Original Article

No Promoting Effect of Ethyl *Tertiary*-butyl Ether (ETBE) on Rat Urinary Bladder Carcinogenesis Initiated with *N*-Butyl-*N*-(4-hydroxybutyl)nitrosamine

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Abstract: The effects of ethyl *tertiary*-butyl ether (ETBE) on two-stage urinary bladder carcinogenesis in male F344 rats initiated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN) were investigated at various dose levels with regard to possible promoting activity. Groups of 30 rats were given drinking water containing 500 ppm BBN, as an initiator, for 4 weeks and starting one week thereafter received ETBE by gavage (daily, 7 days/week) at dose levels of 0 (control), 100, 300, 500 or 1000 mg/kg/day until experimental week 36. No statistically significant differences in incidences of preneoplastic lesions, papillomas, and carcinomas of the urinary bladder were evident in rats treated with 100–1000 mg/kg/day ETBE as compared with control values. Furthermore, the average numbers of preneoplastic or neoplastic lesions per unit length of basement membrane in rats given 100–1000 mg/kg/day ETBE were also comparable to control values. However, papillomatosis of the urinary bladder was found in 4 out of 30 rats (13%) in the group given 1000 mg/kg/day ETBE, and soft stones in the urinary bladder were found in 3 out of these 4 rats. The results thus demonstrated that ETBE did not exert promotional activity on urinary bladder carcinogenesis. However, papillomatosis of the urinary bladder developed in small numbers of the rats given ETBE at 1000 mg/kg/day but not in rats given 500 mg/kg/day or lower doses. (DOI: 10.1293/tox.2013-0027; J Toxicol Pathol 2013; 26: 351–357)

Key words: ETBE, initiation/promotion, urinary bladder carcinogenesis, papillomatosis, soft stone, F344 rats

Introduction

Ethyl *tertiary*-butyl ether (ETBE) and a related chemical, methyl *tertiary*-butyl ether (MTBE), are used as fuel oxygenates in gasoline. They act as octane enhancers, with the additional benefit of making gasoline burn more completely, thereby reducing carbon monoxide, unburned hydrocarbons, and other contaminants in exhaust. However, as MTBE possesses high solubility in water, and low biodegradability as compared with other gasoline ingredients, it has the potential to pollute underground water[1–3]. On the other hand, while the technical characteristics of ETBE are comparable to those of MTBE, the much lower water solubility is considered an advantage because the risk of contamination of underground water is lower.

As one goal for achieving the goals of "Kyoto Protocol", fuel derived from renewable sources (biomass or biofuel) was introduced in the near future. Thus, it has been planned to introduce ETBE, derived from biomass ethanol, in Japan. As ETBE was classified to the type II monitored chemical substances according to the former "Law Concerning the Evaluation of Chemical Substances and Regulation of Their Manufacture, etc.," safety assessment was needed for its use as a fuel. This includes comprehensive evaluation of possible carcinogenicity.

In a toxicological review, safety assessment studies to evaluate carcinogenicity of ETBE were deemed to have been insufficient[3]. Tumor promoting potential in urinary bladder carcinogenesis was suspected from the results of a previous medium-term multi-organ carcinogenesis bioassay, since rats exposed to ETBE developed papillomatosis in the urinary bladder[4]. Thus, it was decided that whether ETBE acts as a tumor promoter should be determined using a medium-term urinary bladder carcinogenesis bioassay protocol[5–9]. MTBE, a structurally related chemical to ETBE, did not cause urinary bladder tumor development in carcinogenicity studies in rats and mice[2]. However, it was

reported that an extremely high dose of *tertiary* butyl alcohol (TBA), a common metabolite of ETBE and MTBE, induces stone formation, inflammation and hyperplasia of the urinary bladder in rats and mice in subchronic toxicity studies[10,11].

The objective of the present study was to assess any possible tumor promoting effects of ETBE in the urinary bladder.

Materials and Methods

The present study was performed in compliance with the Good Laboratory Practice (GLP) Standards of Ministry of Health and Welfare of Japan Ordinance No. 21 (March 26, 1997) and in accordance with the Guidelines for Carcinogenicity Studies of Drugs 3.2 (*In Vivo* Additional Tests for Detection of Carcinogenicity) of the Ministry of Health and Welfare of Japan (Notification No.1607, November 1, 1999).

Initiator

N-Butyl-*N*-(4-hydroxybutyl)nitrosamine (BBN)/*N*-n-Butyl-*N*-butan-4-ol-nitrosamine (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was used as an initiator in this bioassay, the specifications of which were as follows: lot number, FIN01; CAS number, 817-11-6; and storage condition, 4.0–10.0°C and shielded from light (in a refrigerator).

Test material

The ethyl *tertiary*-butyl ether (ETBE) used in the present study was manufactured by Nippon Refine Co., Ltd. (Gifu, Japan), and the specifications of it were as follows: appearance, colorless transparent liquid; boiling point, 70°C; vapor pressure, 17 Kpa (25°C); specific gravity, 0.74 (25°C/4°C); solubility, slightly soluble in water (1.2 g/100 g, 20°C); lot no., L-506251; purity, more than 99 wt% (Toray Research Center Inc., Tokyo, Japan); storage conditions, at room temperature and in a dark place.

Preparation of dosing solutions and analyses

The test material was accurately weighed, dissolved in olive oil (Nacalai Tesque, Inc., Kyoto, Japan), adjusted to produce 10.0 w/v%, 5.0 w/v%, 3.0 w/v% and 1.0 w/v% ETBE dosing solutions, which were prepared more than once in each 7-day period, introduced into glass bottles and stored in a refrigerator. Dosing solutions of 10.0 w/v% and 0.05 w/v% dosing solutions were confirmed to be homogeneous and stable for 7 days (information from the sponsor). Analyses of concentrations of the test material in dosing preparations were performed twice during the course of the study, and each concentration of dosing solution was confirmed to be within the acceptable range. All concentration analyses of the test material in the dosing solution were performed by gas chromatography with a headspace autosampler (HS/GC/MS) at Nisso Chemical Analysis Service Co., Ltd. (Odawara, Kanagawa, Japan).

Animals and husbandry

Male 5-week-old F344/DuCrlCrlj rats (SPF animals) from Charles River Laboratories Japan Inc. (Atsugi, Kanagawa, Japan) were allowed an 8-day quarantine/acclimation period, during which health conditions and body weights were monitored. Only after confirmation of normal status were they entered into the study at the age of 6 weeks. The animals were housed 2 to a polycarbonate cage on hardwood chip bedding (Beta Chips, Northeastern Products Corp., Warrensburg, NY, USA) in an environmentally controlled room. Constant conditions of temperature (20–26.5°C), humidity (45–70%), and ventilation (more than 15 times/hr) were maintained, and the room was artificially illuminated to provide 12 hr of lighting (7:00–19:00) each day. Powder diet MF (Oriental Yeast Co., Ltd., Tokyo, Japan) and Ichinomiya city tap water were available *ad libitum*.

All experimental procedures were performed in accordance with the Law for the Humane Treatment and Management of Animals (Law No. 105, October 1, 1973, and amendment, December 21, 1999), "Standards Relating to the Care and Management etc. of Experimental Animals (Notification No. 6, March 27, 1980, Prime Minister's Office, Japan, and amendment, May 28, 2002) and Guideline for Animal Experimentation (May 22nd, 1987, Japanese Association for Laboratory Animal Science).

Experimental design

An outline of the experimental design for the present study is shown in Fig. 1. A randomized block design (BrexNote Net, Yukms Co., Ltd., Tokyo, Japan) was used to allocate 150 rats to 5 groups (30/group). The animals were given drinking water containing 500 ppm BBN[5,8,9] from the commencement of the experiment to week 4. One week after the end of week 4, they were administered ETBE at dose levels of 0 mg/kg/day (group 1) as a control and 100, 300, 500 and 1000 mg/kg/day (groups 2 to 5) by gavage (daily, 7 days/week) for 31 weeks (from weeks 6 to 36). The administered volume (10 mL/kg) of ETBE dosing solution was adjusted for the latest body weight of each rat. The animals were observed daily for abnormalities, and individual body weights were recorded weekly. Food and water consumption was measured over a 2-day period before each weighing. At experimental week 37, all surviving animals were fasted overnight and euthanized under ether anesthesia for examination of preneoplastic and neoplastic lesion development in the urinary bladder.

Urinalysis

Urinalysis of samples collected over a 4-hr period (from 9:00 AM to 1:00 PM) was conducted for 10 animals/group at experimental weeks 9, 20 and 36; a semiquantitative estimation (Multistix; Bayer Medical Ltd., Tokyo, Japan) of protein, glucose, ketones, bilirubin, occult blood and urobilinogen was included. Specific gravity values were measured using a handheld refractometer (model N, Atago Co., Ltd., Tokyo, Japan), and levels of urinary electrolytes (sodium, potassium, magnesium, calcium and chlorine)
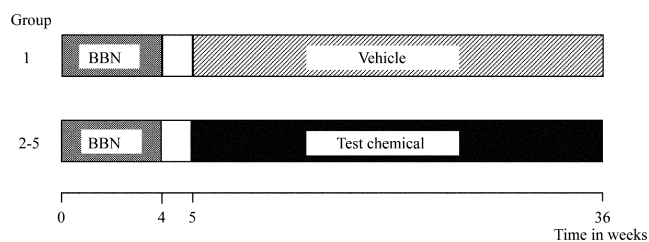
Fig. 1. Experimental design. Animals were given drinking water containing 500 ppm BBM for 4 weeks. One week after the end of week 4, the animals were administered ETBE at dose levels of 0 (control), 100, 300, 500 and 1000 mg/kg/day by gavage (daily, 7 days/week) for 31 weeks from week 6 to 36. All survivors were euthanized at week 37.

were determined using a Hitachi Biochemical Automatic Analyzer 7070 (Hitachi Ltd., Tokyo, Japan). Urine volumes were measured by weighing, and samples were examined macroscopically, with urinary sediments being stained with Labostain (Muto Pure Chemicals Co., Ltd., Tokyo, Japan) for microscopic assessment. In addition, fresh urine samples were obtained, and urinary pH values were measured using a pH meter (Horiba, Ltd., Kyoto, Japan).

Pathological examination

At necropsy, all surviving animals were fasted overnight, and euthanized by exsanguination by drawing blood from the abdominal aorta under ether anesthesia. The urinary bladders were immediately inflated with 10% buffered formalin solution after ligation of the neck, excised, and then immersed in the same fixative. In addition, the kidney, ureter, urethra, prostate, seminal vesicle, thyroid, liver and gross lesions were also excised, and preserved in the fixative. The urinary bladder (after fixation), liver, and kidneys were weighed, and relative organ weights were calculated using the final body weights. After fixation, the urinary bladders were sagittally halved, the luminal surfaces were examined grossly, and the results were recorded. Each bladder half was then divided into four strips for embedding in paraffin, sectioning and staining with hematoxylin and eosin (for all animals). The numbers of hyperplastic and neoplastic lesions were counted under a light microscope and evaluated quantitatively with reference to the total length of the basement membrane, which was measured with a color video image processor (Sumika Technoservice Coporation, Osaka, Japan). However, preneoplastic lesions and/or papillomas of the urinary bladder induced by BBN were masked by the development of papillomatosis (diffuse papillary hyperplasia of the epithelium); these rats were omitted from the evaluation of tumor promoting effect of ETBE on bladder carcinogenesis. All of the rats that were euthanized in extremis or found dead during the treatment period were necropsied, and their tissues and organs were preserved as far as possible in fixative. With these animals, organ weights were not measured, but the urinary bladders were examined histopathologically.

Statistical analysis

The significance of differences in results for each parameter was analyzed and evaluated at P<0.05 or P<0.01. Statistical comparisons between group 1 and groups 2–5 for numerical data obtained for body weights, food consumption, water consumption, organ weights, mean numbers of gross lesions of the urinary bladder and quantitative values for hyperplastic and neoplastic lesions of the urinary bladder were assessed using the Bartlett's test. If homogeneous, the data were analyzed with the Dunnett's multiple comparison test (two sided), and if not, they were analyzed with the Steel's test (two sided). The significance of intergroup differences (between group 1 and groups 2 to 5) in incidences of findings from gross pathology and histopathology was analyzed using the Fisher's exact probability test (two sided), and a comparison of the grade of lesions was performed using the Wilcoxon test (two-sided). Stat Light for Excel 2000 (Yukms Co., Ltd., Tokyo, Japan) was used for statistical analysis.

Results

Antemortem investigations

At the end of the experiment, the survival rates with 100, 300, 500 and 1000 mg/kg/day ETBE (groups 2 to 5) were 97% (29/30), 97% (29/30), 100% (30/30) and 100% (30/30), respectively, and there were no treatment-related changes when compared with the control (group 1) value of 100% (30/30). Improper gavage procedures appeared to have caused one rat to die in group 2 (found at week 26) and one rat to become moribund in group 3 (euthanized at week 22).

During the BBN treatment period (weeks 1–4) and non-treatment period (week 5), no statistically significant body weight changes were found in rats given ETBE (groups 2–5) as compared with the controls (group 1). During the test material treatment period (weeks 6–36), mean body weights in the 1000 mg/kg/day ETBE (group 5) were significantly lower than the control values from week 32 to the end of the experiment. Mean body weights with 500 mg/kg/day ETBE (group 4) were comparable to the control values. Mean body weights with 100 and 300 mg/kg/day ETBE (groups 2 and 3) were significantly lower than the control values from week 16 and 18, respectively, to the end of the experiment.

During the BBN treatment period and nontreatment period, mean food consumptions in rats given ETBE (groups 2–5) were comparable to the control values. During the test material treatment period, mean food consumptions in rats given 300, 500 and 1000 mg/kg/day ETBE (groups 3, 4 and 5) were significantly increased or showed a tendency for increases when compared with the control values from weeks 34, 11 and 9, respectively, to the end of the experiment. Mean food consumption with 100 mg/kg/day ETBE (group 2) was comparable to the control values.

During the BBN treatment period, mean water consumptions in groups 2–5 were comparable to the control (group 1) value, and average BBN intakes in the control

Table 1. Final Body Weights and Relative Organ Weight Data in Rats Initiated with BBN and Then Given ETBE

Group	ETBE (mg/kg/day)	No. of rats	Final body weight (g)	Liver (%)	Kidney (%)	Urinary bladder (%)
1	0	30	420 ± 27	2.34 ± 0.15	0.44 ± 0.02 (n=29)[a]	0.032 ± 0.008
2	100	29	395 ± 33**	2.32 ± 0.09	0.49 ± 0.02**	0.033 ± 0.019
3	300	29	395 ± 23**	2.42 ± 0.09*	0.52 ± 0.03**	0.030 ± 0.009
4	500	30	405 ± 26	2.55 ± 0.09**	0.53 ± 0.02**	0.035 ± 0.015
5	1000	30	389 ± 22**	2.85 ± 0.10**	0.60 ± 0.03**	0.042 ± 0.016*

[a] The Organ from one rat was omitted from the statistical analysis due to formation of a large mass. *, ** Significantly different from the control (group 1) at $P<0.05$ and $P<0.01$, respectively.

Table 2. Incidences and Multiplicities of Hyperplastic Lesions of the Urinary Bladder in Rats Initiated with BBN and Then Given ETBE

Group	ETBE (mg/kg/day)	No. of rats	PN hyperplasia		Papillomatosis
			No. of rats (%)	No./10 cm BM	No. of rats (%)
1	0	30	19 (63)	0.86 ± 0.86	0 (0)
2	100	30	6 (23)**	0.26 ± 0.66**	0 (0)
3	300	30	11 (37)*	0.42 ± 0.61	0 (0)
4	500	30	6 (20)**	0.31 ± 0.78*	0 (0)
5	1000	30	11 (42) (n=26)[a]	0.44 ± 0.61 (n=26)[a]	4 (13)

[a] Four animals were omitted from the evaluation due to development of papillomatosis. PN: Papillary or nodular; BM: Basement membrane. *, ** Significantly different from the control (group 1) at $P<0.05$ and $P<0.01$, respectively.

(group 1) and ETBE treatment groups (groups 2 to 5) were 47–49 mg/kg/day. During the nontreatment period, mean water consumption in groups 2–5 was also comparable to the control value. During the test material treatment period, mean water consumption in rats given 100 to 1000 mg/kg/day ETBE (groups 2 to 5) was significantly increased from weeks 7, 7, 6 and 6, respectively, to the end of the experiment.

In the urinalysis (data not shown) performed at experimental weeks 9, 20 and 36, no occult blood was found in any rat. A significant decrease in urine pH was found at 1000 mg/kg/day at weeks 9 and 20, but not week 36. Significant decreases in urine crystals were noted in rats given 300 mg/kg/day or more at weeks 20 and 36.

Relative organ weights

A significant increase in relative urinary bladder weights was noted with 1000 mg/kg/day ETBE (group 5) but not with 100 to 500 mg/kg/day ETBE (groups 2 to 4) (Table 1). Significant increases in relative liver weight were observed with 300, 500 and 1000 mg/kg/day ETBE (groups 3 to 5). Significant increases in relative kidneys weight were noted with 100–1000 mg/kg/day ETBE (groups 2–5).

Gross pathology

The incidences of nodules (Fig. 2) in the urinary bladder with 100 to 1000 mg/kg/day ETBE (groups 2–5) were 60–80% (18–24/30 rats), and there was no statistically significant difference as compared with the control (group 1) value of 80% (24/30 rats). One rat in group 3 euthanized in extremis at week 22 had nodules in the urinary bladder. Animals found dead after this were included in the effective

numbers for evaluation. Soft stones (Fig. 3A) in the urinary bladder were noted in 3 rats with 1000 mg/kg/day ETBE (group 5).

The average numbers of nodules in each size class (diameters of less than 1 mm, 2 mm, 3 mm, larger than 3 mm) with 300 and 1000 mg/kg/day ETBE (groups 3 and 5) were comparable to control (group 1) values (data not shown). The average numbers of nodules that were less than 1 mm in diameter with 100 and 500 mg/kg/day ETBE (groups 2 and 4) were significantly low, but those of nodules with diameters of 2 mm, 3 mm and larger than 3 mm were comparable to the control values.

No gross pathology findings for organs other than the urinary bladder considered to relate to the test material treatment were observed in the present study.

Histopathology

Incidences of papillary and nodular (PN) hyperplasia, which is a preneoplastic lesion in the urinary bladder (Fukushima *et al*. 1983), were statistically significantly decreased with 100, 300 and 500 mg/kg/day ETBE (groups 2–4), but not with 1000 mg/kg/day ETBE (group 5), as compared with the control (group 1) value (Table 2). Significant decreases in average numbers per unit length (10 cm of basement membrane) of PN hyperplasias were found with 100 and 500 mg/kg/day ETBE (groups 2 and 4), but not with 300 and 1000 mg/kg/day ETBE (groups 3 and 5), as compared with the control value. The incidences of papillomas in the urinary bladder with 100–1000 mg/kg/day ETBE (groups 2–5) were comparable to the control value (Table 3). Significant decreases in the multiplicities (average numbers per unit length) of papillomas were found with 100 mg/kg/day
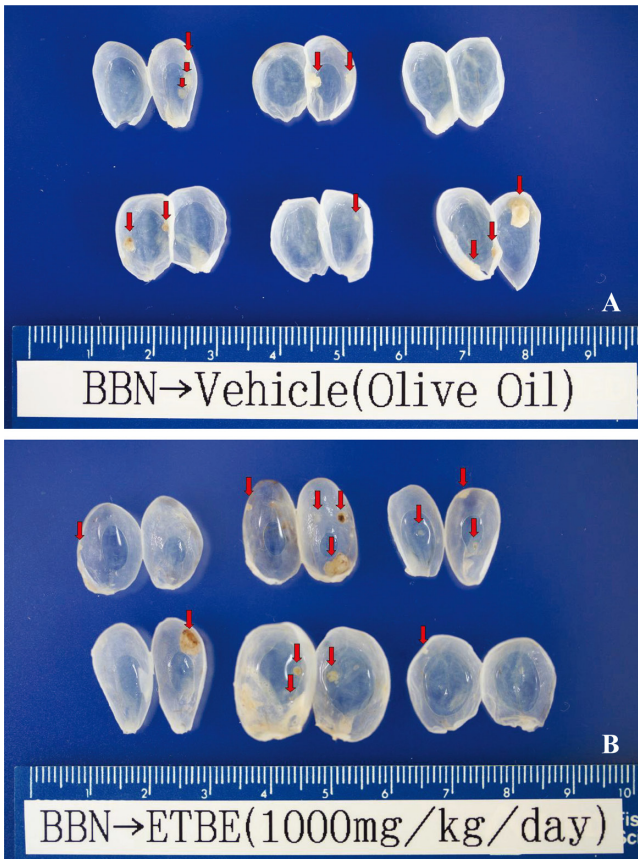
Fig. 2.

ETBE (group 2), but not with 300 to 1000 mg/kg/day ETBE (groups 3–5), as compared with the control value. The incidences and multiplicities of carcinomas in the urinary bladder were significantly increased with 500 mg/kg/day ETBE (group 4), but not with 100, 300 and 1000 mg/kg/day ETBE (groups 2, 3 and 5), as compared with the control values. Those of papillomas plus carcinomas with 100–1000 mg/kg/day ETBE (groups 2–5) were comparable to the control value. Papillomatosis (extensive papillary hyperplasia) (Fig. 3D) in the urinary bladder was observed in 13% (4 out of 30 rats) of animals in the 1000 mg/kg/day ETBE group 5 but was not observed with 100–500 mg/kg/day ETBE (groups 2–4). A carcinoma of the urinary bladder was also found in one of these 4 animals. Three out of the 4 rats with papillomatosis exhibited soft stones in the urinary bladder grossly. Histopathologically, the soft stone in the urinary bladder were eosinophilic, and a lamellar pattern was apparent (Fig. 3B and 3C).

Fig. 2. Representative macroscopic findings of luminal surface of the urinary bladder in rats treated with BBN alone (A) and in rats treated with BBN and then 1000 mg/kg/day ETBE (B). Small to medium size nodules (arrows) were apparent.

Fig. 3. Soft stones (A) in the urinary bladder. Histopathologically, soft stones were eosinophilic (B), and a lamella pattern (C) was apparent at higher magnification. Papillomatosis (extensive papillary hyperplasia) of the urinary bladder (D).
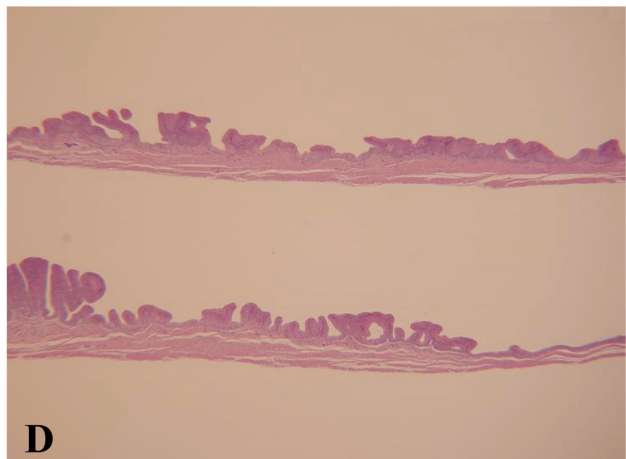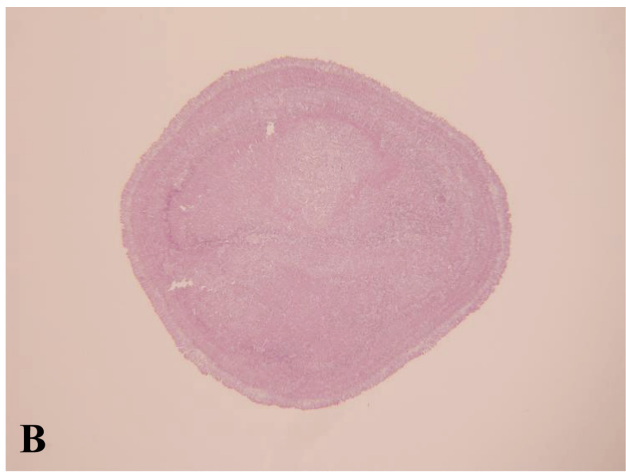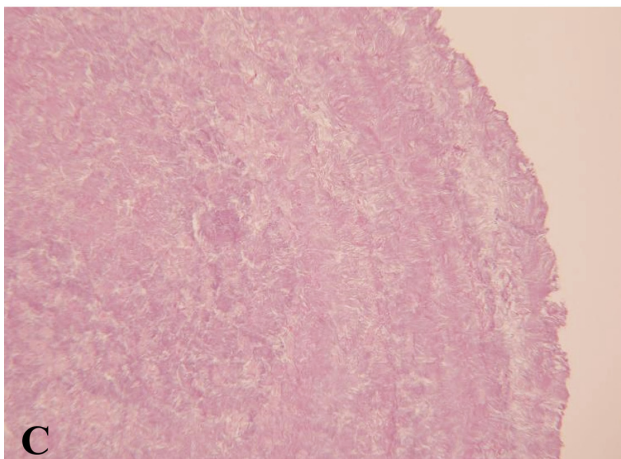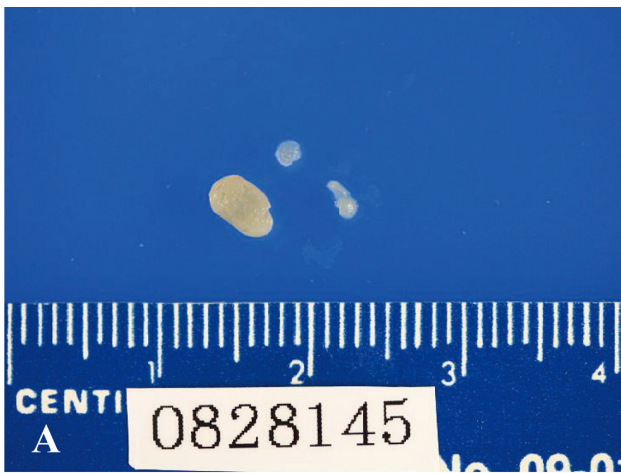

Fig. 3.

Table 3. Incidences and Multiplicities of Neoplastic Lesions of the Urinary Bladder in Rats initiated with BBN and Then Given ETBE

Group	ETBE (mg/kg/day)	No. of rats	Papilloma		Carcinoma		Papilloma or carcinoma	
			No. of rats (%)	No./10 cm BM	No. of rats (%)	No./10 cm BM	No. of rats (%)	No./10 cm BM
1	0	30	21 (70)	1.46 ± 1.42	5 (17)	0.15 ± 0.35	24 (80)	1.62 ± 1.38
2	100	30	13 (43)	0.59 ± 0.83*	7 (23)	0.30 ± 0.62	18 (60)	0.89 ± 1.04
3	300	30	17 (57)	1.02 ± 1.27	6 (20)	0.24 ± 0.51	20 (67)	1.25 ± 1.30
4	500	30	17 (57)	0.74 ± 0.82	14 (47)*	0.50 ± 0.58*	25 (83)	1.24 ± 0.85
5	1000	30	21 (81)[a]	1.77 ± 1.62[a]	9 (30)[a]	0.40 ± 0.71[a]	21 (81)[a]	2.12 ± 1.78[a]

[a] The No. of animals was 26, since 4 rats were omitted from the evaluation due to development of papillomatosis. BM: Basement membrane. * Significantly different from the control (group 1) at P<0.05.

Discussion

High-dose ETBE treatment was found to induce papillomatosis in the urinary bladder in our previous medium-term multi-organ carcinogenesis bioassay[4]. In previous investigations, it was shown that oral administration of uracil to rat induced reversible papillomatosis (diffuse papillary hyperplasia of the epithelium) of the urinary bladder associated with urolithiasis[12–14]. It has been reported that chemicals causing papillomatosis, in general, act as bladder tumor promoters and exert carcinogenicity eventually[6,7,15]. Thus, the potential of ETBE to modify tumor development was investigated in the present medium-term urinary bladder carcinogenesis bioassay using male Fischer rats[5–9]. However, quantitative analysis of hyperplastic and neoplastic lesion developments did not provide any evidence that even 1000 mg/kg/day ETBE acted as a tumor promoter in the present bioassay using BBN as an initiator. However, it was confirmed that 1000 mg/kg/day ETBE induced papillomatosis (also forming soft stones) in the urinary bladder at low incidence, but papillomatosis was not observed in rats given 500 mg/kg/day of ETBE or lower. Since the dose level of 500 mg/kg/day of ETBE was extremely higher than the estimated oral exposure level (0.0059–0.218 mg/kg/day calculated from reported possible contamination levels of drinking water) and the inhalation exposure level (11.4 µg/kg/day calculated from estimated occupational exposure level of 38 µg/m³)[16], the risk to humans must be negligible. Our results are in line with the earlier reports that preneoplastic and/or neoplastic lesions of the urinary bladder did not develop in rodents given 1000 mg/kg/day ETBE[3,17] or a structurally related chemical, MTBE, for 2 years by gavage (4 days administration/week with olive oil as the vehicle)[2,18].

With non-genotoxic substances, it is considered that mechanisms of urinary bladder tumor promoting activity involve cell damage by crystals precipitating in the urine[13–15,19–21]. Previously, it was reported that urinary stones formed as a result of oral administration of uracil and that insertion of paraffin wax pellets caused development of preneoplastic and neoplastic lesions in the urinary bladder[6,13–15,21,22]. In the present study, no crystal and/or precipitate formation was found on urinalyses conducted 3 times during the course of the study. Only soft stone formation was found in rats that developed papillomatosis given 1000 mg/kg/day ETBE. The evidence indicates that rats exposed to 1000 mg/kg/day of ETBE may develop soft stones in the urinary bladder, which cause damage and therefore elevated cell proliferation continuously, and finally induce papillomatosis. In another study, rats given drinking water containing high doses (20 mg/mL or 1520 mg/kg/day and 40 mg/mL or 3610 mg/kg/day for males, 40 mg/mL or 3620 mg/kg/day for females) of TBA, which is a metabolite of ETBE, for 13 weeks exhibited stone formation, inflammation and hyperplasia (males only) in the urinary bladder[11]. However, these alterations were not found at the doses of 5 mg/mL (420 mg/kg/day) for males, and 10 mg/mL (650 mg/kg/day) in a 2-year carcinogenicity study[10,11]. Furthermore, alterations were found in mice administered high doses (20 mg/mL or 3940 mg/kg/day and 40 mg/mL or 8210 mg/kg/day for males, 40 mg/mL or 11620 mg/kg/day for females) of TBA for 13 weeks but not in those administered medium doses (10 mg/mL or 1040 mg/kg/day for males, 10 mg/mL or 1020 mg/kg/day for females)[10,11].

In conclusion, the present investigation of tumor promoting activity of ETBE in a medium-term urinary bladder carcinogenicity assay (BBN method) using male F344 rats indicated no tumor promoting effects, despite confirmation that rats exposed to ETBE at 1000 mg/kg/day may develop papillomatosis in the urinary bladder.

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