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Lack of Promoting Effects of α-Linolenic, Linoleic or Palmitic Acid on Urinary Bladder Carcinogenesis in Rats

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Potential promoting effects of α-linolenic, linoleic and palmitic acids were investigated in a two-stage urinary bladder carcinogenesis model. In experiment 1, male F344 rats were given 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) in their drinking water for 4 weeks and then basal diet containing 10% α-linolenic, 10% linoleic or 10% palmitic acid along with 0.2% butylated hydroxyanisole (BHA) as an antioxidant for 24 weeks. The development of tumors in the urinary bladder was not increased by treatment with any of the fatty acids. In experiment 2, male F344 rats were given 10% α-linolenic, 10% linoleic or 10% palmitic acid along with 0.2% BHA in their diet for 8 weeks without prior BBN treatment. The administration of fatty acids was not associated with any increase in the 5-bromo-2′-deoxyuridine labeling index of the urinary bladder epithelium. Serum and/or urine fatty acid levels increased in the cases of α-linolenic and linoleic acid treatments, but not with palmitic acid. Under the present experimental conditions neither the two polyunsaturated nor the one saturated fatty acid exerted any promoting effect on urinary bladder carcinogenesis.

Key words: Urinary bladder carcinogenesis — Fatty acid — N-Butyl-N-(4-hydroxybutyl)-nitrosamine

Epidemiologically it has become clear that food and nutrition are extremely important factors for human carcinogenesis. In particular, dietary fat intake is related to the risk of breast, colon and prostate cancer development.[1,2] Furthermore, in a number of animal models, promoting effects of dietary fat on breast and colon tumorigenesis have been reported.[3–5] The development of urinary bladder carcinomas in the Western world is four times more frequent than in the Japanese. The epidemiological evidence suggests that the difference in fat intake might be one factor causing this variation in urinary bladder cancer incidences between Western people and Japanese.[6] However, there has been no experimental demonstration of any relationship between dietary fat or fatty acid intake and urinary bladder tumorigenesis.

In previous experimental carcinogenesis studies, fats or oils have been used, and there are some data that indicate promoting effects on carcinogenesis. We considered that a main factor of fat intake correlating with carcinogenesis is the level of fatty acid. Therefore in this study we used free fatty acids instead of fats or oils to exclude other factors contained in dietary fat. In the present experiment we investigated the influence of three fatty acids, α-linolenic acid as an n-3 series polyunsaturated fatty acid, linoleic acid as an n-6 series polyunsaturated fatty acid and palmitic acid as a saturated fatty acid. α-Linolenic acid and linoleic acid are essential fatty acids, the latter being present in many vegetable oils and frequently taken in the diet. Recently fats or oils rich in these fatty acids have often been used in experiments to determine modifying effects in colon or breast carcinogenesis.[4,7] Although n-3 fatty acid was found to inhibit colon and mammary gland carcinogenesis in a recent study,[8] the effect of n-3 fatty acids on urinary bladder carcinogenesis is not entirely clear, either epidemiologically or experimentally. Palmitic acid is a constituent of many vegetable oils and animal fats, and it is the most representative saturated fatty acid. In order to cast light on the relationship between dietary fat and urinary bladder cancer, the three fatty acids were studied using an established two-stage urinary bladder carcinogenesis model in rats.[9]

MATERIALS AND METHODS

Animals A total of 170 male 5-week-old F344/DuCrj (F344) rats were purchased from Charles River Japan Inc., Hino, Shiga. They were housed, 2–3 rats (Experiment 1) or 5 rats (Experiment 2) per cage, in a room maintained with a 12 h (7:00–19:00) light-dark cycle, at a constant temperature of 25±1°C, and relative humidity of 55±5%. The animals were observed daily, and were used at 6 weeks of age for the following experiments.

Chemicals N-Butyl-N-(4-hydroxybutyl)nitrosamine (BBN) was obtained from Tokyo Kasei Co., Ltd. (Osaka). Butylated hydroxyanisole (BHA) was from

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Wako Pure Chemical Company (Osaka). α-Linolenic acid (18:3, n-3) was from Eastman Kodak Company (Rochester, New York). Linoleic acid (18:2, n-6, purity: 75%) and palmitic acid (16:0, grade: guaranteed reagent) were from Kishida Chemical Co., Ltd. (Osaka).

Experiment 1 One hundred and forty-five male F344 rats were used. The animals were administered drinking water with (Groups 1–5, 20 rats in each group) or without (Groups 6–10, 9 rats in each group) 0.05% BBN for the first 4 weeks. Then they were given powdered basal diet (Oriental MF; Oriental Yeast Co., Tokyo) containing 10% α-linolenic acid and 0.2% BHA (Groups 1 and 6), 10% linoleic acid and 0.2% BHA (Groups 2 and 7), 10% palmitic acid and 0.2% BHA (Groups 3 and 8) or 0.2% BHA only (Groups 4 and 9) for 24 weeks. The animals of control groups (Groups 5 and 10) were given powdered basal diet without any supplement. The powdered basal diet (MF) without fatty acid supplement contains 5.6% fat, and the fatty acid composition is α-linolenic acid (3.1%), linoleic acid (45.9%), palmitic acid (14.9%) and others (36.1%). BHA was used as an antioxidant in order to counteract the influence of fatty acid oxidation. The diet was prepared freshly every month and stored in sealed bags at room temperature. Diet and water were available to the rats *ad libitum*. The total observation period was 28 weeks. Body weights were measured weekly up to week 4 and every four weeks from weeks 8 to 28. Total BBN intake was measured for the entire 4-week exposure period. Food consumption was measured every eight weeks from weeks 10 to 26. At the end of the experiment, all rats were killed under ether anesthesia for pathological examination.

Experiment 2 Twenty-five male F344 rats were used, divided into 5 groups of 5 rats each. They were given powdered basal diet containing 10% α-linolenic acid and 0.2% BHA (Group 1), 10% linoleic acid and 0.2% BHA (Group 2), 10% palmitic acid and 0.2% BHA (Group 3) or 0.2% BHA only (Group 4) for 8 weeks without BBN treatment. The animals of the control group (Group 5) were given powdered basal diet only. Diet and water were available to the rats *ad libitum*. The total observation period was 8 weeks. Body weights were measured weekly and food consumption every other week. For the measurement of pH, glucose, protein and blood in urine, fresh urine samples were obtained by forced urination in the morning (9:00–11:00 a.m.). The urine measurement was performed using Ames reagent strips for urinalysis (Miles-Sankyo Co., Ltd., Tokyo) at weeks 2, 4, 6 and 8. In addition, for analysis of the fatty acid composition in the urine, samples were obtained from all rats at week 8. For urine collection, rats were housed individually in metabolic cages without food for 4 h in the morning (8:00–12:00). All rats received a single i.p. injection of 5-bromo-2′-deoxyuridine (BrdU) (Sigma Chemical Co., St. Louis, MO) at 100 mg/kg body weight 1 h before being killed for pathological examination. At this point, serum was obtained from all rats for analysis of the fatty acid composition by gas chromatography.

Pathological examination In experiment 1, the rats were killed under ether anesthesia, and their kidneys were removed, weighed and fixed with 10% buffered formalin (pH 7.4). The urinary bladders were inflated with a constant amount of 10% buffered formalin (pH 7.4), and then weighed and divided into 12–16 pieces. Urinary bladder and kidney tissues were embedded in paraffin for light microscopic histological examination of sections stained with hematoxylin and eosin (H & E). Histopathological lesions of the urinary bladder epithelium were classified into 3 categories: papillary or nodular (PN) hyperplasia, papilloma and carcinoma, as described previously.[10] In experiment 2, urinary bladders were inflated and fixed with Carnoy solution, and tissue slices were embedded in paraffin. Epithelial cells incorporating BrdU were demonstrated in histological sections by the avidin-biotin complex immunohistochemical method[11] with anti-BrdU monoclonal antibody (Dako Japan Co., Ltd., Kyoto). Numbers of labeled cells in 5000–8000 cells per animal were counted by light microscopy and labeling indices were expressed as percentage values.

Data evaluation Statistical analysis of histopathological lesion incidences was conducted by using Fisher's exact probability test, and analysis of BrdU labeling index data by using Student's t test. Other data were evaluated by analysis of variance (ANOVA).

RESULTS

Experiment 1 BBN intake (Groups 1–5) and food consumption (Groups 1–10) were similar among the experimental groups. Final body and relative kidney weights were also similar among the experimental groups, although the relative urinary bladder weights of the BBN-treatment groups (Groups 1–5) tended to be greater than these of the BBN-untreated groups (Groups 6–10) (data not shown).

Histological findings for the urinary bladder epithelium are summarized in Table I (Groups 1–5). The incidence of PN hyperplasias was significantly increased in Group 2 given linoleic acid, when compared to the controls (Groups 4 and 5), but the number of PN hyperplasias per rat was not increased. No significant differences were seen in the incidences or numbers of papillomas or carcinomas among Groups 1–5. No neoplastic lesion was observed in Groups 6–10 not given BBN.

Experiment 2 The final body, relative urinary bladder and relative kidney weights did not differ significantly among the groups (data not shown). Administration of

Table I. Histopathological Changes of the Urinary Bladder in Rats Treated with BBN Followed by Test Chemicals in Experiment 1

Group	Treatment		No. of rats	PN[a] hyperplasia		Papilloma		Carcinoma	
	Fatty acid	BHA		Incidence (%)	No./rat	Incidence (%)	No./rat	Incidence (%)	No./rat
1	α-Linolenic acid	+	20	5 (25)	0.15	1 (5)	0.05	3 (15)	0.15
2	Linoleic acid	+	20	13 (65)[b]	0.25	6 (30)	0.35	4 (20)	0.25
3	Palmitic acid	+	20	8 (40)	0.25	3 (15)	0.15	5 (25)	0.25
4	–	+	20	6 (30)	0.30	5 (25)	0.30	6 (30)	0.30
5	–	–	20	4 (20)	0.15	2 (10)	0.15	3 (15)	0.15

a) PN, papillary or nodular.
b) Significantly different from Group 4 at $P<0.05$ and Group 5 at $P<0.01$ (Fisher's exact probability test).

Table II. Fatty Acid Composition of Serum and Urine of Rats in Experiment 2

Fatty acid (μg/ml)	Treatment									
	α-Linolenic acid		Linoleic acid		Palmitic acid		BHA only		Basal diet	
	Serum	Urine	Serum	Urine	Serum	Urine	Serum	Urine	Serum	Urine
Myristic acid	7[a]	0.8	10	0.8	9	1.4	12	1.0	13	0.8
Palmitic acid	265	7.1	386	6.7	436	9.9	450	5.2	505	4.5
Stearic acid	149	5.6	179	6.2	136	6.8	142	2.8	162	2.5
Oleic acid	198	3.4	213	1.6	133	1.9	221	0.6	248	0.9
Linoleic acid	489	5.4	935[b]	4.3	441	3.2	528	0.7	634	1.4
Linolenic acid	246[b]	4.3[b]	17	0.0	12	0.0	19	0.0	23	0.0
Eicosadienoic acid	4	0.0	13	0.0	2	0.3	5	0.0	7	0.0
Arachidonic acid	147[b]	3.0	349[b]	5.2	241	5.4	230	0.7	284	1.0
Eicosapentaenoic acid	74	0.0	14	0.0	36	0.0	36	0.0	48	0.0
Docosahexaenoic acid	34	0.0	50	0.0	73	0.6	76	0.0	93	0.0

a) Mean (n=5).
b) Significantly different from the other four groups at $P<0.05$ (ANOVA).

Table III. BrdU Labeling Indices for Urinary Bladder Epithelium of Rats in Experiment 2

Group	Treatment		No. of rats	Labeling index (%)
	Fatty acid	BHA		
1	α-Linolenic acid	+	5	0.04±0.02[a]
2	Linoleic acid	+	5	0.06±0.04
3	Palmitic acid	+	5	0.23±0.20
4	–	+	5	0.17±0.17
5	–	–	5	0.20±0.21

a) Mean±SD.

fatty acids did not change the pH of the urine. Other urinalysis parameters were also similar among the experimental groups throughout the experimental period (data not shown).

Data for the fatty acid composition of the serum and urine are shown in Table II. In the rats given α-linolenic acid, this fatty acid was significantly increased in both serum and urine. In the rats given linoleic acid, linoleic acid was significantly increased in serum. However, palmitic acid administration caused almost no alteration in the fatty acid composition. Arachidonic acid was significantly decreased in the serum of rats given α-linolenic acid and increased in the serum of rats given linoleic acid.

BrdU labeling indices for urinary bladder epithelium are shown in Table III. Numbers of BrdU-positive cells were not significantly increased by fatty acid treatments, as compared with control values (Groups 4 and 5).

DISCUSSION

In experiment 1, administration of any of the fatty acids failed to increase the development of urinary bladder tumors, despite the fact that the doses of fatty acids used for admixture in feed in this experiment (10%) were exceedingly large. Body weight reduction was not observed, although the food intake by the treated rats tended to be decreased in each fatty acid group in this study. A greater fatty acid content might further de-

crease the feed intake and thus result in body weight reduction. Therefore, our result indicates that the fatty acids do not promote rat urinary bladder carcinogenesis when given at maximum tolerated doses. In previous studies a 25% high-fat diet was used,[3] but the total fatty acid volume was almost the same as in the present study. In experiment 2 the examination of whether fatty acids cause cell proliferation in the urinary bladder epithelium without BBN initiation treatment revealed no increase after eight weeks' feeding of any of the fatty acids. This time point was chosen because cell proliferation in chemical carcinogenesis has been reported to become most active at week 8.[12] Thus, fatty acids do not appear to be mitostimulatory for the rat urinary bladder epithelium.

This is of interest in the light of the results for fatty acid composition, with α-linolenic acid and linoleic acid increasing in the serum and/or urine. Arachidonic acid, an n-6 series fatty acid, was increased in the serum by administration of linoleic acid, a member of the same series which may give rise to prostaglandin via an arachidonic acid intermediate.[3] The promoting effect of a high-fat diet on carcinogenesis was suggested to be the result of accelerated formation of arachidonic acid and subsequently of prostaglandin.[13] However, no proliferative effect on the urinary bladder epithelium was observed in the present study despite the observed serum elevation.

Several reports have suggested that saturated fats may influence the initiation phase of carcinogenesis, whereas polyunsaturated fatty acids act in the promotion phase.[14] Moreover, lipid oxidation products of polyunsaturated fatty acids have also been found to exert genotoxic effects in short-term assays.[15,16] In this study, fatty acid hydroperoxides, which correlate with the initiating role of polyunsaturated fatty acids, can be essentially ignored because we gave a supplement of BHA as an antioxidant. Recent studies with experimental animals have revealed enhancement of chemically induced colonic tumorigenesis by n-6 polyunsaturated fatty acid, and a protective effect of n-3 polyunsaturated fatty acid.[8,17] Our results, however, indicate that neither n-6 and n-3 polyunsaturated fatty acids nor saturated fatty acid have any modifying effects on rat urinary bladder tumorigenesis. In the colon carcinogenesis case, the promoting effects of fatty acids may be related to bile acids and bacteria, which are not factors that need be considered in the urinary bladder.[18–20]

Several investigations have indicated that urinary bladder carcinogenesis can be influenced by saccharin, coffee and other food factors,[21–23] and moreover, promoting effects of various sodium salts and co-promoting effects of L-ascorbic acid on rat urinary bladder carcinogenesis have been described.[24,25] Whether fatty acids could exert co-promoting effects on urinary bladder carcinogenesis remains unclear, although the fact that no promotion resulted from the combinations with BHA would suggest that this might be unlikely.

In conclusion, we have demonstrated that the three fatty acids, α-linolenic, linoleic and palmitic acid, have no promoting effects on rat urinary bladder carcinogenesis in an established two-stage model. The results do not provide any experimental support for the epidemiological data suggesting a relationship between dietary fat and urinary bladder carcinogenesis,[6] although further studies are necessary to confirm the findings in other models.

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