Fatty Acid Synthase Gene Is Up-regulated by Hypoxia via Activation of Akt and Sterol Regulatory Element Binding Protein-1

Eiji Furuta,1 Sudha K. Pai,1 Rui Zhan,1 Sucharita Bandyopadhyay,2 Misako Watabe,1 Yin-Yuan Mo,1 Shigeru Hirota,3 Sadahiro Hosobe,1 Taisei Tsukada,1 Kunio Miura,3 Shuichi Kamada,3 Ken Saito,1 Megumi Iizumi,1 Wen Liu,1 Johan Ericsson,4 and Kounosuke Watabe1

1Department of Medical Microbiology, Immunology, and Cell Biology, Southern Illinois University School of Medicine, Springfield, Illinois; 2Department of Developmental Biology, Stanford University, School of Medicine, Stanford, California; 3Akita Red Cross Hospital, Akita, Japan; and 4Ludwig Institute for Cancer Research, Uppsala University, Biomedical Center, Uppsala, Sweden

Abstract
The fatty acid synthase (FAS) gene is significantly up-regulated in various types of cancers, and blocking the FAS expression results in apoptosis of tumor cells. Therefore, FAS is considered to be an attractive target for anticancer therapy. However, the molecular mechanism by which the FAS gene is up-regulated in tumor cells is poorly understood. We found that FAS was significantly up-regulated by hypoxia, which was also accompanied by reactive oxygen species (ROS) generation in human breast cancer cell lines. The FAS expression was also activated by H2O2, whereas N-acetyl-l-cystein, a ROS inhibitor, suppressed the expression. We also found that the hypoxia significantly up-regulated sterol regulatory–element binding protein (SREBP)-1, the major transcriptional regulator of the FAS gene, via phosphorylation of Akt followed by activation of hypoxia-inducible factor 1 (HIF1). Moreover, our results of reporter assay and chromatin immunoprecipitation analysis indicate that SREBP-1 strongly bound to the SREBP binding site/E-box sequence on the FAS promoter under hypoxia. In our xenograft mouse model, FAS was strongly expressed in the hypoxic regions of the tumor. In addition, our results of immunohistochemical analysis for human breast tumor specimens indicate that the expressions of both FAS and SREBP-1 were colocalized with hypoxic regions in the tumors. Furthermore, we found that hypoxia-induced chemoresistance to cyclophosphamide was partially blocked by a combination of FAS inhibitor and cyclophosphamide. Taken together, our results indicate that FAS gene is up-regulated by hypoxia via activation of the Akt and HIF1 followed by the induction of the SREBP-1 gene, and that hypoxia-induced chemoresistance is partly due to the up-regulation of FAS. [Cancer Res 2008;68(4):1003–11]

Introduction
Fatty acids have long chains of lipid-carboxylic acid and play pivotal roles in normal cellular function as well as in homeostasis of the whole body. They are the source of membrane components, such as phospholipids and glycolipids, and also provide precursors of critical signal molecules for proliferation and differentiation (1). Fatty acids also function as a medium to store energy in the adipose tissue (2). In general, normal adult cells acquire fatty acid mainly from dietary source and rarely use the pathway of de novo synthesis, except in the liver, adipose tissue, and lactating mammary gland (3, 4). In striking contrast, many human tumor cells synthesize fatty acids by using the de novo pathway as was originally observed by Medes et al. (5) >50 years ago. Fatty acid synthase (FAS) is the major enzyme of lipogenesis and catalyzes the condensation of acetyl-CoA and malonyl-CoA to produce palmitic acid in the presence of NADPH (6). The FAS gene is highly up-regulated in various types of human malignancies, although this gene is expressed at minimum or undetectable level in most normal tissues, and therefore, FAS overexpression is considered to be one of the most common molecular changes in cancer cells (7–11). Importantly, treatment of tumor cells with pharmacologic inhibitors of FAS leads to cell cycle arrest, followed by apoptosis of the tumor cells (12). We have previously shown that specific blocking of the FAS expression by using siRNA in breast cancer cells caused an accumulation of malonyl-CoA, which led to the inhibition of carnitine palmitoyl transferase-1 as well as up-regulation of ceramide (13). This was also followed by the induction of the proapoptotic genes, BNP3, TRAIL, and DAP kinase 2, which resulted in the apoptosis of the tumor cells (13). These observations suggest that FAS overexpression confers selective advantage to tumor cells by inhibiting apoptosis and promoting cell cycle progression.

How the FAS gene is up-regulated in cancer cells is an intriguing question, although it has been poorly understood. FAS was previously found to be up-regulated by several growth factors and their receptors including epidermal growth factor, Her2 (ErbB2/neu), and keratinocyte growth factor (14–16). Upon binding to each receptor, these factors transmit cellular signals such as mitogen-activated protein kinases (Erk1/2 MAPK2), Janus kinase (JNK), and phosphotyrosinolysiotol 3’-kinase (PI3K) followed by Akt activation (17–20). The activation of Akt is commonly observed in a variety of tumors and seems to contribute to the up-regulation of the lipogenic enzymes. On the other hand, a lack of expression or mutation of the tumor suppressor gene, PTEN, has been well established in various types of tumors, and PTEN blocks the function of Akt by counteracting PI3K through dephosphorylation of this enzyme (21). In fact, we have recently shown that the expression of PTEN has a significant inverse correlation with FAS expression in prostate cancer patients (22), and that the inhibition of the PTEN gene expression in vitro indeed led to the overexpression of FAS, although ectopic expression of PTEN significantly suppressed FAS (22). On the other hand, sterol regulatory–element binding protein (SREBP) have been known to be the key transcription factors to regulate lipogenic genes, and the FAS gene was indeed shown to be significantly activated by SREBP-1. Interestingly, Porstmann et al. (20) recently found that Akt stimulated the synthesis and nuclear localization of activated...
SREBP-1 followed by activation of the FAS gene. Therefore, PTEN-Akt pathway and the downstream effectors play a critical role in the FAS gene regulation in cancer cells. Furthermore, FAS expression has also been found to be controlled by tumor suppressors and oncogenes, including p53, p63, p73, and H-ras (18, 23). Therefore, overexpression of FAS is often associated with phenotypic changes of cell transformation that are induced either by oncogenes or tumor suppressors. These observations strongly suggest that FAS overexpression is actively contributing to the process of cell transformation rather than merely a consequence accompanied with the phenotypic changes.

Because FAS alone is not likely to cause cellular transformation but rather provides growth advantage to tumor cells by blocking proapoptotic genes, it is plausible that the FAS gene is up-regulated by tumor microenvironment, such as hypoxia, a hallmark of tumors, as a survival strategy of tumor cells. This assumption is supported by the observation that the Akt pathway is activated by a hypoxic condition, and that Akt is also capable of activating SREBP-1, which is a key transcription factor of the FAS gene. In this article, we tested a possibility of regulation of FAS expression by hypoxia in breast tumor cells both in vitro and in vivo and found that the FAS gene is indeed up-regulated by hypoxia through induction of Akt followed by activation of hypoxia-inducible factor 1 (HIF1) and SREBP-1. We also found that hypoxia-induced chemoresistance, which is a major clinical obstacle, can be partially overcome by a combination of a FAS inhibitor and a chemotherapeutic drug.

Materials and Methods

Cell culture and reagents. Human breast carcinoma cell lines, MX1, MCF7, MDA-MB231, and MDA-MB157 were purchased from American Type Culture Collection. The cells were maintained in RPMI 1640 supplemented with 10% FBS, streptomycin (100 µg/mL), penicillin (100 units/mL), and 250 mM dexamethasone (Sigma Chemical Co.) and grown at 37°C in a 5% CO2 atmosphere. The culture medium was replaced with DMEM after hypoxia treatment at 37°C in GasPak (BD Diagnostic Systems). Hydrogen peroxide, N-acetyl-cysteine (NAC), cerulenin, YC-1, and cyclophosphamide were purchased from Sigma Chemical Co. LYZ294002 was obtained from Calbiochen. The expression plasmid of Akt1, Addgene plasmid 9008, which expressed activated (myristoylated) form of Akt, was purchased from Addgene (24). siRNA for Akt and scramble sequence for control were obtained from Cell Signaling. siRNA for SREBP were purchased from Santa Cruz Biotechnology.

Western blot. The cells were collected and resuspended in lysis buffer (50 mM/L Tris-Cl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mM/L NaCl and 1 mM/L EDTA). The lysates were boiled for 5 min, resolved by SDS-PAGE on an 8% polyacrylamide gel, and blotted onto nitrocellulose membrane. The membranes were treated with antibodies against FAS (0.2 µg/mL; Immuno-biological Laboratories Co.), β-tubulin (1:1,000; Upstate Biotechnology), HIF1 (1:200; BD Bioscience), SREBP-1 (1:200; Santa Cruz Biotechnology), phospho-Akt (1:200; Ser473; Cell Signaling), Akt (1:200; Cell Signaling), and phospho-SREBP (0.5 µg/mL; ref. 25). The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies and visualized by ECL Plus system (Amer sham Life Sciences).

Quantitative real-time PCR. Total RNA was isolated from the cells and reverse transcribed. The cDNA was then amplified with a pair of forward and reverse primers for the following genes: FAS (5’-CAGCCCATCCAGA-TTGTGGCTACCATAGCG-3’ and 5’-CTCCTCAAGTGGCTGACGAG-3’), SREBP (5’-CCTGCTGTTACATAAGGCTGC-3’ and 5’-GACTGGTGCTTCATCATT-CAATG-3’), and β-actin (5’-TGAGACCCCTCAAGCAGCGG-3’ and 5’-CCTGATGAAGGCGACATGTGGTG-3’). PCR reactions were performed using DNA engineopticon2 system (MJ Research) and the DNA SYBR Green qPCR kit (Finnzyme Corp.). The thermal cycling conditions composed of an initial denaturation step at 95°C for 5 min followed by 40 cycles of PCR using the following profile: 94°C, 30 s; 63°C, 30 s; and 72°C, 30 s.

Reactive oxygen species assay. The cells were cultured in RPMI medium and fluorophore dichlorodihydrofluorescein diacetate (DCFDA; Sigma Chemical Co.) was added directly to the medium at a final concentration of 50 µmol/L. The culture was further incubated at 37°C for 1 h, and the cells were washed with PBS. The stained cells were visualized under fluorescent microscope and photographed. The amount of staining was quantified by the MCID software.

Reporter assay. To generate the reporter plasmid for chloramphenicol acetyl transferase (CAT) reporter assay, the promoter region of FAS (from +4 to −1,328 bp) was amplified where the forward primer included the Hind III linker and reverse primers included Btg II linker. The PCR products were cloned into the pBLCAT3 plasmid. A series of deletions were constructed by Erase-a-Base System (Promega) according to the manufacturer’s protocol. These plasmids were transfected to breast cancer cell line MCF7 by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 48 h, the cells were collected and then subjected to CAT assay as described previously (26). The reaction was done, and acetylated [3H]Chloramphenicol was quantified with a Phosphorimager (Packard Instruments). The luciferase reporter plasmid of the FAS promoter was a gift from Dr. Verhoeven (Catholic University of Leuven, Leuven, Belgium; ref. 27). To delete eight bases of the SREBP binding/E-Box sequence on the FAS promoter from this plasmid, QuickChange Site-Directed Mutagenesis kit (Stratagene) was used according to the manufacturer’s protocol. The luciferase reporter plasmids were transfected to MCF7 as described above. Luciferase activities were then measured by using Dual-Luciferase Reporter Assay System (Promega) and Luminometer (Berthold Detection Systems). For each transformation experiment, the Renilla expression plasmid phRG-TK (Promega) was cotransfected as an internal control, and promoter activities were normalized accordingly.

Chromatin immunoprecipitation. MCF7 cells were cultured in T75 flask and fixed with 1% formaldehyde for 10 min at room temperature. To stop the reaction, 125 mM/L glycine was added to the culture medium, and the cells were washed with PBS and harvested. Cells were then suspended in cell lysis buffer (5 mM/L PIPE, 85 mM/L NaCl, and 0.5% NP40) and homogenized with a type A Dounce homogenizer. The cell nuclei were collected and lysed with Nuclei lysis buffer (50 mM/L Tris-HCL, 10 mM/L EDTA, and 1% SDS). The chromatin was sonicated on ice to an average length of 400 bp. The sample was then centrifuged at 4°C, and the precipitates were resuspended in chromatin immunoprecipitation (ChIP) dilution buffer (167 mM/L Tris-HCl, 167 mM/L NaCl, 1.1% Triton X-100, and 0.01% SDS). After preclearing the sample with Protein G agarose beads (DynaBiotech) followed by brief centrifugation, the supernatant was transferred to a new tube and anti-SREBP-1 (Santa Cruz Biotechnology) antibody was added. After 24 h of incubation at 4°C, Protein G agarose beads were added, and the sample was incubated for 3 h at 4°C. The beads were then washed with washing buffer (100 mM/L Tris-HCl, 500 mM/L NaCl, 1% NP40, and 1% deoxycholic acid), and DNA-protein complexes were eluted with elution buffer (100 mM/L NaHCO3 and 1% SDS). DNA protein was decrosslinked followed by phenol extraction, and the purified DNA was subjected to PCR using both specific (5’-TACATCGGCCCCGGGCGGAGCAGCAGAGCCATCCGCCGAGC-3’ and 5’-ACACAGCAGCAGCAGAGCCATCCGCCGAGC-3’) and nonspecific primers (5’-CAGCCCATCCAGA-TTGTGGCTACCATAGCG-3’). The PCR products were visualized after electrophoresis on 8% acrylamide gel followed by staining with ethidium bromide.

Immunohistochemistry. Human breast cancer specimens were obtained from surgical pathology archives of the Akita Red Cross Hospital. All of the tissue sections were obtained by surgical resection. For immunohistochemical staining, 4-µm-thick sections were cut out from the formaldehyde-fixed and paraffin-embedded tissue specimens and mounted on charged glass slides. The sections were baked at 60°C for 1 h, deparaffinized by two changes of xylene, and rehydrated in graded alcohol solutions. For antigen retrieval, the sections were heated in 10 mM/L sodium citrate (pH 6.0) at 85°C for 30 min. The slides were treated with 3% H2O2 to block endogenous peroxidase activity and then incubated overnight at 4°C with anti-FAS rabbit polyclonal antibodies.
antibody (0.2 μg/mL; Immuno-biological Laboratories Co.), anti–SREBP-1 rabbit polyclonal antibody (1:200; Santa Cruz Biotechnology), or anti–carbonic anhydrase 9 (CA9) mouse monoclonal antibody (1:100; R&D Systems, Inc.). The sections were then incubated with horseradish peroxidase–conjugated anti-rabbit or mouse IgG for 30 min at room temperature, and 3,3′-diaminobenzidine substrate chromogen solution [Envision-plus kit (Dako Corp.) or ABC staining system (Santa Cruz Biotechnology)] was applied. Finally, the sections were counterstained with hematoxylin. Results of the immunohistochemistry were judged based on the intensity of staining, comparing the tumor cells and the normal glands on the same slide. Grading of the FAS, SREBP-1, and CA9 expression levels was done by two independent persons without prior knowledge of the patient data. The cases were then divided into those that showed positive staining and those that showed reduced expression of the two genes.

Animal model. Breast cancer cell line, MDA-MB231, was suspended to 30 million/mL with PBS, and equal volume of Matrigel (BD Biosciences) was mixed with the tissue. The cell suspension (0.1 mL) was injected into the mammary fat pad of 4-week-old female nude mice. A disc of 17B-estradiol (Invitrogen) was also embedded under the skin of these mice. After 3 weeks, 0.2 mL of pimonidazole (Hypoxyprobe-1 kit; Chemicon; 2,000 μg/mL) was injected to the mouse via i.p. After 2 h of the injection, the mouse was euthanized and the tumor excised and snap frozen. The tumor sample was cut into a 4-μm slice and mounted on charged glass slides. Immunohistochemical analyses were performed for these slices using anti-pimonidazole and anti-FAS antibodies.

In situ apoptosis assay. The cells were grown in 96-well plates and fixed with 4% paraformaldehyde in PBS followed by permeabilization with 0.2% Triton-X 100/0.1% sodium citrate at 4°C. The cells were then washed extensively and terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling assay was performed using the In Situ Cell Death Detection kit/TMR Red (Roche Applied Science). The reaction was stopped after 1 h, and the number of apoptotic cells in each well was counted under a confocal microscope.

FAS enzyme assay. The enzyme activity of FAS was assayed as described previously (28). Briefly, the cells were grown in 12-well plates with or without cyclophosphamide and cerulenin. After 24 h, the cells were collected and resuspended with 0.25 mol/L Sucrose buffer (0.25 mol/L sucrose, 1 mmol/L EDTA, 5 mmol/L Tris-HCl, and 1 mmol/L DTT), and the cells were then homogenized by a type A Dounce homogenizer. FAS activity was measured spectrophotometrically by monitoring oxidation of NADPH (Sigma). Fifty microliters of the cell extract were added to a 500 μL reaction mixture containing 0.1 mol/L K2HPO4 (pH 7.0), 0.3 mmol/L NADPH, and 0.05 mmol/L Acetyl-CoA, and the absorbance at 340 nm was monitored for 3 min to measure background of NADPH oxidation. Malonyl-CoA

Figure 1. Hypoxia significantly augments the expression of FAS. A, human breast cancer cell lines, MX1, MCF7, and MDA-MB157 were culturated in three sets of 24-well plates under normoxic (N) or hypoxic (H) conditions for 48 h. One set of cells (in triplicate) was collected, and RNA was prepared. The samples were then subjected to qRT-PCR using primers for the FAS and β-actin genes. Another set of cells was collected, and the cell lysates were subjected to Western blot analysis using anti-FAS and antitubulin antibodies (inset). B, the last set of plates was used for assaying the amount of ROS using DCFDA dye. The cells were treated with the dye for 1 h followed by washing the wells with PBS. The stained cells were visualized under fluorescent microscope and photographed. The amount of staining was quantified by using a MCID software.
(0.2 mmol/L) was then added to the reaction mixture, and absorbance at 340 nm was again monitored for 3 min to measure FAS activity.

**Statistical analysis.** For *in vitro* experiments, one way ANOVA was used to calculate the *P* values. Descriptive statistics comparing the expression of FAS, SREBP-1, and CA9 were analyzed by standard *m*2 test. For all of the statistical tests, the significance was defined as having a *P* value of <0.05. In all cases, SPSS software was used.

**Results**

**Hypoxia induces the expression of the FAS gene via reactive oxygen species.** To examine the effect of hypoxia on the expression of the FAS gene, we cultured three breast cancer cell lines, MX1, MCF7, and MDA-MB157, under normoxic or hypoxic conditions. The RNA and cell lysates were prepared from these samples, and the level of FAS expression was measured by quantitative reverse transcription-PCR (qRT-PCR) and Western blot. As shown in Fig. 1, our results indicate that the transcription of the FAS gene was significantly increased in hypoxia compared with that in normoxia (Fig. 1A). Protein level of FAS was also strongly increased in hypoxic condition (Fig. 1A, inset), although the amount of FAS induced in these three cell lines at protein level (7.1-, 4.5-, and 3.0-fold, respectively) seems to be less than that at RNA level (8.7-, 4.6-, and 2.7-fold, respectively). These apparent differences may be due to the instability of the mRNA or the FAS protein. We also examined the amount of reactive oxygen species (ROS) in these cells under normoxic and hypoxic conditions and found that hypoxia significantly augmented the generation of ROS in all these cell lines (Fig. 1B), which is in good agreement with previous reports (29). These results suggest that the expression of the FAS gene is positively controlled by hypoxia, which is also associated with the amount of ROS in the cell. To further corroborate our results, we tested the effect of H2O2 on the FAS expression in MDA-MB157, which displayed the lowest level of FAS. As shown in Fig. 2A, the addition of H2O2 in the culture medium significantly augmented the expression of FAS at both RNA and protein levels in a dose- and time-dependent manner. Other cell lines, MCF7 and MDA-MB231, also showed a similar trend and increased the FAS expression by 3- to 4-fold in response to H2O2 (Fig. 2B). On the other hand, addition of an ROS scavenger, NAC, significantly suppressed the expression of the FAS gene as well as ROS production in MX1, which showed the highest level of FAS expression among the tested cell lines (Fig. 2C, first and second panels).
Figure 3. Hypoxia-induced FAS expression is mediated via Akt, HIF1, and SREBP-1. A, MCF7 cells were cultured in 24-well plates under hypoxia or normoxia conditions for 48 h and with or without H2O2 for 24 h. Cells were then collected and subjected to Western blot analyses using antibodies for FAS, HIF1, phospho-SREBP (p-SREBP), SREBP-1, phospho-Akt (p-Akt), total Akt (Akt), and Tubulin (left). The MCF7 cells were also cultured in the presence or absence of YC-1 (HIF1 inhibitor) under hypoxic or normoxic conditions for 48 h. Cell lysates were subjected to Western blot analyses for HIF1 and FAS expression (right). B, MCF7 cells cultured under normoxia or hypoxia with or without the treatment of LY294002 were subjected to qRT-PCR to quantify the expression of the FAS and SREBP genes (first and second panels). Another set of culture with the same treatment was also subjected to Western blot analysis (third panel). siRNA for Akt1 and SREBP-1, or the expression plasmid of active form of Akt1 were transfected to MCF7 cells. The cells were then incubated under normoxic or hypoxic conditions for 48 h. The cells were collected and subjected to qRT-PCR analysis for FAS expression (fourth panel). C, CAT reporter constructs with various lengths of the FAS promoter were transfected to MCF7, and the cells were continued to be cultured under hypoxic or normoxic conditions for 48 h and with or without H2O2 for 24 h. Cells were then collected, and the cell lysates were subjected to CAT assay (left). The luciferase reporter plasmid with 195 bases of FAS promoter with or without deletion of E-box was transfected to MCF7, and the cells were cultured under hypoxia or normoxia for 48 h and with or without H2O2 for 24 h. Cells were then collected and assayed for luciferase activities (right). D, for ChIP assay, MCF7 cells were cultured under normoxia or hypoxia for 24 h. The cells were lysed and the lysate was pulled down with anti–SREBP-1 antibody. The DNA was then subjected to quantitative PCR using nonspecific (NS) or SREBP binding site–specific primers (S). The ratio of the DNA was calculated based on cyclic threshold value for each reaction.
panels). Because inhibition of the FAS expression has been known to cause apoptosis (13), we also examined the effect of NAC on cell death. As shown in Fig. 2C (third panel), the treatment of the cell with NAC significantly induced apoptosis to the similar level as it was when treated with a specific inhibitor of FAS, cerulenin. To further confirm our results, we tested the effect of NAC on the FAS up-regulation under hypoxia and found that NAC indeed significantly blocked the up-regulation of FAS (Fig. 2C, fourth panel). Collectively, these results suggest that the expression of the FAS gene is up-regulated by hypoxia through the generation of ROS.

**Hypoxia up-regulates the FAS gene expression through SREBP-1.** To understand the mechanism of the hypoxia-induced expression of the FAS gene, we first examined the status of HIF1, SREBP, and Akt under normoxic and hypoxic conditions. HIF1 has been known as a key transcriptional regulator induced by hypoxia (30). SREBP is the major transcription factor of the FAS gene and has been known to be up-regulated under hypoxia (31). In fission yeast, SREBP was indeed found to function as an oxygen sensor (32). Akt is a key signal molecule for cell survival, and apoptosis and has been shown to be up-regulated under hypoxia (33). As shown in Fig. 3A (left), our results of Western blot analysis indicate that expressions of FAS, HIF1, SREBP-1, and phospho-SREBP (T426) were indeed up-regulated under hypoxia as well as in the presence of H2O2 in MCF7 cells. We also found that Akt was strongly phosphorylated at Ser435 in the same set of samples treated with hypoxia or H2O2, although the amount of total Akt was somewhat decreased, suggesting that PI3K/Akt pathway and SREBP-1 are involved in the activation of FAS by hypoxia and ROS (Fig. 3A, left). Because HIF1 was also up-regulated by hypoxia and H2O2, we next examined whether HIF1 was involved in the activation of FAS by adding a HIF1 inhibitor, YC-1, in the cultured cells under hypoxic condition. As shown in Fig. 3A (right), the hypoxic condition strongly up-regulated HIF1, and this up-regulation was blocked by YC-1. Interestingly, the YC-1 treatment also blocked the expression of FAS as well as phospho-SREBP-1, suggesting that HIF1 is also involved in the up-regulation of FAS and SREBP-1. The results of qRT-PCR analysis also indicate that FAS and SREBP-1 were significantly increased by the treatment of hypoxia (Fig. 3B, first and second panels). Furthermore, the results of both of our qRT-PCR and Western blot analyses indicate that the up-regulation of FAS, p-Akt, HIF1, and SREBP-1, as well as p-SREBP, were blocked by LY294002 (Fig. 3B, first, second, and third panels), suggesting that the induction of the FAS expression by hypoxia is mediated through activation of Akt followed by up-regulation of HIF1 and SREBP-1. To further verify our results, we tested the effect of siRNA specific to SREBP-1 and Akt as well as the effect of ectopic expression of an activated form of Akt on the FAS expression. We found that ectopic expression of Akt significantly augmented the FAS expression under normoxia, whereas both siRNA significantly blocked the up-regulation of FAS under the hypoxic condition (Fig. 3B, fourth panel). Therefore, both Akt and SREBP-1 coordinately regulate the up-regulation of hypoxia-induced FAS expression.

To identify the exact hypoxia-responding sequence on the FAS gene promoter, we generated a series of CAT reporter plasmids containing up to −1,328, −616, and −115 base of the FAS promoter, and CAT reporter activities were measured under normoxic or hypoxic conditions as well as in the presence or absence of H2O2. As shown in Fig. 3C (left), both hypoxia and H2O2 significantly increased the FAS promoter activity even when the promoter sequence was deleted to −115 bases. Because this region includes the SREBP binding/E-box sequence, to assess the functional significance of these sequences, we generated luciferase reporter plasmids with or without the SREBP binding sequence and tested their responsiveness to hypoxia and H2O2. The results of the reporter assay indicate that deletion of the SREBP binding/E-box sequence significantly reduced the responsiveness of the FAS promoter to hypoxia and H2O2 (Fig. 3C, right). Therefore, these results suggest that hypoxia induced the FAS gene by activating Akt followed by induction of SREBP-1, which then binds to the SREBP binding site of the FAS promoter. To examine further whether SREBP-1 indeed binds to the SREBP binding site under hypoxia, we performed ChIP assay by precipitating SREBP-chromatin complex using anti–SREBP-1 antibody (Fig. 4).

![Figure 4](cancerres.aacrjournals.org) FAS and SREBP-1 express in hypoxic regions of tumor in vivo. A, MDA-MB231 cells were transplanted into mammary fat pad of nude mice. The tumors were grown for 3 wk, and mice were injected with pimonidazole through i.p. After 2 h, tumors were excised and sliced on slides. These slides were then analyzed by immunohistochemistry using antibodies for FAS and pimonidazole. Photos are shown for two representative regions (a–b and c–d). B, to examine the relationship of FAS and hypoxia in tumor, human breast tumor samples from 29 patients were sectioned and subjected to immunohistochemical analysis using antibodies for FAS, SREBP-1, and CA9. Representative photos for each antibody staining with consecutively sectioned slides are shown. C, for each slide, fields of high and low expression of CA9 were randomly chosen and divided into two groups (CA9-positive and CA9-negative). Using consecutive slides of the identical samples, these regions were then analyzed by immunohistochemistry using anti-FAS and anti–SREBP-1 antibodies. Each sample was then further divided according to positive and negative expression of FAS and SREBP-1 antibodies. To evaluate the significance, the expression of FAS and SREBP-1 in relation to CA9, χ² test was performed.
In vitro which is consistent with our expressed preferentially in the region of hypoxia in breast cancer, As shown in Fig. 5 chemotherapeutic drug commonly used for the treatment of breast resistance. We first examined the effect of cyclophosphamide, a tion of a FAS inhibitor overcomes the hypoxia-induced chemo- high level of FAS protects tumor cells from apoptosis, we sought our results suggest that FAS is induced by hypoxia and that the hypoxia is known to induce chemoresistance (33, 35, 36). Because Rapidly growing tumors are often under hypoxic conditions, and major clinical problem for the treatment of cancer patients.

The level of FAS expression correlates with hypoxia in vivo. To validate our in vitro results of the hypoxia-induced FAS expression, we examined the relationship between the expression level of FAS and hypoxic regions in an animal xenograft model. We first transplanted human breast cell lines, MDA-MB231, into nude mice and grew the tumor for 3 weeks. We then injected pimonidazole, which reacts with hypoxic cells, to the mice through i.p. After 2 h, tumors were excised and stained with antibodies for FAS and pimonidazole. We found that FAS expression colocalized with the area reactive to anti-pimonidazole, suggesting that hypoxic areas strongly expressed FAS in these tumors (Fig. 4A). To further validate these results in a clinical setting, we performed immunohistochemical analysis for clinical samples from 29 breast cancer patients using antibodies for FAS, SREBP-1, and CA9, which is a hypoxia marker (34). We first stained the samples with anti-CA9 and randomly chose positive and negative fields for each specimen. These samples were then stained with antibodies for FAS and SREBP-1. We then inspected the staining intensity of FAS and SREBP-1 in these CA9-positive and CA9-negative regions. As shown in Fig. 4B and C, of 29 CA9-positive regions, 21 were FAS positive (72%) and 8 were FAS negative. On the other hand, 20 of 29 CA9-negative samples (69%) were also FAS negative (P = 0.0038). CA9-positive regions were also significantly correlated with SREBP-1 expression (P = 0.0037). These results indicate that FAS was expressed preferentially in the region of hypoxia in breast cancer, which is consistent with our in vitro data. Taken together, our results of in vitro and in vivo experiments strongly suggest that the expression of the FAS gene is significantly induced by hypoxia, and that this induction is mediated by the generation of ROS followed by the activation of Akt and SREBP-1.

Inhibition of FAS overcomes hypoxia-induced chemoresistance. Development of resistance to chemotherapeutic drugs is a major clinical problem for the treatment of cancer patients. Rapidly growing tumors are often under hypoxic conditions, and hypoxia is known to induce chemoresistance (33, 35, 36). Because our results suggest that FAS is induced by hypoxia and that the high level of FAS protects tumor cells from apoptosis, we sought a possibility that inhibition of FAS expression by low concentration of a FAS inhibitor overcomes the hypoxia-induced chemoresistance. We first examined the effect of cyclophosphamide, a chemotherapeutic drug commonly used for the treatment of breast cancer, on MCF7 cells under hypoxic or normoxic condition. As shown in Fig. 5A, cyclophosphamide induced apoptosis in MCF7 in a dose-dependent manner under normoxic condition. However, when the cells were treated with cyclophosphamide under hypoxic condition, cells became significantly resistant to cyclophosphamide, indicating that hypoxia induced chemoresistance. We then treated MCF7 cell with a combination of a FAS inhibitor (cerulenin), a PI3K inhibitor (LY294002), and cyclophosphamide under hypoxic or normoxic condition followed by apoptosis assay. We found that a combination of these drugs synergistically enhanced the degree of apoptosis under normoxic condition (Fig. 5B). Importantly, the treatment of the cells with the combination of cyclophosphamide and cerulenin or LY294002 under hypoxia condition blocked hypoxia-induced resistance to cyclophosphamide. These results suggest that a combination of cerulenin and other chemotherapeutic drugs such as LY294002 synergistically induces tumor cell death, and that hypoxia-induced chemoresistance is partially blocked by suppression of the FAS expression or the Akt pathway. We also examined the enzymatic activity of FAS and found that the FAS activity was indeed significantly higher under hypoxic condition compared with that under normoxia even in the presence of cyclophosphamide and cerulenin (3.8 ± 1.1 versus 1.1 ± 0.2, respectively), and that the activity was inversely correlated with the degree of apoptosis. Although currently available FAS inhibitors are relatively toxic, using these drugs at a low concentration with a combination of other drugs may be a rational strategy for the treatment of chemoresistant tumors.

Discussion

Although the de novo pathway of fatty acid synthesis is quite active during embryogenesis, normal adult cells acquire fatty acids mainly from dietary source and rarely use the de novo pathway because nutritional fatty acid strongly suppresses the expression of the genes involved in fatty acid synthesis (3, 4). However, cancer cells are no longer sensitive to this nutritional signal and prefer to use the de novo pathway. In fact, linoleic and arachidonic acid, potent suppressors of the FAS gene of normal hepatic and adipocytic cells, have been shown to have no significant inhibitory effect on the expression of the FAS gene in breast cancer cells (37). Therefore, what triggers the reactivation of the FAS gene in cancer
cells and whether they use the same signal pathway as the normal cells are critical questions to understand the role of FAS in tumorigenesis. When primary tumor grows >1 mm in size, it can no longer obtain oxygen and nutrients by diffusion and requires to promote angiogenesis by inducing proangiogenic genes as a survival strategy (36). Therefore, tumor cells at an early stage are usually under hypoxic condition and at a risk of apoptosis. The reactivation of the FAS gene has been observed at a relatively early stage in various types of cancer, and these results suggest that the FAS gene is up-regulated by a common factor of cancer microenvironment such as hypoxia. In this report, we have shown that the FAS gene in cancer cell is indeed significantly up-regulated by hypoxia, and that this up-regulation is due to the activation of the Akt and HIF1 followed by up-regulation of SREBP-1.

Due to the high rate of proliferation and oxygen consumption, tumors are often under hypoxic condition, which is a hallmark of cancer. The hypoxic microenvironment is normally proapoptotic; however, tumor cells adapt themselves by inducing various enzymes to circumvent the problem. This induction is mediated by an activation of the known hypoxia-sensing pathways such as HIF1 and PI3K/Akt (38–40). In this context, it should be noted that Akt has been shown to stabilize HIF1 in both breast and prostate cancer cells (41, 42). Beitner-Johnson et al. (43) also showed that hypoxia dramatically increased phospho-Akt (Ser473) in PC3 cells, and this activation of Akt was completely abolished by wortmannin, a PI3K inhibitor. It is worth noting that Akt was also found to be up-regulated by H2O2 (44). Consistent with these results, we have shown that hypoxia and H2O2 indeed induced activation of Akt (Ser473) and HIF1, and that this activation was accompanied by the up-regulation of SREBP-1, a major factor involved in the regulation of the FAS gene. In cancer cells, it has been shown that PI3K/Akt signaling significantly augmented the expression of SREBP-1 in response to oncogenic signaling, including overexpression of various growth factors (11). Furthermore, we have previously shown that the tumor suppressor, PTEN, which inhibits Akt by dephosphorylation, significantly suppressed the expression of the FAS gene (22). Therefore, the activation of the Akt pathway followed by induction of SREBP-1 is considered to be one of the major pathways of reactivation of the FAS gene in cancer cell, and this reactivation is triggered at least by the hypoxic condition of tumor microenvironment. This notion is also strongly supported by our results of immunohistochemical analysis on clinical samples where FAS expression was significantly colocalized with the CA9-stained hypoxic area. It is known that Akt is quickly phosphorylated under hypoxic condition and that this activation of Akt results in up-regulation of HIF1 (45–47). Our results indeed showed that LY294002 inhibited hypoxia-induced HIF1 as well as the expression of FAS and p-SREBP-1 (Fig. 3B). Our results also indicate that HIF1 inhibitor, YC-1, strongly blocked phosphorylation of SREBP-1 (Fig. 3A), which is in good agreement with the recent finding by Li et al. (48) that HIF1 plays a key role in activation of SREBP-1 in vivo. Therefore, the hypoxia-induced FAS expression is considered to be mediated via phosphorylation of Akt followed by activation of HIF1 and SREBP-1.

Hypoxia generally induces apoptosis in normal cells partly due to malfunction of the respiratory system in mitochondria, which requires oxygen for ATP production (49). However, cancer cells have an unusual tolerance to hypoxic condition because they use the glycolysis pathway to generate ATP even under normoxic condition, which has been known as the Warburg effect (50). On the other hand, hypoxia was shown to cause an increase of NADH/NADPH ratio in a cell due to increased flux of glycolysis, and this change of redox balance induces inactivation of PTEN followed by activation of Akt (21). Therefore, up-regulation of FAS may be partly due to increased glycolysis and the following Akt activation. It is likely that the increased activity of FAS enhances lipidogenesis, which consumes more NADPH and rebalances redox so that cells can compensate for the shortfall of oxygen.

As we and others previously reported, inhibition of the function or expression of FAS results in apoptosis of tumor cells (7–11). This cell death is considered to be caused by the suppression of CPT1 followed by accumulation of ceramide, which in turn activates proapoptotic genes such as BNIP3 (13). It should be noted that BNIP3 was found to be one of proapoptotic genes induced by hypoxia, and that specific blocking of the FAS expression by siRNA significantly increased the expression of BNIP3 followed by apoptosis (13, 51). In fact, we have shown that the expressions of FAS and BNIP3 are indeed inversely correlated in breast cancer patients (13). Therefore, FAS may act as an “antiapoptotic” gene under hypoxia. This notion is consistent with the previous observations of immunohistochemical analysis on human tumor samples where overexpression of FAS was found to be a relatively early event (7–11). We also reported that the expression of FAS was inversely related to that of PTEN in human breast tumor specimens, and the expression of higher FAS and lower PTEN is correlated to poor survival of patients, suggesting that the PTEN inactivation followed by Akt activation induced the FAS expression (22). Although the direct involvement of FAS in the initial step of tumorigenesis is yet to be determined, overexpression of FAS in tumors seems to be a survival strategy of the cancer cells to block apoptosis caused by hypoxic condition.

Because inhibition of FAS causes tumor cell apoptosis, FAS is considered to be a promising target for cancer therapy. The pharmacologic inhibitors of FAS such as cerulenin [(2R, 3S)-2,3-epoxy-4-oxo-7, 10-trans-trans-12-dodecadienamide], C75, and Orlistat have been shown to significantly suppress the cellular FAS level and also to induce apoptosis in a variety of human cancer cells including breast, prostate, colon, and ovarian cancer, although their specificity of action and potential side effects remains to be of some concern for actual clinical use (7–11). On the other hand, traditional chemotherapeutic agents commonly used for breast cancer treatment such as cyclophosphamide, carboplatin, and doxorubicin often become ineffective due to chemoresistance, particularly under hypoxic condition (52). The exact mechanism of the hypoxia-induced chemoresistance has not been well understood; however, one possible mechanism is the activation of the Akt pathway and following expression of antiapoptotic genes including FAS (33). Our results of the in vitro experiments clearly indicate that a FAS inhibitor, cerulenin, indeed partially overcame the hypoxia-induced chemoresistance of cyclophosphamide. Although the existing FAS inhibitors are still somewhat toxic, a use of lower concentration of these drugs in combination with the current chemotherapeutic drugs may enhance the therapeutic effect by reducing the hypoxia-induced chemoresistance.

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References


Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelman, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O2 consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = −0.16 and the normal differential is 65 per cent M and 35 per cent L, then

\[ 0.65 (+0.27) + 0.35 (-0.16) = +0.12 \]

a figure identical to the observed +0.12 for normal leukocytes.
Fatty Acid Synthase Gene Is Up-regulated by Hypoxia via Activation of Akt and Sterol Regulatory Element Binding Protein-1

Eiji Furuta, Sudha K. Pai, Rui Zhan, et al.


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