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EFFECT OF DIETARY STEARIC ACID ON THE GENESIS OF SPONTANEOUS MAMMARY ADENOCARCINOMAS IN STRAIN A/ST MICE

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Strain A/ST female mice maintained on a high fat (15%) diet in which stearic acid was the major lipid component developed initial spontaneous mammary adenocarcinomas at an older age than mice fed a low fat (4.5%) stock diet. Mice placed on the SA diet at weaning developed tumors at 15.7 ± 0.87 months compared to 12.7 ± 0.43 months for those retained on the stock diet ($p < .05$). Placing mice on the SA diet at 11.5 months resulted in a smaller but significant increase in the latency period (5.0 ± 0.86 vs 3.0 ± 0.57 months ± 0.57 mo), ($p < .05$). Fatty acid analyses of non-tumorous mammary tissue from mid-pregnant mice and of tumor tissues showed that feeding large amounts of 18:0 did not result in increases in the proportion of 18:0. Significant reductions in the percentages of polyunsaturated fatty acids (PUFA) was found in tissues on mice fed the SA diet. The percentage of 18:2 was reduced in both types of tissues; 20:3 and 20:4 was reduced in tumor tissues. Distribution of C_{18} fatty acids in plasma membranes of tumors of mice fed the two diets were similar; percentages 18:2 was higher in plasma membranes of non-tumorous tissues of mice fed the SA diet. These results suggest that dietary stearic acid interferes with the availability of certain PUFA required for tumor production.

Numerous studies have shown that high-fat diets promote tumor growth and increase the incidence of mammary tumors in mice and rats (Tannenbaum, 1942; Carroll and Khor, 1970; Chan and Cohen, 1975; Hopkins and West, 1976). Although polyunsaturated fats were more effective than saturated fats in promoting the development of spontaneous, 7.12-dimethyl (α)-anthracene (DMBA)-induced and transplanted mammary tumors (Carroll and Khor, 1971; Rao and Abraham, 1976; Hillyard and Abraham, 1979; Hopkins *et al.*, 1978), neither total fat nor the overall degree of unsaturation can be used as a single indicator of tumorigenicity of a diet (Hopkins and West, 1977; King *et al.*, 1979). Dietary fat appears to affect the developmental stage of tumorigenesis by providing a more favorable environment for the growth of latent tumor cells (Carroll, 1975; Hopkins *et al.*, 1978). It has been suggested that prostaglandins, derived from polyunsaturated fats, promote the neoplastic process by affecting immune reactions and limiting host responsiveness during early stages of tumor growth (Kollmorgen *et al.*, 1979; Rolland *et al.*, 1980; Fischer *et al.*, 1981; Carter *et al.*, 1983). Other workers have related the tumor-promoting effect of high-fat diets to alterations in the plasma membrame and cell structure of preneoplastic cells (Schlager and Ohanian, 1979).

A few studies have attempted to identify the role of individual dietary fatty acids. Hillyard *et al.* (1980) observed enhanced growth of transplanted tumors in rats when as little as 0.1% linoleate was added to a fat-free diet. Chan *et al.* (1983) reported that the major factor influencing the incidence of tumors induced by N-nitrosomethylurea is the total oleate and linoleate content of a high-fat diet. Tinsley *et al.* (1981), using statistical methods to isolate the effects of individual dietary fatty acids on the incidence of spontaneous mammary tumors in C3H mice, concluded that linoleate, but not oleate, was essential for tumor development. Increased amounts of stearate were associated with lower tumor incidence.

In the present study a 15% fat diet containing over 13% stearic acid was used to determine the effect of this saturated fatty acid on the genesis of spontaneous mammary adenocarcinomas in strain A/St mice. The level of linoleate was sufficient to support normal growth of the animals, allowing the experiments to continue over a 2-year period. Fatty acid distributions of tumors and mammary gland tissues of mice fed the experimental diet were compared to those in tissues excised from mice fed a low-fat (4.5%) stock diet.

MATERIAL AND METHODS

Strain A/St mice now maintained by brother: sister inbreeding in our laboratory (Strong, 1936), were housed in plastic cages in a controlled environment (21-24°C, 24 h light: dark cycle) and fed a 4.5% fat stock diet of Purina Lab Chow (Ralston Purina, St. Louis, MO) and water *ad libitum*. Information including dates of birth and of tumor occurrence was recorded and stored in a computer file. On weaning, one male and 3 female litter-mates were kept in breeder cages until females had produced two litters (approximately 5-6 months). Females were then transferred to larger retirement cages, each of which held 14-17 mice. Twenty-six female mice were placed on an experimental (SA) diet containing 14% stearic acid (95% purity), 1% safflower oil, and 85% "fat-free" test diet (US Biochemical Corp., Cleveland, OH) at weaning. Twenty-seven "retired" females from the same litters were retained on the low-fat stock diet (Table I). Another group of 23 "retired" females were placed on the SA diet at 11½ months of age (Table I). Sixty-five females retired during the same time period and retained on the stock diet were used as a reference group.

Adequacy of the SA diet was determined by observing 12 female mice placed on the diet at weaning. Over a period of one year, weights of these animals did not differ significantly from those of mice fed the stock diet.

Animals were checked weekly for tumors. Tumors that reached 1-2 cm in diameter were surgically re-

Abbreviations: HAN, hyperplastic alveolar nodules; PUFA, Polyunsaturated fatty acids; SA, stearic acid diet; 18:0 etc., first number refers to number of carbons, second number to number of double bonds in fatty acids.

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TABLE 1 – FATTY ACID COMPOSITION OF DIETS

Fatty acid	Stock diet (4.5% fat)		SA diet (15% fat	
	Dietary[1] fat	Total diet	Dietary fat	Total diet
14:0	4.1	0.2	–	–
16:0	23.7	1.2	6.0	0.9
16:1	3.7	0.2	–	–
18:0	6.3	0.3	87.0	13.1
18:1	26.3	1.3	0.9	0.1
18:2	30.6	1.5	5.1	0.8
18:3	2.7	0.3	–	–
20:0	0.8	0.01	0.1	0.1
Total saturated fatty acids	35.2	1.7	93.1	14.1

[1]Figures represent the percentage total fatty acids.

moved and analyzed as described below. The age of each mouse was recorded when its first tumor was removed.

Non-tumorous mammary tissue was obtained from female mice placed on the SA diet at weaning and from those fed the stock diet. Mammary tissue was removed from mice bred at 2½ months of age and killed, by cervical dislocation, at the 15-17th day of the gestation period. Excised tumors and mammary gland tissues were immediately placed in ice-cold isolatin medium (0.25 M sucrose, 0.1 M $KHPO_4$, pH 7.0). All tissues were analyzed immediately or stored briefly at –20°C.

Tumors were cleaned of any adhering necrotic tissue or blood, washed, cut into fine pieces, suspended in 5 vol isolation medium and homogenized in an Elvehjem glass homogenizer. Connective tissue was removed by filtering the homogenate through 4 layers of cheesecloth. One-half of the filtrate was set aside for fatty acid analysis; the remainder was used for preparation of plasma membranes. Mammary gland tissue was treated similarly.

Plasma membranes were isolated by a modification of the procedure described by Emmelot *et al* (1974). The tissue homogenate was centrifuged at 1,500 *g* for 10 min, then the pellet was resuspended in a calcium-fortified buffer (2 mM $CaCl_2$, 1.0 mM $NaHCO_3$, pH 7.5) and centrifuged at 750 *g* for 10 min. The supernate, containing the plasma membranes, was removed and centrifuged at 1,500 *g*. The resulting pellet was resuspended in calcium-fortified buffer and centrifuged at 1,500 *g*. This step was repeated 3 times. The final pellet, in calcium-fortified buffer, was mixed with 2 × volume of sucrose solution (d 1.34) and subjected to a discontinuous (d 1.16-1.22) sucrose gradient at 105,000 *g* for 90 min. Plasma membranes were obtained from the interface of the d 1.16 and d 1.18 layers. Purity of the plasma membranes was verified by microscopic examination and determination of activities of marker enzymes (Emmelot and Bos, 1969; Emmelot *et al.*, 1974). Membrane preparations were assayed for NADPH cytochrome C oxidoreductase (Scottocasa *et al.*, 1967), NADH cytochrome C oxidoreductase (Mahler, 1955), RNA (Munro and Flick, 1966) and DNA (Ceriotti, 1955).

Fatty acid analyses. Plasma membranes and total tissue fractions were saponified by refluxing in 15% KOH in 85% methanol at 85°C for 90 min. After acidification with concentrated HCl, fatty acids were extracted with hexane:ether (1:1 vol/vol), washed with 0.37% KCl, and dried over anhydrous sodium sulfate. The solvent was evaporated under N_2 and the resulting fatty acids were methylated by treatment with diazomethane (Schlenk and Gellerman, 1960).

Methyl esters of fatty acids were purified by thin-layer chromatography on plates coated with silica gel G impregnated with 0.02% rhodamine B using hexane:ether:acetic acid (70:30:1; v/v/v) as the developing solvent. Methyl esters were scraped from the plates and eluted from the silica gel with hexane:ether (1:1, vol/vol). Fatty acids were analyzed quantitatively by GLC at 180°C in a Packard Model 7500 (Packard, Downers Grove, IL) using a flame ionization detector and a 10 ft × 0.08 in. (305 cm × 2 mm) glass column packed with 5% diethylene glycol succinate on 80-100 mesh chromosorb W-AW (Alltech Associates, Deefield, IL) with an N_2 flow rate of 40 ml/min.

The fatty acid methyl esters were identified by comparing retention times to those of standards (Supleco, Bellefonte, PA) using a best-fit computer program. Data obtained from standards and adjustments for any variation in overall retention time of individual samples were provided. Further verification of the identity of fatty acid methyl esters was based on cochromatography with standards on a 10 ft × 0.08 in. (305 cm × 2 mm) SE-30 glass column before and after hydrogenation. Identity of some fatty acid methyl esters was verified by GLC analysis of methyl esters separated by argentation TLC.

Statistical significance was assessed using student's *t*-test.

RESULTS

Seventeen of 26 female mice placed on the high-fat SA diet at weaning and 20 of 27 mice retained on the low-fat stock diet developed spontaneous mammary adenocarcinomas by the time they were 24 months old.

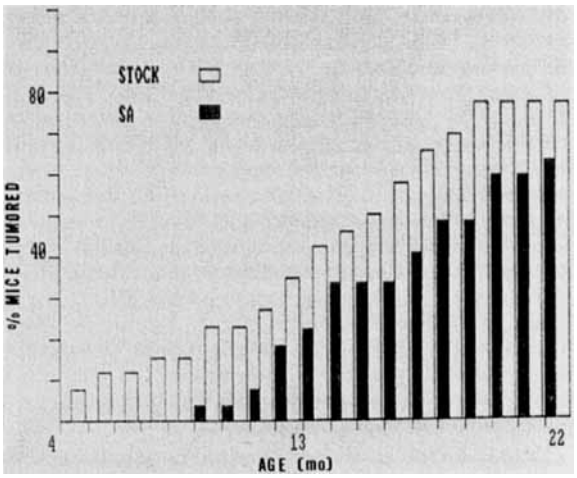

FIGURE 1 – Effect of high stearic acid diet on the formation of spontaneous mammary tumors. Stearic acid was present in the diet at a level of 13% (14.1% saturated fatty acids) and was instituted at weaning. Stock diet contained 4.5% fat (1.7% saturated fatty acids) instituted at weaning. Differences in age of initial tumor formation in two groups were significant at $p < 0.05$.

The average age at tumor development of the mice fed the SA diet was 15.7 ± 0.87 months compared to 12.7 ± 0.43 months for females retained on the stock diet. This difference was significant at $p < 0.05$ (Fig. 1). The time to 50% incidence for the mice fed the SA diet was 18 months compared to 15 months for those fed the stock diet. Three females fed the SA diet, but none fed the stock diet, lived beyond the 24-month experimental period. Observations of over 1,200 mice in our colony over a period of 7 years reveal that approximately 70% of those fed the stock diet develop mammary tumors at a mean age of 12.6 months (median age of 12.5 months). In this colony, few live beyond 2 years of age.

Feeding the SA diet for a shorter period of time also resulted in a delay in tumor development. Sixteen of 23 mice placed on the SA diet at 11.5 months of age, just prior to the age of maximal tumor onset, developed mammary tumors after an average of 5.0 ± 0.86 months. Forty-three of 60 mice that were born during the same time period and retained on the stock diet developed tumors after 3.0 ± 0.57 months. This difference was significant at $p < 0.05$ (Fig. 2). Fifty percent of

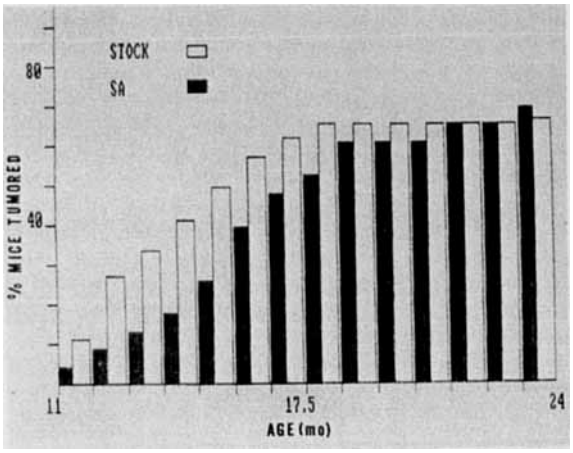

FIGURE 2 – Effects of high stearic acid diet on the formation of spontaneous mammary tumors. Both groups of animals were maintained on the stock diet for 11.5 months, after which the SA diet was instituted. Diets were the same as in Figure 1. Differences in age at tumor formation were significant at $p < 0.05$.

the mice on the SA diet that developed tumors had done so by the time they were 16.0 months old compared to 14.1 months for the mice on the stock diet. The time to 50% incidence for the mice fed the SA diet was 18 months compared to 15 months for those fed the stock diet.

Fatty acid analysis showed a reduction in the percentage of PUFA in tissues removed from mice fed the SA diet compared to that seen in tissues from mice fed the stock diet. The percentage of 18:2 and 20:4 in mammary tumor tissues removed from mice fed the SA diet was significantly lower (Table II). The percentage of 18:2 was one-fourth and that of 20:4 was one-half of that recovered from tumors of mice fed the stock diet. Although stearic acid accounted for 87% of the fatty acids in the SA diet, the percentages of 18:0 in the tumors removed from mice fed the two diets were

TABLE II – FATTY ACID COMPOSITION OF TISSUES AND PLASMA MEMBRANES OF MAMMARY ADENOCARCINOMAS OF MICE FED DIFFERENT DIETS[1]

Fatty acid	Total tissue		Plasma membranes	
	Stock diet (20)[2]	SA diet (13)	Stock diet (8)	SA diet (11)
<14	0.2±0.1	2.4±0.6[3]	10.7±4.8	10.9±4.1
14:0	2.7±0.4	2.1±0.3	18.2±2.7	7.9±1.9[3]
16:0	22.5±1.0	19.4±0.9[3]	12.3±2.3	11.8±1.0
16:1	4.1±0.4	7.4±0.4[3]	3.4±0.6	4.6±0.7
18:0	8.0±0.9	8.3±0.8	9.4±2.1	10.2±0.9
18:1	35.1±1.4	46.2±3.1[3]	9.7±1.9	12.6±1.5
18:2	11.0±1.2	2.7±0.4[3]	1.9±0.7	1.4±0.2
20:3	1.7±0.5	0.8±0.3	4.5±1.4	8.1±0.5[3]
20:4	4.4±0.6	2.3±0.5[3]	0.1±1.5	1.5±0.3[3]
22:1	0 ±0	0.1±0	2.5±0.8	5.1±1.2
22un	1.0±0.2	1.3±0.4	1.2±0.9	4.0±1.1
24un	3.6±1.0	4.4±1.1	19.4±5.2	14.5±3.0

[1]Results are percentages of total fatty acids, reported as mean ± SEM. – [2]Numbers in parentheses = number of tumors used. – [3]$p < 0.05$, compared to stock diet.

similar. The percentages of both 18:1 and 16:1 were elevated in tumors of mice fed the SA diet. The major fatty acid component in tumors from both groups of animals was 18:1.

Distributions of C_{18} fatty acids in plasma membranes of tumors of mice fed the two diets were similar. Percentages of 20:3 and 20:4 varied in plasma membranes of tumors from mice fed SA diet or the stock diet. The percentage of 20:3 in plasma membranes of tumors of mice fed the SA diet was twice that of those fed the stock diet (8.1 ± 0.5 vs 4.5 ± 1.4). In both groups 20:4 was low, but was significantly higher in plasma membranes of mice fed the SA diet (0.1 ± 1.5 vs 1.5 ± 0.3).

To determine the effect of diet on the tissues from which tumors develop, the fatty acids of the total tissue fraction and plasma membrane fractions of non-tumorous mammary tissue excised from mid-pregnant female mice were analyzed (Table III). These tissues have been reported to closely resemble HAN outgrowths according to Kopelovich *et al.* (1966). As was observed

TABLE III – FATTY ACID COMPOSITION OF TISSUES AND PLASMA MEMBRANES OF MAMMARY TISSUES OF MICE FED DIFFERENT DIETS[1]

Fatty acid	Total tissue		Plasma membranes	
	Stock diet (5)[2]	SA diet (11)	Stock diet (6)	SA diet (10)
<14	0.8±0.5	0.9±0.3	3.4±0.9	11.8±4.9
14:0	1.8±0.4	6.5±1.2[3]	5.6±0.9	8.8±2.0
16:0	22.4±1.0	15.5±0.9[3]	13.3±1.3	11.9±1.1
16:1	7.5±0.5	6.4±0.8	5.9±0.5	5.4±0.9
18:0	3.9±0.3	4.6±0.3	13.6±1.3	5.5±0.5[3]
18:1	39.7±2.0	46.8±2.9	5.9±0.7	21.9±3.6[3]
18:2	15.4±0.8	2.8±0.5[3]	0.8±0.2	6.0±1.4[3]
20:3	1.5±1.1	1.1±0.3	6.7±0.8	2.2±0.6[3]
20:4	0.6±0.1	0.7±0.1	4.8±2.9	0.3±0.1
22:1	0.7±0.3	0.4±0.1	5.3±1.7	0.8±0.3[3]
22un	0.9±0.5	2.7±1.3	10.4±0.9	3.7±1.0[3]
24un	1.1±0.8	5.5±1.4	15.2±2.4	12.4±3.4

[1]Results are percentage of total fatty acids, reported as mean ± SEM. – [2]Numbers in parentheses = number of animals used. – [3]$p < 0.05$, compared to stock diet.

in the tumor tissues, the percentage of 18:0 in the mammary gland tissue of mice fed the SA diet was not significantly higher than in the tissues of mice fed the stock diet. The predominant fatty acid in tissues of both diet groups was 18:1. A significant reduction in the percentage of 18:2 in mammary gland tissues of mice fed the SA diet was found (2.9 ± 0.5 compared to 15.4 ± 0.8). Mammary tissues from mice fed the SA diet had a lower percentage of 16.0. Percentages of 20:3 and 20:4 were similar.

Analysis of fatty acids isolated from plasma membranes of mammary glands removed from mice fed the SA diet revealed significant shifts in the distributions of the C_{18} fatty acids when compared with those from mice fed the low-fat stock diet (Table III). Although mammary gland tissues of mice fed the stock diet had higher percentages of 18:2, the percentage of 18:2 in fatty acids recovered from plasma membranes of mice fed the SA diet was 6.0 ± 1.4 compared to only 0.8 ± 0.2 in those from mice fed the stock diet. The percentage of 20:3 in fatty acids from plasma membranes of mice fed the SA diet was approximately one-third that of mice fed the stock diet (2.2 ± 0.6 *vs* 6.7 ± 0.8).

Unsaturated fatty acids of 24 carbon chain length were found in similar amounts in tissues and plasma membranes of mice fed either diet. These long-chain fatty acids have been reported to be components of proteolipids (Skipski *et al.*, 1971) and of phospholipids in plasma membranes (van Hoeven *et al.*, 1975).

DISCUSSION

Results of this study demonstrate that a high-fat diet *per se* does not stimulated the development of spontaneous mammary adenocarcinomas in mice. In fact, when stearic acid was used as the major lipid component in a high-fat diet, the development of initial tumors was delayed. The significant increase in the latency period in mice shifted to the SA diet at 11½ months of age indicates that the effect of dietary fat on mammary carcinogenesis in mice is not necessarily a long-term effect. This would be consistent with previous observations that dietary fat acts as a promoter in the preneoplastic stage of tumor development. Stearic acid was chosen as the fatty acid to be investigated because it can be acted on by the animal's desaturase-elongation system, but cannot be converted into linoleic acid and other prostaglandin-active fatty acids. These desaturase systems are related and subject to dietary regulation (Kurata and Privett, 1980). Thus, a large amount of stearic acid might interfere with the synthesis of arachidonic acid from the available linoleic acid.

Unlike many of the saturated fat diets used in studying tumorigenic properties of fat, the SA diet contained sufficient linoleic acid to produce normal growth, prevent EFA deficiency symptoms, and sustain mice in a study that would extend over a period of 2 years. Both the stock and the SA diet contained less than then 3% linoleic acid considered optimal for tumor production (Carroll, 1975); still the production of tumors in mice fed the SA diet was delayed when compared to that in animals fed the low-fat stock diet.

The distributions of C_{18} fatty acids of lipids extracted from tumor tissues of mice fed the stock diet were similar to those reported by Rao and Abraham (1975). In these experiments, tumorous and non-tumorous tissues of mice fed a diet containing over 87% stearic acid had stearate concentrations similar to those found in tissues of mice fed the low-stearate, low-fat stock diet. Instead, 18:1 was the major fatty acid component in both groups. The distribution of C_{18} fatty acids of tumors taken from SA-fed mice was similar to that observed in transplanted mammary adenocarcinomas of C3H mice fed a fat-free diet (Rao and Abraham, 1975) that had low tumorigenicity.

If dietary fat, or more specifically linoleic acid, does promote the neoplastic process by limiting the host responsiveness during the early stages of tumor development, the results of fatty acid analysis of mammary gland tissues of mid-pregnant mice are of particular significance. These tissues have a lower percentage of 18:2 available for the synthesis of membranes or arachidonic acid and the prostaglandins. The higher percentage of 18:2 found in the plasma membranes of mammary gland tissues suggests that available 18:2 may be used preferentially for the synthesis of membranes, thus maintaining the fluidity of the membrane. If so, less 18:2 would be available for the tumor-enhancing process.

These results are in agreement with those of Tinsley *et al.* (1981) who found that increasing levels of stearate in high-fat diets were associated with decreased tumor incidence and increased time to tumor development in C3H mice. Other workers have reported that stearic acid differs from other saturated fatty acids in its physiological and biochemical effect. For example, when compared to other saturated fatty acids, stearate has a greater effect on liver lipids, plasma cholesterol and the cholesterol content of the liver (Caster *et al.*, 1975). Stearate is also more effective than palmitate or oleate in inhibiting fatty acid synthesis in Ehrlich cells (McGee and Spector, 1974) hepatocytes (Goodridge, 1973) and fibroblasts (Jacobs and Majerus, 1973). Further, stearate has been reported to inhibit the growth of normal and neoplastic rat mammary epithelial cells (Wicha *et al.*, 1979). Concentrations of other lipid components of tumors and mammary gland tissues of mice fed the SA diet are currently being determined.

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