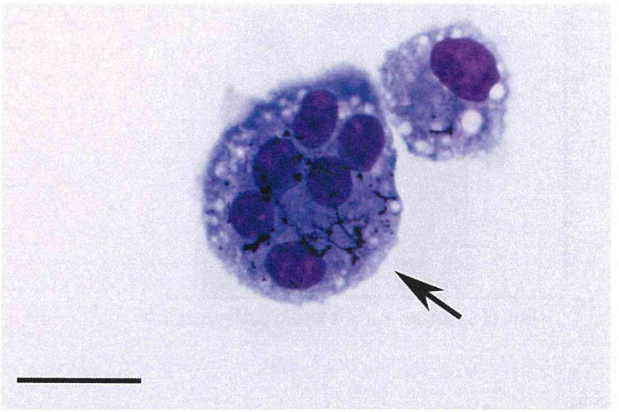

Fig. 7. Alveolar macrophages in the BALF from a rat exposed to 5 mg/m^3 MWCNTs at the end of the 4-week postexposure period. A multinucleated macrophage with phagocytosed MWCNTs is shown (arrow). Bar indicates 30 μm. May-Grünwald-Giemsa stain.

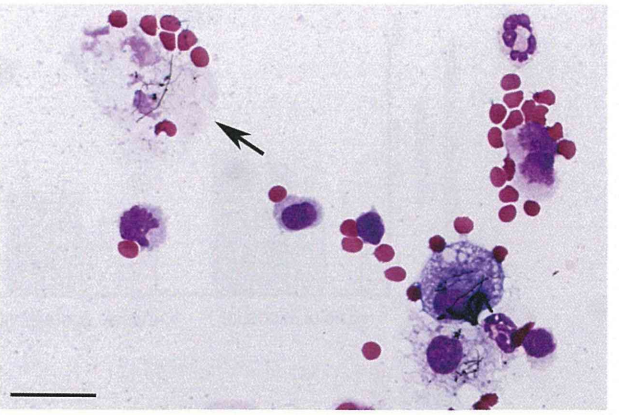

Fig. 8. Alveolar macrophages in the BALF from a rat exposed to 5 mg/m^3 MWCNTs at the end of the 2-week exposure period. The arrow indicates a macrophage with phagocytosed MWCNTs that has lost its cytoplasmic contents. Bar indicates 30 μm. May-Grünwald-Giemsa stain.

exposure period and approximately 41.2 μg/lung at the end of the 4-week postexposure period: this data was obtained using an MWCNT-measuring method in our research center (unpublished data). Therefore, most MWCNTs remained in the lung, and only a small amount of MWCNTs was delivered out of the lungs during the 4-week postexposure period.

The incidence of MWCNT deposition in the peritracheal lymph node increased after the 4-week postexposure period, suggesting that MWCNTs are transported to lymph nodes outside the lung. In our previous study using intratracheally instilled MWCNTs (MWNT-7), MWCNT deposition in the posterior mediastinal lymph node (peritracheal lymph node) was observed 7 days after intratracheal instillation, and MWCNT deposition in the parathymic lymph node was observed 91 days after intratracheal instillation[20]. These data suggest that MWCNTs deposited in the alveoli migrated through the lymphatic drainage systems for pulmonary dust clearance: the deep-set drainage system consisting of the periarterial, perivenous and peribronchiolar lymph vessels and the pleural drainage system following the surface of the lung segments and lobes.

Deposition of MWCNT fibers in the respiratory tract caused foreign body reactions to occur. In the nasal cavity and nasopharynx of rats exposed to 1 or 5 mg/m^3 MWCNTs, goblet cell hyperplasia occurred. Goblet cell hyperplasia regressed when exposure to MWCNTs was discontinued. It is likely that the enhanced mucus secretion by goblet cells played a role in the clearance of MWCNTs from the nasal cavity.

In the lung, exposure to the highest levels of MWCNTs, 5 mg/m^3, caused the formation of granulomatous changes. These granulomatous changes were characterized by aggregation of macrophages containing phagocytosed MWCNTs with a small amount of fibrosis. The low amounts of collagen formation in these lesions indicate that they were early stage granulomas. However, granuloma formation increased slightly and was progressing toward a chronic lesion with giant cells during the 4-week postexposure period. Thus, if the postexposure period was lengthened, persistent MWCNT deposition could have effects not seen in this short-term study. As discussed above, during the 4-week postexposure period, very few MWCNTs were removed from the lung; one possible factor could be that granuloma formation by macrophages with phagocytosed MWCNTs would hinder clearance of MWCNTs from the lung.

Increased levels of multinucleated macrophages were observed in rats exposed to 1 or 5 mg/m^3 MWCNTs. Since MWCNT deposition persisted in the lung, the levels of these large multinucleated macrophages phagocytosing MWCNTs tended to remain elevated throughout the 4-week postexposure period. It is known that multinucleated giant cells, which are frequently found in conditions of granulomatous inflammation, are formed by amitotic nuclear division or by fusions of macrophages[21, 22]. Asakura *et al.* (2010) reported that exposure of Chinese hamster lung cells to MWCNTs resulted in the formation of polyploidy, bi- and multinucleated cells and suggested that MWCNTs physically interfere with biological processes during cytokinesis[23]. Therefore, it is possible that the increases in multinucleated macrophages in the present study were caused by changes associated with granulomatous inflammation and the mitotic inhibition of alveolar macrophages containing phagocytosed MWCNTs. In addition, morphological examination of the BALF showed toxic effects on alveolar macrophages in the rats exposed to 1 and 5 mg/m^3 MWCNTs. Taken together, the decrease of alveolar macrophages in the BALF could reflect the cytotoxicity of phagocytosed MWCNTs and the formation of multinucleated macrophages. The toxicity of phagocytosed MWCNTs could also be a factor hindering clearance of MWCNT fibers from the lung.

Finally, inflammatory parameters in the BALF were elevated in rats exposed to 1 and 5 mg/m^3 MWCNT: Elevated neutrophil and lymphocyte counts and elevated levels of total protein and albumin in the BALF were observed. While

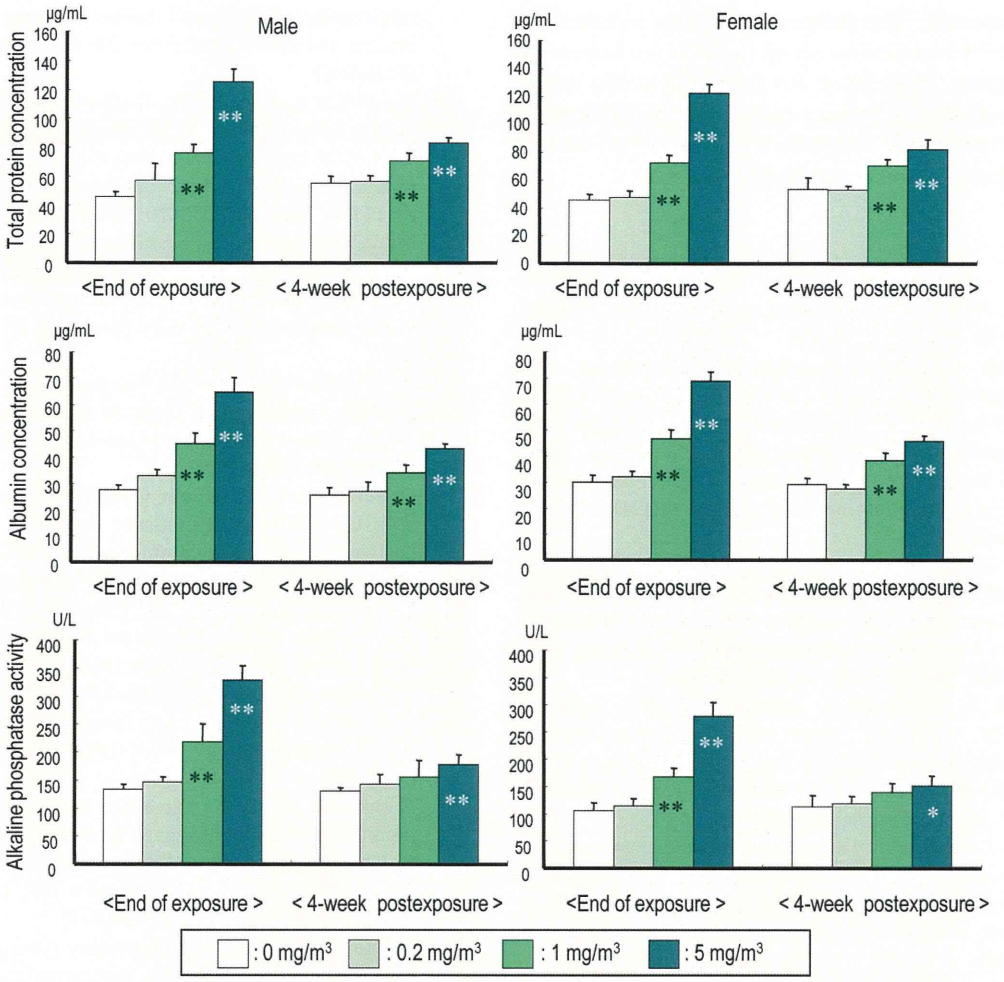

Fig. 9. Total protein and albumin concentrations and ALP activity in the BALF from rats at the end of the 2-week exposure and 4-week postexposure periods. Error bars indicate the SD of 5 rats. *: $p<0.05$ by Dunnett's multiple comparison test. **: $p<0.01$ by Dunnett's multiple comparison test.

the changes in these inflammatory parameters were comparatively weak, and clear inflammatory cell infiltration was not found in the lower or upper pulmonary tracts by histopathological examination, it is notable that changes in the inflammatory parameters were still present at the end of the 4-week postexposure period. These results indicate that the inflammatory changes at the end of the 4-week postexposure period were caused by the persistence of MWCNT deposition in the lung. In addition, ALP activity in the BALF was elevated in rats exposed to 1 and 5 mg/m^3 MWCNTs and was still present at the end of the 4-week postexposure period. Therefore, MWCNT exposure induced persistent pulmonary damage, although its severity was weak.

In conclusion, F344 rats of both sexes were exposed by inhalation to 0.2, 1 or 5 mg/m^3 MWCNT aerosol for 6 h/day, 5 days/week for 2 weeks using a whole-body exposure system. We found persistent deposition of MWCNTs in the lungs of all MWCNT-exposed groups, and MWCNTs migrated to the lymphatic drainage systems for pulmonary dust clearance. Foreign body reactions included goblet cell hyperplasia in the nasal cavity and the nasopharynx and granulomatous formation with slight fibrosis in the alveoli. Macrophage levels in the BALF were decreased, and the levels of multinucleated macrophages were increased, possibly in response to the toxicity of phagocytosed MWCNTs. There was also a persistent pulmonary inflammatory reaction to MWCNT exposure. No histopathological or inflammatory changes in the rats exposed to 0.2 mg/m^3 MWCNTs were observed; therefore, the NOAEL for inhaled MWCNTs in this study was 0.2 mg/m^3. However, our results suggest that MWCNTs might persist in the lung for long periods of time and could eventually cause severe pulmonary toxicities. Moreover, migration of MWCNTs to the lymphatic system outside the lung raises concern about toxic effects of inhaled MWCNTs on the visceral pleura. Therefore, further, long-term studies are essential to reveal the toxicities in the lung, pleura and other organs caused by inhalation exposure to MWCNTs.

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GENOTOXICITY AND CARCINOGENICITY

Hepatotumorigenicity of ethyl *tertiary*-butyl ether with 2-year inhalation exposure in F344 rats

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Abstract Carcinogenicity of ethyl *tertiary*-butyl ether (ETBE) was examined with inhalation exposure using F344/DuCrlCrlj rats. Groups of 50 male and 50 female rats, 6 week old at commencement, were exposed to ETBE at 0, 500, 1,500 or 5,000 ppm (v/v) in whole-body inhalation chambers for 6 h/day, 5 days/week for 104 weeks. A significant increase in the incidence of hepatocellular adenomas was indicated in males exposed at 5,000 ppm, but not in females at any concentration. In addition, significantly increased incidences of eosinophilic and basophilic cell foci were observed in male rats at 5,000 ppm. Regarding non-neoplastic lesions, rat-specific changes were observed in kidney, with an increase in the severity of chronic progressive nephropathy in both sexes at 5,000 ppm. Increased incidences of urothelial hyperplasia of the pelvis were observed at 1,500 ppm and above, and mineral deposition was apparent in the renal papilla at 5,000 ppm in males. There were no treatment-related histopathological changes observed in any other organs or tissues in either sex. The present 2-year inhalation study demonstrated hepatotumorigenicity of ETBE in male, but not in female rats.

Keywords Ethyl *tertiary*-butyl ether · ETBE · Inhalation · Hepatotumorigenicity · Rat · Kidney

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Introduction

Ethyl *tertiary*-butyl ether (ETBE) is chemically synthesized from bioethanol and isobutane. To support the Kyoto Protocol for reducing CO_2 emissions, the Petroleum Association of Japan decided to use ETBE as a gasoline blending component. It is also added to unleaded gasoline as an oxygenate to increase oxygen in fuel as an octane enhancer and to decrease exhaust emissions, particularly of carbon monoxide, unburned hydrocarbons, polycyclic aromatics and oxides of nitrogen and particulate carbon. While the use of oxygenated motor fuels can have beneficial environmental consequences, humans can be exposed to oxygenates by inhalation and oral route (Ahmed 2001; McGregor 2006, 2007). Exposure of the public to ETBE is principally by inhalation of fumes while refueling vehicles (McGregor 2007).

Subchronic inhalation toxicity of ETBE has been reported in rats and mice. Medinsky et al. (1999) exposed F344 rats and CD-1 mice to ETBE vapor at concentrations of 0 (control), 500, 1,750 or 5,000 ppm for 6 h/day, 5 days/week for 13 weeks. They found degenerative changes in testicular seminiferous tubules in male rats at 1,750 and 5,000 ppm, and increases in the incidence of regenerative foci, rates of renal cell proliferation and α2u-globulin-containing protein droplets in kidneys of all treated male rats. Furthermore, increases in the incidence of centrilobular hepatocyte hypertrophy and rates of hepatocyte cell proliferation were apparent in the livers of male and female mice at 5,000 ppm. While no carcinogenicity study has so far been reported by the inhalation route for ETBE, methyl *tertiary*-butyl ether (MTBE), a related chemical, was tested in rats and mice. When Bird et al. (1997) exposed F344 rats and CD-1 mice to MTBE vapor for 24 and 18 months, respectively, they found an increased incidence of renal

tubular cell tumors in male rats with increases in the incidence and severity of chronic progressive nephropathy (CPN) in male and female rats and an increased incidence of hepatocellular adenomas in female mice.

In order to assess health risks of ETBE exposure by the inhalation route, we here performed a 2-year inhalation study using F344 rats of both sexes in accordance with standard test guidelines and good laboratory practices (GLPs).

Materials and methods

This study was conducted with reference to the Organisation for Economic and Co-operation and Development (OECD) Guideline for Testing of Chemicals 451; "Carcinogenicity Studies" (OECD 1981) and in accordance with the OECD principles of GLP (OECD 1998). The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (NRC 1996), and the present studies were approved by the ethics committee of the Japan Bioassay Research Center (JBRC).

Test material

ETBE used in the present study was manufactured by Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), with specifications as follows: appearance, colorless transparent liquid; boiling point, 70 °C; vapor pressure, 17Kpa (25 °C); solubility, slightly soluble in water (1.2 g/100 g, 20 °C); lot number, L-506251; purity, >99 % (measured by Toray Research Center Co., Ltd., Tokyo, Japan); and storage conditions, at room temperature and in a dark place. Stability of test material was measured using gas chromatography (Agilent Technologies 5890A) before the beginning and at the end of the test material administration and confirmed to be acceptable by comparison of the data from the two time points, no significant difference being evident.

Animals

F344/DuCrlCrlj rats of both sexes were obtained at the age of 4 week from Charles River Japan, Inc (Kanagawa, Japan). The animals were quarantined and acclimated for 2 weeks and then divided by stratified randomization into four body weight-matched groups, each comprising 50 rats of both sexes. The animals were individually housed in stainless-steel wire-hanging cages (150 W × 270 D × 176 H mm) which were placed in stainless-steel inhalation exposure chambers. Four whole-body inhalation chambers of 8,600 liters in volume installed in a barrier system animal room were used in the present studies. The environment in each exposure chamber was maintained at a temperature of 20–24 °C and a relative humidity of 30–70 % with 12 air changes/h. Fluorescent lighting was controlled automatically to give a 12-h light/dark cycle. All rats were given sterilized commercial pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and filtered and ultraviolet-irradiated tap water ad libitum. The body weights measured immediately before the first exposure to ETBE or clean air were 124 ± 6 (mean ± SD) g for male and 97 ± 3 g for female rats.

Experimental design

Groups of 50 rats of both sexes were exposed to airflow containing ETBE vapor in whole-body inhalation chambers at a target concentration of 500, 1,500 or 5,000 ppm for 6 h/day, 5 days/week for 104 weeks. Fifty rats of both sexes, serving as respective controls, were handled in the same manner as the ETBE-exposed groups, but were exposed to clean air in an inhalation chamber. The exposure concentrations were decided based on the results of a preliminary 13-week inhalation study performed presenting our laboratory.

In the preliminary 13-week inhalation exposure study, both sexes of F344/DuCrlCrlj rats were exposed to ETBE at the concentrations of 0 (control), 50, 150, 500, 1,500 or 5,000 ppm by whole-body inhalation for 13 weeks. No mortality was found in any groups. A significant inhibition of body weight gain was found in the 5,000 ppm group males. Average body weights at the end of the treatment in males of the 50, 150, 500, 1,500 and 5,000 ppm-exposed groups were 94, 95, 95, 97 and 89 %, respectively, and those in female were 95, 97, 96, 94 and 95 %, respectively, of the control values. Food consumption of the 5,000 ppm-exposed males was low in the early phase of the exposure period. In hematology, slight but significant decreases in red blood cell count and hemoglobin concentration, and slight but significant increase in platelet counts were observed in the 5,000 ppm-exposed males. In blood biochemistry, protein, parameters related to lipids, and urea nitrogen were significantly altered in the 5,000 ppm-exposed males. In organ weights, significant increases in kidney weights were observed in both sexes of rats exposed at 1,500 ppm or above, and significant increases in liver weights were noted in both sexes at 5,000 ppm. On histopathological examination, an increased incidence of regeneration of proximal renal tubules in kidneys was observed in the 5,000 ppm-exposed males. Based on these findings, the concentration of 5,000 ppm was estimated to be the maximum tolerated dose (MTD) and was selected as the highest dose for the present 2-year study. The lowest dose was set at 500 ppm, with 1,500 ppm as the middle level, because no treatment-related changes were found in either sex at 500 ppm in the preliminary 13-week study.

Vapor generation and chamber concentrations of ETBE

Airflow containing ETBE vapor at target concentrations of 500, 1,500 or 5,000 ppm was produced by a vaporization technique. The saturated vapor–air mixture was generated by bubbling clean air through ETBE liquid in a temperature-regulated glass flask (50 ± 5 °C) and by cooling it through a thermostatted condenser at 20 ± 3 °C. The airflow containing the saturated vapor was diluted with clean air and then warmed to 25 ± 3 °C in a thermostatted circulator which served to stabilize the vapor concentration by complete gasification of ETBE. The flow rate of the vapor–air mixture was regulated with a flow meter, further dilution with humidity- and temperature-controlled clean air being conducted in a spiraling line mixer before supply to inhalation chambers. Concentrations of ETBE vapor in inhalation chambers were monitored by gas chromatography every 15 min.

Clinical observations and histopathological examinations

The animals were observed daily for clinical signs and mortality. Body weights and food consumption were measured once a week for the first 14 weeks and every 4 weeks thereafter. Urinary parameters were measured in the last week of the 2-year study period with Ames Reagent Strips (Siemens Healthcare Diagnostics, IL, USA). All rats underwent complete necropsy. For hematology and blood biochemistry, blood was collected from the abdominal aorta under etherization at the end of the study period after overnight fasting. The blood samples were analyzed with an automatic blood cell analyzer (ADVIA 120, Bayer Co. NY, USA) for hematology and an automatic analyzer (Hitachi 7080, Hitachi, Ltd., Ibaraki, Japan) for blood biochemistry. Organs were removed, weighed and examined for macroscopic lesions at necropsy. The organs and tissues designated in the OECD test guidelines (Organization for economic co-operation and development (OECD) 1981) were examined for histopathology in all the rats. After fixation in 10 % neutral-buffered formalin and embedding in paraffin, tissue sections of 5 μm in thickness were prepared and stained with hematoxylin and eosin (H & E) for histopathological examination.

Statistical analysis

Incidences of neoplastic lesions were analyzed for any dose–response relationship as indicated by a significant positive trend by Peto's test (Peto et al. 1980) and for significant differences from the clean air-exposed group by Fisher's exact test. Incidences and severity of pre- and non-neoplastic lesions and urinary parameters were analyzed with the chi-square test. Date for body weights, food consumption, hematological, blood biochemical parameters and organ weights were analyzed by Dunnett's test. A two-tailed test was used for all statistics except for Peto's test. In all cases, a p value of 0.05 was applied as the level of significance.

Results

Concentrations of ETBE vapor in inhalation chambers

Concentrations of ETBE vapor in the inhalation chambers were maintained constant throughout the 2-year exposure period. Mean ± SD of the concentrations of ETBE in the 500, 1,500 and 5,000 ppm-exposed groups were 501 ± 5, 1,501 ± 13 and 4,999 ± 48 ppm, respectively.

Survival, body weight and clinical sign

The survival rates of animals at the end of the study period are shown in Table 1. Decrease was found in males of the 5,000 ppm group, attributable to an increased number of deaths due to CPN (no control males vs. 11 rats in the 5,000 ppm males). In females, the survival rates in the 1,500 and 5,000 ppm groups were lower than the control group value. Deaths caused by pituitary tumors in the 5,000 ppm female group (6 rats) were more frequent than in the control group (1 rat). On observation of clinical signs, no exposure-related change was found in any groups. Body weights in males of the 5,000 ppm group were significantly lower than in the control group throughout the study period (Fig. 1; Table 1). In addition, body weights in females of the 1,500 and 5,000 ppm groups were significantly lower than the control group after weeks 30 and 10, respectively (Fig. 2; Table 1). Final body weights in the 500, 1,500 and 5,000 ppm groups were 94, 94 and 75 % (statistically significant) for males and 95, 91 and 78 % (statistically significant) for females, respectively, of the control values. Significantly lowered food consumption in males was indicated in all treated groups during the early phase of the study period (before week 7) (data not shown). Food consumption in females of all treated groups was comparable to that in the control group.

Hematology and blood biochemistry, and urinalysis

No significant change in hematological parameters was observed in any ETBE-treated group in either sex, except for a decrease in mean cell volume (MCV) of the 5,000 ppm group in females (data not shown).

In blood biochemistry, significant increases in urea nitrogen, creatinine, and inorganic phosphorus were

Table 1 No. of surviving animals, terminal body weight and organ weight of male and female rats in the 2-year inhalation study of ETBE

Dose (ppm)	0 (control)	500	1,500	5,000
Males				
No. of animals examined	50	50	49[a]	50
No. of surviving animals[b] (rate %)	44 (88.0)	38 (76.0)	39 (79.6)	30 (60.0)
Terminal body weight (g)[c]	426 ± 55	402 ± 113**	402 ± 55**	321 ± 30**
Organ weights				
Kidneys (g)	2.81 ± 0.29	3.03 ± 0.49*	3.28 ± 0.56**	3.44 ± 0.47**
Kidneys (%)[d]	0.71 ± 0.09	0.85 ± 0.19**	0.90 ± 0.19**	1.18 ± 0.23**
Females				
No. of animals examined	50	50	50	50
No. of surviving animals[b] (rate %)	38 (76.0)	39 (78.0)	30 (60.0)	30 (60.0)
Terminal body weight (g)[c]	276 ± 30	262 ± 30*	251 ± 16**	214 ± 18**
Organ weights				
Kidneys (g)	1.81 ± 0.18	1.90 ± 0.20	1.92 ± 0.13*	2.13 ± 0.28**
Kidneys (%)[d]	0.72 ± 0.17	0.80 ± 0.14**	0.83 ± 0.06**	1.08 ± 0.16**

* Significantly different from the control at $p < 0.05$ by Dunnett's test

** Significantly different from the control at $p < 0.01$ by Dunnett's test

[a] One rat died because of accident

[b] No. of surviving animals at the end of the 2-year administration period

[c] Mean ± SD for body weights of the surviving animals at the end of the 2-year administration period

[d] Relative organ weights were calculated with the following equation. Absolute organ weight/fasted body weight × 100

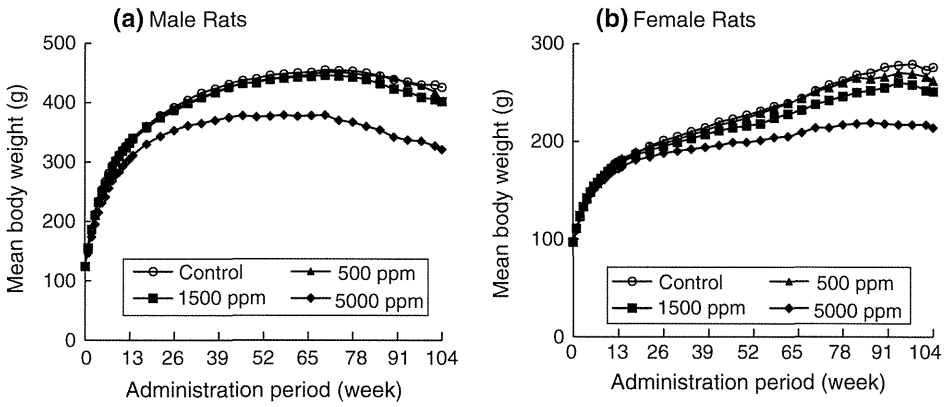

Fig. 1 Growth *curves* of male rats (**a**) and female rats (**b**) inhaling ETBE-containing or flesh air for 2 years

observed in all treated groups in males (data not shown). Significant increases in total cholesterol, triglyceride, phospholipid, γ-GTP and calcium and a significant decrease in A/G ratio were also observed in the 1,500 and 5,000 ppm groups in males. Furthermore, significant increases in potassium and significant decreases in total protein, albumin, AST and chlorine were noted at 5,000 ppm in males. In females, significant increases in total cholesterol, phospholipid, urea nitrogen and potassium and significant decreases in albumin, the A/G ratio, AST, ALT and sodium were observed in the 5,000 ppm group.

No significant change on urinalysis was observed in any ETBE-treated groups in either sex, except for a lowering of urine pH values in all treated groups in males and in the 5,000 ppm group in females, and an increase in urine protein in the 5,000 ppm group in females (data not shown).

Organ weights

Significantly increased absolute and relative weights were noted in kidneys of all treated groups in males (Table 1). In females, absolute kidney weights were significantly increased in the 1,500 and 5,000 ppm groups. Significantly

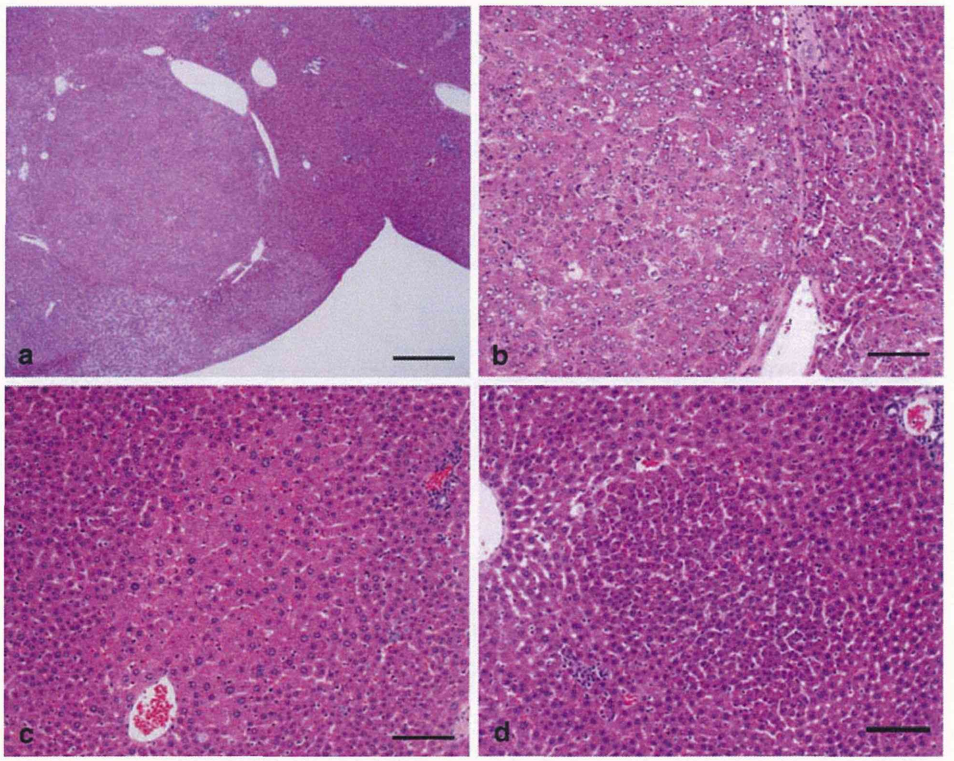

Fig. 2 Principal neoplasms induced by ETBE. Each section was stained by H&E. **a** Hepatocellular adenoma in a male rat exposed to 5,000 ppm. Note expansive tumor compression of adjacent parenchyma. *Bars* indicate 500 μm. **b** High magnification of **a** *Bars* indicate 100 μm. **c** Acidophilic cell focus in a male rat exposed at 5,000 ppm. The hepatocytes are slightly enlarged with acidophilic (eosinophilic) cytoplasm. *Bars* indicate 100 μm. **d** Basophilic cell focus in a male rat exposed at 5,000 ppm. The hepatocytes are smaller than normal. *Bars* indicate 100 μm

increased relative kidney weights were apparent in all treated groups.

Histopathology

Neoplastic and proliferative lesions

Data for incidences of neoplastic and proliferative lesions in the liver are shown in Table 2. Diagnoses of hepatocellular adenoma, hepatocellular carcinoma and proliferative lesions were based on the criteria of "Pathology of the Fischer Rat" (Gary et al. 1990). The incidences of hepatocellular adenomas in males exhibited a significantly positive trend by Peto's Test, and the incidence (9/50, 18 %) in the 5,000 ppm group in males was significantly increased as compared to that (0/50, 0 %) of the control group by Fisher's exact test. A hepatocellular carcinoma was found in only one male rat in the 5,000 ppm group. In addition, a significantly increased incidence of eosinophilic cell foci and a significantly increased incidence of basophilic cell foci, considered to be pre-neoplastic lesions, were found at 5,000 ppm in males (chi-square test). No increase in the incidence of tumors or proliferative lesions was indicated in the livers of any treated groups in females.

Data for incidences of neoplastic lesions in other organs or tissues besides the liver are shown in Table 3. No treatment-related significant increase was found in any other organs or tissues in either sex. Despite deaths caused by pituitary tumors in the 5,000 ppm female group, the total incidences of pituitary adenomas in females did not reach statistical significance as compared to the controls in any of the treated groups. Therefore, it was judged that the incidence of pituitary adenomas was not affected by administration of ETBE. On the other hand, a significantly decreased incidence of fibromas of the subcutis was found at 5,000 ppm in males, along with reduction in fibroadenomas of the mammary gland in the 5,000 ppm grope of females, by Fisher's exact test. A significantly decreased incidence of C-cell adenomas of the thyroid gland was found at 1,500 ppm in females by Fisher's exact test. Incidences of C-cell hyperplasia in females showed a significant decrease at 500 and 5,000 ppm.

Table 2 Incidences of neoplastic and proliferative lesions in the liver of F344 rats exposed to ETBE by inhalation for 2 years

Group name (ppm)	Male				Female			
No. of animals examined	Control 50	500 50	1,500 49	5,000 50	Control 50	500 50	1,500 50	5,000 50
Neoplastic lesions								
Hepatocellular adenoma	0	2	1	$9^{FF\uparrow\uparrow}$	1	0	1	1
Hepatocellular carcinoma	0	0	0	1	0	0	0	0
Proliferative lesions								
Clear cell focus	5	0	0	4	2	0	0	3
<+>	⟨5⟩			⟨4⟩	⟨2⟩			⟨3⟩
Eosinophilic cell focus	31	28	36	39**	2	1	4	2
<+>	⟨31⟩	⟨28⟩	⟨36⟩	⟨30⟩	⟨2⟩	⟨1⟩	⟨4⟩	⟨2⟩
<2+>				⟨9⟩				
Basophilic cell focus	18	10	13	33**	36	31	32	28
<+>	⟨18⟩	⟨10⟩	⟨13⟩	⟨31⟩	⟨32⟩	⟨24⟩	⟨27⟩	⟨23⟩
<2+>				⟨2⟩	⟨4⟩	⟨7⟩	⟨5⟩	⟨5⟩
Vacuolated cell focus	0	0	0	0	1	2	2	0

Values indicate number of animals bearing lesions

The values in angle bracket indicate the severity grade of lesions: +, slight; 2+, moderate

Significant difference: ^{FF} $p < 0.01$ by Fisher's exact test, $^{\uparrow\uparrow}$ $p < 0.01$ by Peto's test, ** $p < 0.01$ by Chi-square test

Non-neoplastic lesions

Treatment-related significant increases in non-neoplastic lesions were found in kidney (Table 4).

A significant increase in the degree of CPN was indicated in the 5,000 ppm group in both sexes as compared to the respective controls. CPN was evaluated for severity with reference to the criteria of the report from working group on long-term holding of experimental animals in the Japanese Association for Laboratory Animal Science (Kawai 1980). A significantly increased incidence of urothelial hyperplasia of the pelvis was found in the kidneys at 1,500 and 5,000 ppm of males, and the incidence of mineral deposition in the renal papilla in the 5,000 ppm group was significantly elevated in males. Mineral deposition featured linear basophilic material in Henle's loops in the papilla. In males, urothelial hyperplasia of renal pelvis was characterized by multilayer thickening of the papillary epithelium with protrusion into the pelvis and occurred in small parts of renal papilla.

No exposure-related histopathological changes were observed in any other organs or tissues besides the liver and kidney of the ETBE-exposed rats of either sex, except for increases in incidence of mineral deposition in the stomach and arteries at 5,000 ppm in males. Mineral deposition in arteries was mainly observed in the walls of arteries efferent from the left ventricle of the heart.

Discussion

In the present 2-year inhalation study, an increased incidence of hepatocellular adenomas was observed in male rats, which was considered to be causally related to the ETBE exposure, on the basis of the following evidence. First, the incidences of hepatocellular adenomas in males were increased in an exposure concentration-dependent manner, as indicated by a significant positive trend with Peto's test. Second, statistical significance by Fisher's exact test was attained at the concentration of 5,000 ppm compared with the control group. Third, the incidences of hepatocellular adenomas in males of the 5,000 ppm-exposed group exceeded the maximum incidences of historical control data of the JBRC. Maximum and average incidences of hepatocellular adenomas in control groups from 48 carcinogenicity studies performed in the JBRC are 8 and 1.8 %, respectively. In addition, a significantly increased incidence of the eosinophilic cell foci and basophilic cell foci was observed in male rats at the exposure concentration of 5,000 ppm (chi-square test). On the other hand, no significant increase in incidence of malignant liver tumors was indicated in male rats exposed to ETBE, only one hepatocellular carcinoma being found in a male rat in the 5,000 ppm-exposed group.

As histopathological features of hepatocellular adenomas found in the 5,000 ppm-exposed rats were similar to those commonly observed in F344 rats, the increased incidence in male rats exposed to ETBE might be caused

Table 3 Incidences of neoplastic lesions in the other organs or tissues besides the liver of F344 rats exposed to ETBE by inhalation for 2 years

Group name (ppm)	Male				Female			
No. of animals examined	Control 50	500 50	1,500 49	5,000 50	Control 50	500 50	1,500 50	5,000 50
Subcutis								
Fibroma	8	7	3	2^{F}	1	0	0	0
Lung								
Bronchiolar-alveolar carcinoma	1	3	1	0	0	0	0	0
Spleen								
Mononuclear cell leukemia	6	8	11	8	8	7	6	4
Pancreas								
Islet cell adenoma	7	7	6	2	0	2	1	0
Pituitary								
Adenoma	13	10	18	6	18	14	11	$13^{\uparrow *}$
Thyroid								
C-cell adenoma	7	4	9	5	7	5	1^{F}	3
C-cell carcinoma	3	4	1	2	1	2	0	0
Adrenal								
Pheochromocytoma	9	8	3	4	2	2	2	0
Testis								
Interstitial cell tumor	42	44	45	46	–	–	–	–
Uterus								
Endometrial stromal polyp	–	–	–	–	8	15	9	8
Adenocarcinoma	–	–	–	–	2	3	1	4
Endometrial stromal sarcoma	–	–	–	–	2	2	3	2
Mammary gland								
Fibroadenoma	1	0	0	1	8	10	4	2^{F}
Preputial gland								
Adenoma	1	1	1	3	1	1	0	0
Peritoneum								
Mesothelioma	0	2	3	0	0	0	0	0

Values indicate number of animals bearing lesions

Significant difference: $^{F}p < 0.05$ by Fisher's exact test, $^{\uparrow}\ p < 0.05$ by Peto's test (* by standard method only)

by enhancement of spontaneous occurrence in this strain of rats. Promotion of pre-neoplastic lesion and hepatocellular adenoma development was earlier reported in the livers of male F344 rats orally administered ETBE at daily dose of 1,000 mg/kg body weight following multi-organ initiation with carcinogens (Hagiwara et al. 2011). Genotoxicity of ETBE has been examined by several test systems including gene mutation tests using *Salmonella typhimurium* and Chinese hamster ovary cells (CHO), chromosomal aberration test using CHO, and in vivo micronucleus tests in bone marrow cells of mice orally treated at dose of up to 5,000 mg/kg body weight and of mice exposed by inhalation at concentration of up to 5,000 ppm, 6 h/day for 5 days. None of the results from those studies suggested that ETBE is genotoxic (McGregor 2007). Therefore, non-genotoxic mode of action is presumed for the enhanced incidence of hepatocellular adenomas observed in male rats exposed to ETBE in the present study.

Some reports have pointed out non-neoplastic toxic effects of ETBE on the liver in rodent studies. In the preliminary 13-week inhalation exposure study for the present 2-year study, a significant increase in liver weights was indicated in rats exposed to ETBE at the concentration of 5,000 ppm, although there did not appear to be any associated histopathological changes. A significant increase in liver weight was noted in rats of the 13-week inhalation exposure study of ETBE conducted by Medinsky et al. (1999) at the concentration of 5,000 ppm. Centrilobular hypertrophy of the liver was noted after 6 months and in our recent 2-week studies of rats treated with ETBE orally (unpublished data), and Medinsky et al. (1999) also described centrilobular hepatocyte hypertrophy and