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INDUCTION OF CYTOCHROME P450-DEPENDENT MONOOXYGENASE IN SERUM-FREE CULTURED Hep G2 CELLS

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Abstract—We examined the induction of cytochrome P450-dependent mixed-function monooxygenase (MFO) in the human hepatoma cell line Hep G2 by means of several factors. The MFO activities induced in the cells cultured in medium containing five commercial sera varied significantly, and the activity in the cells cultured in the absence of serum was about twice as high as that in cells supplemented with serum. The activity of ethoxycoumarin *O*-deethylase was highest 12 hr after adding 3-methylcholanthrene, and it was induced by several polycyclic aromatic hydrocarbons such as benzo(*a*)anthracene and benzo(*a*)pyrene, which are usually found in urban air as environmental contaminants. Furthermore, an extract from the total suspended particles collected using a high volume air sampler, which was mutagenic in the Ames assay using *Salmonella typhimurium* TA98, induced the same enzyme activities in Hep G2 cells. These findings suggest that serum-free culture allows the stable and highly sensitive measurement of induced MFO activity, and that studies of MFO induction by environmental samples using human hepatoma Hep G2 cells should provide helpful information regarding the risk associated with environmental contaminants.

Key words: Hep G2 cells; serum-free culture; cytochrome P450-dependent mixed-function monooxygenase; mutagenicity; polycyclic aromatic hydrocarbons; total suspended particles

MFOs† catalyze the biotransformation of endogenous substrates, such as fatty acids, steroids, and eicosanoids, and the detoxification of exogenous organic chemicals. However, these enzymes also catalyze the bioactivation of xenobiotics, leading to the formation of highly reactive, electrophilic derivatives, such as mutagens and carcinogens [1–3]. Investigations into the bioactivation of xenobiotics *in vitro* have been performed using primary cultured hepatocytes isolated from animal livers [4–6]. However, primary cultures of hepatocytes undergo rapid de-differentiation within 24–72 hr of culture, and the activity of the MFO system is particularly unstable in cultured hepatocytes from humans and other animals [7, 8]. Furthermore, normal human hepatocytes are not available for routine experiments. Some human P450 species have notably different catalytic activities towards various carcinogens from those predicted on the basis of studies with experimental animals, and the gene expression sometimes differs between human P450s and animal orthologues, although some of structural and functional characteristics are shared [9, 10]. Thus, the extrapolation of procarcinogen metabolism data from rodents to humans may not be relevant when assessing human risk.

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† Abbreviations: MFO, cytochrome P450-dependent mixed-function monooxygenase; FBS, fetal bovine serum; PAH, polycyclic aromatic hydrocarbon; MC, 3-methylcholanthrene; PB, sodium phenobarbital; ECOD, 7-ethoxycoumarin *O*-deethylase; AROD, 7-alkyloxyresorufin *O*-dealkylase; MROD, 7-methoxyresorufin *O*-demethylase; EROD, 7-ethoxyresorufin *O*-deethylase; PROD, 7-pentoxyresorufin *O*-depentylase; BROD, 7-benzyloxyresorufin *O*-debenzylase; PCB; polychlorinated biphenyl; p,p′-DDT, *p,p′*-dichlorodiphenyltrichloroethane; γ-HCH, γ-hexachlorocyclohexane; and TSP, total suspended particles.

Hep G2 [11] is a highly differentiated human hepatoma cell line that 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 (glucuronic acid 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 [12–15]. These cells are useful for studying cytochrome P450 and the regulation of MFO activities in liver cells of human origin [16]. Hep G2 cells historically have been cultured in medium containing serum. However, there are many substances in serum, such as complement, blood coagulation factors, and unknown materials. In addition, there are also differences in constitutive materials among commercial lots of serum [17]. Hammond and Fry [18] demonstrated that the MFO activities of rat hepatocytes are maintained at higher levels in serum-free medium, and Doostdar *et al.* [19] have shown that the MFO activities of Hep G2 cells are influenced by the composition of the culture medium. Thus, we prepared a serum-free medium for Hep G2 cells, which was supplemented with growth factors, hormones, nutrients, and egg yolk low-density lipoprotein, to optimize the culture conditions under which stable MFO activities could be obtained [20].

We estimated the suitability of our serum-free culture conditions for MFO induction of comparing the MFO activities in Hep G2 cells cultured in medium in the presence and absence of serum. In addition, we examined the potential MFO-inducing activity of PAHs, which are known contaminants [21, 22] of the environment and of environmental air samples.

MATERIALS AND METHODS

Materials

7-Ethoxycoumarin, 7-hydroxycoumarin, MC, resorufin sodium salt, and β-glucuronidase (type H-3) were obtained from the Sigma Chemical Co. (U.S.A.). Five

lots of FBS were from Boehringer (Germany), Bocknek Laboratories Inc. (Canada), Whittaker Bioproducts Inc. (U.S.A.), and Flow Laboratories Inc. (U.K.). PB was from Wako Pure Chemical Industries Ltd. (Japan). Methoxy-, ethoxy-, pentoxy-, and benzyloxyresorufin were synthesized as described by Mayer *et al.* [23]. All other reagents were of reagent grade.

Cell culture and induction protocol

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° as described [20]. When the cells were cultured in medium containing serum, 10% (v/v) FBS was added instead of growth factors. For induction, the cells were plated in ϕ60-mm culture dishes coated with collagen type I (porcine Achilles' tendon) at a density of about 1×10^6 cells/dish. When the cells reached confluence, the medium was renewed, and test samples dissolved in DMSO were added to the cells 12–16 hr before the experiments. DMSO, at a final concentration of 0.2% in the medium, had no effect upon metabolism and enzyme induction.

Analytical methods

Drug-metabolizing enzyme activities were measured according to a modification of the method of Dawson *et al.* [24]. The cells were washed with phosphate-buffered saline, scraped off dishes using a rubber policeman, and then resuspended into Eagle's minimal essential medium (phenol red-free) supplemented with 1 mM substrate, 30 mM D-glucose, and 12 mM HEPES. The cells were shaken gently at 37° for 3 hr, and the reactions were stopped by adding a half-volume of ice-cold acetone. The sediment obtained by centrifugation was assayed for protein. After removing the acetone under a stream of N_2, the pH of the aqueous layer was adjusted to pH 4.8 with sodium acetate-buffered saline, and β-glucuronidase (700 units/mL) was added to hydrolyze conjugated metabolites to the corresponding aglycones. The aqueous layer was incubated at 37° for 16 hr, and then the metabolites were extracted twice with ethyl acetate. Next, the pooled organic phases were evaporated, and the residues containing hydroxycoumarin or resorufin were dissolved in 0.2 M glycine/NaOH-buffered saline (pH 10.4) or ethanol, respectively. The fluorescence intensity was measured using a fluorescence spectrophotometer (Shimadzu, RF-520) at 370 nm_{exc}/454 nm_{emis} or 530 nm_{exc}/585 nm_{emis} to detect hydroxycoumarin or resorufin, respectively. Protein was assayed as described by Lowry *et al.* [25]. The enzyme activity is expressed as nanomoles per milligram of whole cell protein per 3 hr.

Preparation of the extract from total suspended particles collected by a high volume air sampler

At the front entrance of our Institute (the location refers to the above correspondence address), TSP were collected on a silica fiber filter (QR-100, Toyo Roshi Co., Ltd., Japan) set in a high volume air sampler for 24 hr (Jan. 19 to Jan. 20, 1994). The estimated number of cars passing within the period was 50,000. The total volume of air was 1139 m^3, and the total weight of particulate matter was 126.5 mg. The filter paper was extracted with dichloroethane using a Soxhlet extractor for 8 hr. The extract was dried *in vacuo* and dissolved in 2 mL of DMSO [26].

Assay of mutagenicity

The Ames test was performed essentially according to the method of Maron and Ames [27] using *Salmonella typhimurium* strain TA98. For metabolic activation, commercially available rat liver S9 fraction (Oriental Yeast Co., Ltd., Japan) was used.

Statistical analysis

Statistical differences between treatment groups were determined by the analysis of variance using Tukey's comparison. Pairwise comparisons between treated groups and the controls were performed by means of Dunnett's *t*-test.

RESULTS

Effect of commercial sera on the induction of ethoxycoumarin O-deethylase activity in Hep G2 cells

The ECOD activities in Hep G2 cells that were cultured in medium supplemented with or without five commercial sera, and incubated for 16 hr with MC are shown in Table 1. Sera A and B were packaged by the same company, but were from different lots. The induced ECOD activities in the cells cultured with sera A, C and D were significantly different from those with sera B and E. The ECOD activity with serum E was 64% of that with serum D which gave the highest induction of all sera. The cells cultured in serum-free medium supplemented with growth factors induced significantly higher ECOD activity than that in serum-supplemented medium. These findings suggested that induced MFO activity is affected by the serum added to the culture medium, and that medium containing serum cannot provide a suitable environment in which to obtain reliable data regarding enzyme induction.

Table 1. Ethoxycoumarin *O*-deethylase (ECOD) activities in Hep G2 cells cultured in medium with and without five commercial sera

	ECOD activity (nmol/mg whole cell protein/3 hr)	
Serum	Control	MC*
A	0.09 ± 0.02	3.48 ± 0.35†
B	0.11 ± 0.03	2.80 ± 0.20‡
C	0.09 ± 0.01	3.52 ± 0.27†
D	0.09 ± 0.05	3.59 ± 0.04†
E	0.12 ± 0.03	2.29 ± 0.18‡
Serum-free	0.23 ± 0.03	4.93 ± 0.38§

Results are the means ± SD of four experiments.

* Incubation with 2.5 μM 3-methylcholanthrene for 16 hr.

† Significantly different from B, E, and serum-free, $P < 0.01$ (Tukey's comparison).

‡ Significantly different from A, C, D, and serum-free, $P < 0.01$ (Tukey's comparison).

§ Significantly different from all sera, $P < 0.01$ (Tukey's comparison).

Induction of ECOD and EROD activities in Hep G2 cells cultured in medium with or without serum

The induction of ECOD activity in Hep G2 cells cultured in the absence or presence of serum (using serum E in Table 1) is shown in Fig. 1. The activities in cells cultured in both media were highest 12 hr after adding MC, and the activity in serum-free cultured cells was about twice as high as that in cells cultured in the presence of serum. PB induced ECOD activity after 48 hr in Hep G2 cells, and the level was about 40% of that induced by MC. The activities of MROD and EROD in serum-free cultured Hep G2 cells incubated with MC were 2.5-fold (4.10 ± 0.56 nmol/mg whole cell protein/3 hr, $P < 0.01$) and 8.2-fold (6.47 ± 0.44, $P < 0.01$) higher than that of control (1.60 ± 0.60, 0.79 ± 0.44), respectively. These activities were not affected by exposure to PB for 16 hr. Furthermore, the activity of EROD in serum-free cultured cells was 1.6 times higher than that of medium containing serum (serum A in Table 1; MC, 4.03 ± 0.43, $P < 0.01$; control, 0.41 ± 0.18). The activities of PROD and BROD in Hep G2 cells were not affected by exposure to MC and PB for 16 hr. Similar to ECOD induction, the activity of EROD in Hep G2 cells cultured in serum-free medium was highest 12 hr after adding MC (data not shown). These findings suggested that serum-free culture allowed the stable and sensitive production of MFO activity in cultured cells.

Induction of ECOD and EROD in Hep G2 cells treated with polycyclic aromatic hydrocarbons or organochlorine compounds

We estimated the potency of twelve PAHs, which are air pollutants in TSP [28], as well as three organochlorine compounds, to induce ECOD and EROD in Hep G2 cells cultured without serum (Table 2). Benzo(*a*)anthracene, 2,3-benzofluorene, benzo(*a*)pyrene, 7-methylbenzo(*a*)anthracene, and pyrene significantly induced both ECOD and EROD activities against these controls. Benzo(*b*)fluoranthene, γ-HCH, and PCB (kanechlor 500) significantly induced only ECOD activity in Hep G2 cells. However, the other PAHs or organochlorine compounds, which have mutagenic or carcinogenic activities [29], did not affect the induction of ECOD and EROD activities under the same conditions. At 100 μM benzo(*a*)pyrene or 7-methylbenzo(*a*)anthracene, both ECOD and EROD activities were decreased compared with 10 μM values. We assumed that the cell viability was decreased due to PAH toxicity. On the other hand, 100 μM pyrene significantly induced ECOD and EROD activities.

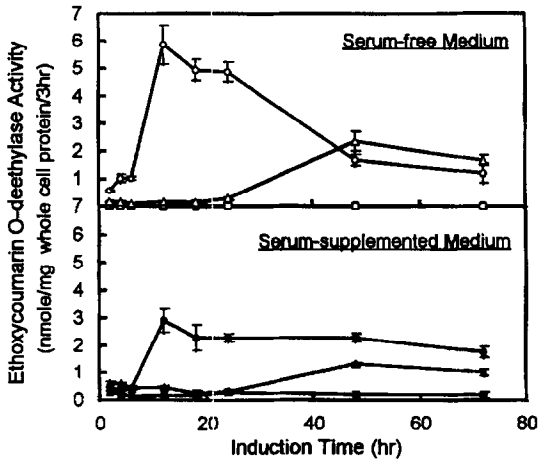

Fig. 1. Induction of ethoxycoumarin *O*-deethylase activity in Hep G2 cells cultured without (□, △, ○) and with serum (serum E in Table 1, ■, ▲, ●). Hep G2 cells were incubated with 2 mM phenobarbital (△, ▲) or 2.5 μM 3-methylcholanthrene (○, ●) (control: □, ■). Results are the means ± SD of three experiments.

MFO-inducing activity and mutagenicity of the extract from TSP

We investigated the ability of the extract from TSP collected using a high volume air sampler to induce ECOD and EROD in serum-free cultured Hep G2 cells (Fig. 2). The mutagenic activity of the same sample using the *S. typhimurium* TA98 strain with and without S9-Mix is shown in Table 3. Six microliters (TSP 0.38 mg equivalent) of the extract induced 17- and 8-fold higher ECOD and EROD activities, respectively, than that of the control. Although the level of EROD activity further increased in the presence of 60 μL (3.8 mg) of the TSP extract, ECOD activity decreased due to toxicity. The extract was mutagenic in *S. typhimurium* TA98 with or without metabolic activation by S9-Mix. Without S9-Mix, a dose of 100 μL (6.3 mg) was toxic to *Salmonella*. Since the ECOD activity induced by 0.6 μL (0.038 mg) of the sample was 3-fold higher than that of the control, and 1 μL (0.063 mg) had no mutagenic activity, the sensitivity of Hep G2 cells to environmental chemicals was higher than that of *Salmonella* in the Ames test. The induction of drug-metabolizing enzymes in Hep G2 cells and the mutagenicity in the Ames test suggested that the extract contained xenobiotics that subsequently would be metabolized in cells, and that TSP in the air contained mutagens or carcinogens.

DISCUSSION

Hep G2 cells have been applied to many studies of cholesterol or fatty acid metabolism [30–32], and are useful as an *in vitro* model of drug metabolism [13–16]. However, the regulation of cytochrome P450 metabolic activity in cultured hepatocytes and Hep G2 cells depends upon the composition of the culture medium [19, 33]. Furthermore, medium containing serum, in which Hep G2 cells are commonly cultured, does not provide a stable environment for studying metabolic activity. On the contrary, serum-free medium can provide us with reproducible and highly sensitive results, since the cells are cultured constantly under the same conditions (Table 1). Hep G2 cells cultured in the presence of serum in collagen-coated dishes showed the same level of activities as those in uncoated dishes (data not shown). Coating dishes with collagen type I did not affect MFO induction. Thus, the inducer may be absorbed onto the proteins such as bovine serum albumin in serum, or an inhibitor that suppresses the induction of drug-metabolizing enzymes may be a serum constituent.

Rodents have not only MC- but also PB-type induction, which involves the cytochrome P4502B enzymes that play a major role in the metabolism of xenobiotics such as aflatoxin B_1. PB selectively induces hepatic P4502B1 and P4502B-catalyzed PROD activity in rats *in vivo* [34], but in cultured hepatocytes and liver cell

Table 2. Induction of ethoxycoumarin *O*-deethylase (ECOD) and ethoxyresorufin *O*-deethylase (EROD) activities in Hep G2 cells by polycyclic aromatic hydrocarbons

	Ethoxycoumarin *O*-deethylase (nmol/mg whole cell protein/3 hr)			Ethoxyresorufin *O*-deethylase (nmol/mg whole cell protein/3 hr)		
	PAH or organochlorine concentration			PAH or organochlorine concentration		
	1.0 μM	10.0 μM	100 μM	1.0 μM	10.0 μM	100 μM
Anthracene	0.22 ± 0.05	0.20 ± 0.00	0.26 ± 0.06	0.16 ± 0.21	0.07 ± 0.02	0.08 ± 0.04
Benzo(*a*)anthracene	0.45 ± 0.07	2.38 ± 0.36*	3.66 ± 0.38*	0.18 ± 0.07	0.77 ± 0.01*	1.00 ± 0.09*
Benzo(*b*)fluoranthene	0.59 ± 0.05*	0.50 ± 0.07*	0.38 ± 0.09	0.15 ± 0.02	0.12 ± 0.07	0.01 ± 0.11
2,3-Benzofluorene	0.26 ± 0.08	1.21 ± 0.13*	2.14 ± 0.18*	0.27 ± 0.07	0.60 ± 0.04*	0.82 ± 0.11*
Benzo(*a*)pyrene	0.35 ± 0.06	1.90 ± 0.18*	0.75 ± 0.12*	0.11 ± 0.04	0.60 ± 0.06*	0.16 ± 0.01
9,10-Dimethylanthracene	0.18 ± 0.09	0.21 ± 0.09	0.24 ± 0.14	0.09 ± 0.07	0.08 ± 0.04	0.04 ± 0.04
Fluoranthene	0.21 ± 0.06	0.22 ± 0.06	0.30 ± 0.06	0.05 ± 0.02	0.15 ± 0.13	0.10 ± 0.05
1-Methylanthracene	0.20 ± 0.05	0.13 ± 0.11	0.35 ± 0.08	0.11 ± 0.09	0.07 ± 0.08	0.15 ± 0.11
7-Methylbenzo(*a*)anthracene	0.55 ± 0.06†	1.77 ± 0.12*	0.98 ± 0.12*	0.22 ± 0.08	0.63 ± 0.05*	0.20 ± 0.00
Pyrene	0.20 ± 0.05	0.27 ± 0.12	2.12 ± 0.23*	0.09 ± 0.01	0.10 ± 0.04	0.46 ± 0.05*
2-Nitrofluorene	0.18 ± 0.15	0.34 ± 0.04†	0.06 ± 0.03	0.04 ± 0.09	0.03 ± 0.04	0.06 ± 0.03
1-Nitropyrene	0.25 ± 0.03	0.23 ± 0.12	0.42 ± 0.15	0.03 ± 0.10	0.01 ± 0.04	0.03 ± 0.01
p,p'-DDT	0.32 ± 0.06	0.26 ± 0.02	0.24 ± 0.03	0.05 ± 0.06	0.02 ± 0.05	0.07 ± 0.07
γ-HCH	0.28 ± 0.01	0.34 ± 0.04†	0.56 ± 0.03*	0.10 ± 0.03	0.07 ± 0.02	0.02 ± 0.05
PCB (kanechlor 500)	0.26 ± 0.05	0.26 ± 0.00	0.39 ± 0.02*	0.02 ± 0.05	0.04 ± 0.02	0.06 ± 0.07

Cells were cultured in serum-free medium, and incubated with the above PAHs for 16 hr. Results are expressed as the means ± SD of three experiments. Control values: ECOD, 0.23 ± 0.03 nmol/mg whole cell protein/3 hr; EROD, 0.13 ± 0.05 nmol/mg whole cell protein/3 hr. Abbreviations: p,p'-DDT, *p,p'*-dichlorodiphenyltrichloroethane; γ-HCH, γ-hexachlorocyclohexane; and PCB, polychlorinated biphenyl.

* Significantly different from control, $P < 0.01$ (Dunnett's *t*-test).

† Significantly different from control, $P < 0.05$ (Dunnett's *t*-test).

lines PB seems to act rather like a PAH inducer, selectively inducing the P4501A subfamily instead [35, 36]. However, typical PB-like induction of P4502B1 has been identified in cultured rat hepatocytes [37, 38]. Doostdar *et al.* [39] have reported that the effects of PB on monooxygenase induction in Hep G2 cells are not statistically significant. There is no convincing evidence in the literature that PB induces P4502B in humans *in vivo*, despite the presence of the *CYP2B6* gene in human liver. However, it is possible that a therapeutic dose of PB in humans is insufficient to induce P4502B. Therefore, we limited this study to PAH-inducible P4501A enzymes.

We exposed the cells only once to chemicals. Doostdar *et al.* [39] have reported that incubating Hep G2 cells with chemicals for 3 days induces MFO activities such as EROD or PROD. However, because the amount of the extract prepared from the environment is usually small, the induction of enzyme activity should be measurable

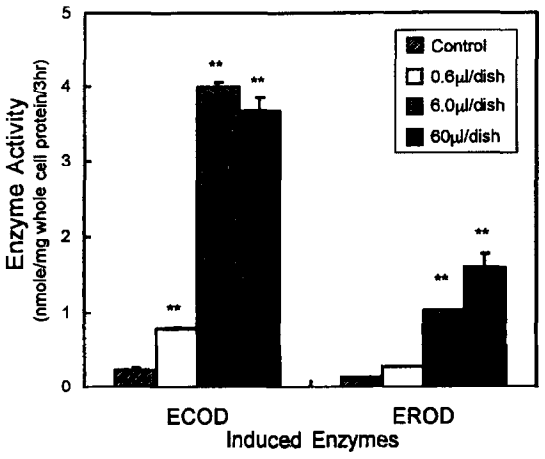

Fig. 2. Induction of ethoxycoumarin *O*-deethylase and ethoxyresorufin *O*-deethylase activities in Hep G2 cells by the extract from TSP collected using a high volume air sampler. Hep G2 cells were cultured in serum-free medium and incubated with the extract obtained from 126.5 mg TSP and dissolved in 2 mL of DMSO. Results are the means ± SD of three experiments. Key: (**) significantly different from the control, $P < 0.01$ (Dunnett's *t*-test).

Table 3. Mutagenic activity of the extract from total suspended particles collected using a high volume sampler, in *S. typhimurium* TA98 with and without S9-Mix

		Revertants per plate	
	μL	S9–*	S9+*
Extract	100	130†	442
	10	145	105
	1	38	33
SP‡		30	36
POS§		356	245

The extract was prepared from 126.5 mg TSP and dissolved in 2 mL DMSO.

* S9–, without S9-Mix; S9+, with S9-Mix.

† Toxic.

‡ SP, spontaneous revertants.

§ POS, positive control. S9–, 0.1 μg/plate 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide. S9+, 0.5 μg/plate 2-aminoanthracene.

after one dose of the extract. We found that several PAHs induced ECOD and AROD activities in Hep G2 cells, but nitro-PAHs, which are environmental contaminants [40], did not. Furthermore, several PAHs such as 9,10-dimethylanthracene, fluoranthene, and 1-methylanthracene also did not affect the induction of either ECOD or EROD activity (Table 2); however, these chemicals may have the potential to induce other cytochrome P450 isozymes.

Since carcinogens may potently induce cytochrome P4501A type MFO, the inducing potential of a chemical should be quantified to ascertain its likelihood of being a carcinogenic hazard [41, 42]. Furthermore, inducers of cytochrome P4501A type MFO may have three consequences for toxicity or carcinogenicity. First, they are likely to be substrates of cytochrome P4501A and will be metabolically activated to mutagenic, carcinogenic, or immunogenic reactive intermediates. Second, the induction will increase the levels of the activating cytochrome P4501A enzymes and of the Ah receptor, thereby enhancing substrate activation, enzyme induction, and potential toxicity. Third, the activity of protein kinases and of epidermal growth factor increases, as does arachidonate metabolism [43]. We demonstrated the MFO-inducing activity and mutagenicity of air extract prepared from environmental samples in Hep G2 cells and *Salmonella*. However, we could not determine which compound(s) induced the MFO activity or the reverse mutation. Our results should not be interpreted as indicative of a correlation between MFO-inducing activity and mutagenicity. However, we are convinced that the results from ongoing studies using the human-derived cell lines will clarify these relationships.

MFO activity of rat hepatocytes in primary culture decreases drastically [7]. P450 expression or regulation is not stable in rodent hepatocytes. Furthermore, differences in cytochrome P450 between rodents and humans have been demonstrated [9, 10]. Thus, the effects of environmental contaminants upon human health cannot be evaluated precisely by extrapolating rodent data. The use of human cell lines such as Hep G2, which retains many differentiated functions, may be useful for evaluating the effects of environmental contaminants upon human health and, therefore, could be applied to human risk assessment.

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