Fatty acid synthase is a potential molecular target for the chemoprevention of breast cancer

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The purpose of this investigation was to determine whether fatty acid synthase (FAS) is a potential molecular target for the chemoprevention of breast cancer by evaluating the effect of the FAS inhibitor triclosan on rat mammary carcinogenesis. At 50 days of age, 60 female Sprague-Dawley rats received 50 mg/kg methyl-nitrosourea (MNU) i.p. to initiate mammary carcinogenesis. One week later, half of the rats were fed triclosan at a level of 1000 p.p.m. in an AIN-93G diet for the remainder of the experiment. The other 30 control rats were fed an AIN-93G diet without triclosan. Twelve weeks after MNU treatment, 70% of control rats had mammary adenocarcinomas compared with only 43.3% of the triclosan group (P < 0.05). The control rats had an average of 2.7 ± 0.3 tumors/rat compared with 1.8 ± 0.3 in the triclosan group (P < 0.05). Western analysis showed that the tumors in the control rats expressed high levels of FAS. Immunohistochemistry showed that sections of tumors that stained strongly for FAS also showed strong staining for proliferating cell nuclear antigen. Furthermore, at biologically relevant dose levels, triclosan inhibited the activity of FAS in mammary tumor homogenates. These results indicate that triclosan suppresses rat mammary carcinogenesis by inhibiting FAS and suggest that FAS is a promising molecular target for breast cancer chemoprevention.

Introduction

Fatty acid synthase (FAS) is a multi-enzyme complex catalyzing the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA (1,2). Expression of FAS is linked to specific functions such as the production of lecithin in fetal lungs (3), the synthesis of lipids of milk in the lactating breast, and the conversion and storage of energy in liver and in adipose tissue (4). FAS is also expressed in hypothalamic neurons and is possibly involved in the regulation of food intake (5). Cer-}


cellular target for breast cancer chemoprevention.

High levels of FAS expression have been found in many human cancers including breast, prostate, colon, ovary, endometrium, thyroid, oral cavity, esophagus, bladder, retino-blastoma and melanoma (4,10–14), and expression levels often correlate with poor prognosis (4,12,14). Recently, a number of studies have pointed to the potential importance of FAS in breast cancer. Two studies have shown that FAS expression in malignant breast tumors is associated with a 4-fold increase in risk of death (12,15). In one of these, there was a 9-fold increased risk of death when high levels of FAS expression occurred together with a high proliferative index (>17%) (15). FAS expression appears to play an important role in the growth and survival of breast cancer cells since FAS inhibitors decrease cell proliferation and induce apoptosis in breast cancer cell lines (16). Growth of s.c. xenografts of MCF-7 breast cancer cells were shown to be inhibited in nude mice treated with the FAS inhibitor C75 without comparable toxicity in normal tissues (17), suggesting that FAS may be a useful target for chemotherapy.

Recent studies have suggested that FAS may also be involved in the process of malignant transformation. In breast epithelial cells, HER2 over-expression and v-H-ras-mediated transformation lead to up-regulation of FAS and sensitize the cells to apoptosis induced by FAS inhibitors (18,19). These results suggest that FAS inhibitors may be potential chemopreventive agents for breast cancer development. In a previous study, we showed that the cyclooxygenase-2 (COX-2) inhibitor celecoxib is a potent inhibitor of rat mammary carcinogenesis (20). Celecoxib treatment also decreased fat deposition in these rats that were fed a high fat diet and significantly down-regulated FAS expression in liver and visceral adipose tissues (unpublished observations). These results prompted us to speculate that suppression of the fatty acid synthesis pathway may contribute to the chemopreventive effects of celecoxib in mammary carcinogenesis. No studies, however, have demonstrated that FAS is, indeed, a molecular target for breast cancer chemoprevention.

Triclosan is a broad-spectrum antimicrobial agent because of its ability to inhibit bacterial FAS (21). It has been safely used in a variety of personal care products for >20 years (22). This antibiotic has also been shown to inhibit mammalian and avian FAS activity by inhibiting enoyl-reductase of the FAS multi-enzyme complex (23). The objective of the present study was to provide evidence that FAS is a molecular target for the prevention of breast cancer by examining the effects of triclosan on mammary carcinogenesis in rats.

Materials and methods

Tumor induction protocol

Female Sprague-Dawley rats (43 days old), purchased from Charles River Laboratories (St Constant, Quebec, Canada), were housed at 22 ± 2°C, 50% humidity with a 12-h light-dark cycle. Tap water was provided ad libitum throughout the experiment. The rats were acclimatized for 1 week on an
AIN-93G diet. At 50 days of age, they were given a single i.p. injection of 50 mg/kg methyltritosrosine (MNU, Sigma Chemical, St Louis, MO) dissolved in 0.05% acetic acid in normal saline and used within 30 min of preparation. A week later, the animals were randomized into control and experimental groups (30/group). The control group was maintained on the AIN-93G diet, and the experimental group was fed the AIN-93G diet supplemented with 1000 p.p.m. triclosan (Protameen Chemicals, Totowa, NJ). Both diets were in the form of pellets. Animals were weighed and palpated for mammary lesions weekly. Moribund animals, those with tumors >20 mm in diameter, or those remaining 12 weeks after MNU administration were killed and the lesions dissected. All internal organs were examined at autopsy. Tumors were fixed in 10% formalin, embedded in paraffin, sectioned and processed for histopathological evaluation by hematoxylin and eosin staining.

Western blot analysis
Mammary tumors were homogenized on ice in a glass–glass tissue grinder with PBS containing 10 μg/ml leupeptin, 10 μg/ml pepstatin A and 10 μg/ml apronin. The homogenates were centrifuged at 16 000 × g for 10 min and the supernatants were used for western analysis. Twenty micrograms of protein were fractioned by 10% SDS-PAGE. Proteins were transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 5% dried non-fat milk in TTBS (10 mM Tris-HCl, pH 8.0, 100 mM NaCl and 0.05% Tween 20) overnight at 4°C. Then, incubated with a mouse monoclonal anti-FAS antibody (BD Transduction Laboratories, Mississauga, Canada) at 1:250 dilution or a mouse monoclonal anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:500 dilution for 2 h at room temperature, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Santa Cruz Biotechnology) at 1:2000 dilution for 45 min at room temperature.

Immunohistochemical staining for FAS and proliferating cell nuclear antigen (PCNA)
Serial sections (5 μm) from paraffin-embedded mammary tumors were subjected to heat-induced epitope retrieval followed by exposure to primary antibodies. Detection was with the LSAB2 system (DakoCytomation, Mississauga, Canada). The anti-FAS antibody was used at 1:100 dilution and the mouse monoclonal antibody against PCNA (DakoCytomation) was diluted 1:5. The specificity of the staining was ensured by replacing the primary antibody with a negative serum (from the LSAB2 system). In all cases, no staining was observed.

FAS activity analysis
FAS activity of mammary tumors was measured using a method described previously (24). Briefly, tumors were homogenized on ice in 20 mM Tris–HCl, pH 7.5 containing 1 mM DTT and 1 mM EDTA using a glass–glass tissue grinder. Homogenates were centrifuged at 12 000 × g for 10 min and the supernatants were used for measuring FAS activity. Thirty micrograms of protein in a volume of 20 μl were added to 125 μl of 100 mM potassium phosphate, pH 7.0, containing 100 mM KCl, 0.5 mM NADPH and 0.25 μM triclosan. The reaction mixtures were prewarmed for 15 min at 37°C, and reactions were started by the addition of 4.5 μl of a substrate mixture containing 25 nmol of acetyl-CoA, 25 nmol of malonyl-CoA and 0.05 μCi (5 μl) of [2-14C]malonyl-CoA (NEN Life Sciences Products, Boston, MA). Reactions were carried out at 37°C for 10 min and were stopped by the addition of 1 ml of ice-cold 1 N HCl/methanol (6:4, v/v). Fatty acids were extracted with 1 ml of petroleum ether (Sigma) and incorporation of radioactivity into the fatty acids was assessed by scintillation counting.

Statistical analysis
Tumor incidences were compared using the χ2 test. Differences in average numbers of tumors/rat were determined using Mann-Whitney test and differences in final body weights and FAS activities were determined using Student’s t-tests.

Results

Tumor incidence and body weights
The effects of 1000 p.p.m. dietary triclosan on the cumulative mammary tumor incidence in Sprague-Dawley rats over a period of 12 weeks following administration of 50 mg/kg MNU are shown in Figure 1. This dose of triclosan has been demonstrated to be non-toxic after long-term oral administration in Sprague-Dawley rats (22). The drug produced a significant reduction in the final mammary tumor incidence (43.3% in the triclosan group versus 70.0% in the control group, P < 0.05) as well as the average numbers of tumors/rat (1.8 ± 0.3 in the triclosan group versus 2.7 ± 0.3 in the control group, P < 0.05). As expected from our own work (25) and that of others (26), histopathological examination of the tumors showed that they were adenocarcinomas.

Body weights of the two groups began to diverge somewhat 7–8 weeks after MNU administration. The food intake at that time, however, was not significantly different in the control (15.2 ± 0.5 g/rat/day) and triclosan (14.0 ± 0.6 g/rat/day) groups. However, in order to minimize body weight differences, we began to feed the control rats 10% less than the amount consumed by the triclosan-treated rats. Thereafter, the difference in body weights between the two groups was always <10%. At the termination of the experiment, the body weights of the two groups were not significantly different (352.0 ± 8.5 g vs 367.7 ± 7.4 g for the triclosan-treated and control groups, respectively). At autopsy, no gross changes in any tissues other than the mammary glands were observed.

FAS and PCNA expression in tumors
FAS was abundantly expressed in mammary tumors as shown by western blot analysis (Figure 2A), although the expression levels varied among the tumors. Immunohistochemical analysis of tumor sections showed that FAS was localized in the cytoplasm, and its expression showed cellular heterogeneity (Figure 2B). Regions of tumors showing strong staining of FAS also had high levels of PCNA expression (Figure 2B).

In vitro inhibition of FAS activity
FAS enzyme activity in mammary tumor homogenates from rats fed the control diet showed a dose-dependent inhibition by triclosan (Figure 3).

Discussion
The purpose of this investigation was to determine whether the FAS inhibitor triclosan inhibits rat mammary carcinogenesis, and thus, whether FAS is a potential molecular target for the chemoprevention of breast cancer. We used a well-established
model in which mammary adenocarcinomas are induced in rats by a single i.p. injection of the direct-acting carcinogen MNU (27). 1000 p.p.m. triclosan in the diet significantly inhibited tumorigenesis, leading to a 33% decrease in tumor multiplicity as well as a 38% reduction in tumor incidence. An unexpected small difference in body weights between the triclosan-treated and control groups occurred 7--8 weeks after MNU treatment. The food intake at that time, however, was not significantly different in the two groups, suggesting that the difference in body weights may be the metabolic outcome of FAS inhibition as reported previously for another FAS inhibitor, C75 (28,29). Throughout the course of the experiment, the difference in body weights of the two groups was kept to <10% by feeding the control rats 10% less than the amount consumed by the triclosan-treated rats. At the termination of the experiment, the body weights of the two groups were not significantly different. A 12% reduction in body weight gain induced by chronic food restriction has been shown to have no effect on mammary tumor occurrence in Sprague-Dawley rats (30). The inhibitory action of triclosan on mammary tumorigenesis, therefore, was unlikely to have been caused by the small differences in body weight that occurred during the course of the experiment.

As expected from previous studies (31), western blot analysis showed that FAS was abundantly expressed in the mammary tumors induced by MNU in rats fed the control diet, although the expression levels varied among the tumors. Previous studies have also demonstrated a link between FAS and cell proliferation. For example, studies of human endometrial carcinomas have shown that regions of tumors displaying the highest FAS expression are those regions with the highest cell proliferation as measured by Ki-67 expression (32). Similar observations have also been reported in colorectal neoplasia (33). In the present study, examination of the expression of both FAS and PCNA in mammary tumors by immunohistochemistry showed that FAS is highly expressed in the regions showing high levels of PCNA expression, suggesting that FAS is also associated with cell proliferation in our model system.

Long-term oral administration of 1000 p.p.m. triclosan in Sprague-Dawley rats has previously been shown to lead to blood levels of the drug ranging from 26.3 to 88.6 p.p.m. (90--300 μM) (22). When we incubated mammary tumor homogenates from rats fed the control diet with triclosan within this dose range, we observed significant inhibition of FAS activity, suggesting that the enzyme would have been inhibited in vivo in the present tumorigenesis experiment. Studies have shown that inhibition of FAS in human breast cancer cells leads to decreased cell growth and increased apoptosis. For example, 20 p.p.m. triclosan in the culture medium inhibited the growth and decreased the viability of MCF-7 and SKBr-3 cells (23). Furthermore, s.c. xenografts of MCF-7 cells in nude mice treated with the FAS inhibitor C75,
displayed inhibition of fatty acid synthesis, increased levels of apoptosis and inhibition of tumor growth (17). In distinction to these previous studies using breast cancer cells, however, we have shown here that inhibition of FAS inhibits the development of mammary tumors. Commensurate with our findings, we have recently shown that, like pre-neoplastic lesions of the colon and oesophagus (11,34), putative pre-neoplastic intra-ductal proliferations (IDPs) induced by MNU in the mammary glands of Wistar-Furth rats also over-express FAS (unpublished observations). Thus, triclosan probably inhibits mammary carcinogenesis by inhibiting FAS expressed in IDPs, thereby suppressing the progression of these lesions to neoplasia. Our observations also suggest that the down-regulation of FAS we observed following treatment of rats with ceceolizib (unpublished observations) might have contributed to the inhibitory effects of this drug on the mammary carcinogenesis we demonstrated previously (20).

In summary, we have shown that administration of the FAS inhibitor triclosan to rats suppresses mammary tumorigenesis. Our observations suggest that FAS is a molecular target for breast cancer chemoprevention. Furthermore, as FAS is over-expressed in many neoplasms, it may also be a useful target for chemoprevention of other common cancers such as colon and prostate.

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References


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