Increased Fatty Acid Synthase is a Therapeutic Target in Mesothelioma

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ABSTRACT
Many common human cancer tissues express high levels of fatty acid synthase (FAS), the primary enzyme for the synthesis of fatty acids, and the differential expression of FAS between normal and neoplastic tissues has led to the consideration of FAS as a target for anticancer therapy. To investigate the potential of targeting FAS for the treatment of pleural mesothelioma, we first determined whether FAS is overexpressed in human mesothelioma. By immunohistochemistry, we found 22 of 30 human mesothelioma tissue samples tested to express significantly increased levels of FAS compared with normal tissues, including mesothelium. To further explore FAS as a therapeutic target in mesothelioma, we established a nude mouse xenograft model for human mesothelioma using the H-Meso cell line. The i.p. xenografts of this cell line have high levels of FAS expression and fatty acid synthesis pathway activity and grow along mesothelial surfaces in a manner similar to the growth pattern of human mesothelioma. Growth of these tumor xenografts was essentially abolished in mice treated with weekly i.p. injections of C75, a synthetic, small molecule inhibitor of FAS, at levels that resulted in no significant systemic toxicity except for reversible weight loss. These results suggest that FAS may be an effective target for pharmacological therapy in a high proportion of human mesotheliomas.

INTRODUCTION
Mesothelioma is an uncommon malignant neoplasm derived from mesothelial cells that is most often caused by exposure to asbestos. The incidence of mesothelioma in the United States is approximately 2000–3000 cases per year, and this incidence is increasing steadily (1). Mesothelioma, although slow-growing, spreads diffusely along the mesothelial surfaces of the pleura, peritoneum, or pericardium and is thus difficult to treat by surgical resection. Furthermore, mesothelioma generally responds poorly to radiation therapy or conventional chemotherapy (2, 3), and the 3-year survival for this cancer is less than 5% (4). Thus, there is a need for novel therapeutic approaches to treat this cancer.

One therapeutic target that has not previously been considered for mesothelioma treatment is the pathway for the endogenous synthesis of fatty acid. FAS, the principal enzyme in this pathway, is highly expressed in many common human tumors (5). This is in contrast to normal human tissues, in which FAS is down-regulated due to our ingestion of high levels of dietary fatty acids. The preferential expression of FAS in cancer cells has recently been exploited as a target for anticancer chemotherapy. For example, significant antitumor activity against human breast (6) and prostate (7) xenografts that express high levels of FAS has been achieved using a novel pharmacological inhibitor of FAS, C75. The present study was undertaken to assess the feasibility of treating mesothelioma with pharmacological inhibitors of FAS. For this purpose, we first evaluated a series of mesothelioma tumors for FAS expression using immunohistochemistry. Encouraged by a finding that mesotheliomas frequently overexpress this enzyme, we then developed a nude mouse xenograft model of a FAS-overexpressing human mesothelioma cell line to test the hypothesis that FAS inhibition will exhibit antineoplastic activity.

MATERIALS AND METHODS
Mesothelioma Tissues and Immunohistochemistry for FAS. Paraffin-embedded samples from 30 cases of mesothelioma were obtained from surgical pathology, autopsy, and consultation files at the Johns Hopkins Bayview Medical Center. All patients had a previous history of exposure to asbestos. Immunohistochemistry for FAS was performed on tissue sections using a mouse monoclonal antihuman FAS antibody (8) at 1:2000 on the Dako Immunostainer and the LSAB2 detection kit. FAS expression was evaluated independently and semiquantitatively for both intensity and percentage area of tumor stained by two pathologists (E. G. and F. P. K.). The fraction of positive cells was scored using a four-tiered scale (<10% = 1, 11–50% = 2, 51–80% = 3, and >80% = 4), and staining intensity was scored from 0 to 3+ as described previously (9). The overall FAS score was the product of both the intensity and fraction of positive cells score. Cases with an overall score of ≤3 were

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3 The abbreviations used are: FAS, fatty acid synthase; CPT-I, carnitine palmitoyltransferase I.
considered negative. Adjacent lung and soft tissues and mesothelium were also stained for FAS, and the same grading system was used for assessment.

**Fatty Acid Synthesis Inhibitor.** C75 was synthesized in the laboratory of C. A. Townsend in the Department of Chemistry (Johns Hopkins University, Baltimore, MD; Ref. 10). C75 is an \( \alpha \)-methylene-\( \gamma \)-butyrolactone with an 8-hydrocarbon side chain. C75 is a slow-binding inhibitor of mammalian FAS.

**Cell Lines, Culture Conditions, and Metabolic Labeling.** The human mesothelioma cell line H-Meso (11) was maintained in DMEM with 10% fetal bovine serum. Cells were screened periodically for *Mycoplasma* contamination (Gen-probe). All inhibitors were diluted from stock 5 mg/ml solutions in DMSO. For fatty acid synthesis activity determinations, 5 \( \times \) 10^4 cells/well in 24-well plates were pulse labeled with [U-\( ^{14} \)C]acetate after exposure to drug or vehicle in triplicate for each concentration. Lipids were then extracted and quantified as described previously (12). Pathway activity was determined after 2 h of inhibitor exposure.

**Xenograft Studies.** The i.p. xenografts of human mesothelioma cell line H-Meso in nu/nu female mice (Harlan) were used to study the antitumor effects of C75 in *vivo*. All animal experiments complied with institutional animal care guidelines. Approximately 10^7 (0.1 ml packed) H-Meso cells were xenografted from culture in DMEM supplemented with 10% fetal bovine serum.

To compare fatty acid synthesis activity in tumor with that in normal tissue, tumor xenografts and liver tissue from three mice were *ex vivo* labeled with [U-\( ^{14} \)C]acetate and lipids were extracted and counted as described previously (12). In a parallel experiment, to study FAS expression *in vivo*, tumor and normal tissues from a xenograft were fixed in neutral-buffered formalin and processed for routine histology, and immunohistochemistry for FAS was performed as described above. To test FAS inhibitory treatment on this mesothelioma xenograft model, we began i.p. C75 treatment 2 weeks after tumor inoculation. Six mice were treated i.p. with an initial dose of 40 mg/kg C75 in 0.1 ml of RPMI 1640, followed by weekly doses of 30 mg/kg C75 in 0.1 ml of RPMI 1640. Five mice were treated with vehicle control. Dosing was based on a single dose LD_{50} determination of 40 mg/kg in BALB/c mice; a dose of 30 mg/kg has been well tolerated in outbred nude mice. The experiment was terminated after 1 month because the control group underwent a 15% increase in weight due in part to a combination of tumor and malignant ascites.

**RESULTS**

By immunohistochemistry, 22 of the 30 (73%) mesothelioma cases were scored as FAS positive, whereas 8 (27%) were scored negative. Of the 22 FAS-positive cases, 13 (43%) showed high levels of expression defined as an overall score of \( \geq 6 \), whereas 9 (30%) had moderate levels of FAS expression, with scores of 4–5 (Table 1). FAS expression is not limited to any histological subtype because epithelial, mixed, and sarcomatous mesotheliomas in our series all displayed similar levels of FAS expression. Fig. 1 illustrates immunohistochemical localization of FAS in clinical cases of malignant mesothelioma. Both epithelial (Fig. 1, A and B) and sarcomatoid (Fig. 1C) mesotheliomas show intense cytoplasmic reactivity, whereas histologically benign mesothelial cells (Fig. 1D) have undetectable levels of FAS. Variable FAS expression was noted in normal adipose tissue and in reactive type II pneumocytes adjacent to the tumors in the lung (data not shown). In comparison with carcinomas, high levels of FAS appear to be more common in mesothelioma than in breast cancer (8, 13, 14) but less frequent than in colon cancer (15), where high levels of expression are ubiquitous.

Based on the high levels of FAS expression in human mesothelioma, we chose the H-Meso human mesothelioma cell line as a model system both for its high level of endogenous fatty acid synthesis *in vitro* and *in vivo* and for its ability to grow in athymic nude mice, recapitulating human disease. One month after i.p. inoculation of H-Meso cells, multiple tumors stud the internal surfaces of the abdominal peritoneum, bowel, and mesentery, similar to the disease in humans (Fig. 2, A and C). In addition, the xenograft expresses high levels of FAS by immunohistochemistry similar to clinical tumor tissue (Fig. 2, C and D). These high levels of FAS expression by immunohistochemistry are reflective of high levels of endogenous fatty acid synthesis. The H-Meso xenograft has over a 15-fold increased fatty acid synthesis activity compared with the liver as measured by *ex vivo* [U-\( ^{14} \)C]acetate incorporation into total lipids (Fig. 3A). This level of fatty acid synthesis *in vitro* for mesothelioma is even higher than that observed previously in breast and prostate cancer cells (data not shown).

To establish our ability to pharmacologically inhibit FAS, we treated H-Meso cultures with cerulenin, a broad spectrum inhibitor of type I and II FAS (16, 17), and C75, a novel, chemically stable, inhibitor of FAS (10). Both C75 and cerulenin inhibit fatty acid synthesis in H-Meso cells (by approximately 30% and 70%, respectively) *in vitro* as measured by [U-\( ^{14} \)C]acetate incorporation into lipids (Fig. 3B). This level of fatty acid synthesis inhibition in other human cancer cells results in significant cytotoxicity *in vitro* (6). However, we were unable to demonstrate *in vitro* cytotoxicity of these agents on H-Meso cells using clonogenic assays due to the poor colony-forming ability of the H-Meso cells. Hence, C75 was tested for antitumor activity directly in H-Meso xenografts.

### Table 1 FAS expression in subtypes of malignant mesothelioma

<table>
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<tr>
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<th>Epithelial mesothelioma</th>
<th>Mixed mesothelioma</th>
<th>Sarcomatous mesothelioma</th>
<th>Totals</th>
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<tr>
<td>High FAS expression</td>
<td>9 (30%)</td>
<td>2 (7%)</td>
<td>2 (7%)</td>
<td>13 (43%)</td>
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<tr>
<td>Low FAS expression</td>
<td>7 (23%)</td>
<td>1 (3%)</td>
<td>1 (3%)</td>
<td>9 (30%)</td>
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<tr>
<td>Negative FAS expression</td>
<td>5 (17%)</td>
<td>1 (3%)</td>
<td>2 (7%)</td>
<td>8 (27%)</td>
</tr>
<tr>
<td>Totals</td>
<td>21 (70%)</td>
<td>4 (13%)</td>
<td>5 (17%)</td>
<td>30 (100%)</td>
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To test the effect of C75 on H-Meso xenografts, tumor-bearing mice were treated with C75 beginning 2 weeks after tumor inoculation. By 1 month, all control animals (Fig. 1A) had widespread studding of the mesentery by tumor nodules, consisting of mesothelioma cell clusters ranging in size from <1–5 mm. One untreated animal also had 0.8 ml of malignant ascites. Among the treated animals, none had ascites, two had no evidence of tumor, and three had single tumor nodules ranging in mass from 0.1–1.1 g. None of the treated animals developed the multiple tumor seeding of the abdomen seen in control animals. A representative C75-treated animal with no gross or microscopic tumor is shown in Fig. 2.

Similar to the previous experience of treating breast and prostate xenografts (6, 7), transient reversible weight loss was noted. Histological analysis of normal host tissues failed to show evidence for significant acute or chronic toxicity other than a slight increase in fibrous adhesions in the abdominal cavity of treated mice. One animal in the C75 treatment group died within 24 h of the first dose; no deaths occurred with subsequent treatments. It could not be determined whether this death was due to the effect of the drug or to other causes.

DISCUSSION

Our finding of FAS overexpression in mesothelioma tissues parallels observations of increased FAS expression in a variety of common human cancers including breast, prostate,
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rather than the reduction of fatty acid synthesis likely due to accumulation of the FAS substrate, malonyl-CoA (18, 19). The link between apoptosis and FAS inhibition is through inhibition of macromolecular synthesis and apoptosis synthesis at the FAS step ultimately leads to cancer cell death cancers has not yet been elucidated, inhibition of fatty acid ical advantage of endogenous fatty acid synthesis by human ical preparations.

Fig. 3 The H-Meso xenograft has high levels of fatty acid synthesis inhibited by C75 and cerulenin. A, fatty acid synthesis is about 15-fold higher in the H-Meso xenograft than in the liver from the same animals as measured by ex vivo [U-14C]acetate incorporation into lipids (n = 3; P < 0.004, t test). Within 2 h of drug administration, C75 (10 \( \mu \)g/ml) and cerulenin significantly inhibit fatty acid synthesis in H-Meso cells in vitro (8). The results of Student’s t test comparing treated cells with control, in triplicate, are as follows: 5 \( \mu \)g/ml C75, P = 0.062; 10 \( \mu \)g/ml C75, P = 0.051; 5 \( \mu \)g/ml cerulenin, P = 0.003; and 10 \( \mu \)g/ml cerulenin, P = 0.001 (GraphPad Prism Software). Error bars, SE.

Although malonyl-CoA has only recently been implicated in apoptosis, this is not surprising given its key role as a regulator of intermediary metabolism. In addition to its role as a substrate for fatty acid synthesis, malonyl-CoA is a potent inhibitor of CPT-I, the rate-limiting enzyme in fatty acid oxidation (22). CPT-I is located on the outer membrane of the mitochondria, where it esterifies long-chain acyl-CoAs to carnitine, allowing their entry into the mitochondria for oxidation. Physiologically, malonyl-CoA inhibits CPT-I during fatty acid synthesis to prevent the concomitant oxidation of newly synthesized fatty acid. Inhibition of CPT-I with etomoxir has produced apoptosis in vitro (23), and the recent association of CPT-I with Bcl-2 on the mitochondrial surface supports this observation (24). In our studies, inhibition of acetyl-CoA carboxylase with 5-(tetradecyloxy)-2-furoic acid prevented the rise in malonyl-CoA after FAS inhibition and significantly reduced the apoptotic response (6).

Most importantly, our studies suggest that C75 could have significant anticancer activity in a significant proportion of mesothelioma patients. The possibility of treating mesothelioma with pharmacological inhibitors of FAS has great significance because human mesothelioma remains largely refractory to conventional treatment. Treatment of H-Meso xenograft mice with the FAS inhibitor C75 led to significant reductions in mesothelioma tumor burden in all treated animals and also altered the growth pattern of the tumor from diffuse abdominal involvement to more localized tumor masses. Whereas direct i.p. delivery of the C75 to the tumor xenografts may increase the efficacy of this agent, it is also notable that systemic C75 has been found to be effective in the treatment of s.c. human tumor xenografts (6, 7). Additional experiments are need to determine whether local delivery, systemic delivery, or a combination of drug delivery modalities will be most effective for treatment of mesothelioma by inhibitors of FAS.

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REFERENCES

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