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Induction of Cytochrome P450 in Hep G2 Cells and Mutagenicity of Extracts of Sediments from a Waste Disposal Site Near Osaka, Japan

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Usually, environmental chemicals are toxicologically evaluated by means of a mutagenicity assay, such as the Ames test, or sister chromatid exchange using microorganisms or animal cell lines. In the Ames test or mutagenicity tests, liver S9 fraction used for the metabolic activation of chemicals is also prepared from rodents. However, some human drug-metabolizing enzymes, namely cytochrome P450s, have notably different catalytic activities towards various chemicals, and the gene expression sometimes differs between human P450s and animal orthologues, although some structural and functional characteristics are shared (Gonzalez 1989, Guengerich 1992). Thus, applying metabolic data about chemicals from rodents to humans may not be relevant when assessing human risk. Therefore, we have introduced a model to evaluate the biological effects of the chemicals on humans, which was as close to normal humans as possible. Hep G2 is a highly differentiated human hepatoma cell line, which retains many of the cellular functions often lost by cells in culture. This cell line also has the enzymes involved in phase I (MFO) and phase II (glucuronate and sulfate conjugation) metabolism of xenobiotics, and it has been used as an *in vitro* system instead of human normal hepatocytes to study drug metabolism and toxicity (Grant *et al.* 1988, Doostdar *et al.* 1991). We demonstrated that polycyclic aromatic hydrocarbons (PAHs) induced MFO activity, and an extract from the total suspended particles collected using a high volume air sampler, which was mutagenic in the Ames assay using *Salmonella typhimirium* TA98, also induced the same enzyme activities in Hep G2 cells cultured in serum-free medium (Nakama *et al.* 1995). The enzymes metabolizing the xenobiotics are induced by exposing the cells to some chemicals, in which promutagens form carcinogens upon metabolic activation with drug-metabolizing enzymes. Therefore, we assumed that MFO-inducing activity indicates the biological effect of environmental chemicals.

In this study, we estimated the effects of sediments in a sea-based solid waste disposal site on MFO induction in Hep G2 cells, Simultaneously, to investigate the correlation among amounts of PAHs, mutagenic activity, and MFO-inducing activity of sediments, the same samples were analyzed by HPLC to determine PAHs and were assayed with the Ames test to estimate the mutagenicity.

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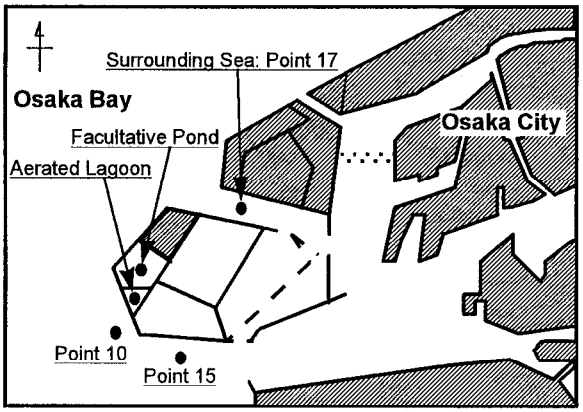

Figure 1. The location of the North Port Solid Waste Disposal Site, which was being constructed outside the breakwaters in the sea west of Osaka City in Japan. The shaded portion is land. ● indicates sampling points.

MATERIALS AND METHODS

Sediment samples were collected from the facultative pond and the aerated lagoon in the North Port Solid Waste Disposal Site and surrounding areas, located outside the breakwaters in the sea west of Osaka City in Japan (Figure 1). The sediments of the facultative pond and the aerated lagoon were collected in 1992/9/2, 1993/1/22, and 1993/9/21, and those from the surrounding sea points in 1993/7/21. Sediment extracts were prepared according to a modification of the method of Yoshikura *et al.* (1991). Wet sediment samples were dried in a drying-oven for 30 min at 120°C. Hydrophobic organic compounds in sediments were extracted from 20 g dry sediments with acetone three times. After adding 200 mL n-hexane and 400 mL water, the organic phases were desulfurized twice with 20 mL of 6.6 % tetrabutyl-ammonium sulfite and 20 g sodium sulfite. Then, the organic phases were washed with 500 mL water twice and concentrated *in vacuo* by a rotary-evaporator. Finally, the condensate was dissolved in 2 mL dimethylsulfoxide (DMSO) . Thus, 1 mL of the extract was equivalent to 10 g of dry sediment.

Anthracene, fluoranthene, pyrene, and benzo(a)pyrene were analyzed using a Shimadzu LC-10A HPLC system (Shimadzu Co., Japan) with a Model RF-550 spectro-fluorometric detector. Reversed-phase HPLC was done on a Shim-pack CLC-ODS column (6 x 150 mm, Shimadzu Co.) and developed with acetonitrile-water (8:2, v/v) at 1.2 mL/min. The detector was set at 334 $nm_{exc.}$/ 384 $nm_{emis.}$ or 365 $nm_{exc.}$/ 440 $nm_{emis.}$ to detect anthracene and pyrene or fluoranthene and benzo(a)pyrene, respectively.

The human hepatocellular carcinoma cell line, Hep G2 (ATCC HB 8065) at passage 76 was supplied by the American Type Culture Collection (U.S.A.). The cells were maintained in serum-free medium supplemented with growth factors, in a humidified 5 % CO_2 - 95 % air atmosphere at 37°C as described (Nakama and

Yamada 1993). For induction, the cells were plated in ϕ60mm culture dishes at a density of about 1 x 10^6 cells/dish. When the cells reached confluence, test samples dissolved in DMSO were added to the cells 16 hr before experiments. DMSO at a final concentration of 0.2 % in the medium had no effect on metabolism and enzyme induction. Ethoxycoumarin O-deethylase (ECOD) activities were measured as described (Nakama *et al.* 1995). The enzyme activity is expressed as nanomole per milligram of whole cell protein per 3 hr. The Ames test was performed essentially as described (Maron and Ames 1983) using *Salmonella typhimurium* strain TA98. Metabolic activation was achieved using commercially available rat liver S9 fraction (Oriental Yeast Co., Ltd. Japan).

RESULTS AND DISCUSSION

Anthracene, fluoranthene, benzo(a)pyrene, and pyrene were analyzed by reversed-phase HPLC with a spectra-fluorometric detector. The amounts of PAHs in the sediment extracts are shown in Table 1. The sum of 4 PAHs is indicated as 'Total PAHs'. The contamination at the aerated lagoon by PAHs was lowest in all samples. Conversely, the surrounding sea points showed the highest contamination, especially in the sample from point 17, which included 806.81 ng/g (dry sediment) of total PAHs. The distribution of PAHs in all sediments was generally fluoranthene ≈ pyrene > benzo(a)pyrene > anthracene. There were no changes with time in the PAH contents among samples from the same point. These suggested that the amount of PAHs dissolving out from the disposal waste and continuously accumulating in the sediment is less than that in the surrounding sea.

Table 1. Determination of polycyclic aromatic hydrocarbons in the sediment extracts by HPLC.

Date	Samples	Anthracene	Fluoranthene	Benzo(a)pyrene	Pyrene	Total PAHs[a]
				(ng/g:dry sediment)		
92/9/2	FP	5.68	37.60	5.34	30.15	78.77
93/1/22	FP	4.56	33.44	2.27	21.50	61.76
93/9/21	FP	4.38	46.75	6.63	37.46	95.21
92/9/2	AL	1.32	11.87	4.07	11.75	32.02
93/1/22	AL	0.69	10.79	3.11	10.80	25.39
93/9/21	AL	0.58	6.01	1.90	6.50	14.99
93/7/21	SS:10	9.54	49.60	25.40	30.42	114.96
93/7/21	SS:15	6.14	30.95	29.21	34.58	100.89
93/7/21	SS:17	50.23	308.91	162.20	285.48	806.81

[a] Sum of 4 PAHs.
Abbreviations: FP, Facultative pond; AL, Aerated lagoon; SS, Surrounding sea

The mutagenicity of the sediment extracts in *Salmonella typhimurium* TA98 strain with S9Mix is shown in Table 2. The number of reverse-mutation colonies was more than twice that of spontaneous mutation for only 4 sediments, 80µ1 (equivalent to 0.8g dry sediment)/plate from the facultative pond (93/9/21), 40µ1

(0.4g)/plate and over from surrounding sea point 10, and 20µl (0.2g)/plate and over from surrounding sea points 15 and 17. The sample from surrounding sea point 17, which contained the highest total PAH (Table l), was also the most mutagenic among all the samples. The correlation between the mutagenic activity and the amount of total PAHs determined from Table 1 is shown in Figure 2. Although the plots were concentrated in a small area, the mutagenic activity of the sediment extracts correlated closely with the amount of total PAHs (R=0.97). If the mutagenic activity of the sediment was dependent on only the amount of total PAHs shown in Table 1, at about 50ng of PAHs, the mutagenicity would double the number of reverse-mutations compared with spontaneous colonies. However, since the sediment extract should contain other chemical contaminants such as organochlorine compounds, pesticides or nitrated PAHs, the mutagenicity of the sediment cannot be sufficiently accounted for by the total PAHs.

Table 2. Ames assay of the sediment extracts with *Salmonella typhimurium* TA98 in the presence of SBMix.

Date	Samples	(µl/Plate) 20	40	80
		(Revertants/Plate)		
92/9/2	Facultative pond	37	38	39
93/1/22	Facultative pond	36	38	47
93/9/21	Facultative pond	40	45	54
92/9/2	Aerated lagoon	28	46	41
93/1/22	Aerated lagoon	29	32	47
93/9/21	Aerated lagoon	23	27	32
93/7/21	Surrounding sea: Point 10	35	52	56
93/7/21	Surrounding sea: Point 15	50	62	85
93/7/21	Surrounding sea: Point 17	105	137	171
	Spontaneous control	25		
	Positive control[a]	245		

[a] 0.5µg 2-aminoanthracene/plate.
1 mL of the extract was equivalent to 10 g of dry sediment.
The results were expressed as the means of two experiments.

The induced activity of ECOD, which is a cytochrome P4501A1type drug-metabolizing enzyme induced by PAHs, by extracts from the sediments is shown in Table 3. The typical drug-metabolizing enzyme inducer, methylcholanthrene, induced about 6 times more ECOD activity than that of the control. Similar to the findings of the Ames assay, surrounding sea point 17 had the highest inducing activity among all the samples; 0.6µl (equivalent to 6mg dry sediment)/plate of this extract induced ECOD activity that was 3.7-fold higher than that of control, and 6µl(60mg)/plate induced 7.4-fold the activity. However, the addition of 60µl

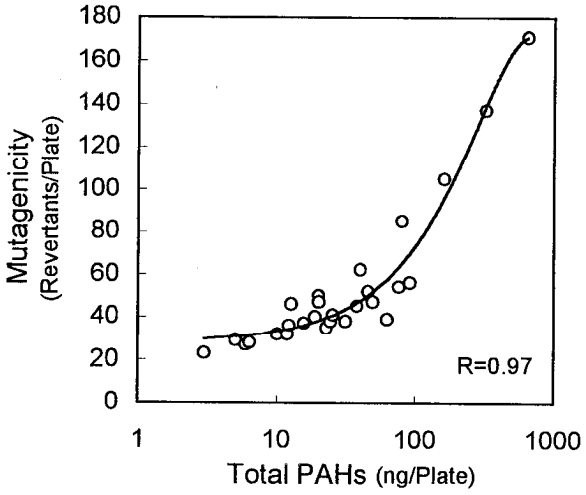

Figure 2. The correlation between amounts of PAHs and mutagenic activity of the sediment extracts. All data were plotted. 'Total PAHs' indicates the sum of 4 PAHs analyzed by HPLC.

Table 3. Ethoxycoumarin O-deethylase activities in Hep G2 cells induced by the sediment extracts.

Date	Samples	(μl/ϕ60mm dish) 0.6	6	60
		(nmole/mg: whole cell protein/3 hrs)		
92/9/2	Facultative pond	1.41	2.64	5.63
93/1/22	Facultative pond	1.09	3.14	6.85
93/9/21	Facultative pond	1.27	3.21	6.28
92/9/2	Aerated lagoon	0.95	2.87	6.37
93/1/22	Aerated lagoon	1.11	2.00	6.34
93/9/21	Aerated lagoon	0.92	1.76	3.54
93/7/21	Surrounding sea: Point 10	2.33	4.52	7.64
93/7/21	Surrounding sea: Point 15	1.89	4.49	5.05[b]
93/7/21	Surrounding sea: Point 17	3.52	6.96	2.94[b]
Control		0.94		
Methylcholanthrene(2.5μM)[a]		6.43		

[a] Positive control; [b] Toxic

1 mL of the extract was equivalent to 10 g of dry sediment.

The results were expressed as the means of two experiments.

(600mg)/plate decreased the ECOD activity. We assumed that the cell viability decreased due to the toxicity of chemicals contained in the extract. The ECOD-inducing activity of sediment extracts is not able to account for only the 4 kinds of PAHs determined with HPLC; however, the amount of total PAHs may broadly

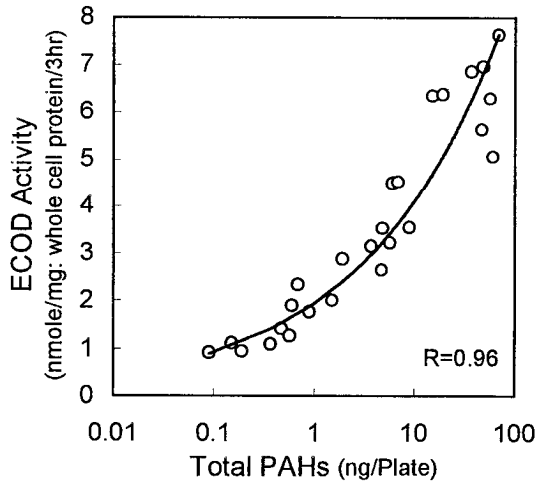

Figure 3. The correlation between the amounts of PAHs and the MFO-inducing activity of the sediment extracts. We omitted one plot in which the toxicity was assumed to appear. 'Total PAHs' indicates the sum of 4 PAHs analyzed by HPLC.

indicate the pollution level of the sediment. Thus, we investigated the correlation between the ECOD-inducing activity and the amount of total PAHs (Table 1) as the pollution level of sediment as shown in Figure 3. The ECOD-inducing activity of the sediment extracts correlated closely with the amount of total PAHs (R=0.96). If the ECOD-inducing activity of the sediment is dependent on only the amount of total PAHs shown in Table 1, about 1 ng of total PAHs doubled the ECOD activity compared with control in Hep G2 cells. However, Lipp *et al.* (1992) showed that polychlorinated dibenzo-*p*-dioxins (PCDDs) intensely induced cytochrome P4501A in Hep G2 cells. Even if the sediment extracts contained only a small amount of PCDDs or it analogues, cytochrome P4501A in Hep G2 cells should be induced by these extracts. However, since all sediment extracts which were prepared from different sediments showed correlation between PAHs concentration and ECOD-inducing activity or mutagenicity, the sediment extracts should not contain PCDDs enough to markedly induce cytochrome P4501 A in Hep G2 cells.

Toxic evaluation of environmental contaminants by machine analysis, such as TEQ (toxic equivalent) which is the sum of contaminant's TEF (toxic equivalency factor), is represented as the additive effects of determined contaminants. All environmental chemicals, which include decomposition products of contaminants by photo-degradation or microbial degradation, can not be determined by machine analysis such as GUMS or LC/MS. Thus, the additive toxic evaluation by machine analysis shows only a partial toxicity of contaminants. On the other hand, toxic evaluation by biological assay represents comprehensive findings of additive, synergistic and/or inhibitory effects of all chemicals in the contaminant. However, the biological assay shows no specific information about the chemicals in the contaminants, which is needed to control the contaminants exhausting from a

source point. Thus, to compensate each defect, both the machine analysis and biological assay are important for evaluating environmental contamination.

Several methods have been proposed for monitoring xenobiotic pollutants in the aquatic environment using rainbow trout, in which the induction of hepatic microsomal monooxygenase (Kleinow *et al.* 1986) or hepatic cytochrome P450 isozymes (Pesonen *et al.* 1992) are used as the biological indicator of pollution by environmental chemicals. Ueng *et al.* (1995) have shown that the sediment extracts from the polluted Damsui River in Taiwan markedly induced Tilapia *(Oreochromic mossambicus)* hepatic monooxygenases and cytochrome P4501A1, and that the induction of fish monooxygenases may serve as a biological monitor for PAHs and PCBs. Sleiderink *et al.* (1995) have shown that the induction of cytochrome P4501A in dab *(Limanda limanda)* was a useful biomarker of environmental contaminants such as PCBs. In these experiments, the intensity of hepatic cytochrome P4501A induction in fish is a biomarker of environmental contamination, and these methods allow us to estimate the effects of environmental contaminants on organisms living in the contaminated area. However, employing human cell lines such as Hep G2 should be useful for studying the effects of extensive environmental samples, such as extracts from drinking water, river water or suspended particles in air. They could also be used to evaluate the effects of environmental contaminants upon human health, and thus be applied to human risk assessment. Moreover, serum-free medium can provide reproducible and highly sensitive results since the cells are constantly cultured under the same conditions (Nakama *et al.* 1995).

We demonstrated the MFO-inducing activity and mutagenicity of the sediment extracts in Hep G2 cells and *Salmonella*. A comparison between the mutagenicity in the Ames assay and the ECOD-inducing activity in Hep G2 cells showed that the sensitivity of ECOD-inducing activity on the sediment extracts was far superior to that of the Ames assay. Furthermore, the total PAH concentration that doubled the activity compared with the control was about 2% of that in the Ames assay. However, although several PAHs were analyzed by HPLC, we could not determine which compounds induced MFO activity or the reverse mutation. Our findings should not be interpreted as indicative of a correlation between the MFO-inducing activity and mutagenicity. However, the findings from ongoing studies using the human-derived cell lines should clarify these relationships.

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