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Regular Article

Polycyclic Aromatic Hydrocarbons Activate CYP3A4 Gene Transcription through Human Pregnane X Receptor

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Summary: Aryl hydrocarbon receptor (AhR) activators have been shown to induce members of the cytochrome P450 (P450) 1 family. Here we demonstrate that the AhR activators induce CYP3A4 through human pregnane X receptor (PXR). AhR activators, polycyclic aromatic hydrocarbons (PAHs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) increased CYP3A4 reporter activity and CYP3A4 mRNA expression in HepG2 cells. The CYP3A4 reporter activity was also increased by treatment with cigarette tar. The increased CYP3A4 reporter activity was clearly knocked down by the introduction of human PXR-small interfering RNA, but not by that of human AhR-small interfering RNA. The CYP3A4 reporter activity enhanced by overexpression of human PXR was further increased by treatment with PAHs and TCDD as well as by treatment with rifampicin. These results suggest that PAHs contained in cigarette smoke induce CYP3A4 in human liver.

Keywords: AhR activator; polycyclic aromatic hydrocarbons; TCDD; CYP3A4 induction; PXR

Introduction

Members of the cytochrome P450 (CYP) supergene family of monooxygenases play an important role in efficient detoxification, by converting pollutants, plant toxins, carcinogens, and drugs to products that can be excreted in urine or bile.[1,2] Human CYP3A4 is of particular significance in this respect because it is involved in the metabolism of approximately two-thirds of clinically relevant drugs.[3] A number of compounds, including pesticides, herbal supplements, vitamins, and drugs, activate *CYP3A4* gene transcription both in the liver and in the small intestine.[4,5] This process of induction constitutes the molecular basis for a number of important drug interactions in patients taking multiple medications.

Pregnane X receptor (PXR; NR1I2) is the principal regulator of *CYP3A4* gene expression and binds as a heterodimer with retinoid X receptor α (RXRα) to regulatory DNA sequences. These sequences include AG(G/T)TCA-like direct repeats spaced by 3 bases (DR3) located at −8 kb upstream from the transcription start point and identified as distal nuclear receptor-binding element 1 (dNR1) and the everted repeats separated by 6 bases (ER6) located in the CYP3A4 proximal promoter (prER6).[6] Recently, we identified a distinct PXR response element as an essential distal nuclear receptor-binding element (eNR3A4) for CYP3A4 induction.[7] PXR is activated by a number of structurally and chemically diverse ligands such as various xenobiotics [rifampicin (RIF) and clotrimazole],[8] pesticides (pyributicarb and endosulfan),[9,10] natural and synthetic

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steroids (dexamethasone),[8] bile acids (lithocholic acid)[11] and herbal medicines (St. John's wort, *Ginkgo biloba*, and *Sophora flavescens*).[12–14] As a result, CYP3A4 induction by these PXR activators leads to the accelerated metabolism of the drugs themselves and the efficacy of concomitant drugs is decreased. Interestingly, there are significant species differences in the activation by PXR ligands between humans and rodents.[15] Drugs such as RIF and clotrimazole activate human PXR but are weak activators of rodent PXR. In contrast, dexamethasone and pregnenolone 16α-carbonitrile activate rodent PXR but are weak activators of human PXR.

Cigarette smoke contains thousands of chemical compounds including a number of carcinogenic polycyclic aromatic hydrocarbons (PAHs) and is known to affect drug therapy in both pharmacokinetic and pharmacodynamic events.[16,17] PAHs induce CYP1A1 and CYP1A2, which mediate the rate-limiting step in the metabolism of many drugs, including theophylline and clozapine, as well as in the bioactivation of procarcinogens. These P450 inductions are also expected to cause drug interaction in this respect. In fact, it was reported that cigarette smoking reduced the therapeutic efficacy of theopylline.[18] The molecular mechanisms by which PAHs or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) transcriptionally activates the *CYP1A1* and *CYP1A2* genes involve the binding of the ligand to aryl hydrocarbon receptor (AhR).[19]

Diazepam (DZ) is a drug that relieves anxiety and controls agitation; it has long been controversial whether cigarette smoking reduces its therapeutic efficacy. Norman *et al.* reported that cigarette smoking strongly decreased the elimination half-life and AUC of DZ, but Klots *et al.* reported no effect.[20,21] DZ is mainly metabolized to 3-hydroxydiazepam and *N*-desmethyldiazepam by CYP3A4 and CYP2C19, respectively. However, there have been no reports about the induction of CYP3A4 and/or CYP2C19 by AhR activators, PAHs or TCDD. Recently, we found a novel event in CYP3A4 induction by AhR activators.

In the present study, we investigated the effect of the AhR activators on transcriptional activation of the *CYP3A4* gene and clearly demonstrated that the AhR activators induced CYP3A4 through PXR pathways in HepG2 cells.

Materials and Methods

Materials: 3-Methylcholanthrene (3-MC) and RIF were purchased from Sigma-Aldrich (St. Louis, MO). Benzo[*a*]-pyrene (B[*a*]P) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). TCDD was purchased from Wako Pure Chemicals (Osaka, Japan). Tar was provided from Japan Tobacco Inc. (Tokyo, Japan). The chemicals used for this study were dissolved in dimethylsulfoxide (DMSO). All other reagents used were of the highest quality available. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Hyclone Laboratories (Logan, UT). MEM Non-Essential Amino Acids and Antibiotic-Antimycotic were purchased from Invitrogen (Paisley, UK).

Cell culture: HepG2 human hepatoma cells and Huh7 human hepatoma cells were obtained from American Type Culture Collection (Manassas, VA). LS174T human colon adenocarcinoma cells were obtained from the Institute of Development, Aging, and Cancer, Tohoku University (Sendai, Japan). HepG2-derived cell lines stably expressing the *CYP3A4*-luciferase reporter gene, 3-1-20 cells, were as reported previously.[22] These cells were cultured in DMEM supplemented with 10% fetal bovine serum, MEM Non-Essential Amino Acids, and Antibiotic-Antimycotic. The cells were seeded at the appropriate cell density for each experimental condition. The next day, the cell medium was exchanged for the medium containing chemicals (0.1% DMSO) and cultured for 48 h. Adenovirus infection and small interfering RNA (siRNA) transfection were carried out as described previously.[23]

Construction of recombinant adenovirus: Construction of the *CYP3A4* gene reporter adenovirus (AdCYP3A4-362-7.7k) and human PXR-expressing adenovirus (AdhPXR) was performed previously.[9] Control adenovirus, β-galactosidase-expressing adenovirus (AdCont; AxCALacZ), was provided by Dr. Izumi Saito (Tokyo University, Tokyo, Japan). The titer of adenoviruses, 50% titer culture infectious dose (TCID50), was determined as reported previously.[9] Multiplicity of infection (MOI) was calculated by dividing the $TCID_{50}$ by the number of cells.

Small interfering RNA-mediated protein knockdown: Double-stranded siRNAs (25-mer) targeting human AhR (hAhR), human PXR (hPXR), and control siRNA were obtained from Invitrogen (Carlsbad, CA). The corresponding target mRNA sequences for the siRNAs were as follows: hAhR-siRNA, 5′-uuaagucggucucuaugccgcuugg-3′; hPXR-siRNA, 5′-uuucaucugagcguccaucagcucc-3′; control siRNA 5′-uagucauugcacacugcacaguagc-3′. Cells were transfected with each siRNA using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions.

Luciferase gene reporter assay: The cells were washed with Dulbecco's phosphate buffered saline (D-PBS) obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and suspended in Passive Lysis Buffer from Promega (Madison, WI) in a microcentrifuge tube. The cell suspension was centrifuged at 12,000 × *g* for 5 min at 4°C, and the cell extract was used for luciferase assay. Luciferase assay was performed according to the manufacturer's instructions using the Luciferase Assay System and GloMax™ 96 Microplate Luminometer (Promega). Resulting data are presented as ratio of luminescence of treated cell samples to that of control. Luminescence of each sample was normalized by its protein concentration determined with the Protein Assay Kit from Bio-Rad Laboratories (Hercules, CA).

Table 1. Sequences of the oligonucleotide primers used for PCR amplification

	Primer sequence
Human CYP3A4	
sense	5′-GATTGACTCTCAGAATTCAAAAGAAACTGA-3′
antisense	5′-GGTGAGTGGCCAGTTCATACATAATG-3′
Human CYP1A1	
sense	5′-ACTGCTTAGCCTAGTCAACCTG-3′
antisense	5′-CAATCAGGCTGTCTGTGATGTC-3′
Human GAPDH	
sense	5′-GAGTCAACGGATTTGGTCGT-3′
antisense	5′-TTGATTTTGGAGGGATCTCG-3′

RNA isolation and quantitative real-time polymerase chain reaction analysis: Total RNA was isolated from 3-1-20 cells using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. cDNA was prepared from 0.5 μg of total RNA with MMLV reverse transcriptase (Promega) using oligo (dT) primer (Greiner Japan, Tokyo, Japan) and porcine RNase inhibitor (Takara Bio, Shiga, Japan). Quantitative real-time polymerase chain reaction (PCR) was performed using Permix Ex Taq (Perfect Real Time, Takara Bio) in a Thermal Cycler Dice Real Time System (Takara Bio). All samples were quantified using a comparative Ct method for relative quantification of gene expression, normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequences of primers are shown in **Table 1**.

Statistical analysis: Data were evaluated by the paired Student's *t*-test. When the p value was less than 0.05, the difference was considered to be significant.

Results

Transcriptional activation of the CYP3A4 reporter gene by tar: We investigated whether tar as a major component of cigarette smoke activates the CYP3A4 transcription using HepG2-derived cells stably expressing the *CYP3A4* gene reporter (3-1-20 cells) as reported previously.[22] The cells were treated with 1 to 200 μg/ml tar for 48 h and reporter activities were determined. As shown in **Figure 1**, tar increased *CYP3A4* gene reporter activity in a dose-dependent manner. In contrast, nicotine, which is one of the major components of cigarette smoke, had no effect (data not shown).

Transcriptional activation of the CYP3A4 reporter gene by PAHs: Tar, a by-product of the gasification process, is a complex mixture consisting of a wide range of different compounds in which PAH is one of the major compounds.[24] Therefore, we examined the effect of typical PAHs and TCDD on CYP3A4 reporter activity, which are known as ligands for AhR. RIF was used as a positive control. 3-1-20 cells were treated with RIF (10 μM), 3-MC (1 μM), B[*a*]P (1 μM), and TCDD (10 nM) for 48 h. As shown in **Figure 2**, RIF, 3-MC, B[*a*]P, and TCDD increased CYP3A4 reporter activity by 18.6 ± 2.5-fold,

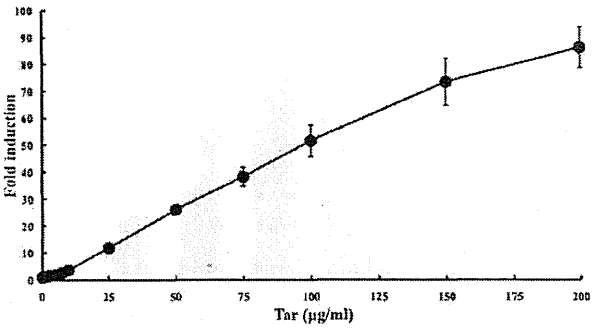

Fig. 1. Effect of tar on CYP3A4 reporter activity in 3-1-20 cells
Clone 3-1-20 was seeded at 1 × 10^4 cells in 96-well tissue culture plates with 0.1 ml of DMEM 1 day before tar treatment. The cells were treated with 1–200 μg/ml tar for 2 days and the reporter activity was measured by luciferase assay. Reporter activities are expressed as fold increase compared with that in the vehicle-treated cells. Results represent the mean ± S.D. of three separate experiments.

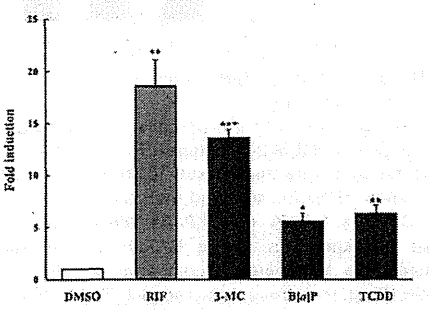

Fig. 2. Effects of 3-MC, B[a]P and TCDD on CYP3A4 reporter activity in 3-1-20 cells
Clone 3-1-20 was seeded at 3 × 10^4 cells in 48-well tissue culture plates with 0.2 ml of DMEM 24 h before RIF, 3-MC, B[*a*]P, or TCDD treatment. The cells were treated with 10 μM RIF, 1 μM 3-MC, 1 μM B[*a*]P, or 10 nM TCDD for 48 h and the reporter activity was measured by luciferase assay. Reporter activities are expressed as fold increase compared with that in the vehicle-treated cells. Results represent the mean ± S.D. of three separate experiments. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.005$, difference from the vehicle-treated cells based on unpaired Student's *t*-test.

13.6 ± 0.8-fold, 5.57 ± 0.8-fold, and 6.3 ± 0.8-fold, respectively. Furthermore, we investigated the effect of PAHs on endogenous CYP3A4 mRNA expression in 3-1-20 cells. As a result, all chemicals used in this study increased CYP3A4 mRNA expression (**Fig. 3A**). The CYP3A4 mRNA expressions of 3-MC and B[*a*]P were higher than that of RIF. On the other hand, CYP3A4 mRNA expression induced by TCDD was equivalent to that by RIF. In addition, 3-MC, B[*a*]P, and TCDD strongly increased endogenous CYP1A1 mRNA expression, whereas RIF had no effect (**Fig. 3B**). In particular, 3-MC and TCDD increased CYP1A1 mRNA expression by 365.7 ± 39.3-fold and 378.3 ± 37.0-fold, respectively. These results suggest that PAHs induce

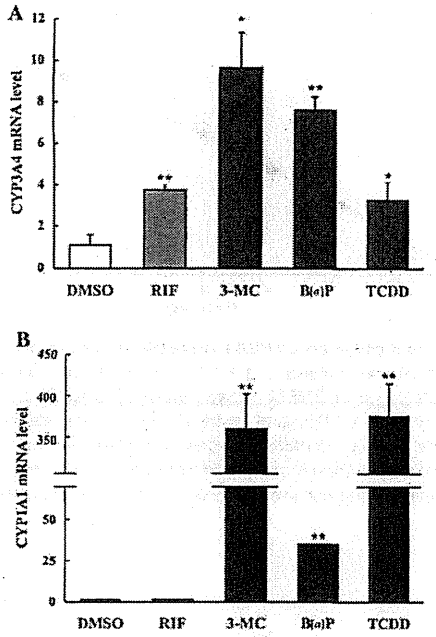

Fig. 3. Effects of 3-MC, B[a]P and TCDD on CYP mRNA expression in 3-1-20 cells

Clone 3-1-20 was seeded at 3×10^4 cells in 48-well tissue culture plates with 0.2 ml of DMEM 24 h before RIF, 3-MC, B[*a*]P, and TCDD treatment. The cells were treated with 10 μM RIF, 1 μM 3-MC, 1 μM B[*a*]P, or 10 nM TCDD for 48 h and then assayed using real-time RT-PCR. CYP3A4 mRNA or CYP1A1 mRNA expression was normalized by expression of the GAPDH housekeeping gene and presented as fold increase compared with that of vehicle-treated cells. Results represent the mean ± S.D. of three separate experiments. (A) CYP3A4 mRNA level, (B) CYP1A1 mRNA level. *, $p < 0.05$, **, $p < 0.005$, difference from the vehicle-treated cells based on unpaired Student's *t*-test.

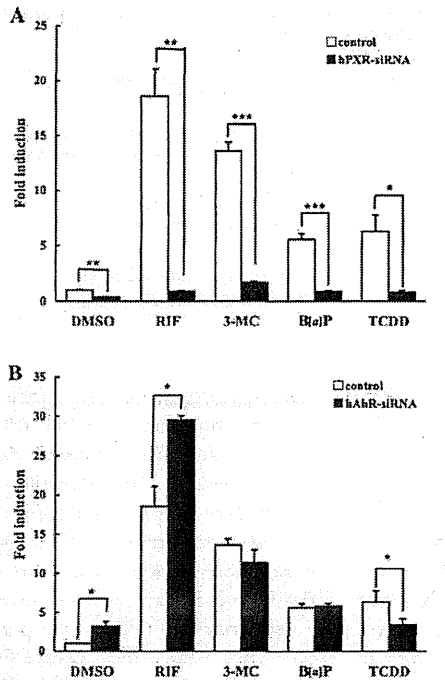

Fig. 4. Effects of hPXR-siRNA and hAhR-siRNA on CYP3A4 reporter activity in 3-1-20 cells

Clone 3-1-20 was seeded at 3×10^4 cells in 48-well tissue culture plates and the cells were treated with hPXR-siRNA (A) or hAhR-siRNA (B). After 48 h, the cells were treated with 10 μM RIF, 1 μM 3-MC, 1 μM B[*a*]P, or 10 nM TCDD for 48 h and the reporter activity was measured by luciferase assay. Reporter activities are expressed as fold increase compared with that in the vehicle-treated cells transfected with the control siRNA. Results represent the mean ± S.D. of three separate experiments. *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.005$, difference from the control siRNA-transfected cells based on unpaired Student's *t*-test.

CYP3A4 expression in 3-1-20 cells. In addition, these compounds also increased CYP3A4 mRNA expression in HepG2 cells (data not shown).

Effects of PXR and AhR on activation of CYP3A4 reporter gene by PAHs: PXR is widely known as a major transcription factor mediating CYP3A4 induction, whereas AhR is well known as a transcription factor mediating CYP1A1 induction by PAHs. Therefore, to clarify whether PXR or AhR mediates the CYP3A4 activation by PAHs, we utilized knockdown of PXR or AhR using hPXR-siRNA or hAhR-siRNA and overexpression of PXR using AdhPXR, hPXR-expressing adenovirus. CYP3A4 reporter activities induced by 3-MC, B[*a*]P, and TCDD were significantly decreased by the introduction of hPXR-siRNA (**Fig. 4A**). In contrast, knockdown of the AhR using hAhR-siRNA did not affect the CYP3A4 reporter activity induced by 3-MC or B[*a*]P, whereas CYP3A4 reporter activities induced by TCDD were slightly decreased by the introduction of hAhR-siRNA (**Fig. 4B**). Then, we examined the effect of overexpression of PXR using AdhPXR on CYP3A4 reporter activity induced by PAHs in 3-1-20 cells. As shown in **Figure 5**, we found that the CYP3A4 reporter activation treated with 3-MC, B[*a*]P, and TCDD as well as RIF was increased by the introduction of AdhPXR in 3-1-20 cells.

Effect of PAHs on CYP3A4 reporter activity in Huh7 cells and LS174T cells: Next, we investigated whether the PAHs induce CYP3A4 reporter activity in different cell lines. Huh7 cells and LS174T cells were used, which are *in vitro* models of hepatic and intestinal cells, respectively. As shown in **Figure 6**, the CYP3A4 reporter activation upon treatment with 3-MC, B[*a*]P, and TCDD as well as RIF was increased by the introduction of AdhPXR in Huh7 cells. On the other hand, RIF increased CYP3A4 reporter activity, whereas 3-MC, B[*a*]P, and TCDD had no effect on the activity in LS174T cells.

Discussion

In this study, we investigated the effect of PAHs on transactivation of the *CYP3A4* gene using 3-1-20 cells. 3-MC

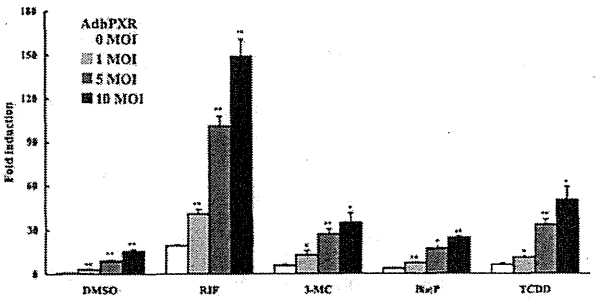

Fig. 5. Effect of AdhPXR on CYP3A4 reporter activity in 3-1-20 cells

Clone 3-1-20 was seeded at 3×10^4 cells in 48-well tissue culture plates pre-incubated for 24 h and then treated with AdhPXR (MOI of 0, 1, 5, 10). Forty-eight hours after infection, these cells were treated with 10 μM RIF, 1 μM 3-MC, 1 μM B[*a*]P, or 10 nM TCDD for 48 h and the reporter activity was measured by luciferase assay. Reporter activities are expressed as fold increase compared with that in the vehicle-treated cells uninfected with AdhPXR. Results represent the mean ± S.D. of three separate experiments. *, $p < 0.05$, **, $p < 0.005$, difference from the uninfected cells based on unpaired Student's *t*-test.

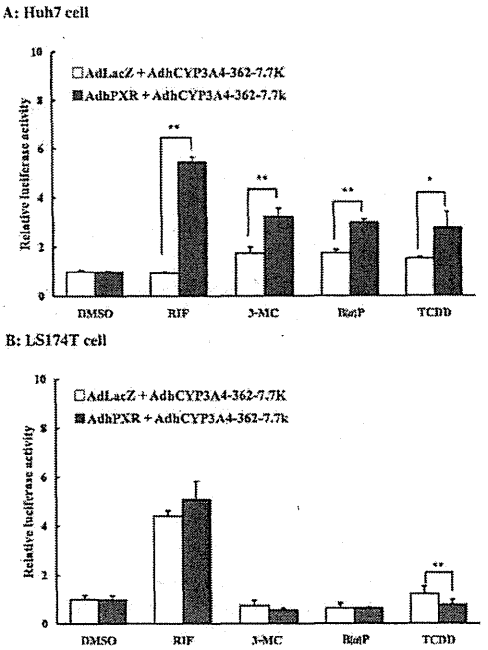

Fig. 6. Effect of AdhPXR on CYP3A4 reporter activity in Huh7 cells and LS174T cells

Huh7 cells (A) and LS174T cells (B) were seeded at 2×10^4 cells in 24-well tissue culture plates pre-incubated for 24 h and then were treated with AdLacZ (10 MOI) or AdhPXR (10 MOI) and Ad-CYP3A4-362-7.7k (5 MOI). Forty-eight hours after infection, these cells were treated with 10 μM RIF, 1 μM 3-MC, 1 μM B[*a*]P, or 10 nM TCDD for 48 h and the reporter activity was measured by luciferase assay. Reporter activities are expressed as fold change compared with that in the vehicle-treated cells. Results represent the mean ± S.D. of three separate experiments. *, $p < 0.05$, **, $p < 0.005$, difference from the corresponding AdLacZ-infected cells based on unpaired Student's *t*-test.

and B[*a*]P, which are typical PAHs, activated the *CYP3A4* reporter gene (**Fig. 2**). The expression of endogenous CYP3A4 mRNA was also induced by PAHs in 3-1-20 cells (**Fig. 3**). These results indicate that PAHs are potent inducers of CYP3A4. However, the CYP3A4 induction profiles by RIF and PAHs were different between CYP3A4 reporter activity and CYP3A4 mRNA expression level. It is reported that the distal promoter region, from −7836 nt to −7200 nt, and the proximal promoter region, from −362 nt to +11 nt, of the *CYP3A4* gene play important roles for CYP3A4 induction.[25] Recently, Matsumura *et al.* discovered that a novel enhancer region from −11.4 to −10.5 kb, designated the constitutive liver enhancer module of CYP3A4 (CLEM4), is involved in the constitutive activation of the *CYP3A4* gene in HepG2 cells.[26] Furthermore, Liu *et al.* identified a functional nuclear receptor responsive element (F-ER6 at −11368 to −11351) in Huh7 cells.[27] Taking these reports together, for intrinsic CYP3A4 induction, all these *cis*-elements might be required in human liver. However, the cell line 3-1-20 was established by Noracharttiyapot *et al.* by insertion of the luciferase reporter plasmid, pGL3-CYP3A4-362-7.7k, which includes both −7836 to −7200 of the distal promoter region and −362 to +11 of the proximal promoter region of the *CYP3A4* gene, the elements reported to be necessary for high response to RIF, into HepG2 cell chromosome.[22] Therefore, the differences of these CYP3A4 activation patterns by RIF and PAHs might be attributable to other enhancer regions not included in pGL3-CYP3A4-362-7.7k. In addition, CYP1A1 plays critical roles in the metabolism of PAHs.[28] Thus, 3-MC and B[*a*]P were initially metabolized by CYP1A1 induced through AhR activation in this culture condition. Subsequently, the metabolites of 3-MC and B[*a*]P might cause CYP3A4 induction. On the other hand, the induction profile was different among AhR ligands. Although CYP3A4 induction potency is similar between 3-MC and B[*a*]P, the CYP1A1 induction potency of B[*a*]P was far lower than that of 3-MC. This result may suggest that AhR is not directly involved in the *CYP3A4* gene activation by 3-MC and B[*a*]P.

CYP3A4 induction by xenobiotics and hormones is mediated by PXR,[29–31] constitutive androstane receptor (CAR, NR1I3),[32] vitamin D receptor (VDR, NR1I1),[33] and glucocorticoid receptor-α (GRα, NR3C1)[34] in the liver. In particular, chemical-induced activation of the *CYP3A4* gene is mainly mediated by PXR through binding to the *CYP3A4* 5′-flanking region,[29,31,35] whereas PAHs cause transactivation of *CYP1A1* and *1A2* genes *via* AhR.[19] Therefore, we knocked down hPXR expression using hPXR-siRNA. When hPXR-siRNA was introduced into the 3-1-20 cells, the activation of the *CYP3A4* reporter gene by PAHs as well as RIF was significantly decreased (**Fig. 4A**), whereas the knockdown of AhR by hAhR-siRNA had no effect on the activation of the *CYP3A4* reporter gene by RIF and PAHs (**Fig. 4B**). The same results were observed when hPXR-

siRNA was introduced by adenovirus (data not shown). Furthermore, the overexpression of PXR using AdhPXR increased CYP3A4 reporter activity by PAHs as well as RIF in an infection dose-dependent manner (**Fig. 5**). Therefore, these results strongly suggest that 3-MC and B[*a*]P enhance the transactivation of the *CYP3A4* gene through PXR activation but not through AhR activation. On the other hand, knockdown of AhR by hAhR-siRNA decreased the CYP3A4 reporter activity by TCDD (**Fig. 4**). This reason for this is unclear. The *CYP3A4* gene might be transactivated partially by TCDD through the AhR pathway owing to its strong AhR activation potency. Further studies are needed to resolve this issue.

It is known that the drug-induced expression of the *CYP3A4* gene in the liver is predominantly regulated through PXR.[36] In contrast, VDR controls the CYP3A4 transactivation with the secondary bile acid, lithocholic acid (LCA), in the intestine.[37,38] In addition, recently, a novel PXR functional *cis*-acting PXR-binding element designated eNR3A4 has been discovered to be an essential element for RIF-inducible CYP3A4 transactivation in human liver.[7] This element is located approximately 7.6 kb upstream from the transcription initiation site of the *CYP3A4* gene, to which hPXR binds as a heterodimer with human RXRα. Furthermore, Pavek *et al.* reported that eNR3A4 has negligible or no effect on CYP3A4 transactivation through VDR.[39] Thus, these findings indicate that eNR3A4 is a key regulatory element for the xenobiotic induction of CYP3A4 through hPXR in the liver. In this study, we show that PAHs increased CYP3A4 reporter activity in PXR-overexpressing hepatoma cells, whereas this phenomenon was not observed in PXR-overexpressing intestinal cells (**Fig. 6**). These results indicate that eNR3A4 might have been involved in the activation of the *CYP3A4* gene through hPXR by PAHs in the liver. Since the difference of the HepG2 cells and the LS174T cells in an induction mechanism of the *CYP3A4* gene by PAHs is unknown, further analysis is still in progress.

Numerous studies of induction by treatment with PAHs have been carried out to date. PAHs as well as TCDD have been believed to strongly induce CYP1A1 and CYP1A2 through the AhR pathway. However, there are no data about CYP3A induction by them in experimental animals. Recently, it was reported that 3-MC induces CYP3A4 in HepG2 cells.[40] In addition, when the reporter assay was measured in pGL3-CYP3A4-362-7.7k-transformed rat hepatoma Reuber H35 cells, PAHs and TCDD did not increase the reporter activity (data not shown). Together with these results, it is suggested that PAHs and TCDD activate human PXR, but not rat or mouse PXRs. Further studies are needed to clarify this issue.

In conclusion, we have demonstrated that PAHs activate *CYP3A4* gene transcription through the activation of hPXR in HepG2 cells. Thus, PAHs may contribute to CYP3A4 induction in human liver. More detailed study on the molecular mechanism behind the CYP3A4 induction by PAHs may provide important information on the drug-drug interaction.

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鉄存在下 HepG2 細胞における TNF-α/actinomycin D 処理による肝障害モデルの構築

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TNF-α/actinomycin D-mediated HepG2 cells in the presence of iron as a model of hepatocyte injury

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We examined the contribution of iron to the cytotoxicity of tumor necrosis factor (TNF)-α combined with actinomycin D (ActD) as a model of hepatocyte injury in HepG2 cells. In general, hepatocytes are resistant to TNF-α. However, a transcriptional inhibitor such as ActD can sensitize then to TNF-α. In the present study, we show that low levels of ActD (0.5 nM) sensitized HepG2 cells to the cytotoxic effects of TNF-α (20 ng/mL) for 48 h. Iron plays a critical role in catalyzing the formation of potent oxidants. To assess the toxicological significance of this TNF-α/ActD interaction, ferric-nitrilotriacetate (Fe-NTA, 2 μM) was added to the cells. Treatment with Fe-NTA significantly increased the sensitivity to the TNF-α/ActD-mediated cell death. TNF-α/ActD-mediated cell death in the presence of a lower concentration of iron did not result in DNA fragmentation. We suggest that iron increased the sensitivity to the cytotoxicity of TNF-α/ActD in HepG2 cells. It is likely that TNF-α/ActD/Fe-NTA-mediated cell death contributes to the non-apoptotic death of cells via oxidative stress caused by iron. Our experimental model may be useful for studying hepatic drug metabolism using TNF-α as a model of hepatocyte injury, especially in HepG2 cells.

Key words —— Tumor necrosis factor, cytotoxicity, actinomycin D, oxidative stress, iron

緒　　言

創薬において，医薬品候補化合物の開発中止における重大な要因は，ヒトに対する効能不足および毒性発現とされている．[1] すなわち，非臨床試験で有効とされていた新薬候補化合物が，臨床試験において有効性が認められない，または強い副作用により開発中止を余儀なくされている．[2-4] その中でも，薬物暴露により肝臓に生じる毒性（薬物性肝障害）は，非常に高頻度で発現する副作用である．[5] 実際，薬物性肝障害は開発中止，警告あるいは販売中止に至る主要な薬剤関連有害事象となっている．[6] 薬物性肝障害は非臨床試験および市販後試験のいずれの段階においても発現し得るため，肝障害ポテンシャルおよび肝障害機序を可能な限り早期かつ正確に評価し，リスク・ベネフィット分析等を通じて，医薬品開発に関する go/no-go の決定は，新薬の生産性に大きく貢献すると考えられる．近年の実験動物から得られた知見として，穏やかな炎症状態が毒性への閾値を低下させ，副作用への個々の感受性を上昇させるとの報告があり，[7] 肝障害に対する炎症反応の寄与が注目されている．また，Wei らにより，非ステロイド性抗炎症薬（NSAIDs）のスリンダクが，中用量の Lipopolysaccharide（LPS）供処理により肝障害が誘発されるとの報告がなされた．[8,9] 加えて，そのスリンダク誘発性肝障害は，tumor necrosis factor (TNF)-α により増強されることが示唆されている．TNF-α は本来，生体防御機構に関わる炎症性サイトカインであり，肝において炎症性変化の中心的役割を演じている．一方で，近年の TNF-α による肝細胞死誘導のメカニズムの研究により，この TNF-α は肝細胞の TNF-α 受容体に結合し reactive oxygen species（ROS）産生とともに c-jun N-terminal kinase（JNK）経路を活性化し，炎症を起こした肝細胞の細胞死を誘導し，炎症を収束させると考えられている．[10] これは，活性化された JNK が，caspase-8 に対する阻害作用をもつ cellular FLICE-inhibitory protein（c-Flip）を抑制し引き起こされると考えられている．[10] しかしなが

ら，*In vitro*では多くの細胞がTNF-αの細胞死誘導に対して耐性であり，特に肝細胞や肝がん細胞においては顕著である．近年の研究から，それには，nuclear factor-kappa B（NF-κB）によるJNK経路の抑制が寄与しており，[11] また，NF-κBの発現量をノックダウンした細胞では，少量のTNF-αでも細胞死が起こることが知られている．[12] 従って，薬物性肝障害を起こしやすいモデル細胞を樹立するには，NF-κB抑制によるJNK経路の感受性を高めることが必要である．一方，転写阻害剤であるactinomycin D（ActD），D-galactosamineおよびα-amanitinの存在下で，TNF-αによる細胞死が誘導されることが明らかとなっている．[13] また，酸化ストレスの誘導が細胞死に重要であることが報告されているが，[14] 近年TNF-α刺激による細胞死においても，活性酸素による細胞シグナル制御（ROSシグナル）が関与することが明らかになってきている．[15] そこで本稿では*in vitro*における薬物性肝障害評価系の構築を目的として転写阻害剤ActDおよび酸化ストレス誘発剤鉄ニトリロ三酢酸（Fe(Ⅲ)-nitrilotriacetic acid，Fe-NTA）を肝モデル細胞として汎用されるHepG2細胞へ添加し，TNF-α誘導性肝細胞障害モデルの構築を試みた．

実験方法および実験材料

1．細胞培養

HepG2細胞は，東北大学加齢医学研究所医用細胞資源センターより供与されたものを用いた．この細胞を10% fetal calf serum（FCS, WAKO社製）およびAntibiotic-Antimycotic［100 U/mL penicillin G sodium, 100 μg/mL amphotericin B（Invitrogen社製）］，0.45% glucose，2 mM-glutamine含有Dulbecco's modified Eagle's medium，（DMEM, WAKO社製）中で，5% CO_2-95% airを気相とし，37℃でインキュベーションを行った．

2．Fe-NTA調製

Awaiらの方法に従った．[16] 硝酸鉄（Ⅲ）九水和物（WAKO社製）は1 M HCl溶液を用いて50 mMに調整した．また，NTA（Nacalai tesque社製）は1M NaOH溶液（Nacalai tesque社製）を用いて150 mMに調整した．50 mM硝酸鉄（Nacalai tesque社製）および150 mM NTAを1:3で混合し，$NaHCO_3$を用いてpH 7.4に合わせた後，0.45 μm membrane filterでろ過滅菌し，Fe-NTAとし使用した．

3．MTT assay

細胞毒性試験はMTT試薬［3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide］，Nacalai tesque社製）を用いて，同社のプロトコールに従って行った．細胞を96 well-plate（Becton, Dickinson社製）に2×10^4 cells/wellの密度で播種し，CO_2インキュベーター内で24時間前培養した後，ActDを処置し，30分後TNF-α，さらに1時間後Fe-NTAを細胞に処理した．48時間培養後，MTT溶液を各wellに10 μLずつ添加し，定色反応を4時間行った．可溶化液（0.04 M塩酸を含むイソプロピルアルコール）を100 μL添加し，沈殿したホルマザンをピペッテイングにより可溶化し，その後マイクロプレートリーダー（TOSOH社製）を用いて570 nmの吸光度測定を行った．

4．アガロースゲル電気泳動法によるDNA断片化の検出

100 mm培養ディッシュで培養した細胞を回収し，滅菌済みDulbecco's phosphate-buffered saline（DPBS, WAKO社製）200 μLに浮遊させ，1.5 mLチューブに移した．遠心分離（250×g, 10分間）後上清を取り除き，細胞ペレットに細胞溶解バッファー［0.1 M Tris-HCl, 0.1 M-2-{2-[bis(carboxymethyl) amino]ethyl} (carboxymethyl) amino) acetic acid (EDTA)，5% Triton］100 μLを加え，細胞を溶解させ，DNA断片を抽出した．4℃，10分間放置後，15,000 rpm，5分間遠心分離し，上清を新たな1.5 mLチューブに取り，TEバッファー300 μL，フェノール/クロロホルム（Nacalai tesque社製）400 μL加え再度，遠心分離（15,000 rpm，5分間）した．上清を新たな1.5 mLチューブに取り，Ribonuclease A（RNase A, Invitrogen社製）溶液を1 μL加え，37℃，1時間温置した後，proteinase K（Nacalai tesque社製）溶液を8 μL加え，20℃で一晩放置した．遠心分離後（15,000 rpm，15分間），上清を除去し，70% エタノール1 mL加え，再度遠心分離した．上清を除去後TEバッファー20 μL添加し，1.2% アガロースゲル電気泳動を行った．

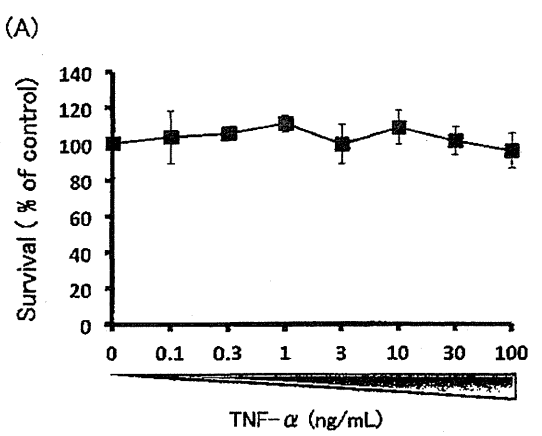
5．統計学的解析

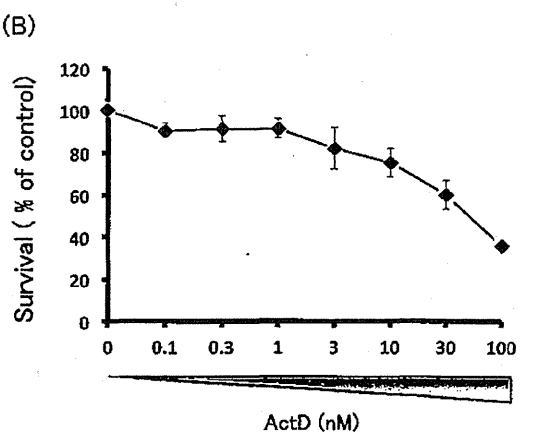
得られた実験値は平均値±標準偏差（mean±S.D.）で示し，比較検定にはStudentのt検定を行った．統計学的有意差は危険率1%を基準として判定した．

結　　果

1．TNF-α (A)，ActD (B)，Fe-NTA (C) 単独処理での HepG2 細胞への細胞毒性

我々はまず，TNF-α，ActD，および Fe-NTA 単独処置による細胞毒性を検討した．HepG2 細胞において，TNF-α 単独添加 (0.1～100 ng/mL) の 48 時間後では有意な細胞毒性を示さなかった (Fig. 1A)．また，HepG2 細胞は TNF-α 誘発性肝障害に対する耐性を示し，それは ActD 等により減少することが知られている．[17] そこで，ActD を 0.1～100 nM の範囲で処理し，48 時間後の細胞毒性を検討した．その結果，Fig. 1B に示すように量依存性で細胞毒性が増大し，100 nM の ActD では生存率は 36%に低下した．一方，0.1～1 nM Act D 処置群では細胞毒性は示さなかった．さらに，我々は酸化ストレスモデルを構築する目的で代表的な遷移金属である鉄を導入して検討を行った．一般に鉄イオン単独での細胞内導入率は低いことから，導入効率の良い Fe-NTA を使用し，HepG2 細胞にそれを 0.5～150 μM の範囲で曝露した．48 時間で 0.5～10 μM では有意な細胞毒性は認められなかったが，50～150 μM では有意な毒性が認められた (Fig. 1C)．





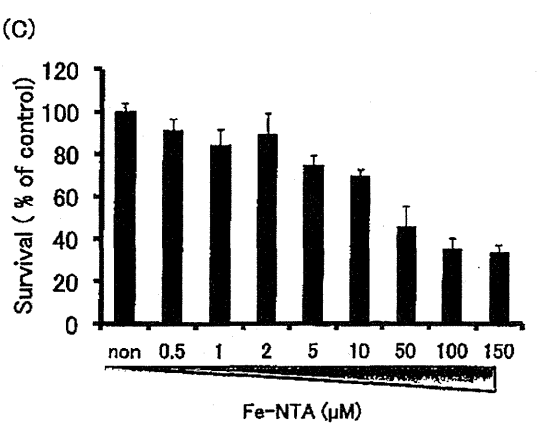


Fig. 1. Changes in cell viability of the treatments with TNF-α (A), ActD (B) and Fe-NTA (C) in HepG2 cells.

These cells were preincubated for 24 h with DMEM. After the addition of TNF-α (0.1～100 ng/mL), ActD (0.1～100 nM) and Fe-NTA (0.5～150 μM) to the culture medium, the cells were incubated for 48 h. The cell survival was measured by the MTT assay as described in materials and methods. Data represent the means ± SD (n = 4 experiments).

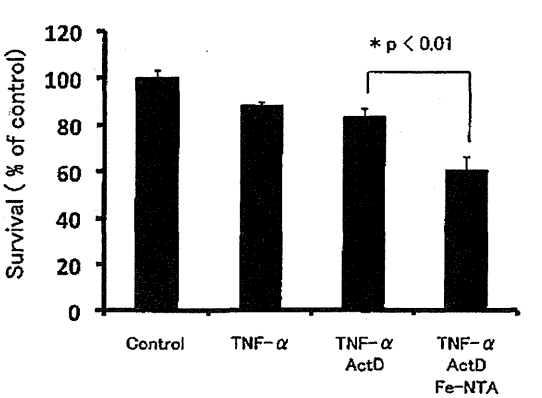


Fig. 2. Effect of iron on TNF-α/ActD-induced cytotoxicity in HepG2 cells.

These cells were incubated with TNF-α (20 ng/mL) combined with ActD (0.5 nM) for 48 h in the presence of Fe-NTA (2 μM). Cell survival was measured by the MTT assay as described in materials and methods. The surviving fraction was determined by dividing the absorbance of treated cells by that of control cells. A: Control, B: TNF-α, C: TNF-α/ActD, D: TNF-α/ActD/Fe-NTA. Data represent the mean ± SD (n = 4 experiments). *$p < 0.01$, compared to cells treated with TNF-α /ActD (Student's t-test).

2．鉄存在下での ActD-感受性 HepG2 細胞における TNF-α の細胞毒性

本研究において，ActD (0.5 nM)，Fe-NTA (2 μM) では HepG2 細胞における細胞毒性は認められなかったことから，TNF-α (20 ng/mL) /ActD (0.5 nM) /Fe-NTA (2 μM) の低添加量を選択し，48 時間後での鉄存在下 TNF-α /ActD による HepG2 細胞における細胞毒性を検討した．ActD は TNF-α 処理 30 分前に細胞に添加した．その 1 時間後に Fe-NTA を処理した (Fig. 2)．その結果，TNF-α /ActD では無処置細胞に比べ細胞生存率は 82% であるが，鉄存在下での TNF-α /ActD/Fe-NTA 群においては顕著な細胞死が観察され，細胞生存率は 60%まで低下した (Fig. 2)．

3．HepG2 細胞での TNF-α /ActD/Fe-NTA 処理による DNA の断片化

上記に示すように鉄存在下 TNF-α /ActD が有意な細胞死を認めたため，この肝細胞死の形態がアポトーシスあるいはネクローシスかについて検討した．

本研究においてアガロースゲル電気泳動法で DNA 断片化に基づく細胞死の判定を行ったところ，アポトーシス陽性対照として用いた 10 μM camptothecin 処置群においては，ラダーリングは

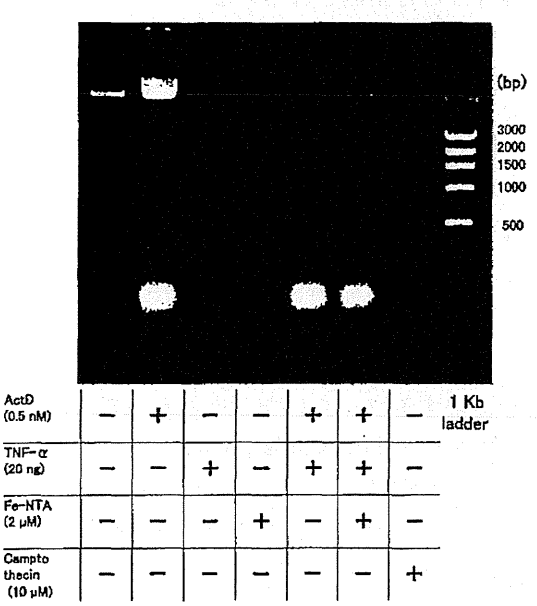


Fig. 3. Effects of TNF-α/ActD/Fe-NTA-treated HepG2 cells on DNA laddering.

These cells were incubated with TNF-α (20 ng/mL) combined with ActD (0.5 nM) for 48 h in the presence of Fe-NTA (2 μM). DNA was prepared from supernatant of 15,000 rpm of cell homogenates and electrophoresed as described in materials and methods. DNA fragments were used as molecular markers. DNA laddering was absent in control cells.

認められたが，低濃度 TNF-α /ActD/Fe-NTA 処置群においては整数倍のラダーパターン (180-200 bp) は見られなかった．この結果より，本鉄存在下での TNF-α /ActD 誘導細胞死はネクローシス様の細胞死であることが示唆された (Fig. 3)．

考　　察

TNF-α は腫瘍部位に出血壊死を誘導する因子として見いだされたが，現在では炎症での生体防御機構に広く関わる炎症性サイトカインとして理解されている．[4] 肝細胞においても，炎症反応を惹起するサイトカインとして中心的な役割を演じ，生体の恒常性の維持に重要な役割を果たしている．また，TNF-α は，過剰に生産されると肝障害を誘発することから，TNF-α は肝障害 mediator とも考えられている．[23] 我々は，薬物性肝障害モデル細胞として，薬物代謝や肝毒性研究に汎用されるヒト肝癌由来細胞株の HepG2 細胞を使用してきた．しかしながら，この細胞は NF-κB による JNK 経路の抑制を示すことが知られているため，転写阻害剤である ActD および酸化ストレス誘発材である Fe-NTA により，TNF-α 誘発性肝障害に感受性を持たせることを試みた．ActD は，転写阻害剤として知られており，*in vivo*, *in vitro* でアポトーシスを誘導する．[18] Leist らは [13] HepG2 細胞への ActD 333 nM，24 時間処理で TNF-α の感受性増大を報告しているが，Fig. 1B に示すように著者らの検討ではこれらの ActD 濃度では細胞障害が強く不適であったため，0.5 nM 処置を行った (Fig. 1B)．また，肝障害においては，活性酸素による酸化ストレスが起こることが知られており，[24] 2 価のフリー鉄は Fenton/Haber-Weiss 反応により有毒なヒドロラジカル (·OH) を生じ酸化ストレスを生起する．[19] HepG2 細胞へ Fe-NTA を添加しない ActD/TNF-α に比べ，Fe-NTA 添加群では，明らかに TNF-α による細胞障害が認められた (Fig. 2)．これらの結果から 2 価鉄と ActD が TNF-α 誘導細胞に対する感受性因子に重要な役割を演じることが示された．

スリンダク/LPS 誘導性肝障害などの炎症反応が関与する薬物性肝障害において，TNF-α が媒介する経路は重要な役割を担っている．[9] それは，TNF-α による death receptor を介した経路の他に JNK 活性化経路，さらに NF-κB 活性化によるアポ

トーシス抑制経路が主なものである．一般に，肝細胞における TNF-α の細胞死に対する耐性は，NF-κB の肝細胞と炎症細胞における作用の違いに起因すると考えられている．すなわち，NF-κB は，肝細胞においては TNF-α 誘導性の細胞死シグナルに対して拮抗する生存シグナルを伝えるが，一方，炎症細胞においては，TNF-α の産生を誘導する二面性をもつことから説明されている．本モデルにおいては，ActD により NF-κB 経路が阻害され，細胞死が誘導された可能性が示唆される．

また，細胞死は形態学的にアポトーシスとネクローシスに区別され，TNF-α による肝障害においても，肝細胞ではアポトーシスおよびネクローシスが関与し細胞死を誘導することが報告されている．[13,20] 従って，その細胞死を識別することは，その原因を探索する上で重要な手がかりとなる．DNA 断片化（DNA fragmentation）は，アポトーシスの生化学的指標とされており，通常，アポトーシスが生じた細胞より抽出した DNA をアガロースゲル電気泳動で泳動後，180～200 bp の整数倍の“ラダー（ladder）”として検出されている．[21] 一方，ネクローシスによる DNA の断片化はアポトーシスに比べて少ないのが特徴である．ActD はアポトーシスを誘導するが，Kleeff らは ActD 100～1000 ng/mL では DNA 断片化が認められるが，10 ng/mL では認められないと述べている．[22] 本条件下 ActD は 0.5 nM を使用しているため，この ActD の濃度ではアポトーシスを誘導しないと考えられた．実際，この条件下での TNF-α /ActD/Fe-NTA 添加の細胞死誘導は，アポトーシスの特徴である DNA の断片化は見られず，非アポトーシスによることを示唆した（Fig. 3）．スリンダクなどの薬物による TNF-α を媒介した肝障害においても，その細胞死は，主にネクローシスであることが示唆されている．[8] また，そのような薬物性肝障害は，ROS の発生が重要な因子となることが報告されている．[24,25] そこで，我々の肝障害モデルでは，Fe-NTA 添加により，細胞内の H_2O_2 を反応性の強い·OH に変換させ（Fenton 反応），ROS の産生能を増加させている．これにより，薬物およびその代謝物により産生された ROS に対する感受性が増大し，薬物性肝障害を感度よく予測することができると考えられる．

これまでカスパーゼを介するアポトーシスが分子生物学的および生化学的に解明されてきており，ネクローシスは偶発的なものと考えられてきた．しかし，近年，TNF-α による細胞死が細胞の種類によってアポトーシスではなく，ネクローシス様の細胞死を誘導することが知られており，[26,27] “偶発的でない制御されたネクローシス”の存在が示唆されていた．その分子機構は全く不明であったが，最近，TNF-α によって誘導されるネクローシス様の細胞死は RIPK1 および RIPK3（RIPK; receptor-interacting protein kinase）というキナーゼに依存性であることが明らかとなり，[27] ネクローシスもアポトーシスと同様に高度に遺伝子によって制御されたプログラムネクローシス（necroptosis）の存在が認知されるようになった．TNF-α 受容体の活性化は細胞死および細胞生存という相互に排他的な 2 つのシグナル伝達経路を誘発するが，[28] 前者はさらに，カスパーゼに依存性のアポトーシスと RIPK に依存性のネクローシスという 2 つの形態の細胞死につながるシグナル伝達経路に分岐する．近年，薬物性肝障害における TNF-α など炎症性サイトカインの寄与が示唆されているが，[6] このシグナル伝達経路の均衡の崩れが肝障害に寄与する可能性が考えられる．また，酸化ストレスにおいても，その強弱によりアポトーシスあるいはネクローシスが誘導されると推測されている．[29] ヒト白血病 T 細胞株である Jurkat 細胞において，H_2O_2 は 0.7 μM 以下では細胞増殖に作用し，1～3 μM 以下ではアポトーシス，そして 3 μM 以上ではネクローシスを生じることが報告されている．[30] 薬物性肝障害においても，ネクローシスとアポトーシスの発生の差異は，酸化ストレスによっても担われると推測される．すなわち，TNF-α および酸化ストレスに対して，高感受性である TNF-α /ActD/Fe-NTA による本モデルは，薬物性肝障害モデルとして有用な情報を提供するツールになると考えられる．更なる検討により，薬物性肝障害の病態をより正確に評価できる系が確立されれば，創薬において，医薬品の開発プロセスにおける精度良い予測を可能にし，創薬シーズのヒット率の上昇に寄与できると考えられる．

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Regular Article

Evaluation of Human Embryonic Stem Cell-derived Hepatocyte-like Cells for Detection of CYP1A Inducers

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: There is a great deal of interest in differentiation of human embryonic stem cells (hESCs) into hepatocyte-like cells for application in pharmaceutical screening. Cytochrome P450 (CYP) 1A is involved in the metabolic activation of procarcinogenic compounds as well as in detoxification of drugs. We differentiated hESCs into hepatocyte-like cells (hESC-derived hepatocyte-like cells) and examined whether CYP1A was induced in these cells by typical inducers of CYP1A. hESC-derived hepatocyte-like cells expressed albumin, α-fetoprotein, CYP3A4, CYP3A7, CYP1A1, CYP1A2, and UDP-glucuronyl transferase (UGT) 1A1 mRNA. The levels of CYP1A1, CYP1A2, and UGT1A1 mRNA expression were increased by omeprazole and 3-methylcholanthrene. Furthermore, the enzyme activity of CYP1A was also increased by these compounds. In conclusion, hESC-derived hepatocyte-like cells are available for the detection of CYP1A inducers.

Keywords: hESCs; differentiation; hESC-derived hepatocyte-like cells; CYP1A; induction

Introduction

Human hepatocytes and liver tissues are used for nonclinical tests in pharmaceutical screening. However, some problems remain, including difficulty in obtaining cells due to limitations of tissue availability as well as individual differences between such specimens. Furthermore, the expression levels of many proteins decrease rapidly in culture.[1)] An alternative is to use hepatoma cell lines; however, these cells normally contain low levels of metabolic enzymes, and usually poorly reflect the phenotype of human hepatocytes *in vivo*.[2)]

Human embryonic stem cells (hESCs) are able to grow indefinitely and have the ability to differentiate into most body cell types.[3)] Hepatocyte-like cells, which are differentiated from hESCs, would be useful for regenerative medicine and drug discovery.[4)] Therefore, there is a great deal of interest in methods of differentiating hESCs into hepatocyte-like cells for application to pharmaceutical screening.

In humans, enzymes belonging to the cytochrome P450 1 family (CYP1A) play important roles in the detoxification of therapeutic agents in the liver. On the other hand, CYP1A is involved in the metabolic activation of procarcinogenic compounds, such as polycyclic aromatic hydrocarbons and aromatic amines found in cigarette smoke and cooked foods.[5,6)] For example, CYP1A1 are known to metabolize benzo[*a*]pyrene and 3-methylcholanthrene (3-MC) to their ultimate carcinogenic forms.[5,7)] Therefore, it is desirable to remove drug candidates that are capable of inducing expression of the CYP1A subfamily in pharmaceutical screening.

To increase the efficiency of drug discovery, it is important to establish a new assay system that can detect enzyme induction by drug candidates without using human liver samples. In the present study, we differentiated hESCs into hepatocyte-like cells (hESC-derived hepatocyte-like cells) expressing CYP1A1 and CYP1A2. Moreover, we demonstrated induction of mRNA levels of CYP1A1 and CYP1A2,

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and metabolic activity by representative CYP1A inducers. In conclusion, hESC-derived hepatocyte-like cells may become a reliable substitute source of normal human hepatocytes, which are useful for clinical research and drug discovery as a tool for enzyme induction studies.

Materials and Methods

Materials: Dexamethasone (DEX), dimethyl sulfoxide (DMSO), omeprazole (OME), 3-MC, phenacetin, (+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexane carboxamide dihydrochloride (Y-27632), oncostatin M (OSM), human fetal liver total RNA, and human normal adult liver total RNA were purchased from Wako Pure Chemical Industries (Osaka, Japan). Human fetal liver total RNA is from 1 donor, who was male and 38 weeks old. Human adult liver total RNA is from 1 donor, who was male and 64 years old. Activin A and hepatocyte growth factor (HGF) were purchased from Funakoshi Co., Ltd. (Tokyo, Japan). Wnt3a was purchased from R&D Systems (Minneapolis, MN). Murine embryonic fibroblasts (MEF) were obtained from Oriental Yeast (Tokyo, Japan). KnockOut Serum Replacement (KSR) and SuperScript III First-Strand Synthesis System for reverse transcription–polymerase chain reaction (RT-PCR) were from Invitrogen Life Technologies (Carlsbad, CA). TaKaRa SYBR Premix Ex Taq was obtained from Takara Bio Inc. (Otsu, Japan). Modified Lanford medium was obtained from Charles River Laboratories Japan Inc. (Yokohama, Japan). Collagen Type I (collagen I)-coated microplates were obtained from Asahi Glass (Chiba, Japan). All other reagents used were of the highest quality available.

Cell culture and differentiation: This study was approved by the Shinshu University Institutional Review Board. KhES-3 cells were provided from Institute for Frontier Medical Science, Kyoto University, Japan, and cultured according to the method reported by Suemori *et al.*[8] The hESCs were used between passages 30 and 40, and maintained in 3% CO_2 at 37°C. When hESCs reached a confluence level of approximately 70%, differentiation was initiated by replacing the medium with medium A (RPMI1640 medium containing 2 mM GlutaMax, 2% B-27, 0.5% FBS, 100 ng/mL activin A, and 50 ng/mL Wnt3a). After 72 h, the medium was changed to medium B (RPMI1640 medium containing 2 mM GlutaMax, 2% KSR, 100 ng/mL activin A and 50 ng/mL Wnt3a) and cultured for 48 h. The cells were then passaged on collagen-coated 24-well plates and cultured in medium C (KnockOut-DMEM containing 20% KSR, 1 mM GlutaMax, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, and 1% DMSO) for 7 days, although Y-27632 was added at 10 μM during first 24 h of culture. Subsequently, the cells were cultured in medium D (Lanford modified medium containing 10 ng/mL HGF, 20 ng/mL OSM, and 100 nM DEX) for 6 days. Finally, the cells were cultured in Lanford modified medium alone for 4 days (**Fig. 1**).

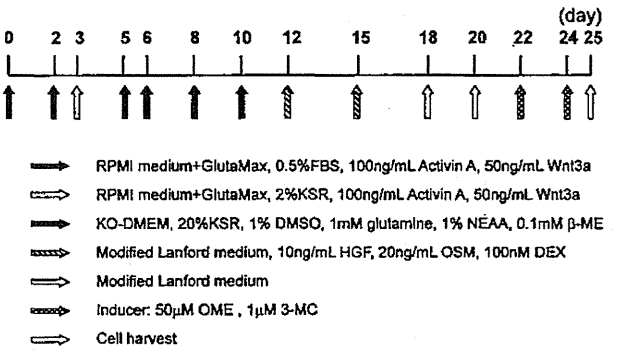

Fig. 1. Protocol for differentiation of hESCs into hepatocyte-like cells and treatment with inducers

RNA extraction and reverse transcription reaction: hESC-derived hepatocyte-like cells were treated with 50 μM OME or 1 μM 3-MC for 72 h. Total RNA was isolated from the cells using ISOGEN (Nippon Gene Co., Ltd.) according to the manufacturer's protocol. First-strand cDNA was generated from 500 ng of total RNA. Reverse transcription reaction was performed using a SuperScript III First-Strand Synthesis System for RT-PCR in accordance with the manufacturer's instructions.

Real-time PCR: For detection of expression levels, mRNAs were analyzed by SYBR Green real-time quantitative RT-PCR. Real-time PCR analysis was performed on an ABI Prism 7300 Real-time PCR System using SYBER® Premix ExTaq (Takara Bio Inc.). PCR was performed in mixtures consisting of 10 μL of SYBER Green PCR Master Mix, 0.4 μL of 10 mM forward and reverse primers, 0.4 μL of dye, 7.8 μL of water, and 1 μL template cDNA in a total volume of 20 μL. The relative expression of each gene was normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used were as follows: GAPDH: forward primer, 5′-GAG TCA ACG GAT TTG GTC GT-3′, reverse primer, 5′-GAC AAG CTT CCC GTT CTC AG-3′; albumin (ALB): forward primer, 5′-GAG CTT TTT GAG CAG CTT GG-3′, reverse primer, 5′-GGT TCA GGA CCA CGG ATA GA-3′; α-fetoprotein (AFP): forward primer, 5′-AGC TTG GTG GTG GAT GAA AC-3′, reverse primer, 5′-TCT GCA ATG ACA GCC TCA AG-3′; CYP3A4: forward primer, 5′-CTG TGT GTT TCC AAG AGA AGT TAC-3′, reverse primer, 5′-TGC ATC AAT TTC CTC CTG CAG-3′; CYP3A7: forward primer, 5′-AGA TTT AAT CCA TTA GAT CCA TTC G-3′, reverse primer, 5′-AGG CGA CCT TCT TTT ATC TG-3′; CYP1A1: forward primer, 5′-CCT CTT TGG AGC TGG GTT TG-3′, reverse primer, 5′-GCT GTG GGG GAT GGT GAA-3′; CYP1A2: forward primer, 5′-CTT TGA CAA GAA CAG TGT CCG-3′, reverse primer, 5′-AGT GTC CAG CTC CTT CTG GAT-3′; UGT1A1: forward primer, 5′-CAG CAG AGG GGA CAT GAA AT-3′, reverse primer, 5′-ACG CTG CAG GAA AGA ATC AT-3′.

Table 1. HPLC timetable

Time (min)	Solvent A (%)	Solvent B (%)
0.0	98	2
0.8	98	2
5.0	10	90
7.5	10	90
7.6	98	2
13.0	98	2

Solvents A and B: 10 mM ammonium acetate in water and 0.1% formic acid in methanol, respectively.

Measurement of enzymatic activity: hESC-derived hepatocyte-like cells were treated with 50 μM OME or 1 μM 3-MC for 72 h, and then incubated with 50 μM phenacetin for 24 h. After incubation, medium was collected and acetaminophen was measured by LC-MS/MS under the conditions described below.

Instrument: An Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump and a degasser linked to a CTC HTS PAL New Wash System Autosampler (AMR Inc., Tokyo, Japan) was used. Mass spectrometric detection was performed on an API 4000 triple quadruple instrument (Applied Biosystems/Sciex, Foster City, CA) equipped with a TurboIonSpray® electrospray ionization (ESI) interface. Data processing was performed with the Analyst 1.4.2 software package (Applied Biosystems/Sciex).

Chromatographic conditions: Chromatographic separation was performed on a reversed-phase CAPCELL PAK C18 MG III column (50 × 4.6 mm i.d., 5 μm; Shiseido Co., Inc., Tokyo, Japan). The column temperature was kept constant at 40°C. The mobile phase consisted of a mixture of 10 mM ammonium acetate in water (A) with 0.1% formic acid in methanol (B) and was delivered at a flow rate of 0.6 mL/min. A stepwise gradient was used as shown in Table 1.

Mass spectrometric conditions: The mass spectrometer was operated using the ESI source in positive ion detection. To optimize all of the MS parameters, standard solutions (100 ng/mL) and an internal standard were infused into the mass spectrometer at a flow rate of 250 μL/min. The ion spray voltage (IS) was set at 4,500 V. The TurboIonSpray probe temperature was maintained at 600°C. The instrument parameters *viz.*, nebulizer gas, curtain gas, auxiliary gas, and collision gas, were set at 60, 15, 80, and 5, respectively. Compound parameters *viz.*, declustering potential, collision energy, entrance potential, and collision exit potential, were 40, 20, 10, and 15, respectively, for acetaminophen and [$^2H_4$]acetaminophen. Zero air was used as source gas, while nitrogen was used as both curtain and collision gas. The mass spectrometer was operated in ESI positive ion mode and detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of m/z 152 precursor ion [M + H] to the m/z 110 product ion for acetaminophen (retention time: 4.7 min) and m/z 156 precursor ion [M + H] to the m/z 114 product ion for [$^2H_4$]acetaminophen (4.7 min). Quadrupoles Q1 and Q3 were set to unit resolution. Data acquisition and quantification were performed using Analyst software version 1.4.2 (Applied Biosystems, MDSSciex, Toronto, Canada).

Calibration standards: Calibration standards to cover the assay range of 1–5,000 nM of acetaminophen were prepared by adding 10 μL of 0.01, 0.05, 0.1, 0.5, 1, 5, 10, and 50 μM working standards to 0.1 μL aliquots of control reaction mixture.

Statistical analysis: Data are expressed as means ± standard deviation (SD). Statistical significance was assessed using an unpaired *t*-test. Differences were regarded as statistically significant at $p < 0.05$.

Results

Basal mRNA levels of various genes in hESC-derived hepatocyte-like cells: hESCs were differentiated into hepatocyte-like cells according to the method described in **Figure 1**. The levels of mRNAs of ALB, AFP, CYP3A4, CYP3A7, CYP1A1, CYP1A2, and UGT1A1 were measured by real-time PCR and compared with those of human adult or fetal liver. The mRNAs of ALB, AFP, CYP3A4, and CYP3A7 were expressed in hESC-derived hepatocyte-like cells, whereas the levels of expression of these genes were lower than those of human adult or fetal liver except for AFP (**Figs. 2A–2D**). In addition, the mRNAs of CYP1A1, CYP1A2, and UGT1A1 were expressed in these cells. The basal mRNA levels of these genes were lower than those of human adult liver, but higher than those of human fetal liver when compared to the reference standard of human adult and fetal liver total RNA (**Figs. 2E–2G**).

Influence of typical CYP1A inducers on CYP1A1, CYP1A2, and UGT1A1 mRNA levels in hESC-derived hepatocyte-like cells: We studied whether the mRNAs of CYP1A1, CYP1A2, and UGT1A1 in hESC-derived hepatocyte-like cells were inducible by typical inducers, such as OME and 3-MC, using real-time PCR. The level of CYP1A1 mRNA expression was markedly elevated from 35- to 48-fold compared to controls by OME and 3-MC (**Fig. 3A**). The level of CYP1A2 mRNA expression was also significantly increased from 3.2- to 3.5-fold compared to controls by OME and 3-MC (**Fig. 3B**).

The level of UGT1A1 mRNA expression was also significantly increased from 6.5- to 8.0-fold compared to controls by OME and 3-MC (**Fig. 3C**).

Influence of typical CYP1A inducers on phenacetin *O*-deethylase activity in hESC-derived hepatocyte-like cells: To evaluate the effect of OME or 3-MC on CYP1A activity in hESC-derived hepatocyte-like cells, we measured the concentration of acetaminophen in medium

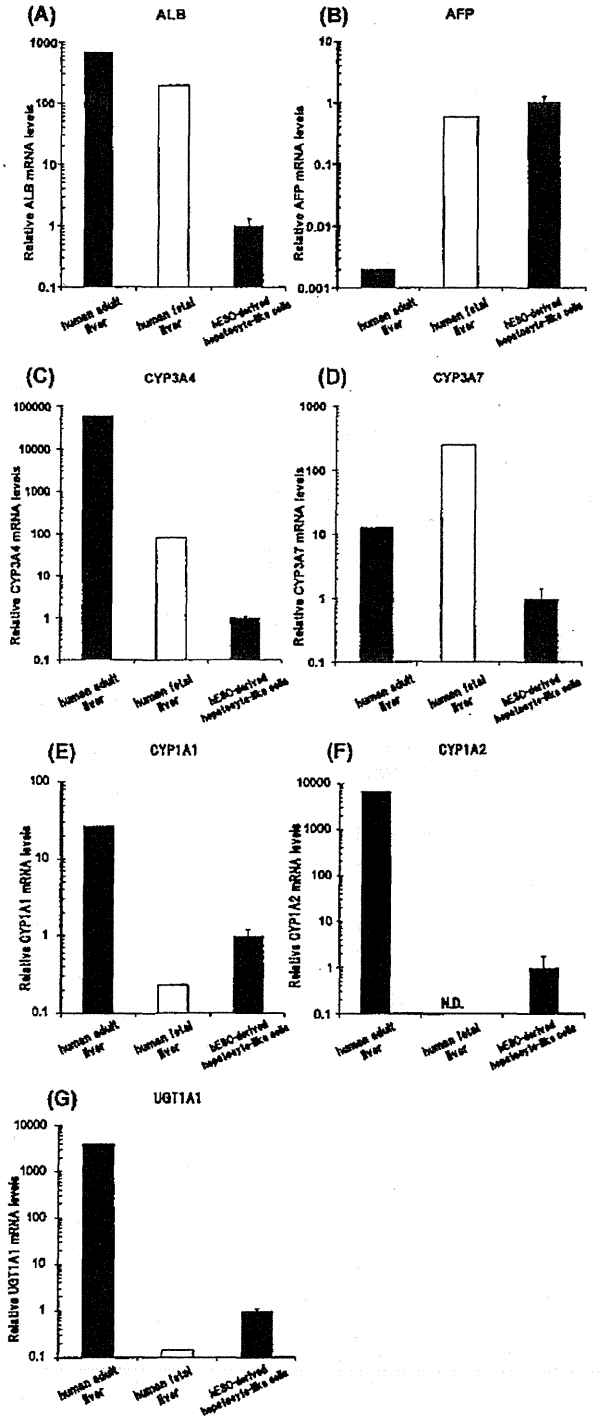

Fig. 2. Albumin (ALB), α-fetoprotein (AFP), CYP3A4, CYP3A7, CYP1A1, CYP1A2 and UGT1A1 mRNA levels in hESC-derived hepatocyte-like cells

hESCs were differentiated into hepatocyte-like cells according to the method described in **Figure 1**. Then, total mRNA was extracted and subjected to real-time PCR. The mRNA levels were normalized relative to that of GAPDH mRNA, and the values are shown as ratios to the average for hESC-derived hepatocyte-like cells treated with 0.1% DMSO. Results are presented as means ± SD from 6 independent experiments. N.D. means not detected.

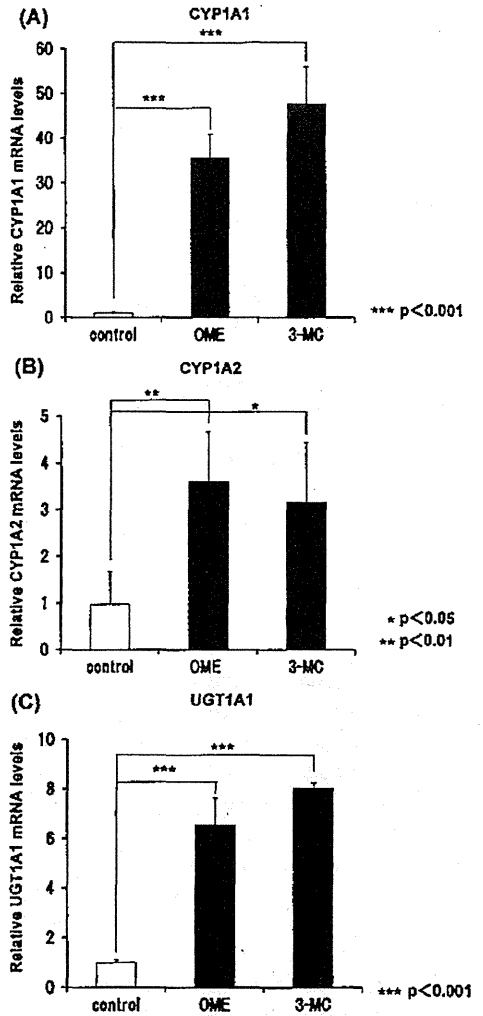

Fig. 3. The change of CYP1A1, CYP1A2 and UGT1A1 mRNA levels treated with typical CYP1A inducers in hESC-derived hepatocyte-like cells

hESC-derived hepatocyte-like cells were treated with 50 μM OME or 1 μM 3-MC for 72 h. Then, total mRNA was extracted and subjected to real-time PCR. The mRNA levels were normalized relative to that of GAPDH mRNA, and the values are shown as ratios to the average for hESC-derived hepatocyte-like cells treated with 0.1% DMSO. Results are presented as means ± SD from 6 independent experiments. Statistical analyses were performed using unpaired *t*-test; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

after incubation with phenacetin, which is a typical substrate for CYP1A. The phenacetin *O*-deethylase activity was elevated by 24-fold compared to controls by treatment with 3-MC, but showed only slight induction by treatment with OME (**Fig. 4**).

Discussion

In the present study, we attempted to differentiate hESCs into hepatocyte-like cells and evaluate whether CYP1A enzyme induction could be observed in these cells. A three-

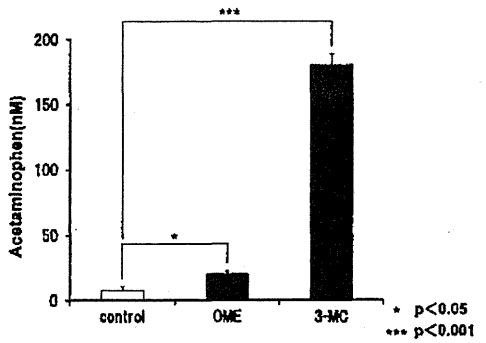

Fig. 4. The change of phenacetin O-deethylase activity treated with typical CYP1A inducers in hESC-derived hepatocyte-like cells

hESC-derived hepatocyte-like cells were treated with 50 μM OME or 1 μM 3-MC for 72 h and then incubated with 50 μM phenacetin for 24 h. After incubation, medium was collected and acetaminophen was measured with LC-MS/MS. Results are presented as means ± SD from 6 independent experiments. Statistical analyses were performed using unpaired *t*-test; $^{*}p < 0.05$ and $^{***}p < 0.001$.

step process is required for induction of hESC differentiation into hepatocytes—the first step involves the differentiation of undifferentiated hESCs into endoderm, the second step involves the differentiation of endoderm into hepatoblast-like cells, and the third step involves maturation into hepatocytes. Previous studies suggested that it is important to add activin A when inducing differentiation of hESCs into endoderm.[9–11] Hay *et al.* reported that Wnt3a can also differentiate hESCs into hepatic endoderm, and its concomitant use with activin A has synergistic effects on efficient differentiation of hESCs into hepatic endoderm.[12] In the present study, we used activin A and Wnt3a concomitantly for initiation of differentiation. In addition, Y-27632, a selective inhibitor of p160-Rho-associated coiled kinase, was used to permit survival of hESCs during passage,[13,14] the use of Y-27632 decreased the death of cells during passage in the present study (data not shown).

The mRNAs of AFP and ALB, used as markers of hepatocytes, were detected in hESC-derived hepatocyte-like cells together with CYP3A4, CYP3A7, CYP1A1, CYP1A2, and UGT1A1 mRNAs (**Figs. 2A–2G**). The levels of ALB and CYP3A4 mRNAs were markedly lower than those in the human adult and fetal liver when compared to the reference standard of human adult and fetal liver total RNAs (**Figs. 2A** and **2C**). On the other hand, the expression level of AFP mRNA was higher than those in the fetal and adult liver when compared to the reference standard of human adult and fetal liver total RNA (**Fig. 2B**). AFP is found to express predominantly in human fetal hepatocytes.[15] Although these results represent comparison to only one standard sample for both human adult and fetal liver, hESC-derived hepatocyte-like cells were shown to be differentiated into more fetal-like cells rather than into adult hepatocyte-like cells. It has been reported that CYP, UGT, and other liver specific genes are expressed in hepatocyte-like cells,[16] but in this report, the mRNA levels of CYP3A4 and CYP1A2 were lower than these in the present study (**Figs. 2C** and **2F**).

In some studies, fibroblast growth factor, bone morphogenetic protein or sodium butyrate were used to differentiate hESCs into hepatocyte-like cells more efficiently.[11,17–19] However, when we used fibroblast growth factor, bone morphogenetic protein, and sodium butyrate to differentiate hESCs into hepatocyte-like cells in preliminary experiments, the degree of differentiation was inefficient in comparison with the conditions used in the present study (data not shown). It is necessary to develop methods to induce maturation of hepatocyte-like cells, *e.g.*, it may be useful to examine use of three-dimensional culture methods reported previously.[20–22] Besides, Takayama *et al.* recently reported that transduction of hepatocyte nuclear factor 4 into hESCs generated functional hepatocytes.[23] Therefore, it might be beneficial to utilize the transduction of a gene which differentiates hESCs efficiently into hepatocytes.

Chemical-induced expression of CYP1A is mainly regulated by the aryl hydrocarbon receptor (AhR), one of several ligand-activated transcription factors.[24–26] OME and 3-MC are typical CYP1A inducers. Although 3-MC is a strong ligand of AhR,[24] OME has been shown to activate AhR without direct binding.[27–30] The induction of UGT1A1 is also regulated by AhR as well as CYP1A1.[31] Treatment of hESC-derived hepatocyte-like cells with OME and 3-MC induced not only CYP1A1 and CYP1A2, but also UGT1A1 (**Figs. 3A–3C**), indicating that these cells possess active AhR. Induction of CYP1A1 and CYP1A2 are caused by binding of AhR to the xenobiotic-responsive element of the CYP1A1 and CYP1A2 genes.[32] Therefore, it is possible that these hESC-derived hepatocyte-like cells are available for evaluation of AhR activator. By the way, we tried to study the mRNA of CYP3A4, but the mRNA of CYP3A4 was not elevated by the treatment of rifampicin (data not shown). The character of the hESC-derived hepatocyte-like cells, *i.e.* the high mRNA levels of AFP, and the low levels of ALB and CYP3A4, suggest that these cells are fetal hepatocyte-like cells. This character is supported by the fact that the mRNA of CYP3A4 was not induced by rifampicin as shown previously.[33,34]

It is important that hESC-derived hepatocyte-like cells have inducible metabolic enzyme activity. The *O*-deethylation of phenacetin is mainly catalyzed by CYP1A2. Similar to the mRNA expression, the level of phenacetin *O*-deethylase activity was also significantly induced by treatment with OME and 3-MC (**Fig. 4**). However, the induction of phenacetin *O*-deethylase activity by OME was considerably weaker than that by 3-MC, although both showed essentially equivalent induction of not only CYP1A1 mRNA level, but also CYP1A2 mRNA level. Although the reason for this discrepancy between mRNA level and enzymatic activity is not yet clear, it may be due to the effects on posttranscrip-

tional regulation, *e.g.*, differences in RNA stability, translation, and protein stability.

Several reports described hESC-derived hepatocytes as having CYP1A2 activity,[12,17,19,23,35] but a few reports have previously shown inducible activity of CYP1A2 in hESC-derived hepatocytes.[23,35] In the present study, basal phenacetin *O*-deethylase activity was low, but as for elevation of phenacetin *O*-deethylase activity treated with CYP1A inducer, the hESC-derived hepatocyte-like cells may be highly sensitive to AhR activator.

In conclusion, although additional investigations are required for the development of practical applications, hESC-derived hepatocyte-like cells appear to be suitable for evaluation of CYP1A inducers.

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