Tumor-Promoting Activity of Phthalate Esters Estimated by *in Vitro* Transformation Using Bhas Cells

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The tumor-promoting activity of four phthalate diesters and three monoesters were tested *in vitro* in a screening assay using a transformed and non transformed BALB/c3T3 cell mixture and in a transformation assay using Bhas cells (v-Ha-*ras*-transfected BALB/3T3 cells). Di-2-ethylhexyl phthalate (DEHP), di-*n*-butyl phthalate (DBP), and their monoesters increased cell proliferation in the screening assay. Mono-*n*-butyl phthalate (MBuP) and mono-2-ethylhexyl phthalate (MEHP) promoted Bhas cell transformation, whereas the activities of DBP and DEHP were weak. The activity of MEHP was about 1/500 that of 12-*O*-tetra-decanoyl-phorbol-13-acetate. MBuP and MEHP therefore showed tumor-promoting activity, whereas all the other phthalate esters tested, including di-isononyl phthalate, had little or no such activity. In addition, MBuP and MEHP were formed by hydrolysis in Bhas cells exposed to DBP and DEHP for 4 days. We postulate that MEHP plays a role in the slight ability of DEHP to promote cell proliferation.

Key words —— tumor-promoting activity, phthalate ester, phthalate monoester, Bhas cells, transformation assay, hydrolysis

INTRODUCTION

Phthalate diesters comprise a family of chemicals synthesized through the esterification of phthalate anhydride with alcohols. These diesters are widely applied as plasticizers for compounds such as polyvinyl chloride (PVC). Medical devices and toys made of PVC contain the predominant plasticizers, di-2-ethylhexyl phthalate (DEHP), di-*n*-butyl phthalate (DBP), and/or diisononyl phthalate (DINP).[1] These compounds have been identified as contaminants of environmental water, soil, and the atmosphere, and have been detected in human serum and plasma.[2]

Some phthalate diesters induce testicular toxicity and other effects on the male and the female reproductive tract.[3] Zeiger *et al.* found that 16 phthalate diesters had no mutagenic activity in the Ames assay.[4] Therefore the hepatocarcinogenic effect of DEHP must be exerted during the promotion phase, since this compound is not genotoxic.[5] Both DEHP and DINP are peroxisome proliferators that induce tumors in the rodent liver.[6] This effect is probably not directly exerted by the diesters but by their monoesters and oxidative products.[7] In addition, phthalate diesters are metabolized to monoesters by hydrolases in many tissues, such as the intestine, pancreas, and blood,[8] where they are absorbed almost entirely as the corresponding monoesters.[9]

Although others have estimated the tumor initiation and/or promotion activity of DEHP, only a few have examined the *in vitro* promoting activity of other phthalate diesters and related monoesters at low doses. The present study screened the tumor-promoting activity of four phthalate diesters and three monoesters *in vitro* using BALB/c3T3 cocultured with transformed cells, as well as transformation assays using Bhas cells (v-Ha-*ras*-transfected BALB/3T3 cells). In addition, we evaluated the influence of monoesters formed by the hydrolysis of phthalate diesters on tumor-promoting activity in Bhas cells.

MATERIALS AND METHODS

Reagents —— DBP and DINP were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Diethyl

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phthalate (DEP) and DEHP were purchased from Wako Pure Chemical Industries (Osaka, Japan). DBP and DEHP were more than 99.0% pure. Mono-*n*-butyl phthalate (MBuP) and mono-2-ethylhexyl phthalate (MEHP) of over 90% purity were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Phthalate di- and monoesters were dissolved in dimethyl sulfoxide.

Cells and Media —— The Kihara Institute for Biological Research, Yokohama City University, supplied BALB/c 3T3 cells and 4-1-1 transformed cloned cells. The growth of BALB/c 3T3 cells is subject to contact inhibition and these cells never form clumps under normal conditions. These cells strain very faintly. In contrast, 4-1-1 cells grow in dense clumps and stain deeply. Both cell lines were cultured in Eagle's minimum essential medium (MEM) with 10% fetal calf serum (FCS). Cocultures were incubated in Dulbecco's modified Eagle (DME)/F12 medium supplemented with Insulin-Transferrin-Ethanolamine-Sodium selenite media (ITES) and 2% FCS. Hatano Research Institute, Food and Drug Safety Center, supplied the Bhas cells carrying the induced v-Ha-*ras* gene to BALB/c 3T3. The DME/F12 medium was supplemented with 5% FCS for transformation experiments.

Screening (Co-Culture) Assay —— A screening assay was used to screen the tumor-promoting activity.[10,11] BALB/c 3T3 cells (1×10^5) and 4-1-1 cells (2×10^3) were evenly mixed and seeded into 24-well plates (0.5 ml/well). On day 5, the plates were fixed with formalin and stained with 1% crystal violet. Absorbance was measured after extracting the strain from each well with hydrochloric acid methanol. Increases in absorbance reflected the growth of 4-1-1 cells. The proliferation rate of each analyte was calculated using the following equation:

Proliferation rate of BALB/c3T3 cells,

$$\% = [(P - C) / (T - C)] \times 100$$

where P, T and C are the absorbance of phthalate ester, 12-O-tetra-decanoyl-phorbol-13-acetate (TPA), and control, respectively.

Transformation Assay —— We used a modification of the protocol recommended by the IARC/NCI/EPA Working Group.[11,12] In brief, Bhas cells (2×10^4) carrying the induced BALB/c 3T3 v-Ha-*ras* gene were seeded into 6-well plates (2 ml/well). Three, 7 and t10 days later the medium was changed to include various concentrations of phthalate esters. On day 14, the medium was changed to DME/F12. On day 17, the cells were fixed with methanol and visualized by Giemsa staining to measure the numbers of transformation foci.

Analysis of Phthalate Esters in the Medium —— Bhas cells were exposed to phthalate esters in DME/F12 medium for 4 days. The medium was acidified to pH 4 with 0.01 N hydrochloride, mixed with acetonitrile (1 ml) followed by 7 ml of 0.1% acetic acid, loaded into Oasis hydrophilic lipophilic balance (HLB) cartridges (3 ml; Waters Co., Milford, MA, U.S.A.), and then rinsed with 5 ml of distilled water-methanol (9 : 1). Phthalate esters remaining in the column were eluted with 10 ml of methanol-ethyl acetate (1 : 9) and evaporated to dryness under nitrogen. The residues were derivatized as described in the previous paper[13] and methylated phthalate monoesters were quantified by GC/MS.

GC/MS Analysis —— GC/MS analysis followed the operating conditions described in a previous paper.[13]

RESULTS

We screened the tumor-promoting potential of four phthalate diesters and three monoesters at low concentrations using BALB/c3T3 cocultivated with transformed cloned cells (Table 1). Cell proliferation positively correlated with concentrations of DBP, DEHP, and their monoesters at concentration of from 5 to 5000 ng/ml, but not with those of DINP, DEP, or MEP. The activity of MEHP was marked higher than that of the other phthalate esters.

The tumor-promoting activity of DBP, DEHP, MBuP and MEHP was further examined for in detail in transformation assays using Bhas cells initiated. The doseresponse curves (100–10000 ng/ml) of the four esters shown in Fig. 1 indicate that two monoesters promoted cell transformation, whereas the diesters had little such activity. In addition, the activity of MEHP 5000 ng/ml (17.1 nmol/ml) was similar to that of TPA 20 ng/ml (0.03 nmol/ml) (data not shown).

Figure 2 shows GC/MS chromatographs of DEHP and MEHP in Bhas cells exposed to DEHP 0.5 μg/ml for 4 days, during which DEHP was hydrolyzed to MEHP. Figure 3 shows the amounts of monoesters formed in cells exposed to 0.5–5.0 mg/ml of DBP and DEHP (1.8–18.0 and 1.3–13.2 nmol/ml, respectively) for four days. The ratios of MEHP formed in the cells were 51% and 21% from 1.3 and 13.2 nmol/ml of DEHP, respectively, and those of MBuP were 80% and 65% from 1.8 and 18.0 nmol/ml of DBP, respectively.

Table 1. Effect of Phthalate Ester Concentration on BALB/c3T3 Cocultivated with Transformed Cells

Concentration (μg/ml)	Proliferation rate of BALB3T3 cells (%)[a]						
	DBP	DEHP	DEP	DINP	MBuP	MEHP	MEP
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
5.0×10^{-4}	−0.1	−0.6	6.4	−2.0	−7.2	−4.1	0.9
5.0×10^{-3}	3.6	2.4	10.1	3.8	−6.3	1.0	8.0
5.0×10^{-2}	3.9	3.9	9.0	2.9	2.7	12.2	6.7
5.0×10^{-1}	13.5	4.5	7.6	1.4	3.2	39.7	2.1
5.0×10^{-0}	−2.2	11.2	2.6	9.6	11.4	56.3	−3.5
TPA 100 ng	100.0	100.0	100.0	100.0	100.0	100.0	100.0

a) Proliferation rate of BALB3T3 cells (%) = (Absorbance of phthalate ester − Absorbance of control)/(Absorbance of TPA − Absorbance of control) ×100

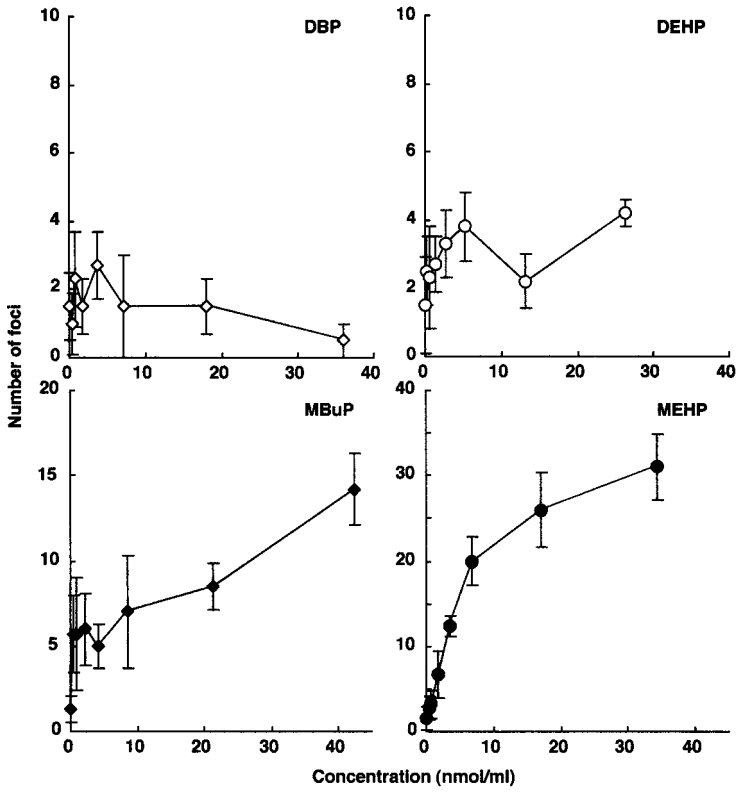

Fig. 1. Effect of Phthalate Esters on the Transformation of Bhas Cells (v-Ha-*ras*-transfected BALB/c3T3 cells)
Bhas cells were incubated with 0.1–10 μg/ml of DBP or its MBuP, DEHP or its MEHP. Results are expressed as mean ± S.D. (n = 6).

DISCUSSION

Contact inhibition between BALB/c3T3 and the transformed cells through gap junctional intercellular communication (GJIC) might be involved in focus formation by tumorpromoters such as TPA.[10,11,14] Among seven phthalate esters tested, DBP, DEHP, and their monoesters positively induced cell proliferation in screening assays in cocultivated transformed and nontransformed BALB/c3T3 cells. In addition, the initiation and promotion stages of chemical carcinogenesis can be detected by transformation assays using BALB/c3T3 cells. Transformation assays using Bhas cells can detect the ability to promote cell proliferation.[12] Bhas cells exposed to MBuP and MEHP underwent transformation, whereas DBP and DEHP had little effect. These results indicated that MBuP and MEHP are tumorpromoters and that DBP and DEHP have little or no activity.

Since DEHP cause hepatic peroxisome proliferation and is considered to be a nonmutagenic car-

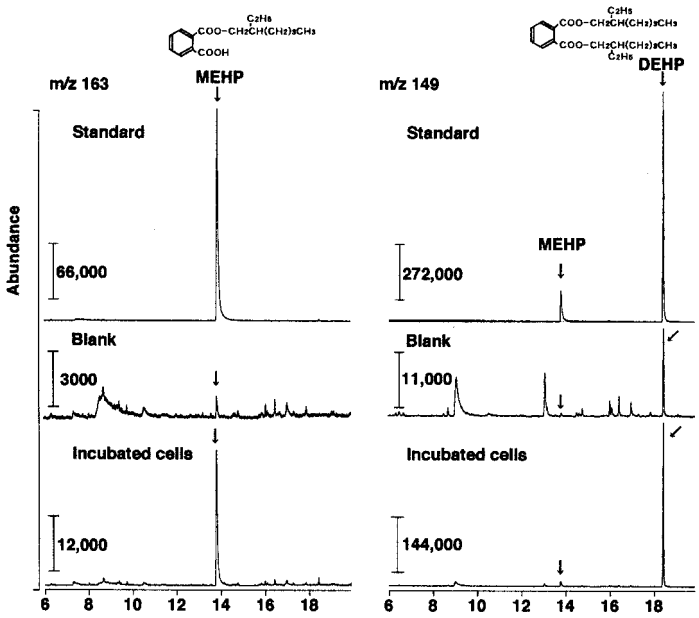

Fig. 2. Typical Selected Ion Chromatograms of DEHP and MEHP in Bhas Cells

Cells were incubated with 5.0 μg/ml (6.4 nmol/ml) of DEHP for 4 days. Details of GC/MS operating conditions: see Materials and Methods in text.

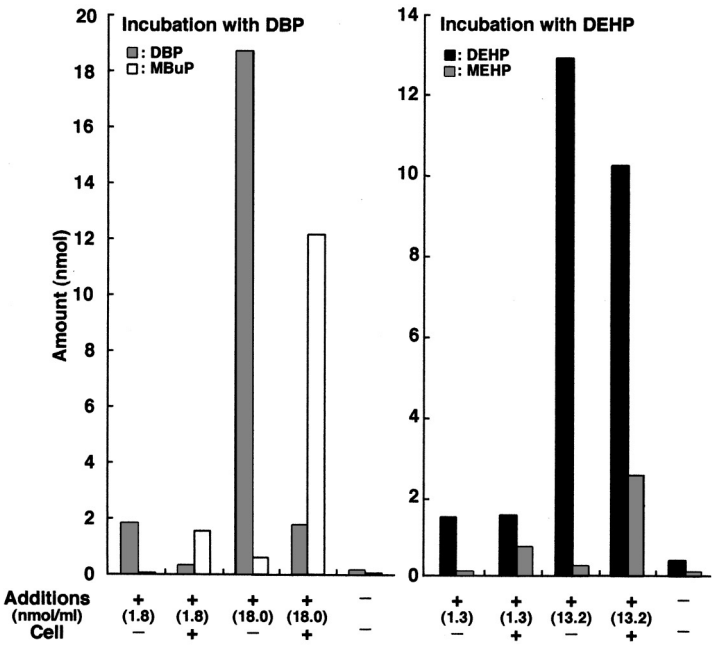

Fig. 3. Amounts of MBuP and MEHP Formed in Bhas Cells during Incubation with DBP and DEHP for 4 Days

Concentration of DBP and DEHP were 0.5–5.0 μg/ml (1.8–18.0 and 1.3–13.2 nmol/ml, respectively).

cinogen, its carcinogenic effect might be exerted during the promotion phase of hepatocarcinogenicity.[5] In addition, DEHP is metabolized to MEHP in the intestine, and MEHP and some of its oxidation products might be the active forms of DEHP responsible for peroxisome proliferation.[6,7] We examined whether MEHP formed *via* DEHP hydrolysis correlates with tumor-promoting activity. We found that the ratio of MEHP formed in Bhas cells reached 21–51% over 4 days, indicating that this metabolite is closely involved in the weak tumor-promoting activity of DEHP in Bhas cells. Tumor-promoter activity in cell transformation assays can be measured as the inhibition of GPIC between

cells.[11,12,14] The inhibition of hepatic GPIC and peroxisomal beta-oxidation in the rat, mouse, and hamster liver correlates with the dose and species tumorigenicity of DEHP and its primary metabolite, MEHP.[15]

In the L5178Y mouse lymphoma assay in the presence of the Aroclor-induced rat liver activation system (S9), DBP positivity probably results from its *in vitro* metabolism.[16] In addition, DBP is quantitatively hydrolyzed to its monoester by esterase in many tissues, such as the small intestine, and by pancreatic lipase.[8,9] However, although 65–80% of DBP in Bhas cells was hydrolyzed to MBuP which had a positive effect in transformation assays, we detected very little tumorpromoting activity of DBP. Therefore we assumed that DBP and the monoester caused Bhas cell transformation *via* a different mechanism from that of DEHP and MEHP. To some extent, MBuP seems to have independent ability to promote tumors.

We reported that DBP and DEHP released from PVC toys are hydrolyzed to MBuP and MEHP in human saliva.[17] The monoesters of these compounds are the active forms that account for the carcinogenesis and reproductive toxicity of DBP and DEHP. In conclusion, the present findings indicate that MBuP and MEHP promote tumor formation in transformed BALB/c3T3 and in initiated Bhas cells, and that the remaining phthalate esters tested had little or no tumor-promoting activity.

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