DNA topoisomerase IIα (TOP2A) inhibitors up-regulate fatty acid synthase gene expression in SK-Br3 breast cancer cells: In vitro evidence for a ‘functional amplicon’ involving FAS, Her-2/neu and TOP2A genes

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Abstract. Fatty acid synthase (FAS), the key metabolic multi-enzyme that is responsible for the terminal catalytic step in the de novo fatty acid biosynthesis, plays an active role in the development, maintenance, and enhancement of the malignant phenotype in a subset of breast carcinomas. We recently described that a molecular bi-directional cross-talk between FAS and the Her-2/neu (erbB-2) oncogene is taking place at the level of transcription, translation, and activity in breast cancer cells. Because Her-2/neu has been linked with altered sensitivity to cytotoxic drugs, we envisioned that FAS gene expression may represent a novel predictive molecular factor for breast cancer response to chemotherapy in a Her-2/neu-related manner. We herein evaluated whether chemotherapy-induced cell damage acts in an epigenetic fashion by inducing changes in the transcriptional activation of FAS gene in breast cancer cells. To evaluate this option, FAS- and Her-2/neu-overexpressing SK-Br3 breast cancer cells were transiently transfected with a FAS promoter-reporter construct (FAS-Luciferase) harboring all the elements necessary for high level expression in cancer cells. SK-Br3 cells cultured in the presence of topoisomerase IIα (TOP2A) inhibitors doxorubicin and etopoxide (VP-16) demonstrated a 2- to 3-fold increase in FAS promoter activity when compared with control cells growing in drug-free culture conditions. We failed to observe any significant activation of FAS promoter following exposure to the anti-metabolite 5-fluorouracil, the alkylating drug cisplatin, or the microtubule interfering-agents paclitaxel and vincristine. Moreover, the up-regulatory effects of TOP2A inhibitors on the transcriptional activation of FAS gene expression were not significantly decreased when the FAS promoter was damaged at the sterol regulatory element binding protein (SREBP)-binding site. Considering that FAS inhibition produces profound inhibition of DNA replication and S-phase progression in cancer cells, we finally asked whether a cross-talk between TOP2A and FAS could exhibit a Her-2/neu-related bi-directional nature. TOP2A protein levels were decreased during treatment with the anti-Her-2/neu antibody trastuzumab while, concomitantly, FAS promoter activity and FAS protein expression were significantly reduced. Of note, when the expression levels of TOP2A protein were analyzed following exposure of SK-Br3 cells to increasing concentrations of the novel slow-binding FAS inhibitor C75, a dose-dependent reduction in TOP2A expression was observed. Although FAS gene is not physically located in the Her-2/neu-TOP2A amplicon, our present findings strongly suggest that a tight functional association between FAS, Her-2/neu and TOP2A genes is taking place in a subset of breast carcinoma cells.

Introduction

After numerous basic research and clinical studies, it now appears that fatty acid synthase (FAS), the key metabolic multi-enzyme that is responsible for the terminal catalytic step in the de novo fatty acid biosynthesis (1), may play a previously unrecognized role in the development, maintenance, and enhancement of the malignant phenotype of several malignancies including breast cancer (2). In fact, infiltrating carcinomas of the breast constitutively express high levels of FAS compared to non-transformed human epithelial tissues, while overexpression and hyperactivity of FAS is associated with more aggressive breast carcinomas and poor clinical outcomes (3-6). Furthermore, increased levels of FAS accompany the development of in situ carcinoma of the breast, suggesting a potential link between FAS up-regulation and increased risk of breast cancer development (7). Accordingly, FAS-dependent endogenous fatty acid biogenesis is a molecular feature that accompanies the malignant transformation of
NIH-3T3 fibroblasts induced by Her-2/neu (erbB-2) oncogene, which is overexpressed not only in invasive breast cancer but also in premalignant atypical duct proliferations and in ductal carcinoma in situ of the breast (8). Moreover, FAS activity plays a necessary role in the oncogenic ability of Her-2/neu, including Her-2/neu-induced anchorage-independent cell growth and increased cell survival (8). It is reasonable to suggest that a molecular bi-directional cross-talk between FAS and Her-2/neu is taking place at the level of transcription, translation, and activity in breast cancer cells. In one hand, high levels of both FAS expression and activity positively correlate with Her-2/neu oncogene amplification and/or over-expression in a wide panel of human breast cancer cell lines (9), thus suggesting that FAS may be a novel down-stream effector of Her-2/neu-promoted tumorigenicity and breast cancer progression. Accordingly, a transcriptome analysis of Her-2/neu revealed that FAS is one of the genes differentially regulated by Her-2/neu in human breast epithelial cells (10). On the other hand, pharmacological FAS inhibitors cerulenin and C75 have been found to suppress Her-2/neu oncoprotein expression in breast Her-2/neu overexpressers (11). Similarly, Her-2/neu expression is dramatically down-regulated when FAS gene expression is silenced by using the highly sequence-specific mechanism of RNA interference (RNAi). Pharmacological and RNAi-mediated inhibition of FAS represses Her-2/neu expression at the transcriptional level, concomitantly up-regulating the expression of PEA3, an Ets factor that specifically reverses the in vitro transformed phenotype of Her-2/neu-overexpressing cancer cells through the attenuation of the Her-2/neu oncogene promoter activity (11). These results, altogether, demonstrate that the response of breast cancer cells to metabolic stress after perturbation of FAS activity is accompanied by the specific suppression of Her-2/neu oncogene, which is amplified in 20-35% of invasive breast cancers and is known to be associated with shortened disease-free and overall survival (12). Therefore, Her-2/neu oncogene could act as a molecular sensor of energy imbalance that participates actively in the maintenance of an abnormally elevated endogenous fatty acid metabolism in breast cancer cells.

Pharmacological and/or small interference RNA-induced inhibition of FAS signaling synergistically sensitizes cultured breast cancer cells to anti-mitotic drugs such as docetaxel (Taxotere™), vinorelbine (Navelbine™) and paclitaxel (Taxol™) (13-15). Moreover, this FAS-related chemosensitization is also synergistic with the anti-Her-2/neu humanized monoclonal antibody trastuzumab (Herceptin™) (11). Because Her-2/neu has been linked with altered sensitivity to cytotoxic drugs according to in vitro studies and clinical trials (16), we recently envisioned that FAS gene expression may represent a novel predictive molecular factor for breast cancer response to chemotherapy in a Her-2/neu-related manner. Here, we evaluated whether chemotherapy-induced cell damage may act in an epigenetic fashion by inducing changes in the transcriptional activation of breast cancer-associated FAS gene. To evaluate this option, Her-2/neu-overexpressing SK-BR3 breast cancer cells were transiently transfected with a FAS promoter-reporter construct (FAS-Luciferase) that harbors all the elements necessary for high level expression in cancer cells. The SK-BR3 cells contain levels of FAS constituting up to 28%, by weight, of the cytosolic protein (17). Remarkably, an increase in the rate of transcription of the FAS gene, and consequently a higher abundance of FAS mRNA, was found to be primarily responsible for FAS overexpression in this breast cancer cell line, whereas increases in FAS message stability and longer half-life of the FAS protein were not detected (18). Taken together, the experimental evidence makes SK-BR3 cells an ideal in vitro model system for studying the regulation of FAS gene. We reveal for the first time that topoisomerase IIa (TOP2A) inhibitors doxorubicin and etopside (VP-16), but not other chemotherapeutic agents, work in an epigenetic fashion by up-regulating the activity of FAS gene promoter in SK-BR3 breast cancer cells. When the expression levels of TOP2A protein were analyzed following pharmacological blockade of FAS activity, a dose-dependent reduction in TOP2A protein expression was observed. These results not only suggest that a previously unrecognized bi-directional cross-talk between TOP2A and FAS seems to occur in Her-2/neu-overexpressors but further reveal TOP2A as a good molecular candidate that actively participates in the linkage between FAS-catalyzed endogenous fatty acid metabolism and DNA replication in breast cancer cells.

Although the clinical and therapeutic importance of FAS expression status to breast cancer management should be resolved in clinical studies, the characterization of a putative ‘functional amplicon’ involving FAS, Her-2/neu and TOP2A genes should enhance our understanding of how FAS-dependent endogenous fatty acid metabolism, once considered a minor anabolic-energy-storage-pathway in normal cells, may be consider a ‘metabolic oncogene’ that actively contributes to the cancer phenotype through previously unexpected bi-directional cross-talks with well-characterized cancer-regulating genes such as Her-2/neu and TOP2A.

Materials and methods

Materials. C75 was purchased from Alexis Biochemicals (San Diego, CA, USA). C75 was dissolved in dimethyl sulfoxide (DMSO) and stored in the dark as stock solution (50 mg/ml) at -20°C. For experimental use, stock solution was diluted with growth medium. In all cases, final concentrations of DMSO were ≤0.1% and did not modify the proliferation of control cells.

The primary antibody for FAS immunoblotting was a mouse IgG, FAS monoclonal antibody (clone 23) from BD Biosciences Pharmingen (San Diego, CA, USA). Anti-c-erbB-2/HER-2/neu (phosphor-specific) Ab-18 (clone PN2A) mouse monoclonal antibody was from NeoMarkers (Lab Vision Corporation, Fremont, CA, USA). Anti-Human Topo II AB rabbit polyclonal antibody was from TopoGEN, Inc. (Columbus, OH, USA). Anti-ß-actin goat polyclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell lines and culture conditions. SK-BR3 breast cancer cells were obtained from the American Type Culture Collection (ATCC) and they were routinely grown in phenol red-containing improved MEM (IMEM, Biosource International, Camarillo, CA, USA) supplemented with 5% (v/v) fetal bovine
serum (FBS) and 2 mM L-glutamine at 37°C in a humidified atmosphere of 95% air and 5% CO₂, unless otherwise specified.

**FAS promoter activity.** Using FuGENE 6 transfection reagent (Roche Biochemicals, Indianapolis, IN, USA) as directed by the manufacturer, overnight serum-starved cells seeded into 24-well plates (~5x10⁵ cells/well) were transfected in low-serum (0.1% FBS) media with 300 ng/well of the pGL3-Luciferase (Promega, Madison, WI, USA) construct containing a luciferase reporter gene driven by either an intact (FAS wtSREBP-BS-Luc) or damaged (FAS ΔSREBP-BS-Luc) 178-bp FAS promoter fragment along with 30 ng/well of the internal control plasmid pRL-CMV, which was used to correct for transfection efficiency. After 18 h, the transfected cells were washed and then incubated in the absence or presence of chemotherapeutic agents as specified. Approximately 24 h after treatments, luciferase activity from cell extracts was detected using a Luciferase assay system (Promega) according to the protocol specified by the manufacturer in a VICTOR™ 1420 multilabel counter (Perkin Elmer). The magnitude of activation in FAS promoter-Lucerase-transfected cells was determined after normalization to the luciferase activity in cells co-transfected with equivalent amounts of the empty pGL3-luciferase vector lacking the FAS promoter (O-Luciferase) and the internal control plasmid pRL-CMV, which was taken as 1.0-fold. This control value was used to calculate the relative (fold) change in transcriptional activities of FAS promoter-Lucerase-transfected cells in response to treatments after normalization to pRL-CMV activity, and the data are shown as the means ± SD from three separate experiments (performed in triplicate).

**Immunoblotting.** Cells were harvested by treatment with trypsin-EDTA solution, washed twice with PBS (-) and stored at -80°C. The cells were lysed in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride) for 30 min on ice, and then a particle-free supernatant solution was obtained by centrifugation at 14,000 x g for 15 min. All operations were at 0-4°C. A sample was taken for measurement of protein content by a BioRad assay (Bio-Rad Laboratories). Equal amounts of protein were heated in SDS sample buffer (Laemmli) for 10 min at 70°C and subjected to electrophoresis on 3-8% NuPAGE Tris-Acetate gels (Novex™) and transferred to nitrocellulose membranes. Non-specific binding on the nitrocellulose filter paper was minimized by blocking for 1 h at room temperature (RT) with TBS-T [25 mM Tris-HCl, 150 mM NaCl (pH 7.5), and 0.05% Tween-20] containing 5% (w/v) nonfat dry milk. The treated filters were washed in TBS-T and then incubated with primary antibodies for 2 h at RT in TBS-T containing 1% (w/v) nonfat dry milk. The membranes were washed in TBS-T, horseradish peroxidase-conjugated secondary antibodies (Jackson Immuno Research, West Grove, PA) in TBS-T were added for 1 h, and immunoreactive bands were visualized with ECL detection reagent (Pierce, Rockford, IL). Blots were reprobed with an antibody for β-actin to control for protein loading and transfer. Densitometric values of protein bands were quantified using Scion imaging software (Scion Corp., Frederick, MD, USA).

**Statistical analyses.** All observations were confirmed by at least three independent experiments. The data are presented as means ± SD. The Student's t test (paired and unpaired) was used to evaluate the statistical significance of mean values. Statistical significance levels were P<0.05 and P<0.005. All Ps are two-tailed.

**Results**

**Topoisomerase IIα (TOP2A) inhibitors specifically activate FAS promoter activity in SK-BR3 breast cancer cells.** SK-BR3 breast cancer cells growing in 24-well plates were transfected with 300 ng/well of the pGL3-Luciferase construct containing a Luciferase reporter gene driven by an intact 178-bp FAS promoter fragment along with 30 ng/well of the internal control plasmid pRL-CMV, which was used to correct for transfection efficiency. After 18 h, the transfected cells were washed and then incubated in the presence or absence of the antimetabolite drug 5-fluorouracil (5-FU; 10 μM), the alkylating agent cisplatin (CDDP; 10 μM), the taxane paclitaxel (PTX; 10 nM), the vinca alkaloid vincristine (VCR; 10 nM), and the topoisomerase IIα (TOP2A) inhibitors doxorubicin (DOX; 1 μM) and etoposide (VP-16; 1 μM). Approximately 24 h after treatments, luciferase activity from cell extracts was detected using a Luciferase assay system according to the protocol specified by the manufacturer. The magnitude of activation in FAS promoter-Lucerase-transfected cells was determined after normalization to the Luciferase activity in cells co-transfected with equivalent amounts of the empty pGL3-luciferase vector lacking the FAS promoter (O-Luciferase) and the internal control plasmid pRL-CMV, which was taken as 1.0-fold. This control value was used to calculate the relative (fold) change in transcriptional activities of FAS promoter-Lucerase-transfected cells in response to treatments after normalization to pRL-CMV activity, and the data are shown as the means ± SD from three separate experiments (performed in triplicate).

**TOP2A inhibitors activate FAS promoter activity in a sterol regulatory element binding protein (SREBP)-independent manner.** Since increased FAS expression in cancer cells appears to be part of a more general change in the genetic program controlling lipogenesis (7,19), it could be argued that TOP2A inhibitors-induced activation of FAS gene promoter may represent a general dysregulation of genes coding for other enzymes of the same lipogenic pathway. Indeed, a common up-stream control system based on sterol regulatory element binding proteins (SREBPs)-transcription factors which bind to sterol regulatory elements (SREs) in promoter regions of target genes, operates for the simultaneous transcriptional up-regulation of all the genetic components
Figure 1. Effects of chemotherapy on the activity of a FAS promoter-reporter construct in SK-Br3 breast cancer cells. SK-Br3 cells were transiently transfected with a plasmid containing a Luciferase reporter gene driven by a 178-bp FAS promoter fragment harboring a well-characterized SREBP-binding site flanked by auxiliary NF-Y and Sp-1 sites (a) or with a similar construct in which the SREBP-binding site was deleted (b). The next day, cultures were treated with chemotherapeutic agents as indicated (see text). After ~24 h of incubation, cells were lysed. Luciferase activity was measured and relative (fold) changes in transcriptional activities of FAS promoter-Luciferase-transfected cells were calculated. The data are shown as the means (columns) ± SD (bars) from three separate experiments (performed in duplicate).
required for the fatty acid synthesis pathway (20). To examine whether the effects of TOP2A inhibitors DOX and VP-16 on FAS gene activation were mediated by the SREBP-binding site present in the proximal FAS promoter, SK-Br3 cells were transiently transfected with a truncated construct in which the region responsible for SREBP binding is deleted (FAS ΔSREBP-BS-Luc; Fig. 1b, top panel). The up-regulatory effects of TOP2A inhibitors on the transcriptional activation of FAS promoter were not significantly decreased when the FAS promoter was damaged at the SREBP-binding site, while the transcriptional activity of the truncated FAS promoter remained unchanged in response to 5-FU, CDDP, PTX, and VCR (Fig. 1b, bottom panel). These findings, altogether, reveal for the first time that TOP2A inhibitors, but not other chemotherapeutic agents, can work in an epigenetic fashion by up-regulating the expression of FAS gene in breast cancer cells. FAS promoter activity was not modulated by TOP2A inhibitors or other chemotherapeutic agents in MCF-7 (no Her-2/neu gene amplification; normal copy number of TOP2A gene) and MDA-MB-361 (Her-2/neu-amplified; physical deletion of TOP2A gene) (21) breast cancer cell lines (data not shown). The fact that TOP2A inhibitors specifically up-regulate FAS promoter activity in SK-Br3 cells (Her-2/neu-amplified; TOP2A gene...
amplification) (21) strongly suggests that a concurrent overexpression of Her-2/neu and TOP2A is necessary to observe the cross-talk between DOX- and VP-16-induced inhibition of TOP2A and FAS promoter activation.

Trastuzumab-induced blockade of Her-2/neu-driven signaling concomitantly down-regulates the expression of TOP2A and FAS. Considering that FAS inhibition produces profound inhibition of DNA replication and S-phase progression in cancer cells, which suggest a direct linkage at a regulatory level between FAS-catalyzed endogenous fatty acid biosynthesis and DNA synthesis in proliferating tumor cells (22-24), we finally asked whether a cross-talk between TOP2A and FAS could exhibit a Her-2/neu-related bi-directional nature. As expected, the anti-Her-2/neu antibody trastuzumab at 10 μg/ml, the target plasma level in human studies, (25) led to a decrease in Her-2/neu-coded p185HER-2/neu oncoprotein (data not shown) and concomitantly abrogated p185HER-2/neu-associated tyrosine phosphorylation in SK-BR3 breast cancer cells (Fig. 2a) as determined by using a monoclonal Her-2/neu (phospho-specific) Ab-18 (clone PN2A), which specifically recognizes the activated, tyrosine phosphorylated (P-Tyr1248) form of p185HER-2/neu. In agreement with earlier studies, TOP2A protein levels decreased during treatment with trastuzumab, in parallel with the decrease in p185HER-2/neu receptor phosphorylation (26). Under the same conditions, FAS promoter activity and FAS protein levels were significantly reduced in response to trastuzumab-induced inhibition of Her-2/neu-driven signaling (Fig. 2a).

Pharmacological inhibition of FAS activity down-regulates TOP2A protein expression. When the expression levels of TOP2A protein were analyzed following a 6 h exposure of SK-BR3 cells to increasing concentrations of the α-methylene-γ-butyrolactone C75, a novel slow-binding inhibitor of FAS activity (27), a dose-dependent reduction in TOP2A protein expression was observed (Fig. 2b). These results not only suggest that a previously unrecognized bi-directional cross-talk between TOP2A and FAS seems to occur in Her-2/neu-overexpressors but further reveal TOP2A as a good molecular candidate that actively participates in the linkage between FAS-catalyzed endogenous fatty acid metabolism and DNA replication in breast cancer cells (22,24).

Discussion

TOP2A is a key enzyme in DNA replication and a molecular target for anti-cancer drugs such as DOX and VP-16. The TOP2A gene is located at chromosome band 17q12-q21, adjacent to the Her-2/neu oncogene, which is the most commonly amplified oncogene in breast cancer. Because this physical proximity to Her-2/neu, copy number aberrations also occur in the TOP2A gene. Indeed, TOP2A is either amplified or deleted, with equal frequency, in almost 90% of Her-2/neu-amplified primary breast tumors (21,28,29). Although all the studies evaluating TOP2A suggest that gene amplification and/or TOP2A protein overexpression might be associated with an increased efficacy of TOP2A inhibitors, the reported studies do not provide the proof of principle needed to authorize the use of TOP2A as a predictive marker for standard practice (30). Our current study indicates that perturbation of TOP2A activity is a novel cellular stress capable of specifically activating FAS promoter in breast cancer cells exhibiting overexpression of Her-2/neu and TOP2A. Therefore, characterization of FAS gene expression may be a clinically valuable molecular surrogate for altered TOP2A activity following TOP2A inhibitors-based chemotherapy in Her-2/neu-overexpressing breast carcinomas. Moreover, our experiments suggest that chemotherapeutic combinations of FAS blockers with TOP2A inhibitors should not be ideal because FAS inhibition leads to decreased target on which TOP2A may act. Accordingly, we previously reported that pharmacological inhibition of FAS activity in combination with anthracyclines, similarly to trastuzumab plus TOP2A inhibitor combinations (26), does not demonstrate synergism in SK-BR3 cells (31).

There have been reports that a region in the long arm of chromosome 17 of SK-BR3 cells spanning 17q22-17q24 (the region to which the human FAS gene has been mapped) is prone to high-level amplification (32,33). However, neither an amplification of the FAS gene nor any gross chromosomal rearrangements have been detected in SK-BR3 breast cancer cells (18). Thus, although FAS gene is not physically located in the Her-2/neu-TOP2A amplicon, our present findings strongly suggest that a tight functional association between FAS, Her-2/neu and TOP2A genes is taking place in a subset of breast carcinomas cells. Therefore, the relationship between Her-2/neu and FAS-dependent endogenous fatty acid metabolism in breast cancer cells is more complex than previously thought, because it involves TOP2A, a key enzyme in the various processes of DNA metabolism, including transcription, recombination, replication, and chromosome segregation during cell division (34,35). Although the clinical and therapeutic importance of FAS status to breast cancer management should be resolved in clinical studies, the characterization of a putative ‘functional amplicon’ involving FAS, Her-2/neu and TOP2A genes should enhance our understanding of how FAS-dependent endogenous fatty acid metabolism, once considered a minor anabolic-energy-storage-pathway in normal cells, may be consider a ‘metabolic oncogene’ that actively contributes to the cancer phenotype through previously unexpected bi-directional cross-talks with well-characterized cancer-regulating genes such as Her-2/neu and TOP2A (Fig. 2c).

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