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In vitro micronucleus test in HepG2 transformants expressing a series of human cytochrome P450 isoforms with chemicals requiring metabolic activation

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ABSTRACT

It is known that many genotoxic chemicals require oxidative metabolism to elicit genotoxicity. Induced rat liver S9 fraction has been employed as a 'metabolite factory' in *in vitro* genotoxicity testing. However, the relevance of the induced rat liver S9 fraction has been called into question due to the differences in the rat and human cytochrome P450 (CYP) activities. In the present study, we used a series of ten transformants expressing major human CYP isoforms such as CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 in HepG2 cells. To elucidate the usefulness and feasibility of these transformants, genotoxicity was tested without using rat S9. Among these transformants, benzo(a)pyrene-induced or cyclophosphamide-produced micronucleus (MN) frequency was markedly increased in transformants expressing CYP1A2 or CYP2C9, respectively. To explore the possibility that these transformants can be used for screening the possible genotoxicity of newly developed drugs, a chemical which is known to enhance genotoxicity in the presence of external metabolic activation system, okadaic acid (OA), was investigated. OA-induced MN frequency was significantly induced in transformants expressing CYP1A2 compared with the other CYP isoforms. The induced MN frequency was suppressed by treatment with a CYP1A2 specific inhibitor and CYP1A2 to siRNA. In control HepG2 cells harboring an empty vector, OA was treated with microsomes expressing CYP1A2 to induce MN. These results demonstrated that this screening system worked well and OA was found to be metabolically activated by CYP1A2 to induce MN. Based on the results obtained in the present study, this system of transformants is useful to elucidate the genotoxicity involving human CYP metabolism in the process of drug discovery.

1. Introduction

Genotoxicity testing is routinely performed to identify potential genotoxic carcinogens. It is well known that many genotoxic chemicals require oxidative metabolism before demonstrating genotoxicity [1], and therefore an activation system is included in standard *in vitro* genotoxicity testing with the purpose of generating electrophilic metabolites that can react with macromolecules such as nucleic acids [2]. In many cases, an induced rat liver S9 fraction is employed as a 'metabolite factory' in *in vitro* genotoxicity testing [3,4]. However, it has been known that the cytochrome P450 (CYP) enzymes in rats and humans differ in their substrate specificities even in the same family [5]. In addition, certain CYP enzymes frequently involved in drug metabolism, such as CYP2C or CYP2D, are more divergent between rats and humans. The relevance of the induced rat liver S9 fraction has been called into question in the identification of hazards to humans [6].

Based on this background, the working group of the International Workshop on Genotoxicity Testing recommended the use of genetically engineered mammalian cell lines stably expressing CYP enzymes as one of the possible remedies [6]. The stable expression system of CYP in cells has made it possible to evaluate the relative risk of a chemical in *in vitro* toxicological testing [7]. Kessova and Cederbaum developed a line, which possesses CYP2E1 activity and used it in a number of mechanistic studies [8]. However, at present genetically engineered cells are primarily used as an analytical tool for elucidating activation and inactivation mechanisms. In general, only one to three foreign factors (enzymes or transporters) have been expressed in a given cell line. For screening purposes, the cells or a set of cell lines expressing the major CYP isoforms are necessary, since several CYP isoforms have been proven to be involved in the metabolism of genotoxic chemicals [9,10]. It is important to clarify the CYP isoforms involved in the metabolic activation, since the catalytic properties and the populations of the CYP isoforms

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Table 1
Characteristics of transformants.

Name of transformant	Expressed CYP isoform	Catalytic reaction measured	V_{max} (pmol/min/mg)
Hepc/1A1.4	CYP1A1	7-Ethoxyresorufin *O*-deethylation	56
Hepc/1A2.9	CYP1A2	7-Ethoxyresorufin *O*-deethylation	2
Hepc/2A6L.14	CYP2A6	Coumarin 7-hydroxylation	812,000
Hepc/2B6.68	CYP2B6	7-Ethoxycoumarin *O*-deethylation	80,000
Hepc/2C8.46	CYP2C8	Taxol 6-hydroxylation	9,400
Hepc/2C9.1	CYP2C9	Tolbutamide 4-hydroxylation	25,000
Hepc/2C19.12	CYP2C19	(S)-Mephenytoin 4′-hydroxylation	140,000
Hepc/2D6.39	CYP2D6	Bufuralol 1′-hydroxylation	14
Hepc/2E1.3-8	CYP2E1	p-Nitrophenol hydroxylation	120
Hepc/3A4.2-30	CYP3A4	Testosterone 6β-hydroxylation	71

From Ref. [11].

are sometimes considered as the determinants of the formation of reactive metabolites in the human body. We previously established a series of HepG2 transformants expressing the cytochromes 1A1, 1A2, 2A6, 2B6, 2C8, 2C19, 2D6, 2E1 and 3A4 with the apparent V_{max} values for characteristic substrates (Table 1) [11]. Since most human hepatic drug metabolism is catalyzed by these CYP isoforms, this HepG2 transformant system would be more suitable for the genotoxic assessment of chemicals than the induced rat liver S9 fraction in the routine screening when considering human hepatic metabolism in the future.

In the present study, we examined the availability of this HepG2 transformant system in *in vitro* genotoxicity testing on two aspects. Firstly, in order to validate the sensitivity of these transformants, we measured the incidence of cells with micronuclei as a genotoxic endpoint in this system after exposure to benzo(a)pyrene (BP) or cyclophosphamide (CP), which are well-studied genotoxic chemicals requiring CYP activation [12–14]. Secondly, we tested whether this system could identify the CYP isoforms responsible for the genotoxicity of a novel chemical. Because the practical application of this transformant system is assumed to get some evidence about which CYP isoform is related to the positive result obtained in *in vitro* genotoxicity testing of a new chemical in the presence of the metabolic activation system without enough information of CYP metabolism. For this part of the study, we selected okadaic acid (OA), which is known to have enhanced genotoxicity in the presence of a metabolic activation system but for which it is not known which CYP isoform(s) play a role in its biotransformation [15–17]. The results from well-studied chemicals and a novel chemical allowed us to utilize this transformant system in routinely performed in *in vitro* genotoxicity testing.

2. Materials and methods

2.1. Chemicals and solvents used

Acridine orange (CAS number 10127-02-3), benzo(a)pyrene (CAS number 50-32-8), dimethyl sulfoxide (DMSO; CAS number 67-68-5), okadaic acid (CAS number 78111-17-8) were obtained from Wako Pure Chemicals (Osaka, Japan); mitomycin C (MMC; CAS number 50-07-7) was from Kyowa Hakko Kogyo (Tokyo, Japan); cyclophosphamide (CAS number 50-18-0) was from Shionogi (Osaka, Japan); furafylline (CAS number 80288-49-9) was from Ultrafine Chemicals (Manchester, UK).

2.2. Cell culture

HepG2 cells and its transformants expressing human CYP isotypes were used [11] and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco® Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) in a humidified atmosphere in 5% CO_2 at 37 °C. Transformants were cultured in DMEM supplemented with 10% FCS, 200 μg/ml G418 (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin.

2.3. Growth rate of the cells

Cells (1×10^5 cells) were seeded on 24-well plates (Falcon®, Beckton Dickinson and Co., NJ, USA) and cultured for 4 days. The number of cells was counted every 12 h using a coulter counter. Doubling time was calculated using the following formula: DT (hour) = T1/log 2(cell number)$_{T1}$/(cell number)$_{T0}$. DT refers to doubling time, and (cell number)$_{T1}$ and (cell number)$_{T0}$ refer to cell number on T1 and T0, respectively.

2.4. Cell treatments

2.4.1. Treatment with chemicals

Approximately 24 h before the treatment, the cells were seeded at 1×10^5 cells/0.5 ml/well in 24-well tissue culture plates. Subsequently, the cells were incubated with various concentrations of the chemicals in a single culture. A stock solution for BP or OA was prepared and frozen. Immediately prior to treatment, these stock solutions were diluted with DMSO as 200× concentrations. A 0.5% (v/v) DMSO or saline was used for a negative control. A 0.5% (v/v) MMC solution (final: 50 ng/ml) was administered as a positive control. After 48 h, cells were harvested for MN analysis. Experiments were repeated at least 3 times independently.

2.4.2. Treatment with a specific inhibitor of CYP1A2

To assess the involvement of CYP1A2 enzymatic activity, furafylline, a mechanism-based inhibitor of CYP1A2 [18], was co-administered with BP, OA, or MMC to HepG2 transformants expressing CYP1A2. Furafylline was dissolved fresh in DMSO and diluted to a 200× concentration to give a final concentration of 0.5% in DMSO. Cells were co-treated with the chemicals and the CYP1A2 inhibitor for 48 h.

2.4.3. Treatment with CYP1A2 siRNA to CYP1A2

CYP1A2 siRNA (siGENOME ON-TARGETplus SMARTpool duplex, CYP1A2) and negative control siRNA (ON-TARGETplus siCONTROL Non-targeting Pool) were obtained from Dharmacon® (GE Healthcare, CO, USA). Transfection complexes were prepared in 500 μl of Opti-MEM® I reduced serum medium (Invitrogen) by mixing 1.5 μl of Lipofectamine™ RNAiMAX (Invitrogen) and 50 nM of siRNA in the wells of a 24-well tissue culture plate. These complexes were incubated for 20 min at room temperature. The cell suspension (0.75×10^5 cells/well) was transferred into the wells and mixed gently with these complexes. After incubation for 24 h at 37 °C, the medium was replaced with fresh medium containing various concentrations of the chemicals or controls and the cells were treated for an additional 48 h.

2.4.4. Treatment with microsomes expressing human CYP1A2

Human CYP1A2 expressing microsomes were used as an external activating system in combination with HepG2 transformants according to Vignati et al. with some modifications [19]. Human CYP1A2 Supersomes™ and Insect Cell Control Supersomes™ were purchased from BD-Gentest (BD Biosciences Co., MA, USA). About 24 h after cultivation, HepG2 transformants expressing an empty vector (Hepc) were treated with various concentrations of the chemicals for 48 h in the presence of 40 pmol/ml human CYP1A2 or Insect Cell Control Supersomes™ and 1 mM of NADPH as a co-factor.

2.5. Micronucleus (MN) analysis

After 48-h treatment, the cells were collected by trypsinization. Cells were incubated in 0.075 M KCl solution (Invitrogen) for 10 min at room temperature and fixed with ice-cold Carnoy's solution (methanol:acetic acid 3:1, v/v). The cells were washed twice with Carnoy's solution and finally resuspended in methanol containing 1% (v/v) acetic acid. The cells were then spread on a microscopic slide. The slides were stained with acridine orange (40 μg/ml) solution, and then scored at 200× magnification. The number of micronucleated cells among 1000 interphase cells per slide was scored. Mitotic cells and multinucleated cells were excluded from the scoring. The diameter of the MN was defined at most half of that of the nucleus.

2.6. Statistical analysis

All data are given as means ± standard deviation (S.D). For the assessment of MN induction, the experiments were repeated at least 3 times independently. Statistical evaluation between groups was carried out using a two-tailed Student's *t*-test, and $p < 0.05$ was considered significant.

Table 2
Doubling time and incidence of cells with micronuclei in the solvent or MMC-treated group in HepG2 and its transformants.

Name of transformant[a]	Expressed CYP isoform	Doubling time[b] (h)	Incidence of cells with micronuclei[c] (%)			
			Saline (10 μl/ml)	DMSO (5 μl/ml)	MMC: (25 ng/ml)	
					MMC	Fold induction[d]
HepG2	–	31.4	1.3 ± 0.3	1.6 ± 0.2	16.1 ± 2.6	12.1 ± 1.7
Hepc	–	27.3	1.1 ± 0.1	1.0 ± 0.3	13.5 ± 1.4	12.0 ± 1.8
Hepc/1A1.4	CYP1A1	28.9	1.1 ± 0.3	0.9 ± 0.3	16.6 ± 3.4	15.8 ± 2.6
Hepc/1A2.9	CYP1A2	23.7	1.3 ± 0.2	1.1 ± 0.6	17.6 ± 3.2	13.8 ± 1.6
Hepc/2A6L.14	CYP2A6	28.5	0.9 ± 0.2	1.0 ± 0.4	16.3 ± 3.7	18.6 ± 1.4*
Hepc/2B6.68	CYP2B6	25.7	1.3 ± 0.6	1.4 ± 0.2	17.0 ± 2.0	13.1 ± 0.6
Hepc/2C8.46	CYP2C8	26.5	1.2 ± 0.0	0.9 ± 0.4	15.4 ± 4.6	13.1 ± 2.9
Hepc/2C9.1	CYP2C9	28.8	1.3 ± 0.3	1.1 ± 0.1	17.9 ± 5.5*	13.7 ± 0.7
Hepc/2C19.12	CYP2C19	33.3	1.6 ± 0.2*	1.1 ± 0.3	18.3 ± 3.2*	11.6 ± 0.6
Hepc/2D6.39	CYP2D6	29.5	1.8 ± 0.3*	1.4 ± 0.6	18.1 ± 2.6	9.9 ± 1.3
Hepc/2E1.3-8	CYP2E1	29.6	1.0 ± 0.2	1.1 ± 0.2	13.5 ± 5.4	13.8 ± 2.2
Hepc/3A4.2-30	CYP3A4	28.1	1.9 ± 0.2*	1.9 ± 0.5	22.6 ± 4.1*	12.0 ± 1.5

[a] Named in the previous study [11].
[b] Cells were seeded on 24-well tissue culture plates and cultured at 37 °C and 5% CO_2 for 72 h. The number of cells was counted after 24 and 72 h and doubling time for 48 h was calculated as described in Section 2. The values are the means of 3 independent experiments.
[c] Cells were seeded on 24-well tissue culture plates and cultured at 37 °C and 5% CO_2. After 24 h, they were treated with chemicals at the indicated concentration for 48 h. After treatment, the cells were collected and sampled. One thousand interphase cells per each treatment were scored and the incidence of cells with micronuclei was calculated. Values are the mean ± S.D. of 3–4 experiments. Data were tested using Student's *t*-test (*$p < 0.05$, compared with Hepc).
[d] Values were normalized with the mean control value of 4 experiments.

3. Results

3.1. Doubling time and MN frequency of HepG2 transformants expressing CYP isoforms treated with control chemicals

The HepG2 and its transformants used in this study are listed in Tables 1 and 2. The doubling time of HepG2 and its transformants was 23.7–33.3 h, during 48 h of saline-treatment, and cell growth was not markedly different between the transformants. In the solvent control groups, the mean frequencies of micronucleated cells (% cell with MN) were 0.9–1.9%. No significant changes were observed among all transformants in the case of DMSO, whereas in the case of saline the significant differences were observed in the transformants expressing CYP2C19, CYP2D6 and CYP3A4 compared to that in the Hepc. MMC treatment significantly increased MN frequency in all the transformants (13.5–22.6%), since MMC directly acts on DNA in cells irrespective of the expression of the CYP isoforms. However, higher MN induction by MMC was seen in the transformants expressing CYP2C9, CYP2C19, CYP2D6 and CYP3A4. These significant differences might be influenced by the relatively high frequencies in the solvent control groups in some transformants. Thus, the relative value (fold induction to the solvent control) was employed in order to assess the effect of the expressed CYP isoforms.

3.2. MN induction by BP and CP

In order to evaluate the applicability of this series of transformants, BP and CP were tested as model chemicals exhibiting CYP-mediated MN induction. HepG2 and its transformants were treated with 2 concentrations of these model chemicals. Among the hepatic CYP isoforms reported, CYP1A1 showed clearly the highest activity in BP metabolism; significant formation of some metabolites was also observed with CYP1A2 and CYP3A4 [12]. CP is efficiently metabolized by CYP2B6, CYP2C9 and CYP3A4 [13,14].

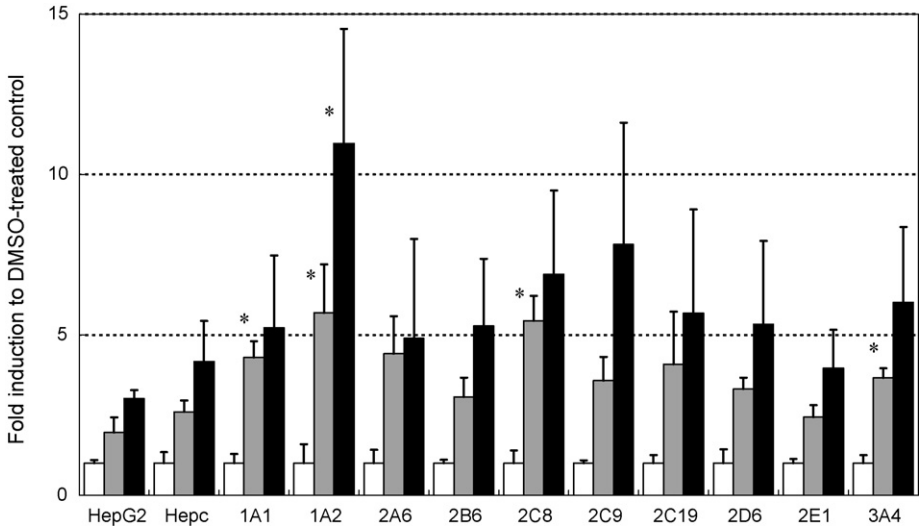

Fig. 1. Micronucleus induction of BP by expression of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4. The cells (1×10^5 cells) were seeded onto a 24-well plate for 24 h, and then treated with 0.5% DMSO (open bars), 200 ng/ml (gray bars) or 400 ng/ml (solid bars) BP. After 48 h, the cells were collected and sampled. One thousand interphase cells per each treatment were scored and the incidence of cells with micronuclei was calculated. Values were normalized with the mean DMSO-treated control value of 3 experiments in each transformant. Each bar represents the mean ± S.D. of 3 experiments. Data were tested using Student's *t*-test (*$p < 0.05$, compared with Hepc).

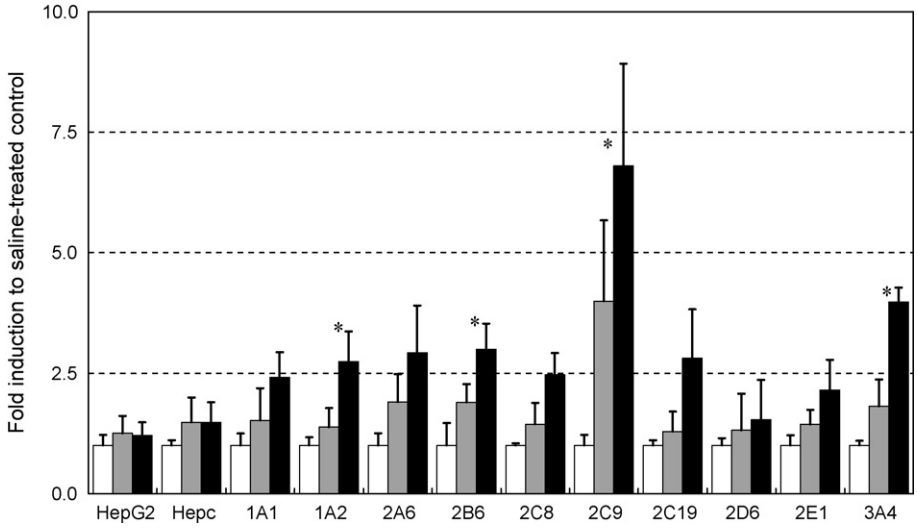

Fig. 2. Micronucleus induction of CP by expression of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4. The cells (1×10^5 cells) were seeded onto a 24-well plate for 24 h, and then treated with 0.5% saline (open bars), 1 mg/ml (gray bars) or 2 mg/ml (solid bars) CP. After 48 h, the cells were collected and sampled. One thousand interphase cells per each treatment were scored and the incidence of cells with micronuclei was calculated. Values were normalized with the mean saline-treated control value of 4 experiments in each transformant. Each bar represents the mean ± S.D. of 3 experiments. Data were tested using Student's *t*-test ($^*p < 0.05$, compared with Hepc).

BP treatment increased the frequency of cells with micronuclei in a dose-dependent manner regardless of the expressed CYP isoforms (Fig. 1). For example, in Hepc harboring an empty vector, the frequencies of MN cells after treatment with 200 and 400 ng/ml BP were approximately 2.6-fold and 4.2-fold higher than that with DMSO, respectively. In the transformant expressing CYP1A2, the fold inductions of % cells with MN by BP compared to the DMSO-treated control were 5.7 and 11.0 and both values were significantly higher than those in Hepc cells. In the transformants expressing CYP1A1, CYP2C8 and CYP3A4, significant differences were also seen in the fold induction of % MN at 200 ng/ml BP. However, significant increase was not observed at 400 ng/ml BP due to the large standard deviations. It was noted that strong cytotoxicity (>50%) was observed at this concentration in the transformant expressing CYP1A1 (data not shown).

CP treatment did not increase the MN frequency in Hepc as well as HepG2, whereas significant MN inductions by CP were seen in the transformants expressing CYP1A2, CYP2B6, CYP2C9 and CYP3A4 at the concentration of 2000 ng/ml (Fig. 2). Specifically in the transformant expressing CYP2C9, the mean fold induction in the % cells with MN at 1000 and 2000 ng/ml CP was approximately 2.6 and 4 times higher than that in Hepc, respectively. As expected, this series of transformants has efficient sensitivity to detect chemicals that can produce MN mediated by CYP.

3.3. MN induction by OA

In order to elucidate the possibility that the set of transformants can be used for screening for the genotoxicity of newly developed drugs, we chose OA because Le Hégarat et al. reported that OA-induced MN were increased in the presence of an external metabolic activation system [15] and as the CYP isoforms involved in the metabolic activation of OA have not been identified, to the best of our knowledge. OA even itself increased the % cells with MN in a dose dependent manner as also reported by Le Hégarat et al. in CHO-K1 cells [16,17], resulting in MN formation in almost all the HepG2 transformants (Fig. 3). However, we found that in the transformant expressing CYP1A2, the fold induction of the % cells

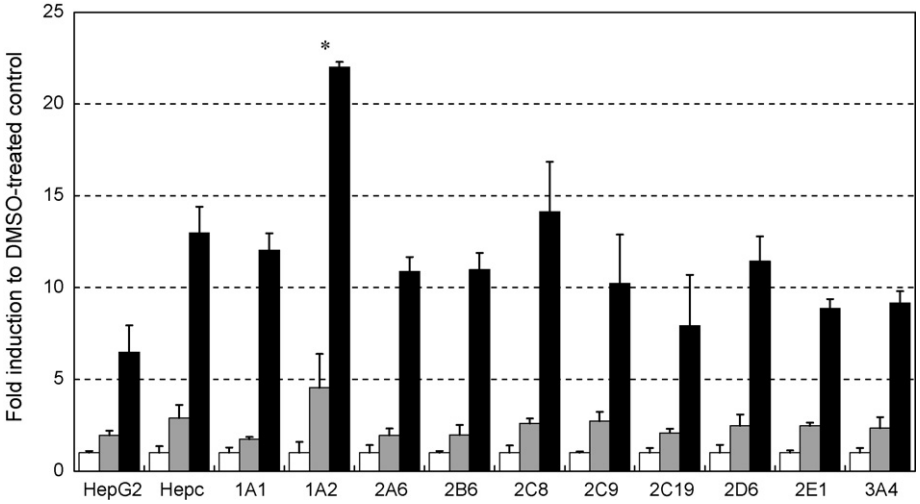

Fig. 3. Micronucleus induction of OA by expression of CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4. The cells (1×10^5 cells) were seeded onto a 24-well plate for 24 h, and then treated with 0.5% DMSO (open bars), 5 ng/ml (gray bars) or 10 ng/ml (solid bars) OA. After 48 h, the cells were collected and sampled. One thousand interphase cells per each treatment were scored and the incidence of cells with micronuclei was calculated. Values were normalized with the mean DMSO-treated control value of 3 experiments in each transformant. Each bar represents the mean ± S.D. of 3 experiments. Data were tested using Student's *t*-test ($^*p < 0.05$, compared with Hepc).

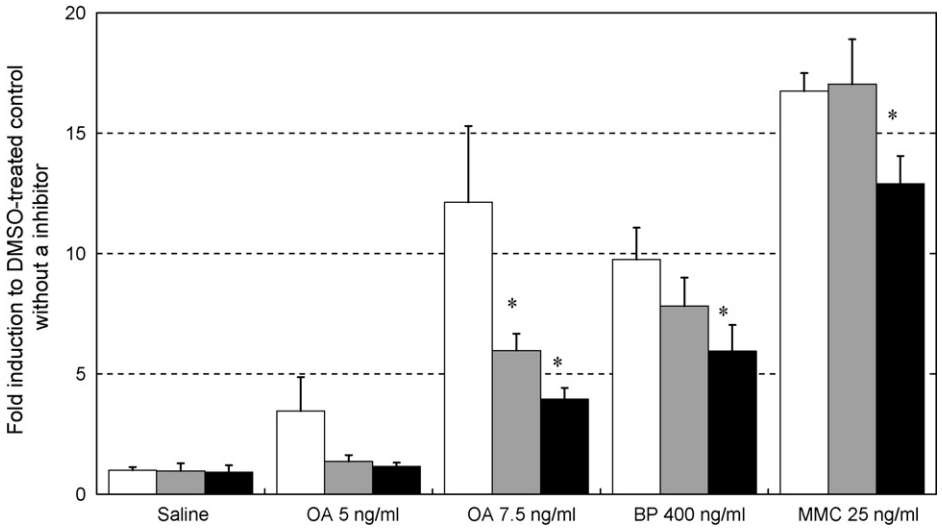

Fig. 4. Effects of furafylline, a CYP1A2 specific inhibitor for micronucleus induction by various chemicals in the transformant expressing CYP1A2. The cells (1×10^5 cells) were seeded onto a 24-well plate for 24 h, and then treated with 0.5% DMSO, 5 or 7.5 ng/ml OA, 400 ng/ml BP and 25 ng/ml MMC in the absence (open bars) or presence of 5 μM (gray bars) or 50 μM (solid bars) furafylline. After 48 h, the cells were collected and sampled. One thousand interphase cells per each treatment were scored and the incidence of cells with micronuclei was calculated. Values were normalized with the mean DMSO-treated control value without an inhibition of three experiments in each transformant. Each bar represents the mean ± S.D. of 3 experiments. Data were tested using Student's *t*-test ($^*p < 0.05$, compared with no inhibition).

with MN to the DMSO-treated control was 4.6 at 5 ng/ml and 22.0 at 10 ng/ml OA. In the other transformants, a significant increase of the % cells with MN after treatment with OA was not observed. This result indicated that CYP1A2 is involved in the metabolic activation of OA. Moreover, this series of transformants can detect the CYP-mediated genotoxicity of novel chemicals.

3.4. Inhibitory effects of the specific inhibitor of CYP1A2 and of siRNA to CYP1A2 on MN induction by OA

Considering the size of substrate pocket, a large molecule such as okadaic acid is expected to be metabolized by CYP3A rather than CYP1A. Therefore, to investigate the involvement of CYP1A2 activity in the induction of MN by OA, we examined the effect of furafylline, a mechanism-based inactivator of CYP1A2 [18], on MN induction with the transformant expressing CYP1A2 (Fig. 4). While furafylline did not affect MN formation in the solvent control, MN formation by OA was reduced to half of that without furafylline but without a statistically significance due to a wide standard deviation. Meanwhile a small but significant decrease in the frequency of micronucleated cells by MMC was detected at a high concentration of furafylline. This might be caused by the repression of cell division since the cell number decreased after co-treatment with 50 μM furafylline and MMC (data not shown), indicating that at high concentration OA-induced MN enhancement might be affected by a certain level of cytotoxicity produced by furafylline.

The involvement of CYP1A2 activity in MN induction by OA was further examined using a siRNA specific to CYP1A2. Non-targeting siRNA did not affect MN induction (Fig. 5), while small but significant decreases in the relative value of MN induction were observed in cells exposed with siRNA to CYP1A2 in OA treatment. The observed repression in OA-induced MN correlated with the

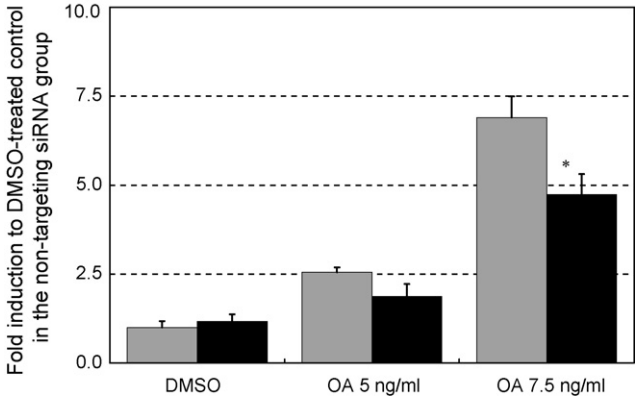

Fig. 5. Effects of siRNA to CYP1A2 on micronucleus induction by okadaic acid in the transformant expressing CYP1A2. The cells (1×10^5 cells) were seeded onto a 24-well plate for 24 h in the presence of 50 nM siRNA for non-targeting (gray bars) or CYP1A2 (solid bars). Medium was changed with a fresh one containing 0.5% DMSO, 5 and 7.5 ng/ml OA. After 48 h, the cells were collected and sampled. One thousand interphase cells per each treatment were scored and the incidence of cells with micronuclei was calculated. Values were normalized with the mean DMSO-treated control value without siRNA of three experiments in each transformant. Each bar represents the mean ± S.D. of 3 experiments. Data were tested using Student's *t*-test ($^*p < 0.05$, compared with non-targeting siRNA).

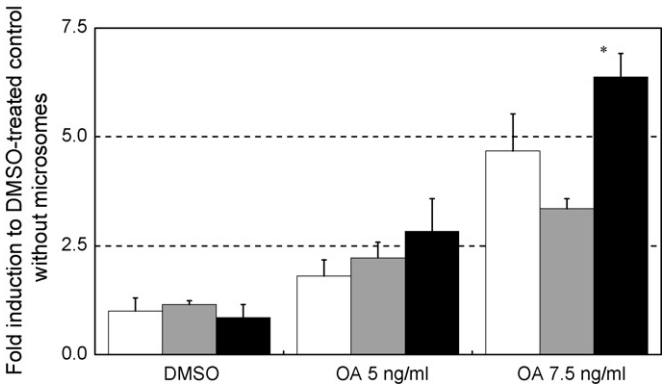

Fig. 6. Effects of external metabolic activation system for CYP1A2 on micronucleus induction by okadaic acid in Hepc. The cells (1×10^5 cells) were seeded onto a 24-well plate for 24 h, and then treated with 0.5% DMSO, 5 or 7.5 ng/ml OA in the absence (open bars) or presence of Insect Cell Control Supersomes™ (gray bars) or Human CYP1A2 Supersomes™ (solid bars). After 48 h, the cells were collected and sampled. One thousand interphase cells per each treatment were scored and the incidence of cells with micronuclei was calculated. Values were normalized with the mean DMSO-treated control value without microsomes of 3 experiments in each transformant. Each bar represents the mean ± S.D. of 3 experiments. Data were tested using Student's *t*-test ($^*p < 0.05$, compared with the control microsomes).

knockdown condition of the CYP1A2, since the MN formation did not seem to be affected by siRNA with CYP1A2 either in the negative (DMSO) or positive (MMC) control group (data not shown).

3.5. Increase of MN formation by co-treatment of OA and microsomes expressing human CYP1A2

We here found that CYP1A2-dependent activation is involved in OA-induced MN formation (Figs. 4 and 5). To further confirm the involvement of CYP1A2 in OA-induced MN formation, the effect of the addition of an external metabolic activation system for CYP1A2 on the MN induction was evaluated by using microsomes expressing recombinant human CYP1A2. The treatment with OA combined with the microsomes expressing human CYP1A2 resulted in the enhancement of MN induction at 5 and 7.5 ng/ml (about 1.5-fold) and a small but significant change was observed at the upper concentration (Fig. 6) when compared to the treatment with or without control microsomes. This result indicated that the OA metabolite(s) generated by CYP1A2 expressed in microsomes contribute to the enhancement of MN formation in the presence of OA.

4. Discussion

In the current genotoxicity testing guidelines, induced rat liver S9 has been adopted for *in vitro* genotoxicity tests as an exogenous activation system for detecting promutagens [2], but the relevance of the induced rat liver S9 fraction has been called into question when considering the risk to humans [4,6]. The International Workshop on Genotoxicity Tests Strategy Expert Group recommended that genetically engineered bacteria, mammalian cells and animal models be used as a currently possible remedy [6]. In the present study, we applied the transformant system with stable expression of a series of human CYP isoforms in the HepG2 cells to examine the genotoxic metabolite of novel chemicals.

We at first compared the ability to induce MN among these transformants by chemicals not requiring metabolic activation. Although MMC treatment resulted in slight differences in the frequency of MN among these transformants, the fold induction of MN frequency from the solvent control group for each transformant was comparable (Table 2). Thus, we used the fold induction to identify which CYP isoform contributed to the production of the genotoxic metabolite of a chemical to induce MN.

Secondly, these transformants were checked for their response to the chemicals in which the CYP isoforms responsible for which genotoxicity has been reported. BP treatment raised the fold induction of MN in the transformants expressing CYP1A1, CYP1A2 and CYP3A4 (Fig. 1) and CP treatment did the same in the transformants expressing CYP1A2, CYP2B6, CYP2C9 and CYP3A4 (Fig. 2). Although not all the CYP isoforms used in the present study were examined with appropriate positive control chemicals, these results suggested that the series of transformants was potentially efficient to identify the CYP responsible for the MN induction by novel chemicals.

It was surprising that the induction of MN with 400 ng/ml of BP in the transformant expressing CYP1A1 was not significantly different from that in Hepc expressing an empty vector (Fig. 1), since a major contribution of CYP1A1 in the metabolism of BP has been reported [12]. We considered that this could be caused by severe cytotoxicity at the higher concentration of BP. After 48-h treatment with BP the number of cells decreased below 50% of that in the DMSO control (data not shown). Thus, it was suggested that severe cytotoxicity from BP caused the repression of cell division, resulting in the absence of an increase in MN in the transformant expressing CYP1A1. In addition, another relevant CYP isoform in the metabolism of BP is CYP1B1 [20]. Unfortunately the transformant expressing CYP1B1 had not been established in the previous study, because of a small contribution of this CYP isoform in human hepatic drug metabolism [11].

Furthermore, we tested the availability of these transformants to explore unknown genotoxic metabolites. Le Hégarat et al. found that OA enhanced formation of MN in the presence of a metabolic activation system [15], although the CYP isoforms involved in the MN induction were not reported. Thus we selected OA as a model chemical to evaluate the ability of our system to investigate which CYP isoform is involved in producing unknown genotoxic metabolites. In the present study, OA significantly increased the fold induction of MN in the transformant expressing CYP1A2 compared with that obtained in the transformant expressing an empty vector (Fig. 3). Furthermore, inhibitory effects of a specific inhibitor of CYP1A and siRNA to CYP1A2 on MN induction by OA were shown (Fig. 5). Moreover, co-treatment with OA and microsomes expressing CYP1A2 showed MN induction in Hepc cells (Fig. 6). These results indicated that MN induction by OA could be associated with the presence of CYP1A2, suggesting that CYP1A2 is involved in the activation of OA.

More effort as for CYP induction is necessary, but the results obtained in the present study demonstrated the availability of these transformants expressing human CYP to elucidate the genotoxic potential of the chemicals that require metabolic activation to create risk to humans. In order to validate these transformants, an additional study is in progress with more chemicals that have been well studied in the metabolic activation or inactivation by CYP enzymes.

For assessing the genotoxicity of chemicals with human metabolism, our system has more appropriate characteristics than other established cell lines used in toxicological testing and reviewed by Sawada and Kamataki [7]. One of major advantage of our system is the variety of human CYP isoforms. In human liver, about 70% of the total CYP could be accounted for by CYP1A2, CYP2A6, CYP2B6, CYP2C, CYP2D6, CYP2E1 and CYP3A proteins [21]. Most human drug metabolism is catalyzed by CYP1A2, CYP2C8, CYP2C19, CYP2D6, CYP2E1 and CYP3A4. When considering replacement of the rat induced liver S9 which is utilized in current genotoxicity testing, increasing the number of the principal CYP isoforms would be desirable in order to cover the diverse cytochrome P450 activities. Another advantage of our system is that the transformants are derived from hepatocytes that possess other factors necessary for the function of CYP. Generally, the reactions catalyzed by cytochrome P450 molecules require the presence of NADPH-cytochrome P450 reductase and cytochrome b_5 to support some CYP-mediated reactions. HepG2 has been shown to have NADPH-cytochrome P450 reductase activity and cytochrome b_5, although the levels are lower than those of human liver [22–24]. Therefore, our system does not need co-expression of reductase and/or cytochrome b_5 with CYP enzymes.

In summary, we have demonstrated the practicality of stable CYP expression cell systems in the assessment of the genotoxicity of chemicals. The transformants expressing the related CYP isoform responded appropriately to known toxic chemicals such as BP and CP. For a model chemical where the CYP isoforms contributing in the genotoxicity were unknown, this system indicated the CYP isoform responsible. Therefore, this cell system may be useful to elucidate genotoxicity related to CYP metabolism. This system could be used at the preclinical stage as a replacement of the induced rat S9 fraction that has been adopted for *in vitro* genotoxicity testing as an exogenous activation system.

Conflict of interest

None.

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