100 mM KCl, pH 6.6 at 25°C in a total volume of 150 μl. Addition of 0.1 μCi (37 kBq) of [2-^{14}C]malonyl-CoA and 25 nmol of nonradioactive malonyl-CoA was followed by incubation at 37°C for 20 min. The fatty acids were extracted and methyl esters were prepared. ^{14}C-labeled fatty acid methyl esters were separated and identified by reversed-phase TLC on Analtech plates with a mobile phase of chloroform/methanol/water, 5:15:3 (vol/vol). Nonradioactive standards (Matreya, Pleasant Gap, PA) were visualized with cyclodextrin spray developed in iodine vapor. ^{14}C-labeled fatty acid methyl esters were detected and quantitated with a Bioscan (Washington, DC) system 2000 imaging scanner with autochanger 3000. For GLC analysis, the reaction was run as above except for a 10-fold increase in enzyme and substrates and no ^{14}C-labeled substrates. The fatty acids were extracted, methyl esters were prepared, and fatty acid methyl esters were analyzed on a Hewlett–Packard 5890A gas chromatograph with a flame ionization detector (9). Cellular fatty acids were identified by comparing their retention times to those of known straight-chain saturated fatty acid methyl ester standards.

For determination of FAS activity in cell lines, 2×10^5 cells were plated in triplicate in standard 24-well plates, grown overnight, scraped, pelleted, and frozen at −80°C. After hypotonic lysis of frozen pellets in 1 mM dithiothreitol/1 mM EDTA/20 mM Tris·HCl, pH 7.5 at 25°C, 20 μl of the lysate was added to a reaction mixture with subsequent addition of [2-^{14}C]malonyl-CoA. The reaction mixture was incubated at 37°C for 20 min and stopped by the addition of 1 ml of chloroform/methanol, 1:1. After a 30-min extraction, the lipids were dried under N_2; twice extracted in 400 μl of hydrated 1-butanol/water, 1:1; pooled; washed; dried under N_2; and assayed for ^{14}C by scintillation counting.

Immunohistochemistry. Anti-OA-519 peptide antibody, with or without preincubation with a 33-fold molar excess of purified OA-519 for 2 hr at 4°C, was applied to sections of formalin-fixed paraffin-embedded human breast cancer tissue. This treatment abolished OA-519 reactivity in the cancer cells. In both instances, anti-OA-519 peptide reactivity was detected by standard biotin–avidin immunohistochemistry with aminoethylcarbazole as the chromagen.

Measurement of Endogenous Acylglycerol Synthesis. Endogenously synthesized acylglycerols and their incorporation into phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol were measured as follows. For each cell type, 2×10^5 cells were plated in triplicate for nonpolar and polar lipid analysis. After overnight growth, each well of cells was incubated with 1 μCi of [U-^{14}C]acetate for 2 hr. ^{14}C-labeled lipids were extracted as above and dried under N_2. For analysis of nonpolar lipids, samples were resuspended in 25 μl of chloroform, spotted on silica gel N-HR (Brinkmann), and chromatographed in hexane/ethyl ether/acetic acid, 90:10:1. Cholesterol, palmitic acid, tripalmitin, and cholesterol palmitate (Matreya) (50 μg of each) were run as controls. For analysis of polar lipids, samples were spotted on silica gel 6A (Whatman) and chromatographed in chloroform/methanol/water, 65:25:4. Cholesterol, phosphatidylethanolamine, phosphatidylcholine, and lysophosphatidylcholine (Matreya) (50 μg of each) were run as controls. ^{14}C-labeled lipids were detected and quantified with Bioscan system 2000 imaging scanner with autochanger 3000. Nonradioactive standards were visualized with rhodamine spray under ultraviolet light. The data represent means of triplicate measurements ± SEM after subtraction of background from lanes run without sample.

Cell Growth Inhibition Assays. For each cell line, 5×10^3 cells were plated in RPMI 1640 with 10% fetal bovine serum in 96-well plates (Corning). After overnight incubation, cerulenin in dimethyl sulfoxide was added to achieve serial dilutions from 40 μg/ml to 0.08 μg/ml in quadruplicate. Controls included cells incubated without cerulenin, cells incubated with vehicle only, and blank wells with medium only. After 24 hr, cells were stained with crystal violet, solubilized in 1% SDS, and read at 570 nm. IC_{50} measurements were determined by linear regression. The IC_{50} data represent means of quadruplicate measurements from at least two experiments ± SEM after subtraction of background from the blank wells run with medium only. For rescue studies, fatty acids were solubilized in dimethyl sulfoxide and added to the medium to achieve the desired concentration 6 hr prior to addition of cerulenin; controls consisted of cells incubated with dimethyl sulfoxide alone.

RESULTS

Purification and Characterization of OA-519. Polyclonal antibodies which detected a prognostically significant molecule in human breast carcinomas (1) were used to follow purification of the prognostic molecule from cytosolic extracts of ZR-75-1 cells, an estrogen and progesterone receptor-positive human breast carcinoma cell line. ZR-75-1 was chosen because its immunologic reactivity was similar to that of primary human breast carcinomas. The prognostic antibodies (anti-OA-519) identified an ≈270-kDa protein (Fig. 1). Sequential size-exclusion and ion-exchange chromatography of hypotonic cell lysates yielded substantially enriched preparations of the 270-kDa protein. The isolated 270-kDa protein specifically and completely inhibited immunostaining of human breast carcinoma tissue by the prognostic anti-OA-519 antibodies (Fig. 2). This indicated immunologic identity between the prognostic molecule detected in sections of human breast carcinoma and the 270-kDa molecule purified from ZR-75-1 cells.

OA-519 is a functional FAS. Two peptides isolated from digests of OA-519, His-Ala-Val-Val-Leu-Glu and Leu-Gln-Gln-His-Asp-Val-Ala-Gln-Glu-Gln-Trp-Xoa-Pro, showed near sequence identity with residues 681–686 and 1208–1220 of rat FAS, also an ≈270-kDa protein (10, 11). Functionally, purified OA-519 behaved like mammalian FAS (12) by oxidizing NADPH in a malonyl-CoA-dependent fashion and by synthesizing fatty acids from acetyl-CoA primer and malonyl-CoA and NADPH substrates. Product analysis by reversed-phase TLC (Fig. 3A) and GLC (9) (data not shown) demonstrated that ≈80% of the total fatty acid production by OA-519 is palmitate, with stearate and myristate each comprising about 10%. This profile of synthesized fatty acids is similar to that reported for human liver FAS (13). In contrast,

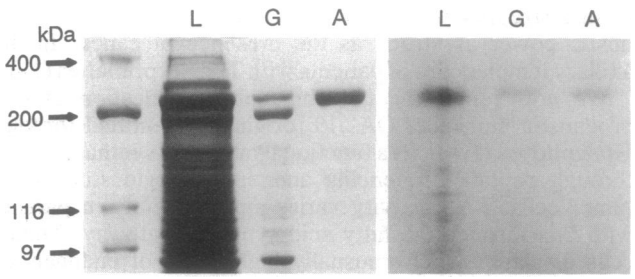

FIG. 1. OA-519 purified from ZR-75-1 cells is an ≈270-kDa polypeptide. (*Left*) OA-519 was purified from a hypotonic lysate of ZR-75-1 cells (lane L) by successive Sephacryl S-200 gel exclusion chromatography (lane G) and HPLC Mono-Q HR 5/5 anion-exchange chromatography (lane A). This resulted in an ≈270-kDa protein that was >95% pure by Coomassie staining after SDS PAGE. (*Right*) A corresponding Western blot with the polyclonal anti-OA-519 peptide antibody (3) shows reactivity with the purified 270-kDa protein.

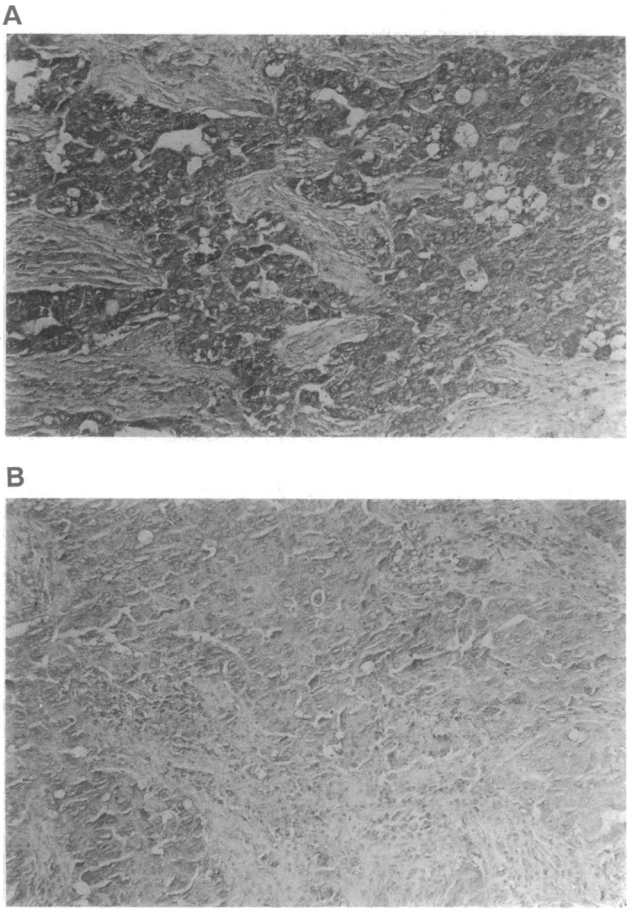

FIG. 2. Purified OA-519 absorbs the immunoreactivity of prognostic anti-OA-519 antibodies with breast carcinoma tissue. (*A*) Section of formalin-fixed, paraffin-embedded human infiltrating duct carcinoma of the breast was immunohistochemically stained with anti-OA-519 peptide antibodies. Note the cytoplasmic localization of OA-519 in the cancer cells. (*B*) Section was stained with anti-OA-519 peptide antibody that had been preincubated with a 33-fold molar excess of purified OA-519 for 2 hr at 4°C. This treatment abolished OA-519 reactivity in the cancer cells. In both instances, anti-OA-519 peptide reactivity was detected by standard biotin–avidin immunohistochemistry with aminoethylcarbazole as the chromagen. (×300.)

analysis of lipids from intact lactating breast epithelial cells reveals predominantly C_{10}–C_{14} fatty acids because a separate chain-terminating enzyme, medium-chain S-acyl FAS thioester hydrolase, releases medium-chain fatty acids from the 4′-phosphopantetheine thiol of FAS (14). The specific activity of OA-519 is 624 nmol of NADPH oxidized per min per mg of protein, which is similar to those reported for FAS purified from a variety of sources, including human liver (15).

Levels of Endogenous Fatty Acid Synthesis Parallel FAS Levels. FAS activity paralleled the overall activity of endogenous fatty acid synthesis in a group of established human breast carcinoma cell lines—SKBR3, ZR-75-1, MCF-7, and MCF-7a (doxorubicin-resistant)—and normal human fibroblasts (HS-27) whose FAS levels span a broad range. Fig. 3*B* shows the relationship between [^{14}C]acetate incorporation into acylglycerols and the FAS levels.

Relative Dependence of Cancer Cells on Endogenous Fatty Acid Synthesis. Not only do cancer cell FAS levels seem to parallel fatty acid synthesis, but cancer cells with higher levels of endogenous fatty acid synthesis appear to depend upon endogenous fatty acid synthesis as well. Cerulenin, a potent noncompetitive inhibitor of FAS, is known to bind covalently to the active site of the condensing enzyme region, inactivating a key enzymatic step in fatty acid synthesis (16).

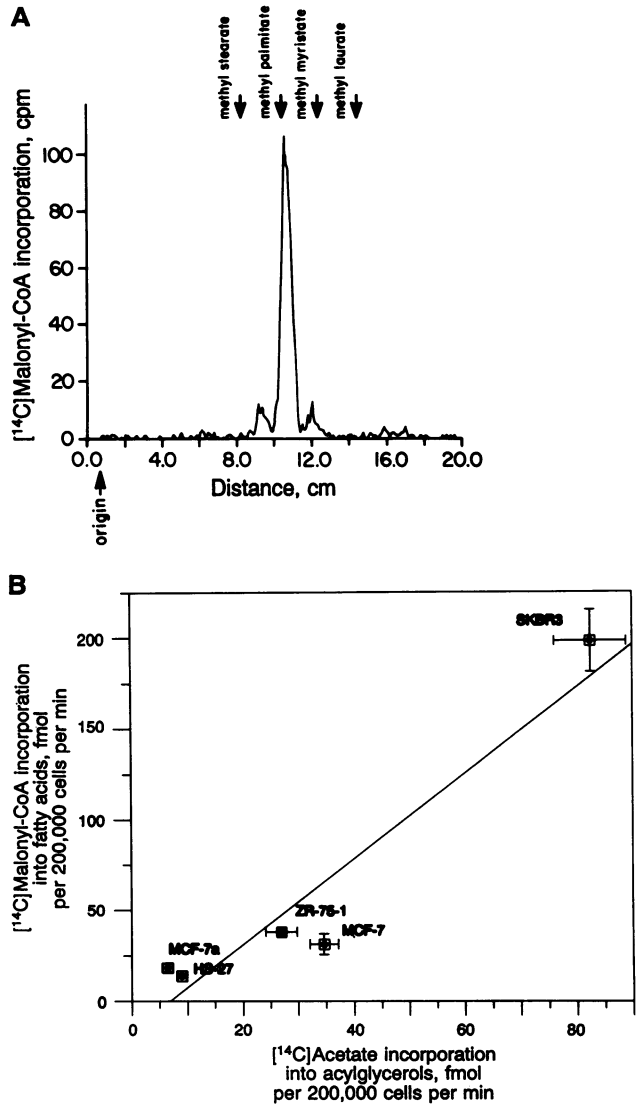

FIG. 3. Purified OA-519 synthesizes fatty acids and FAS activity parallels acylglycerol synthesis. (*A*) OA-519 catalyzes NADPH-dependent synthesis of predominantly C_{16} fatty acid with slight amounts of C_{14} and C_{18} fatty acids from acetyl-CoA and malonyl-CoA. Purified enzyme was incubated with [2-^{14}C]malonyl-CoA, acetyl-CoA, and NADPH, and products were analyzed by reversed-phase TLC. (*B*) FAS activity measured in a group of human carcinoma cell lines and normal fibroblasts parallels the rate of acylglycerol synthesis. FAS activity was quantitated by incorporation of [2-^{14}C]malonyl-CoA into fatty acids. Endogenous-pathway activity was measured by [U-^{14}C]acetate incorporation into acylglycerols in intact cells. Over the course of the experiment, phosphatidylcholine was the predominant acylglycerol with variable amounts of triacylglycerol. Thus, the endogenous fatty acid synthesis pathway is intact in these cell lines, and the level of FAS activity corresponds to the overall pathway activity. Data represent means of triplicate measurements ± SEM.

While cerulenin has been reported to exhibit other effects, these occur in nonmammalian systems and at cerulenin concentrations significantly higher than those which inhibit cancer cell growth (17–19). Recent data further suggest that cerulenin may not inhibit myristoylation of proteins (20). Fig. 4*A* shows that cerulenin inhibited acylglycerol synthesis in a dose-dependent manner in both the breast cancer cell lines and human fibroblasts. Six hours of exposure to cerulenin at 10 μg/ml reduced acylglycerol synthesis at least 50%; parallel experiments showed no effect on cholesterol synthesis (data not shown). In breast cancer cell lines, the IC_{50} values for

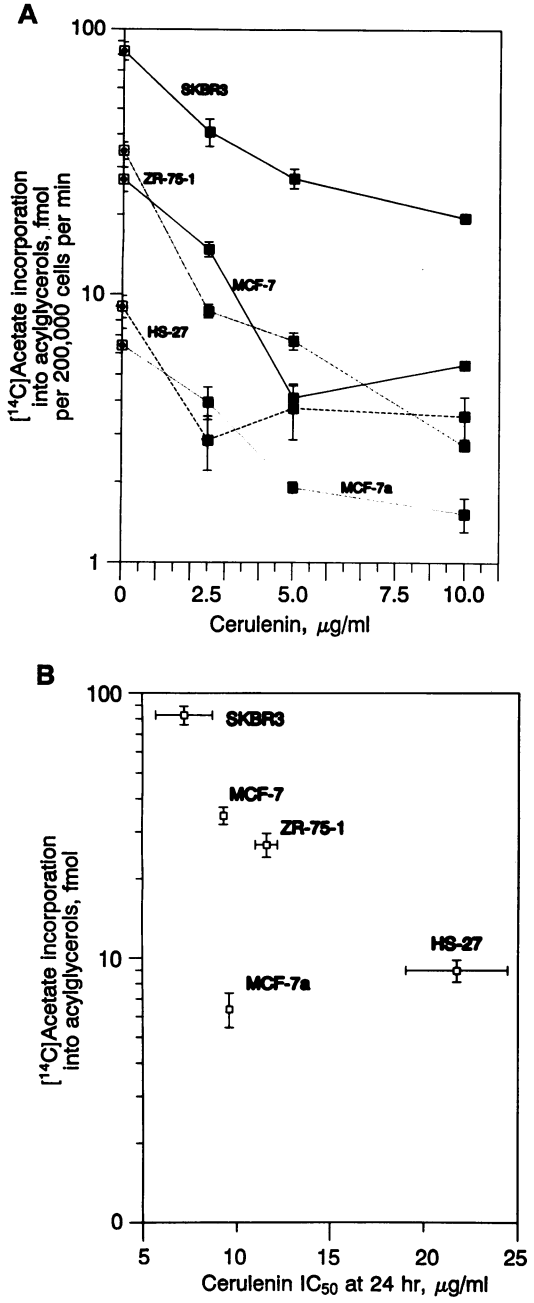

FIG. 4. Cerulenin causes dose-dependent inhibition of [U-^{14}C]acetate incorporation into acylglycerols and inhibits the growth of cancer cells concomitantly with the rate of acylglycerol synthesis. (*A*) Incorporation of [U-^{14}C]acetate into acylglycerols was consistently and significantly inhibited by cerulenin in a dose-related manner. Six hours of cerulenin exposure resulted in at least a 50% reduction in acylglycerol synthesis. Data represent means of triplicate measurements ± SEM. (*B*) Cerulenin IC_{50} parallels the activity of endogenous fatty acid synthesis. IC_{50} measurements were determined by linear regression and represent means of quadruplicate measurements from at least two experiments ± SEM after subtraction of background from wells run with medium only. Acylglycerol determinations are from Fig. 3*B*.

cerulenin-mediated growth inhibition paralleled the levels of endogenous fatty acid synthesis (Fig. 4*B*). The sole exception occurred in MCF-7a cells, which showed unexplained increased sensitivity to cerulenin. These data suggest a relative dependence of some cancer cells on endogenous fatty acid synthesis.

Cerulenin-Induced Growth Inhibition Is Due to Inhibition of Endogenous Fatty Acid Synthesis. Supraphysiologic amounts of fatty acid synthesis end product can overcome growth inhibition caused by FAS blockade. Addition of 14 μM palmitate in dimethyl sulfoxide significantly ameliorated the growth inhibitory effects of cerulenin at up to 5 μg/ml (Table 1). At higher cerulenin concentrations, palmitate only partially reversed the growth inhibition. These data indicate that at low cerulenin concentrations, growth inhibition appears to be due solely to FAS inhibition, since the inhibition is reversed by fatty acids. The partial inhibition seen at higher concentrations may reflect involvement of a secondary mechanism other than FAS inhibition; alternatively, it may signify the inability to achieve palmitate concentrations sufficient to rescue the cells, since high concentrations of free palmitate are themselves toxic. Indeed, palmitate concentrations > 14 μM in this system were growth-inhibitory in the absence of cerulenin. There appears to be a degree of lipid specificity, since exogenous oleate at up to 50 μM was unable to reverse the effects of cerulenin.

Table 1. Exogenous palmitate reverses cerulenin growth inhibition of ZR-75-1 cells

Cerulenin, μg/ml	Cell growth, % control	
	+ Palmitate	− Palmitate
0.23	104.4 ± 2.5	95.0 ± 3.5
0.63	117.9 ± 8.5	87.5 ± 3.4
2.50	104.6 ± 5.5	63.5 ± 2.7
5.00	71.4 ± 8.5	40.4 ± 1.6

Cells (5000 per well) were plated in 96-well plates in 100 μl of RPMI 1640 medium with 10% fetal bovine serum. After overnight incubation, 100 μl of fresh medium was added supplemented with dimethyl sulfoxide (to achieve 39 mM) alone as a control or 14 μM palmitate added from a 5 mM stock solution in dimethyl sulfoxide. After 5 hr of incubation, cerulenin in dimethyl sulfoxide was added as in Fig. 4*B*, to achieve the above concentrations. Cells were stained after 48 hr with crystal violet, solubilized in 1% SDS, and read at 570 nm.

DISCUSSION

The key findings of this paper are the identification of a prognostic molecule in cancer as FAS, the observation that FAS levels reflect levels of endogenous fatty acid synthesis, and the recognition that inhibition of fatty acid synthesis can inhibit the growth of neoplastic cells even when physiologic amounts of exogenous fatty acids are available. Clinical observation lies at the root of these data, suggesting that they will be relevant to human cancer. To date, retrospective diagnostic studies in >400 cases of clinical breast cancer specimens link high levels of FAS (OA-519) with poor prognosis; FAS-positive patients showed shortened disease-free intervals or overall survivals, even in node-negative disease (1–3, 21–24). Preliminary clinical reports also found associations between high levels of FAS and worsened prognosis in adenocarcinomas of the prostate (25–27) and colon (28).

The present studies show that OA-519 is a FAS by structural and enzymatic criteria. The increased FAS protein is accompanied by increased mRNA levels in cancer cells with no evidence of gene amplification (unpublished data), and is likely to occur in the context of increased endogenous fatty acid synthesis *in vivo* as well as in cell lines. Mammalian FAS is a homodimer of 270-kDa monomers which catalyzes seven distinct enzymatic reactions to synthesize fatty acids from acetyl-CoA and malonyl-CoA in the presence of NADPH (12). FAS is one of four key steps of the fatty acid synthetic pathway in humans: acetyl-CoA carboxylase, the rate-limiting enzyme, synthesizes malonyl-CoA; both malic enzyme and the hexose monophosphate shunt produce NADPH; and citrate lyase synthesizes acetyl-CoA. When a normal diet is consumed, this pathway is highly downregu-

lated in liver and adipose tissue, the two main sites of fatty acid synthesis.

Cerulenin—a specific, potent, noncompetitive inhibitor of FAS—administered to human cancer cell lines *in vitro* significantly inhibits the growth of cells, roughly commensurate with their levels of fatty acid synthesis. In contrast, cells with minimal levels of endogenous fatty acid synthesis, which resemble normal tissues *in vivo* (5), are relatively insensitive to FAS inhibition. SKBR3 cells, in which FAS constitutes >20% of cytosolic protein (29), are exquisitely sensitive to FAS inhibition and thus appear dependent on endogenous fatty acid synthesis. These data suggest that fatty acid synthesis is required by some cancers for their growth, despite the presence of physiologic levels of exogenous fatty acids. In tissue culture, supraphysiologic concentrations of palmitate, the end product of FAS, significantly reversed cerulenin-mediated growth inhibition at cerulenin concentrations up to 5 μg/ml. There are three implications of these data. (*i*) The ability to reverse cerulenin effects on *in vitro* tumor growth by palimitate confirms that the mechanism of cerulenin-mediated growth inhibition is through inhibition of FAS. (*ii*) That only supraphysiologic concentrations of free palmitate uncomplexed to albumin [≈10 times that encountered *in vivo* (30, 31)] were effective implies that tumor FAS inhibition will result in tumor growth inhibition *in vivo*. (*iii*) The relative absence of endogenous fatty acid synthesis in normal tissues *in vivo* (5) suggests that fatty acid synthesis represents an exploitable metabolic difference between normal tissues and some cancer cells which may in turn result in a significant therapeutic index. Interestingly, the ability to measure FAS (OA-519) in clinical samples may prove useful in predicting the sensitivity of individual patients to potential therapies based on inhibition of fatty acid synthesis.

The relationship between abnormal fatty acid synthesis and an aggressive tumor phenotype remains unknown. Fatty acids are implicated in tumorigenesis (32), as trophic factors (33, 34), in receptor-mediated signal transduction (35), and as modulators of tumor cell adhesion (36). None of these areas has been investigated from the standpoint of endogenous fatty acid synthesis. It is possible that tumor cells elaborate lipid mediators which may act as autocrine or paracrine factors, ultimately affecting tumor behavior. Similarly, the role of increased endogenous fatty acid biosynthesis in tumorigenesis is unknown. It does appear, however, that for unclear reasons, certain tumors have an apparently obligatory requirement for endogenous fatty acid biosynthesis whereas normal cells do not, leading to the suggestion that inhibition of fatty acid biosynthesis may be a fruitful target for chemotherapy development.

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