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Evaluation of the Genetic Toxicity of Synthetic Chemicals (XIII) - Single Cell Gel Electrophoresis of Benzoyl Chloride, 2-Propyn-1-ol, and 2-Phenoxyethanol in Chinese Hamster Lung Fibroblast -

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ABSTRACT : **Three synthetic chemicals, benzoyl chloride, 2-propyn-1-ol, and 2-phenoxy ethanol were selected for genotoxicity testing, based on production quantity and available genotoxic data. In our previous report, benzoyl chloride induced chromosomal aberrations in Chinese hamster lung (CHL) fibroblast *in vitro* with and without metabolic activation, while 2-propyn-1-ol and 2-phenoxy ethanol induced only with metabolic activation. To compare the genotoxicity of chromosome aberration assay, the single cell gel electrophoresis (comet) assay subjected using CHL cells. As a result, statistically significant differences of tail moment values of benzoyl chloride, 2-propyn-1-ol, and 2-phenoxy ethanol were observed compared with control values on almost all concentrations with S9 or without S9 metabolic activation system. This results suggest that genotoxic results of the comet assay and the chromosome aberration assay show correlationship of genotoxicity in the CHL fibroblast. In summary, the positive result of chromosome aberration of benzoyl chloride, 2-propyn-1-ol, and 2-phenoxy ethanol was also induced DNA damages in comet assay with same cell line. Consequently, comet assay will be useful and more accurate tool to detect and to confirm the genotoxicity especially DNA damages in CHL fibroblast.**

Key words : **Benzoyl Chloride, 2-Propyn-1-ol, 2-Phenoxyethanol, Comet assay, Chromosome Aberration assay**

Introduction

Despite the many toxicological researches on synthetic chemicals, there are few reports on the genotoxicity of some chemicals especially well used in chemical reaction processes in industry. In this respect, our laboratory has great concern to validate the chemical hazards, and conducted the toxicity evaluations of synthetic chemicals, especially in genotoxicity (Ryu *et al.*, 1997, 1998a, b, c, 1999a, b, 2000, 2001a, b, c, d, 2002a, b, c, d, e, f, 2003a, b, 2004a, b; Tice *et al.*, 2000). Among this synthetic chemicals, genotoxicity of 3 synthetic chemicals; benzoyl chloride, 2-propyn-1-ol, and 2-phenoxy ethanol evaluated in this study using Chinese hamster lung (CHL) cells.

Benzoyl chloride (CAS No. 98-88-4) is used in chemical intermediate for stabilizers and pesticides. Animal studies have reported the development of tumors by skin contact and vapors may cause lung injury (Fukuda *et al.*, 1981). OSHA and IARC were classified benzoyl chloride as possible carcinogen. Also, it was mutagenic for *Salmonella typhimurium* TA98 in 5 mg/plate (Chiu *et al.*, 1978). 2-Propyn-1-ol (CAS No. 107-19-7) has been used to prevent hydrogen embrittlement of steel, as corrosion inhibitor, solvent stabilizer, soil fumigant, chemical intermediate. It was also reported that 2-propyn-1-ol induced chromosomal aberrations in CHO cells in vitro with and without metabolic activation, while none induced reverse mutations detectable with the *Salmonella*/mammalian microsome assay. But it did not induce an increase in micronuclei in the mouse bone-marrow micronucleus assay (Blakey *et al.*, 1994). 2-Phenoxyethanol (CAS No. 122-99-6) used in fixative for perfumes or cosmetic, in organic synthesis, as bactericide in conjunction with quaternary ammonium compound, as insect repellent, have been reported that may cause central nervous system depression and kidney damage (Morton, 1990).

Since there are a few researches on genotoxicity and carcinogenicity and based on production quantity, these

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three chemicals were selected for genotoxicity testing. Generally, several assays ascertained induction of DNA damage are now well used to evaluate the genotoxicity of chemicals with rapid and reliable and also frequently adopted as methods for an index of genotoxicity worldwide. To confirm the positive genotoxicity results in chromosome aberration assay, the battery of single cell gel electrophoresis (comet) assay were performed in this study.

Materials and Methods

Cell Culture

A clonal sub-line of a chinese hamster lung (CHL) fibroblast cells was obtained from the National Institute of Health Sciences, Tokyo, Japan. The karyotype of CHL cells consisted of 25 chromosomes. The cells had been maintained by 3-4 day passages and grown in a monolayer with Eagles minimum essential medium (EMEM, Gibco, 410-1100EA) supplemented with 10% fetal bovine serum (FBS, Gibco, 26140-020). These cells were maintained at 37°C in 5% CO_2 atmosphere.

Reagents

Trypsin-EDTA and colcemid were the products of Gibco BRL Life Tech. Inc. (Gaithersburg, USA). 2-Propyn-1-ol (CAS No. 107-19-7) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Benzoyl chloride (CAS No. 98-88-4) and 2-phenoxy ethanol (CAS No. 122-99-6) were obtained from Sigma-Aldrich Co. (St. Louis, USA). They were dissolved in DMSO immediately before use. The final concentration of DMSO used in the medium was below 1%. The preparation of rat liver S-9 fraction for metabolic activation system was previously reported (Maron and Ames, 1983). The S-9 fraction prepared was stored immediately at -80°C before use.

Determination of the 50% growth inhibition concentration

Test article dose levels were determined prior to the main study in a dose range-finding study performed in the absence of a rat liver S-9 activation system. For the growth inhibition assay, CHL cells were seeded at the density of 5×10^4 cells/ml into 96 well plates. Twenty-four hr after seeding, several different doses of sample were separately added and incubated for 2 hr and 24 hr. And then the 50% inhibition concentration (IC_{50}) values were calculated by MTT assay (Mosmann, 1983).

Single cell gel electrophoresis (comet) assay

Preparation of CHL cells for comet assay

For the comet assay, 1.5×10^5 of cells were seeded into 12 wells plate and then treated IC_{50} at 2 hr as maximum dose. After 2 hr, cells were centrifuged for 5 min at $100 \times g$, and gently resuspended with PBS. 100 μl of the cell suspension was immediately used for the test. Cells were mixed with 100 ß° of low melting point agarose (LMPA: 1%) and added to fully frosted slide which had been covered with a bottom layer of 100 μl of 1% normal melting agarose. The cell suspension was immediately covered with coverglass and the slides were then kept at 4°C for 5 min to allow solidification of the agarose. After gently removing the coverglass, the slides were covered with a third layer of 100 μl of 0.5% LMPA by using a coverglass and then the slide were again kept cold at 4°C for 5 min.

Alkaline unwinding/alkaline electrophoresis

The procedure used follows the method described by Singh *et al.*, (1988, 1994) with minor modification. The cells embedded in the agarose on slides were lysed for 1.5 hr in reaction mixture of 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris-HCl (pH 10), and 1% Triton X-100 at 4°C. Slides were then placed in 0.3 M NaOH and 1 mM Na_2EDTA (pH approximately 13) for 20 min to unwinding of DNA before electrophoresis. Electrophoresis was conducted at 25 V (about 1 V/cm across the gels) and approximately 300 mA for 20 min at 4°C. All of the steps described above were conducted under yellow light or in the dark to prevent additional DNA damage.

Evaluation of DNA damage

After the electrophoresis, the slides were washed gently to remove alkali and detergents, which would interfere with ethidium bromide staining, by placing them horizontally and flooding them three times slowly with 0.4 M Tris (pH 7.5) for 5 min. The slides were stained by 50 μl of ethidium bromide in distilled water solution on each slide, and then covering the slide with a coverglass. Image of 100 randomly selected cells (50 cells from each of two replicate slides) was analysed from each sample. All experiments were repeated in an independent test. Measurement was made by image analysis with Komet 3.1 (Kinetic Imaging Limited, Liverpool,

UK) software, determining the mean tail moment (percentage of DNA in the tail times tail length) of the 50 cells. Differences between the control and the other values were tested for significance using one way of analysis of variance (ANOVA).

Results and Discussion

It is well known that carcinogenicity is the most serious effect of toxic chemicals in human health. Since these substances are not limited to the original products, and they may enter the environment and have become widespread environmental pollutants, thus leading to a variety of chemicals that possibly threaten the public health. Nevertheless of the diverse uses of these chemicals in industry, however, there has been no attention to evaluate the toxicity of some chemicals especially used in chemical industry. The detection and the regulation of man-made synthetic chemicals are subjects of great concern because of its close correlation between environmental contamination and human health. Recently, several next generation battery of genotoxicity for the detection of genetic damages in vitro and in vivo were introduced according to the rapid progress in toxicology combined with cellular and molecular biology. Among these methods, the single cell gel electrophoresis (comet) assay which can be detected DNA damages in cell level (Singh *et al.*, 1988, 1994; Ryu *et al.*, 1997, 2001a,d, 2002b; Tice *et al.*, 1991, 2000), mouse lymphoma thymidine kinase gene assay (Clive *et al.*, 1983; Sawyer *et al.*, 1985; Ryu *et al.*, 1999a), FISH (fluorescence in situ hybridization) (Hayashi *et al.*, 1994), PRINS (primed *in situ* hybridization) (Abbo *et al.*, 1993) and transgenic animal and cell line model as a parameter of *lac I* (Big Blue) (Kohler *et al.*, 1991; Ryu *et al.*, 1998 b,c, 1999b, 2000, 2002a) or *lac Z* (Muta Mouse) (Suzuki *et al.*, 1993) gene mutation are newly introduced based on cellular and molecular toxicological approaches (Ryu *et al.*, 2002e,f). Also, in vivo supravital micronucleus assay with peripheral reticulocytes by using acridine orange fluorescent staining (Hayashi *et al.*, 1990, 1992; Ryu *et al.*, 1998a, 2001c) was introduced instead of mouse bone marrow micronucleus assay.

In this study, we performed the comet assay, which is widely used for the detection and measurement of DNA strand breaks (Singh *et al.*, 1988, 1994; Tice *et al.*, 1991, 2000; Anderson *et al.*, 1998; Speit and Hartmann, 1999; Ryu *et al.*, 1997, 2001a,d, 2002b) in order to compare the genotoxicity with the our previous results (Ryu and Kim, 2004) of the analysis of chromosome aberrations of three synthetic chemicals such as benzoyl chloride, 2-propyn-1-ol, and 2-phenoxy ethanol.

We used CHL cells in this experiment because it was reported no diffrences of sensitivity between CHL and CHO (Chinese hamster ovary) cells for *in vitro* chromosome aberration study (Galloway *et al.*, 1997). The IC_{50} values of cell growth of test articles in CHL cells are obtained in the presence and absence of metabolic activation system at 2, 6, or 24 hr exposure as shown in Table 1. The benzoyl chloride revealed high extent of cytotoxicity in CHL fibroblast. The concentration used and detailed previous data of chromosome aberration of three chemicals are summarized in Table 2. The DMSO negative control is revealed only 0.6% and 1.1% spontaneous aberrations in the absence and presence of metabolic activation system in 200 metaphase of CHL cells, respectively. However, the positive controls, cyclophosphamide (10 μg/ml) as an indirect mutagen that require metabolic activation and mitomycin C (0.1 μg/ml) as a direct-acting mutagen, induced remarkable chromosome aberrations (49.4-40.5%) in CHL cells. In our previous report (Ryu and Kim, 2004), benzoyl chloride, the most cytotoxic compound among three chemicals tested, revealed statistically significant aberration frequency in the concentration range of 31-123 μg/ml in the absence of metabolic activation system, and only high dose (43 μg/ml) in the presence of metabolic activation. And, 2-propyn-1-ol and 2-phenoxy ethanol induced chromosomal aberrations with significance at the high concentrations of 20 and 3,100 μg/ml only in the presence of metabolic activation system, respectively.

The *in vitro* comet assay, a sensitive, quick and relatively cheap test, could become a valid alternative to the commonly used in vitro genotoxicity test, in the preliminary evaluation of new chemical entities early phase in the development of new pharmaceuticals (Giannotti *et al.*, 2002). Fig. 1 and Table 2 show the tail

Table 1. 50% Inhibition Concentration (IC_{50}) in Chinese hamster lung fibroblast

Chemical Name	IC_{50}(μg/ml)			
	2hr	2hr	6hr	24hr
	+S9	-S9	+S9	-S9
Benzoyl chloride	12	8	43	123
2-Propyn-1-ol	63	1,000	20	1,450
2-Phenoxy ethanol	3,150	1,850	3,100	1,635

Table 2. Genotoxicity of benzoyl chloride, 2-propyn-1-ol, and 2-phenoxy ethanol evaluated by chromosome aberration assay and comet assay in CHL fibroblast

Test chemicals (CAS No.)	without (-) or with(+) S9 Mix	Chromosome Aberration				DNA damages in Comet assay			
		Conc. (μg/ml)	Treatment (hr)	Total aberration (%)	Significancy-	Conc. (μg/ml)	Treatment (hr)	Tail Moment Mean±SE	Significancy
Benzoyl chloride (98-88-4)	-	123	6	33.0	*	8	2	18.81±10.86	*
	-	62	6	41.5	*	4	2	15.65±8.59	
	-	31	6	11.0	*	2	2	17.78±7.57	*
	+	43	6	17.0	*	12	2	10.36±6.48	
	+	22	6	3.0		6	2	17.32±10.44	*
	+	11	6	1.5		3	2	15.54±7.46	*
2-Propyn-1-ol (107-19-7)	-	1,450	6	3.5		1,000	2	24.48±11.08	*
	-	725	6	2.5		500	2	28.26±11.55	*
	-	363	6	4.0		250	2	13.81±6.62	
	+	20	6	6.5	*	63	2	24.07±11.17	*
	+	10	6	3.0		32	2	22.96±7.10	*
	+	5	6	2.0		16	2	12.38±7.38	*
2-Phenoxy ethanol (122-99-6)	-	1,635	6	2.0		1,850	2	11.36±5.95	*
	-	816	6	1.0		925	2	14.81±8.99	*
	-	408	6	0		463	2	12.96±5.30	*
	+	3,100	6	12.0	*	3,150	2	35.09±16.92	*
	+	1,550	6	1.5		1,575	2	19.39±7.72	*
	+	775	6	5.5		788	2	26.13±10.15	*
DMSO[a]	-		6	0.6±0.6		DMSO	2	13.15±7.65	
	+		6	1.1±0.5			2	6.60±5.22	
MMC[b]	-	0.1	6	40.5±15.8		H_2O_2	2	48.00±8.67	
CP	+	10	6	49.4±22.6		CP	2	21.66±3.96	

*significant at $p<0.05$

Conc. : Concentration, DMSO : Dimethylsulfoxide, CP : Cyclophosphamide, MMC : Mitomycin C

The values of Total aberration percentage are expressed as mean±S.D.

[a]: solvent, [b]: positive control

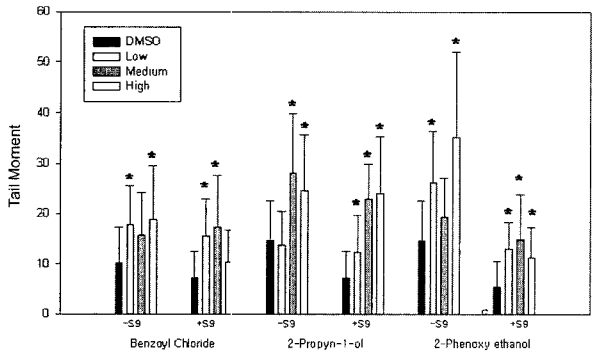

Fig. 1. Comet assay result for benzoyl chloride, 2-propyn-1-ol, and 2-phenoxyethanol. Mean tail moment indicate DNA damage of cells by three chemicals. Values are means±SE from three experiments. In each experiment the tail moment index had been assessed from 50 separately calculated cells. High dose means IC_{50} of each chemical in CHL fibroblast after 2 hr treatment. *significant differences from controls ($p<0.05$).

moment as DNA damage indicator of three chemicals in the comet assay using CHL cells. Three chemicals induced high extent of tail moment values. From this results, statistically significant differences of tail moment values of benzoyl chloride, 2-propyn-1-ol, and 2-phenoxy ethanol were observed compared with control values on almost all concentrations with S9 or without S9 metabolic activation system, despite of high deviation between experiments (Table 2). This results suggest that genotoxic results of the comet assay and the chromosome aberration assay show correlationship of genotoxicity in the CHL cell line.

In summary, the positive result of chromosome aberration of benzoyl chloride, 2-propyn-1-ol, and 2-phenoxy ethanol was also induced DNA damages in comet assay with same cell line. Consequently, comet assay will be useful and more accurate tool to detect and to confirm the genotoxicity especially DNA

damages in CHL fibroblast.

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