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Lack of the Initiation of Benzo[a]pyrene-induced Mouse Forestomach Neoplasia by Di(2-ethylhexyl)phthalate(DEHP)

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Abstract

Carcinogenicity of di(2-ethylhexyl)phthalate(DEHP) to the mouse forestomach and its inhibitory activity for the initiation of benzo[a]pyrene(BP)-induced mouse forestomach neoplasia were studied on the mouse forestomach carcinogenesis regimen. One hundred female ICR mice(6~7 weeks of age) were housed in a polycarbonate cage(5 mice/cage) in a humidity- and temperature-controlled room and subjected to a semipurified diet for a week. Mice were divided into 4 treatment groups(25 mice/treatment): Basal diet, DEHP, BP, and BP+DEHP. On Monday and Wednesday, 0.1ml DEHP mixed with 0.1ml olive oil(for DEHP and DEHP+BP treatment groups) or 0.1ml sailine+0.1ml olive oil(for basal diet group) was intubated, p.o., and on Friday, 2mg BP dissolved in 0.2ml olive oil(for BP and BP+DEHP treatment groups) was intubated, p.o. This cycle was repeated for 4 weeks. Beginning with the first intubation of BP and continuing thereafter, body weight and food intake were recorded once and twice weekly, respectively. All surviving mice were sacrificed 22 weeks after the first dose of BP intubation and countered forestomach tumor. No tumor was formed by DEHP treatment. 5.75 tumors per mouse was formed by BP treatment, whereas its number was reduced to 4.53 by BP+DEHP treatment. Similar results were seen in the tumor incidence. Body weight gain was not affected by DEHP treatment, when compared to that by basal diet treatment. The body weight was significantly reduced by BP treatment, but its reduction was recovered to the level of the basal diet group by BP+DEHP treatment. No significant difference was seen in food intake among all treatment groups. These results indicate that DEHP lacks carcinogenic activity to the mouse forestomach and rather inhibits the initiation of BP-induced mouse forestomach neoplasia.

Key words: di(2-ethylhexyl)phthalate(DEHP), carcinogenesis, forestomach neoplasia

INTRODUCTION

Di(2-ethylhexyl)phthalate(DEHP; Fig. 1) is the diester of *O*-phthalate and is widely used as a plasticizer for plastic manufactures(1). As a result of use, there has been environmental contaminations(2-4), and human exposures which might result in adverse effects for health(5).

It is very well understood that DEHP acts as a hyperplastic agent in the liver of rodents. DEHP induced a marked cellular hypertrophy and hyperplasia in the liver(6,7), hepatic peroxisome proliferation(8,9), and an increase in the activities of some peroxisome-associated enzymes(10,11). Thus, it is suggested that DEHP might act as a carcinogen.

Actually, in rodent carcinogenesis assays, DEHP

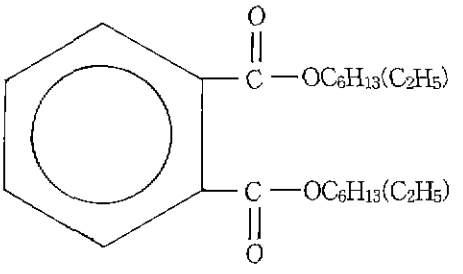

Fig. 1. Chemical structure of DEHP.

enhanced liver tumor formation. Incidences of neoplastic nodules and hepatocellular carcinomas were significantly elevated in rats treated with high doses of DEHP; however, in mice, they were elevated even at low dose(12). Feeding of DEHP for >100 weeks resulted in the oc-

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currence of liver neoplasms in mice and rats at 3,000 ppm and 6,000ppm, respectively(13). Based on animal carcinogenesis experiments, IARC classified DEHP as sufficient evidence for carcinogenicity in animal(13).

By contrast to the carcinogenic activity of DEHP, in chemical carcinogen-induced carcinogenesis models, which are divided into initiation and promotion stages, DEHP shows somewhat different activity from its carcinogenicity. DEHP inhibited the formation of preneoplastic lesions in rat liver promoted by phenobarbital (PB) and dietary choline deficiency(14). It has been reported that no initiating, promoting or syncarcinogenic effect of DEHP was seen in rats induced and promoted by N-2-fluorenylacetamine(FAA) and PB, respectively (15). Similar results were obtained from rat liver carcinogenesis induced by diethylnitrosamine(DEN)(16,17). However, on mouse liver carcinogenesis, DEHP had an enhancing effect when administered shortly after a single exposure to DEN(18,19). Tumor-promoting effect of DEHP in JB6 mouse epidermal cells and SENCAR mouse skin induced by 7,12-dimethylbenz[a]anthracene(DMBA) was also reported by Diwan et al.(20). These results of the studies suggest that effect of DEHP on the carcinogen-induced two-stage-carcinogenesis is dependent upon animals, organ sites and carcinogens used for the test. No report of the carcinogenicity of DEHP on the mouse forestomach, which is a good model to test carcinogenicity of chemicals and the effect of DEHP on the mouse forestomach carcinogenesis induced by BP was published so far.

In the present paper, we investigated whether or not DEHP induces the formation of mouse forestomach neoplasms and inhibits the initiation of mouse forestomach neoplasia induced by BP. Our results show that DEHP is not a carcinogen on mouse forestomach and rather inhibits the initiation of BP-induced mouse forestomach neoplasia under test conditions.

MATERIALS AND METHODS

Materials

DEHP and BP were purchased from Sigma Chemical Company(St. Louis, MO). Olive oil was obtained from a local grocery. All other chemicals used were ACS grade.

Treatment of mice

Mouse forestomach carcinogenesis experiment was conducted according to the protocol described by Ha et al.(21). One hundred female ICR mice(Sprague-Dawley, Madison, WI), 6 to 7 weeks of age, were housed in polycarbonate cages(5 mice/cage) in a temperature- and humidity-controlled facility and permitted free access to water and food(TD86348 semipurified diet; Teklad test diets, Madison, WI). Two weeks later, the animals were randomized by body weight and divided into 4 groups(25 mice/group). They were then subjected to a forestomach tumorigenesis regimen as follows. On Monday and Wednesday each animal was given by gavage (a) 0.1ml DEHP plus 0.1ml olive oil(for DEHP and DEHP+BP treatment groups) or (b) 0.1ml saline plus 0.1ml olive oil(for Basal diet and BP treatment groups); and on Friday animals were given 2mg BP in 0.2ml olive oil(for BP and DEHP+BP treatment groups) or 0.2ml olive oil alone(for Basal diet and DEHP treatment groups). This sequence was repeated for 4 weeks. Beginning with the first intubation and continuing thereafter, body weight and food intake were recorded once and twice weekly, respectively. All surviving mice were sacrificed 22 weeks after the first dose of BP intubation.

Tumor histology

The forestomach was fixed for 24 hours at room temperature in an expanded state produced by i.g., instillation of 4% neutral buffered formalin. The forestomachs were split longitudinally, and presumed tumors 1mm or larger were counted using a dissecting microscope, followed by histological examination(22) for the confirmation of neoplasia.

Statistical analysis

Data were analyzed for statistical significance using, where appropriate, the least significant difference test, one-way analysis of variance(ANOVA), or the χ^2 test.

RESULTS

Body weight and food intake

Fig. 2 shows the effects of DEHP on the body weight gains. The body weight gain of mice treated with DEHP alone was almost same as that of mice subjected to basal diet over an entire experimental period. The body weight of mice treated with BP was significantly reduced($p<0.05$), when compared to that of mice sub-

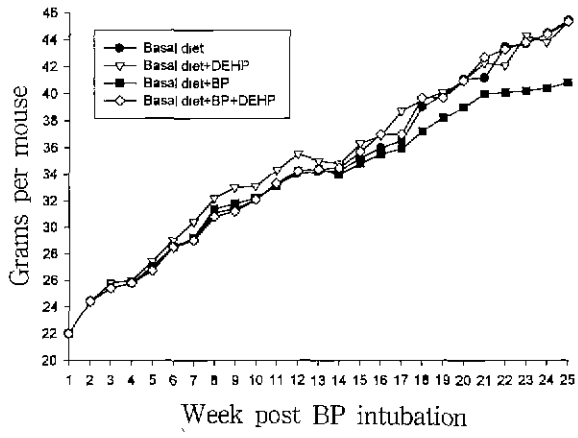

Fig. 2. Cumulative body weight of mice treated with DEHP, BP or BP+DEHP.
Mice were given 0.8ml DEHP, or 8mg BP or 0.8ml+8mg BP in total during the initiation of forestomach neoplasia.

jected to basal diet, beginning 17 weeks after BP intubation. However, the body weight reduction by BP was actually overcomed by DEHP treatment, compared to body weights from BP treatment and BP+DEHP treatment groups.

Table 1 shows the effect of DEHP on an average food intake per week calculated during the entire experimental period of time. Mice treated with DEHP ate more food than mice subjected to basal diet, whereas mice treated with BP ate less food. No food intake difference was seen in the treatment group of basal diet, BP, and BP+DEHP.

Effect of DEHP on forestomach neoplasia

The effect of DEHP on BP-induced neoplasia of the forestomach in female ICR mice is shown in Table 2. DEHP treatment(total dose: 0.8ml/mouse) did not induce

Table 1. Effect of DEHP on the food intake for the mice treated with benzo[a]pyrene

Treatment	Mice in effective	kcal/mouse/week
Basal diet[1]	20	185.4±3.1[2,3]
DEHP	20	188.9±2.5
BP	20	185.0±3.3
DEHP+BP[4]	19	186.7±1.8

[1]Semi-purified diet
[2]Mean±SEM
[3]No significant difference was observed among treatments by ANOVA
[4]0.1ml DEHP with 0.1ml olive oil was intubated, p.o. twice weekly prior to initiation with 2mg BP dissolved with 0.1 ml olive oil over entire experimental period of time

Table 2. Effect of DEHP on the mouse forestomach neoplasia induced by benzo[a]pyrene

Treatment	Mice in effective	Tumor incidence (%)	Tumors per mouse	Tumors per tumor bearing mouse
Basal diet[1]	20	0	0	0
DEHP	20	0	0	0
BP	20	95[2]	5.75±1.09[2,3]	6.05±1.10[2]
DEHP+BP[4]	19	89	4.53±0.88	5.05±0.90

[1]Semi-purified diet
[2]No significant difference was observed between the two treatments for tumor incidence by χ^2 test, and tumors/mouse and tumors/tumor-bearing mouse by t-test
[3]Mean±SEM
[4]0.1ml DEHP mixed with 0.1ml olive oil was intubated, p.o. twice weekly prior to initiation with 2mg BP dissolved with 0.1ml olive oil over entire experimental period of time

any tumor. Likely, no tumor was induced by the basal diet. However, BP-treatment(8mg/mouse) induced substantial number of tumors: 5.75 tumors/mouse, 95% tumor incidence and 6.05 tumors/tumor-bearing mouse. As seen in the treatment group of BP+DEHP and treatment group of BP alone, the tumor formation(tumors/mouse, tumor incidence and tumors/tumor-bearing mouse) was reduced by DEHP treatment, even not statistically significant, facts that DEHP has the ability to inhibit the initiation of forestomach neoplasia induced by BP. These results suggest that DEHP lacked the induction of mouse forestomach neoplasia, but rather suppressed the formation of mouse forestomach neoplasms by BP.

Histological features

No histological abnormality of the forestomachs derived from DEHP and basal diet treated mice was found. However, histological examination of the BP- and BP+DEHP-induced forestomach tumors revealed that they were papillomas with or without focal areas of epidermal hyperplasia.

DISCUSSION

DEHP, which is not genotoxic in either *in vitro* or *in vivo* studies(23), nor to bind to liver DNA *in vivo*(24), is generally recognized as a hyperplastic agent to induce a marked cellular hypertrophy and hyperplasia in the liver(6-9) and thus, it is appeared to be a tumor promoting agent in chemical-induced carcinogenesis models

and a carcinogen in the liver of rats and mice(12,25). Mechanism by which DEHP acts as a carcinogen and a tumor-promoting agent is not well understood. Oxygen radicals(26,27) induced by DEHP is presumed as causative agents.

The data shown in the present study indicated that DEHP did not induce mouse forestomach tumor, but rather it reduced BP-induced mouse forestomach neoplasms even though no significant difference. No information related to the effect of DEHP on mouse forestomach carcinogenesis is available in literatures. Exact mechanisms by which DEHP inhibited the initiation of BP-induced mouse forestomach neoplasia are not known. We presume that the body weight recovering property of DEHP, shown in our results, might be indirectly related to the inhibitory effect of DEHP on the inhibition of the initiation of BP-induced mouse forestomach neoplasia. In addition, recent reports that DEHP inhibited protein kinase C(PKC) activity, which is positively related to tumor promotion in animal models (28) and induced cytochrome P450 in rodents(29,30), can explain the inhibitory activity of DEHP observed in the present study, but no direct evidence related to the forestomach carcinogenesis is available.

Anticarcinogenicity of DEHP has been shown to be a controversial subject, based on various animal studies, and largely relied on experimental conditions, especially animal models. In the case of mouse forestomach model, besides the present study, no DEHP data was available in the literature. Therefore, more research should be conducted to fully elucidate the effect of DEHP on the mouse forestomach carcinogenesis induced by various chemical carcinogens.

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REFERENCES

1. United States International Trade Commission : Preliminary report on U.S. production of selected organic chemicals(including synthetic plastics and resins) March, April, and cumulative totals. U.S. Trade Commission, Washington, DC, Series C/P 83-84(1983)
2. Giust, J. A., Seipelt, C. T., Anderson, B. K., Deis, D. A. and Hinders, J. D : Determination of bis(2-ethylhexyl) phthalate in cow's milk and infant formula by high-performance liquid chromatography. *J. Agric. Food Chem.*, **38**, 415(1990)
3. Sharman, M., Read, W. A., Castle, L. and Gilbert, J. : Levels of di(2-ethylhexyl)phthalate and total phthalate esters in milk, cream, butter and cheese. *Food Addit. Contaminants*, **11**, 375(1994)
4. Wams, T. J. : Diethylhexylphthalate as an environmental contaminant-a review *Sci. Total Environ.*, **66**, 1(1987)
5. Pollack, G. M., Buchanan, J. F., Slaughter, R. L., Kohli, R. K. and Danny, D. S. : Circulating concentrations of di(2-ethylhexyl)phthalate and its de-esterified phthalic acid products following plasticizer exposure in patients receiving hemodialysis. *Toxicol. Appl. Pharmacol.*, **79**, 257 (1985)
6. Daniel, J. W. and Bratt, H. : The absorption, metabolism, and tissue distribution of di(2-ethylhexyl)phthalate in rats. *Toxicol.*, **2**, 51(1974)
7. Oishi, S. and Hiraga, K. : Effects of phthalic acid esters on mouse testes. *Toxicol Lett.*, **5**, 413(1980)
8. Mitchell, A. M., Lhuguenot, J.-C., Bridges, J. W. and Elcombe, C. R. : Identification of the proximate peroxisome proliferator(s) derived from di(2-ethylhexyl)phthalate. *Toxicol. Appl. Pharmacol.*, **80**, 23(1985)
9. Short, R. D., Robinson, E. C., Lington, A. W. and Chin, A. E. : Metabolic and peroxisome proliferation studies with di(2-ethylhexyl)phthalate in rats and monkeys. *Toxicol. Industrial Health*, **3**, 185(1987)
10. Parmar, D., Srivastava, S. P. and Seth, P. K. : Effect of di(2-ethylhexyl)phthalate(DEHP) on hepatic mixed function oxidases in different animal species. *Toxicol. Lett.*, **40**, 209(1988)
11. Moody, D. E. and Reddy, J. K. : Hepatic peroxisome (microbody) proliferation in rats fed plasticizers and related compounds. *Toxicol. Appl. Pharmcol.*, **45**, 579(1978)
12. Kluwe, W. M., Haseman, J. K., Fielding, D. J. and Huff, J. E. : The carcinogenicity of dietary di(2-ethylhexyl) phthalate(DEHP) in Fischer 344 rats and $B6C3F_1$ mice. *J. Toxicol. Eviron. Health*, **10**, 797(1982)
13. Burg, R. V. : Toxicology update. *J. Appl. Toxicol.*, **8**, 75 (1988)
14. DeAngele, A. B., Garrett, C. T and Queral, A. E. : Inhibition of phenobarbital and dietary choline efficiency promoted preneoplastic lesions in rat liver by environmental contaminant di(2-ethylhexyl)phthalate *Cancer Lett.*, **23**, 323(1984)
15. Williams, G. M., Maruyama, H. and Tanaka, T. : Lack of rapid initiating, promoting or sequential syncarcinogenic effects of di(2-ethylhexyl)phthalate in rat liver carcinogenesis. *Carcinogenesis*, **8**, 875(1987)
16. Perera, M. I. R. and Shinozuka, H. : Accelerated regression of carcinogen-induced preneoplastic hepatocyte foci by peroxisome proliferators, BR931, 4-chloro-6-(2.3-xylidino)-2-pyrimidinyl thio(N-B-hydroxyethyl)acetamide, and di(2-ethylhexyl)phthalate. *Carcinogenesis*. **5**. 1193 (1984)
17. DeAngelo, A. B., Queral, A. E. and Garrett, C. T. : Concentration-dependent inhibition of development of GGT positive foci in rat liver by the environmental contaminant di(2-ethylhexyl)phthalate. *Environ. Health Perspect.*,

60, 381(1985)
18. Ward, J. M., Rice, J. M., Creasia, D., Lynch, P. and Riggs, C. : Dissimilar patterns of promotion by di(2-ethylhexyl) phthalate and phenobarbital of hepatocellular neoplasia initiated by diethylnitrosamine in B6C3F1 mice. *Carcinogenesis*, **4**, 1021(1983)
19. Ward, J. M., Ohshima, M., Lynch, P. and Riggs, C. : Di(2-ethylhexyl)phthalate, but not phenobarbital prenobarbital promotes N-nitrosodiethylamine-initiated hepatocellular proliferative lesions after short-term exposure in male B2C3F1 mice. *Cancer Lett.*, **24**, 49(1984)
20. Diwan, B. A., Ward, J. M., Rice, J. M., Colburn, N. H. and Spanglar, E. F. : Tumor-promoting effects of di(2-ethylhexyl)phthalate in JB6 mouse epidermal cells and mouse skin. *Carcinogenesis*, **6**, 343(1985)
21. Ha, Y. L., Storkson, J. and Pariza, M. W. : Inhibition of benzo[a]pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. *Cancer Res.*, **50**, 1097(1990)
22. Lillie, R. D. and Fullmer, H. M : *Histopathologic technique and practical histochemistry*. McGraw-Hill Book Co., New York, 208-3-8(1996)
23. Butterworth, B. E., Bermudez, E., Smith-Oliver, T., Earle, L., Catley, R., Martin, J., Popp, J. A., Strom. S., Jirtle, R. and Michalopoulas, G. : Lack of genotoxic activity of di (2-ethylhexyl)phthalate(DEHP) in rat and human hepatocytes. *Carcinogenesis*, **5**, 1329(1984)
24. Daniken, A. V., Lutz, W. K., Jackh, R. and Schlatter, C. : Investigation of the potential for binding of di(2-ethylhexyl) phthalate(DEHP) and di(2-ethylhexyl) adipate(DEHP) to liver *in vivo* *Toxicol. Appl. Pharmacol.*, **73**, 373(1984)
25. Lake, B. G. : Mechanisms of hepatocarcinogenicity of peroxisome-proliferating drugs and chemicals. 1995. *Ann. Rev. Pharmacol. Toxicol.*, **35**, 483(1995)
26. Reddy, J. K., Azarnoff, D. L. and Hignite, C. E. : Hypolipidemic peroxisome proliferators form a novel class of chemical carcinogens. *Nature*, **283**, 397(1980)
27. Hayashi, F., Tamura, H., Watanabe, T. and Suga, T. : Enhancement by peroxisome proliferators of the susceptibility to DNA-damage in the liver of male F344 rats. *Cancer Lett.*, **92**, 87(1995)
28. Shukla, R. R., Albro, P. W., Corbett, J. T. and Schroeder, J. L. : *In vitro* studies of the inhibition of protein kinase C from rat brain by di(2-ethylhexyl)phthalate. *Chem. -Biol. Interactions*, **69**, 73(1989)
29. Jansen, E. H. J. M., Laan, C. A. and Defluiter, P. : Determination of phthalate-induced rat-liver cytochrome-P450 level by a fluorometric enzymatic assay and by chemiluminescence detection on western blots. *Anal. Chim. Acta*, **303**, 99(1995)
30. Okita, R. T. and Okita, J. R. : Characterization of a cytochrome P450 from di(2-ethylhexyl)phthalate-treated rats which hydroxylates fatty acids. *Arch. Biochem. Biophys.*, **294**, 475(1992)

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