Effect of Branched-Chain Fatty Acids on Fatty Acid Biosynthesis of Human Breast Cancer Cells

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(Received August 22, 2003)

Summary This paper describes the antitumoral activity of branched-chain fatty acid (BCFA) in human breast cancer cells with an emphasis on its effect on fatty acid biosynthesis. First, the relationship between chain-length and antitumoral activity was studied. The highest activity was observed with iso-16:0, and the activity decreased with increase or decrease of the chain-lengths from C16:0. Anteiso-BCFA, as well as iso-series, was cytotoxic to the breast cancer cells. Cytotoxicity of BCFA was comparable to that of conjugated linoleic acid known as antitumoral fatty acid. Incubation of breast cancer cells with BCFA (13-methyltetradecanoic acid) significantly reduced the [14C] acetate incorporation into free fatty acid and fatty acid esters, showing the inhibition of fatty acid biosynthesis by BCFA. Examination of substrate level effect found that BCFA slightly inhibited fatty acid synthetase and acetyl-CoA carboxylase, and significantly the glucose-6-phosphate dehydrogenase which was the main NADPH generating system in breast cancer cells. The present study thus suggests that BCFA synthetically lowers the fatty acid biosynthesis by reducing the precursors, in addition to its direct inhibitory effect on fatty acid synthetase.

Key Words branched-chain fatty acid, biosynthesis, fatty acid synthetase, inhibition, cytotoxicity

The branched-chain fatty acid (BCFA) occurs naturally in a particular bacteria such as Bacilli species (1), or alternatively in some dairy products (2, 3). A saturated BCFA, 13-methyltetradecanoic acid (13-MTD), was purified from a soy-fermentation product as an antitumor compound (4). It was also found that BCFA induced apoptotic cell death of human cancer cells (4).

The regulation of cell growth is a homeostatic balance between stimulatory and inhibitory signals. The negative growth control by induction of apoptosis attracted many investigators’ attention, and provided a strategy for a treatment of malignancies (5–10). Complex signal transduction systems are involved in the induction of apoptosis (11, 12). This leaves the question on the mechanisms of apoptosis induction by BCFA unanswered.

The malignant tissues express high levels of fatty acid synthetase (13–19), which led to the notion that fatty acid synthetase is a target for anticancer drug development (9). It has been proposed that the inhibition of fatty acid synthetase (FAS) triggers the signal transduction flux toward induction of apoptosis, and causes the cell death (6, 8). It thus can be expected that fatty acids exert their biological effects by modulation of fatty acid metabolism, including the pathways of biosynthesis and degradation (4).

Taking all these observations into consideration, the authors postulated that the BCFA modulated the fatty acid metabolism and induced the apoptotic cell death of cancer cells. Therefore, the present study shed light on the mechanisms of anticancer activity of BCFAs with special reference to the fatty acid biosynthesis.

MATERIALS AND METHODS

Chemicals. Radiolabeled chemicals were purchased from Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England or Muromachi Pharmaceutica Ltd., Tokyo, Japan. Other chemicals were all guaranteed grade, and obtained from domestic suppliers.

Cells. Human breast cancer cell lines of MCF-7 and SKBR-3 were purchased from Health Science Research Resources Bank (HSRRB) (Osaka, Japan) and Dainippon Pharmaceuticals Co. (Osaka, Japan), respectively. Other cell lines were from HSRRB unless otherwise stated.

Culture. All cell lines were maintained and subcultured according to the supplier’s recommendations. Culture medium for MCF-7 was MEM containing 10% fetal bovine serum (FBS), 1 mM pyruvate and bovine insulin (1 μg/mL), and was McCoy’s 5A containing 10% FBS for SKBR-3. Cells were suspended in a culture medium, and diluted to a density of 10⁶ cells/

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To this cell suspension was added dimethylsulfoxide to give a final concentration of 10%, and stored in liquid nitrogen. For the cell culture, cells were thawed in 37 ℃ water bath, washed with the culture medium and inoculated in a plastic dish. The cell culture at confluency was dispersed by 0.05% trypsin/0.5 mM EDTA in PBS and used for cytotoxicity assay or another cell culture experiment.

**Cell cytotoxicity assay.** Test substances of free fatty acids were neutralized and dissolved in PBS containing 0.8% Tween 80. Cell cytotoxicity titration curve was constructed with serial dilution of the test substances in a 96-well microplate. Cells seeded at density of 700 cells/well were incubated with serially diluted test substances for 3 d, and the viable cell numbers were determined by MTS assay according to the manufacturer’s instruction (Cell Titer® AQueous Non-Radioactive Cell Proliferation Assay, Promega Co., Madison, USA). Cell cytotoxicity was thus expressed as the relative viability against control cells treated only with the vehicle solutions.

*D* _50_ was defined as the concentration killing 50% of tumor cells, and determined by fitting the following formula to the titration curve:

\[ y = \beta_3 / (1 + \exp(\beta_1 + \beta_2 \log x)) + \beta_4 \]

where *y* = cell viability (%); *x* = concentration of the test substance in the medium; \( \beta_1 - \beta_4 \) = constant. Fitting of the formula for the convergence was done by non-linear regression analysis SAS program (Logitec Analysis Program-Japanese Society of Alternative to Animal Experiments, LAP-JSAE).

**[14C] Acetate incorporation into lipids.** Human breast cancer cell lines (SKBR-3) were grown to 80% confluency in a 5.3 cm Petri dish, and were subsequently incubated with 0.25 mM fatty acid dissolved in PBS containing 0.8% Tween 80 for 24 h. To this cell culture was added [14C] acetate (2.11 MBq/μmol) to give a final concentration of 8.75 μM, and incubated for 4 h. The activities of acetyl-CoA carboxylase, fatty acid synthetase, malic enzyme and glucose-6-phosphate dehydrogenase were measured as detailed previously (21–24). The activity of ATP-citrate lyase was measured according to the procedure described by Takeda et al. (25). With respect to fatty acid synthetase of human cancer cells, the photometric method described above was not applicable because of limited enzyme protein. Thus the incorporation of [14C] malonyl-CoA into fatty acid was measured as described previously (26).

**Protein assay.** Protein concentrations were analyzed by the method of Lowry et al. using bovine serum albumin as standard protein (27).

**Statistical analyses.** The statistical significance was evaluated by Tukey-Kramer tests (28). The criterion for the statistical significance was *p* < 0.05.

## RESULTS

Although the previous studies on anti-tumor activity of soy-fermentation product found the branched-chain fatty acids (BCFAs) to be anti-tumor agents, no information on their structural characteristics relevant to their biological activity has been available. Therefore, the present study first examined the relationship between chain-length and their anti-tumor activity. Figure 1 shows the cytotoxicities of iso-series BCFAs with varied chain-lengths. The highest activity was observed with iso-16:0, and the activity decreased with increase or decrease of the chain-length from C16:0.

Two types of major BCFAs occur in nature: iso-series and anteiso-series (1). Anteiso-15:0 having methyl branching at antepenultimate (anteiso) positions show...
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Fig. 2. D50 of iso- and anteiso-BCFA. MCF-7 cells were treated with the fatty acids. Data are D50 and 95% confidence intervals estimated by non-linear regression analysis.

Fig. 3. Effect of 13-MTD (A) and myristic acid (B) on the growth of normal (○) and tumor (●) lung cells. Normal (WI 38) and tumor (A431) lung cells were respectively cultured with MEM and Dulbecco’s modified Eagle medium (D-MEM) containing 10% PBS. Data are mean of 8 analyses.

Fig. 4. D50 of BCFA and conjugated linoleic acid (CLA). MCF-7 cells were treated with the fatty acids. Data are D50 and 95% confidence intervals estimated by non-linear regression analysis.

Fig. 5. Time course of [14C] acetate incorporation into lipids of SKBR-3 cells. [14C] Acetate was incubated with SKBR-3 cells for 2 to 6 h, and the radioactivities in the lipid classes were measured. Unknown, detected between phospholipid and free cholesterol on the HPTLC chromatography.

Several lines of studies have showed that certain polyunsaturated fatty acids (PUFAs) had a selective cytotoxic effect on tumor cells and minimal or no effect on normal cells (29). By analogy, selective cytotoxicity of BCFA was studied with normal and tumor lung cells (Fig. 3). 13-Methyltetradecanoic acid (13-MTD; iso-15:0) was toxic to both normal and tumor cells with higher cytotoxicity than SCFA, n-C14:0 (compare Fig. 3A and 3B).

Conjugated-linoleic acids (CLAs) have been reported to be antitumoral fatty acids (30, 31). Two types of biologically active CLAs are known: cis-9,trans-11 and trans-10,cis-12-CLA). The former was the principal dietary form of CLA, and used in this experiment as a reference. Comparison of cytotoxicity revealed that cytotoxicity of 13-MTD was almost the same as CLA (Fig. 4). The same experiment on the SKBR-3 cells reproduced the similar results as shown in Fig. 4 (data not shown).

There has been a notion that fatty acid exerts its biological activity by affecting the fatty acid or lipid metabolism (32). We therefore examined the effect of MTD on the lipid biosynthesis from [14C] acetate. SKBR-3 cells were considered to be ideal for these studies because they express the highest cellular levels of FAS yet described in human established cell lines (33). The highest incorporation of [14C] acetate was noted with phospholipids, which increased linearly up to 4 h (Fig. 5). Incorporation of [14C] acetate into other lipids also showed a similar trend of time course as phospholipids with attenuated extent.

Cancer cells treated with 13-MTD, myristic acid (n-14:0) and CLA were incubated with [14C] acetate for 4 h (Fig. 6). The trypan blue dye exclusion test found comparable cell viability between treatments with fatty...
acids. The sum of [14C] label incorporated into free fatty acids and fatty acid esters (phospholipid + triacylglycerol + cholesterol ester) was lower in 13-MTD than myristic acid, showing an inhibition of fatty acid biosynthesis by 13-MTD (Fig. 6A). The incorporation was high with phospholipid, triacylglycerol, free fatty acid, cholesterol and cholesterol ester in the order of magnitude (Fig. 6B). 13-MTD significantly lowered the incorporation of [14C] acetate into triacylglycerol and free fatty acid, compared with myristic acid. A decreased [14C] acetate incorporation was also noted with CLA, but to a rather lower extent compared with 13-MTD. The myristic acid enhanced the transformation of free fatty acid into triacylglycerols more than 13-MTD or CLA. 13-MTD decreased the cholesterogenesis in the breast cancer cells, as well as fatty acid biosynthesis.

13-MTD decreased the fatty acid biosynthesis in breast cancer cells. The in vitro effect of 13-MTD on the enzymes involved in fatty acid biosynthesis was studied (Table 1). Because limited amounts of enzymes were available from tissue culture, the enzyme’s source was from rat liver. Five enzyme activities were assayed: fatty acid synthetase (FAS), acetyl-CoA carboxylase, glucose-6-phosphate dehydrogenase (G6PDH), ATP-citrate lyase and malic enzyme (Table 1). Concentration of fatty acid in the reaction mixture was 0.25 mM comparable to that in the culture medium.

13-MTD inhibited the FAS activity (Table 1). CLA also showed an inhibition of FAS to a greater extent than 13-MTD. Acetyl-CoA carboxylase was inhibited by both 13-MTD and CLA as was the case for fatty acid synthetase. 13-MTD and myristic acid, but not CLA, inhibited G6PDH. ATP-citrate lyase was elevated by vehicle solution, and no difference was noted between fatty acid treatments. Malic enzyme was slightly lowered by all fatty acids tested. No difference between treatments was also notable.

Of the enzymes involved in the fatty acid biosynthesis, FAS, acetyl-CoA carboxylase and G6PDH from rat liver were inhibited in vitro by 13-MTD. We therefore focused on these enzyme activities of human breast cancer cells. In the case of FAS assay, limited amounts of enzyme proteins from cancer cells did not allow to apply the colorimetric method used for the rat liver enzyme. For this reason, FAS activity was assayed by incorporation of [14C] malonyl-CoA into fatty acids (26). A decreasing tendency for FAS was seen with 13-MTD (Fig. 7), while CLA lowered the activity to a greater extent than 13-MTD. 13-MTD suppressed G6PDH from cancer cells as was the case for rat liver enzyme (Fig. 8).
There are two NADPH generating systems: malic enzyme and G6PDH. Activities in SKBR-3 cells were 59.6 \pm 3.0 (\mu mol/min/mg protein) for G6PDH and 2.3 \pm 1.5 for malic enzyme. Thus G6PDH was the main enzyme responsible for the generation of NADPH in this breast cancer cells. CLA consistently showed no inhibition to G6PDH of cancer cells. Acetyl-CoA carboxylase was not assayed due to a limited amount of the enzyme source.

Results mentioned above suggest that 13-MTD slightly inhibits FAS at substrate level. We further studied the effect of 13-MTD on the cellular concentration of enzymes. The human breast cancer cells were incubated with 13-MTD, and the enzyme concentrations were assessed by measurements of the activities. The cells tended to decrease the FAS concentration by 13-MTD treatment, compared with the case of myristic acid (Fig. 9). No significant difference was observed for G6PDH activity between treatments (Fig. 10).

**DISCUSSION**

Higher expression of FAS in tumor cells has led to the notion that FAS may become a therapeutic target for cancer-treatment (9). Several lines of studies have demonstrated the link between cellular proliferation and fatty acid biosynthesis, and inhibition of FAS induces the apoptosis in human cancer cells (5–8). Previous study also showed that BCFA induces apoptotic cell death, and inhibits in vivo growth of human cancer cells (4).

The authors assumed that the FAS inhibition could be responsible for the growth inhibition of cancer cells by BCFAs. This assumption is quite reasonable because it is possible that BCFAs affect the cell replication process through modulation of fatty acid metabolism.

As we assumed, BCFAs inhibited the fatty acid biosynthesis in the cultured SKBR-3 breast cancer cells (Fig. 6). Examination of the effect on the rat liver enzymes involved in fatty acid biosynthesis revealed that BCFA (13-MTD) was inhibitory to FAS, acetyl-CoA carboxylase and G6PDH. CLA, as well as 13-MTD, was an inhibitor to FAS and acetyl-CoA carboxylase, but not to G6PDH. Thus the difference between 13-MTD and CLA appeared to lie in the effect on G6PDH. It was shown that palmitoyl-CoA inhibits yeast G6PDH by dissociating the tetrameric enzyme into an inactive dimeric subunit (34). 13-MTD may decrease the mammalian G6PDH by a similar mechanism.

G6PDH is the first enzyme in the hexose monophos-
phate shunt which provides important precursors for both fatty acid and nucleotide synthesis. For this reason 13-MTD might synthetically lower the fatty acid biosynthesis by reducing the substrate NADPH in addition to its direct inhibitory effect on EAS. Furthermore, G6PDH is an interface enzyme between the glycolytic pathway and hexose monophosphate shunt. Inhibition of G6PDH therefore decreases both substrates for fatty acid and nucleotide biosynthesis. BCFA as 13-MTD may affect the cell dynamics by reducing substrates for both DNA duplication and membrane biogenesis.

13-MTD was shown to be toxic against both cancer and normal lung cells in vitro (Fig. 3). However, this does not necessarily indicate that 13-MTD damages both tumor and normal tissues in vivo. It has been shown that 13-MTD effectively inhibited in vivo growth of various cancer cells without significant side effects (4). Cancer cells expressed higher levels of FAS, and are more dependent on the fatty acid biosynthesis pathway for survival than normal cells (10). This leads to the accepted view that inhibition of EAS is selectively cytotoxic to human cancer cells in vivo (7, 10). Rapid growing normal and cancer cells may even require an increased fatty acid biosynthesis. However, normal tissue does not need to undergo rapid regenerations under normal situations. Therefore, demand for fatty acid synthesis may be smaller in the normal tissues than cancer cells, which may explain in part the selective cytotoxicity of EAS inhibition against cancer cells. Of these tissues, however, rapidly regenerating tissues such as intestinal epithelium or bone marrow may need more fatty acid synthesis than the other tissues even under normal conditions. For this reason, the effect of EAS inhibition may differ with the tissues. Care should be taken when referring to the effect of EAS inhibition on the rapidly proliferating tissues, and no reference to this point has been made in the previous studies.

Inhibition of EAS by cerulenin causes a rapid accumulation of malonyl-CoA, which could be a trigger of the apoptotic cell death (35-37). Furthermore, accumulation of malonyl-CoA inhibits carnitine palmitoyltransferase-I, hence fatty acid oxidation (35). Combined inhibition of fatty acid biosynthesis and oxidation seems to explain the cytotoxicity of cerulenin (35). For this reason, the forthcoming study should focus more on the insight into the detailed mechanism of BCFA cytotoxicity.

REFERENCES

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