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Regular Article

Construction of a System that Simultaneously Evaluates CYP1A1 and CYP1A2 Induction in a Stable Human-derived Cell Line using a Dual Reporter Plasmid

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Summary: Human *CYP1A1* and *CYP1A2* genes are in a head-to-head orientation on chromosome 15 and are separated by a 23-kb intergenic space. To our knowledge, this is the first report on a stable cell line that contains the 23-kb full-length regulatory region and is able to simultaneously assess the transcriptional activation of *CYP1A1* and *CYP1A2* genes. The stable cell line that constitutively expresses the reporter activities was constructed by inserting the dual reporter plasmid containing the 23-kb region between the *CYP1A1* and *CYP1A2* genes into the chromosome. Transcriptional activation of the *CYP1A1* and *CYP1A2* genes was measured simultaneously using luciferase (Luc) and secreted alkaline phosphatase (SEAP) activities, respectively. To demonstrate the utility of the stable cell line, CYP1A1/1A2 induction by the majority of compounds previously identified as CYP1A1/1A2 inducers was measured. The results clearly show that all compounds caused induction of reporter activities. In addition to assessing transcriptional activation of the *CYP1A1* and *CYP1A2* genes by measuring reporter activities, we determined the intrinsic CYP1A1 and CYP1A2 mRNA levels by treating them with the same compounds. The results suggest that this stable cell line may be used to rapidly and accurately predict CYP1A1/1A2 induction.

Keywords: CYP1A1; CYP1A2; XRE; AhR; in vitro reporter assay; stably cell line

Introduction

In humans, the CYP1A subfamily is comprised of 2 members—*CYP1A1* and *CYP1A2*. CYP1A1 and CYP1A2 in the liver play important roles in the detoxification of therapeutic agents and environmental chemicals. These enzymes are also responsible for the metabolic activation of polycyclic aromatic hydrocarbons (PAHs) and aromatic amines that are present in combustion products such as cigarette smoke and charcoal-grilled foods.[1] CYP1A1 and CYP1A2 are induced by exposure to various chemicals including halogenated hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or PAHs such as benzo[*a*]pyrene (B[*a*]P) and 3-methylcholanthrene (3-MC).[2,3] Induction of CYP1A1 and CYP1A2 is under the regulatory control of the aryl hydrocarbon receptor (AhR). Ligand-activated AhR translocates into the nucleus and heterodimerizes with the AhR nuclear translocator (ARNT). The ligand-AhR-ARNT complex then binds to the regulatory *cis*-element xenobiotic-responsive element (XRE), which locates the upstream portion of target genes to activate their transcription.[2–5]

The Food and Drug Administration (FDA) currently suggests that the most reliable method to study induction potency of drugs is by using a primary culture of human hepatocytes that represents the entire repertoire of hepatic drug metabolism enzymes and the genes for maintaining liver-specific functions.[6] However, this method frequently shows large donor-to-donor variability in induction response, resulting in difficulties in data in-

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terpretation.[7] Furthermore, the expression of many proteins has been shown to decrease rapidly during cultivation of human hepatocytes, with drug metabolism enzymes being particularly sensitive.[8] Another disadvantage of using human hepatocytes is their high cost.[9] Thus, the use of human hepatocytes is restricted, and limited access to suitable tissue samples prevents their use in high-throughput screening systems.

Recently, rapid and sensitive bioassays of CYP1A1/1A2 induction are mainly based on application of a luciferase (Luc) gene reporter plasmid that includes the transcriptional regulatory regions of *CYP1A1* and *CYP1A2* genes, respectively. These bioassays require transient transfection of the plasmid DNA construct before each experiment. Consequently, this is difficult to apply in high-throughput screening systems. Additionally, the data often varies between experiments because controlling transfection efficiency is difficult. On the other hand, several researchers have reported on cell-based high-throughput screening systems to assess the induction of CYP1A1 using stable reporter gene expression, which has improved problems associated with the transient expression of the plasmid gene.[10] These systems are constructed by inserting the reporter gene into chromosomes of liver-derived cell lines such as HepG2. The reporter cell lines are then easily maintained as HepG2, and the culture conditions are simpler than those required by hepatocytes. Thus, these stable reporter gene expression systems have the advantage of being more readily available and reproducible than the transient expression systems or hepatocytes used for assessment of CYP1A1/1A2 induction.

The sequence and genomic orientation of the *CYP1A1* and *CYP1A2* loci on chromosome 15 have been reported.[11] The *CYP1A1* and *CYP1A2* genes are located immediately adjacent to each other in a head-to-head orientation. The 2 genes are separated by more than 20-kb of intervening DNA, which possesses 13 candidates for XRE. Yueh *et al.* and Chao *et al.* have been using established stable cell lines harboring a Luc reporter gene integrated in artificial multiple XREs.[10,12] In addition, another reporter cell line has stably integrated approximately 1.6-kb of the 5′-flanking region of the *CYP1A1* gene.[13] In contrast, assessment systems of CYP1A2 induction using stable reporter gene expression remain to be established. We have reported that transcriptional activation of the *CYP1A1* and *CYP1A2* genes is regulated simultaneously through a common regulatory element existing between these 2 genes that act bidirectionally.[14] Thus, transcriptional activation of *CYP1A1* and *CYP1A2* genes is necessary to assess its intensity in a simultaneous measurement method. Furthermore, to reflect the induction *in vivo*, it is important to establish stable cell lines containing the reporter genes and the 23-kb intergenic spacer regions between *CYP1A1* and *CYP1A2* genes. However, current procedures are not assessed under such conditions.

To develop a simultaneous evaluation method for CYP1A1/1A2 induction, we established a stable reporter gene expression in HepG2 for high-throughput screening systems. Stable reporter cell lines are designed to simultaneously assess the transcriptional activation of *CYP1A1* and *CYP1A2* genes and target the full-length regulatory region existing between these 2 genes. In the present study, this system was used to determine whether known CYP1A1/1A2 inducers, such as drugs, endogenous compounds, and PAHs, mediate induction of CYP1A1/1A2.

Materials and Methods

Materials: 3-MC, benzo[*e*]pyrene (B[*e*]P), benz[*a*]anthracene (B[*a*]A), omeprazole (OME), lansoprazole (LPZ), and albendazole (ALB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). α-Naphthoflavone (α-NF), β-naphthoflavone (β-NF), B[*a*]P, 9,10-dihydroanthracene (DHA), 7-methylbenz[*a*]anthracene (7-MB[*a*]A), dibenz[*de,kl*]anthracene (DB[*de,kl*]A), chrysene (Chr), dibenz[*a,h*]anthracene (DB[*a,h*]A), and dibenz[*a,c*]anthracene (DB[*a,c*]A) were obtained from Tokyo Chemical Industry (Tokyo, Japan). TCDD and dibenz[*a,j*]acrydine (DB[*a,j*]AC) was purchased from Wako Pure Chemicals (Osaka, Japan) and dimethylsulfoxide (DMSO) from Nacalai Tesque (Kyoto, Japan). Indirubin (IND) was synthesized as descrived by Hossel *et al.* (1999).[15]

Generation of stable cell lines and cell extract: The dual reporter plasmids of human *CYP1A1* and *CYP1A2* (pd-1A1/1A2) containing approximately 23-kb of DNA segments (from +1039 of the *CYP1A1* gene to +90 of the *CYP1A2* gene) were reported previously.[14] The plasmids pd-1A1/1A2 and pQBI including the neomycin resistant gene (Wako Pure Chemicals) were linearized with *Not*I (NIPPON GENE, Tokyo, Japan), and the resulting fragments were mixed at a molar ratio of 5:1 and ligated using DNA ligation Kit ver. 2.1 (TaKaRa Bio, Kyoto, Japan). The human hepatoma cell line HepG2 was obtained from the RIKEN cell bank (Tsukuba, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM, WAKO Pure Chemicals) supplemented with 10% fetal calf serum (Biowest. Miami, FL, USA) and MEM nonessential amino acids (Invitrogen, Carlsbad, CA, USA). Cells were seeded in 6-well tissue culture plates (BD Biosciences, Heidelberg, Germany) at 3×10^5 cells per well a day before transfection, and the ligation fragment was transfected using Targefect F-1 (TARGETING SYSTEM, El Cajon, CA, USA) according to the manufacturer's instructions. After 2-weeks incubation, the cells were selected with 700–900 μg/mL geneticin (G418, Invitrogen). Medium was changed 3 times for 3 weeks until small colonies were visible. Five out of 12 colonies were further subcloned into 48-well tissue culture plates (BD Biosciences) to obtain monoclonal cells.

G418-resistant clones were chosen for induction testing by treating the cells with 1 μM 3-MC and 10 μM β-NF. The induction response was measured using the Luc and secreted alkaline phosphatase (SEAP) assay method. A clone exhibiting the greatest induction response was selected for this study.

Cell culture and cell extract: A stable cell line was cultured in DMEM containing 10% fetal calf serum, MEM nonessential amino acids, and antibiotic-antimycotic (Invitrogen). The cells were seeded in 24-well tissue culture plates (BD Biosciences) at 1×10^5 cells per well in 0.5 mL of DMEM, and the media containing the various compounds dissolved in DMSO (final concentration, 0.1%) were changed at 24 h intervals. Control cells were treated with 0.1% DMSO. After 48 h exposure to various xenochemicals, portions of the media were collected and used for the SEAP assay. Subsequently, cells were washed with phosphate buffered saline (PBS) and suspended in 0.1 mL of Passive Lysis Buffer (Promega, Madison, MI, USA) in a microcentrifuge tube. The cell suspension was centrifuged at $12,000 \times g$ for 5 minutes at 4°C. The supernatant was processed for Luc assay and determination of protein concentration. Protein concentration was measured using the Bio-Rad Protein Assay (BIO-RAD, Hercules, CA, USA).

Luc and SEAP assays: The Luc assay was performed according to the manufacturer's instructions for Promega using the Luciferase Assay System. Luc activity, corrected by the protein content of the cell lysate, was expressed in relative light units (RLU)/mg protein. SEAP activity was measured using Great EscAPe™ SEAP Chemiluminescence Kit 2.0 (Clontech, Palo Alto, CA, USA). The luminescence obtained from reactions of the Luc and SEAP assays was then monitored by Glomax™ 96 Microplate Luminometer (Promega).

Quantitative Analysis of CYP1A mRNA contents: Total RNA was extracted using TRI REAGENT® (Molecular Research Center, Cincinnati, OH, USA). First-strand cDNA was synthesized from 1 μg total RNA in a 20 μL reaction mixture using Molony Murein Virus Reverse Transcriptase (Promega), oligo(dT)$_{20}$ primer, and Ribonuclease Inhibitor (TaKaRa Bio). cDNA was used to carry out real-time polymerase chain reaction (PCR) using SYBR Premix ExTaq (TaKaRa Bio) to measure mRNA levels of CYP1A1, CYP1A2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The amplification reaction were performed with specific primers for CYP1A1 (forward: 5′-ACTGCTTAGCCTAGTCAACCTG-3′ and reverse: 5′-CAATCAGGCTGTCTGTGATGTC-3′), CYP1A2 (forward: 5′-CATTGGTGCCATGTGCTTC-GGACAG-3′ and reverse: 5′-AAGTCCTGATAGTGCTC-CTGGACTG-3′), and GAPDH (forward: 5′-GAAGGTG-AAGGTCGGAGTCAAC-3′ and reverse: 5′-CAGAGTTA-AAAGCAGCCCTGGT-3′). Quantitative values were obtained above the threshold PCR cycle number (Ct) at which increase in signal associated with an exponential growth for PCR products was detected using Thermal Cycler Dice™ TP800 (TaKaRa Bio). Relative mRNA expression levels in each sample were normalized according to GAPDH expression levels.

Results

Induction of reporter activities derived from dual reporter genes of *CYP1A1* and *CYP1A2* by TCDD in the stable cell line: The stable cell line that constitutively expressed reporter activities was constructed by inserting the dual reporter plasmid containing the 23-kb intergenic spacer region, responsible for transcriptional activation and regulation of *CYP1A1* and *CYP1A2* genes into the chromosome of HepG2 cells. The transcriptional activities of *CYP1A1* and *CYP1A2* genes were measured simultaneously with Luc and SEAP activities, respectively. To characterize the isolated stable cell line, a time-course and dose-dependent induction of reporter activities by TCDD was measured. The reporter activities were determined at 3 time intervals (24, 48, and 72 h) after adding the concentrations ranging from 0.001 to 100 nM of TCDD to the culture medium. TCDD response of Luc and SEAP in the stable cell line increased in a time-course and dose-dependent manner (**Fig. 1**). Maximum induction occurred when cells were exposed to 10 nM for 72 h, during which Luc and SEAP activities were observed at 200- and 30-fold inductions, respectively. In addition, the reporter activities were readily detectable in the stable cell line, even though a low concentration of TCDD (1 nM) for 48 h was used.

The effects of various cell densities on Luc and SEAP activities were also examined. The stable cell line was seeded at densities of 2.5×10^4, 5×10^4, and 10×10^4 cells, and reporter activities were determined after 48 h treatment with 1 nM and 10 nM TCDD, respectively. As expected, enhancement of the reporter activities occurred in a cell density-dependent manner with the 10×10^4 cell density exhibiting the greatest elevation (**Fig. 2**).

Influence of inducer concentration and exposure period on reporter activity: TCDD has a long half-life (5–11 years in humans) owing to its high lipophilicity and little or no metabolism.[16–20] Therefore, in this system, TCDD accumulated in the stable cell line. On the other hand, a number of CYP1A1/1A2 inducers such as PAHs, therapeutic agents, and endogenous compounds are substrates for the enzymes. Among prototypical AhR ligands, 3-MC and IND are rapidly metabolized by drug metabolism enzymes and lose their ligand activity.[21,22] To confirm time- and dose-dependent change of Luc activity, the stable cell line was treated with TCDD, 3-MC, and IND under the same conditions (**Fig. 3**). The inducible potency of 3-MC and IND decreased from 24 to 72 h, whereas TCDD caused induction that remained maximal for the entire 72 h duration of the experiment.

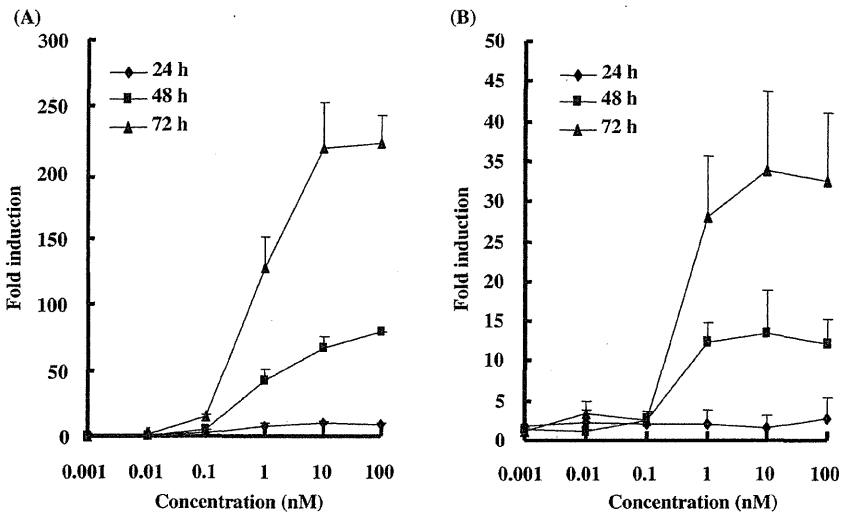

Fig. 1. Time-course and dose-dependent induction of reporter activities by TCDD in the stable cell line
Cells were seeded at 5.0×10^4 cells in 24-well tissue culture plates and treated with TCDD (0.001–100 nM). Cells were harvested at 24 (closed diamonds), 48 (closed squares), and 72 h (closed triangles) after TCDD treatment, and the lysate was used for assays of Luc activity and protein concentration. The assay of SEAP activity used a portion of the media. Luc and SEAP activities were normalized with protein concentration, and the values are each shown as the ratio of the average for the control treated with 0.1% DMSO. Each paint represents the means of 3 samples and the error bars represent standard deviations. (A) Luc activity, (B) SEAP activity.

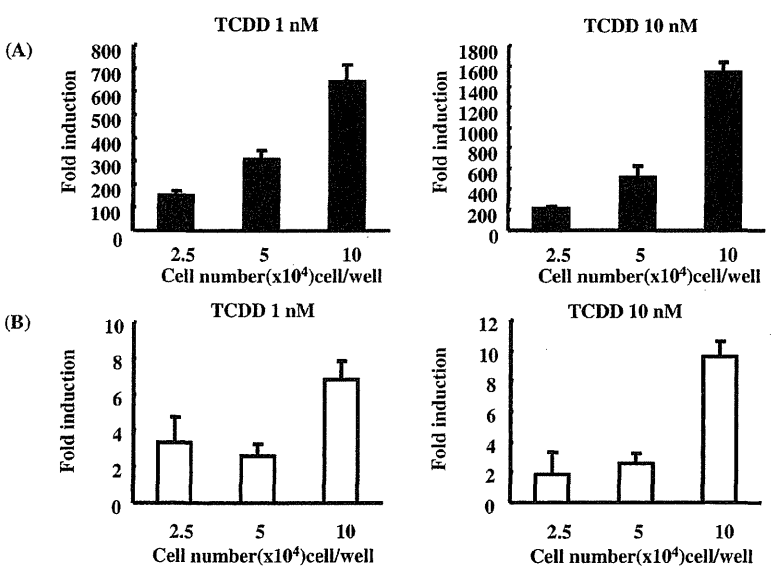

Fig. 2. The effects of various cell densities on reporter activities induced by TCDD in the stable cell line
Cells were seeded in 24-well tissue culture plates at the densities of 2.5×10^4, 5×10^4, and 10×10^4 cells/well 24 h before TCDD treatment. Cells were treated for 48 h and the lysate was harvested. The lysate was used for assays of Luc activity and protein concentration. The assay of SEAP activity used a portion of the media. Luc and SEAP activities were normalized with protein concentration, and the values are each shown as the ratio of the average for the control treated with 0.1% DMSO. The columns represent the means of 3 samples and error bars represent standard deviations. (A) Luc activity, (B) SEAP activity.

Because the stable cell line was treated with low concentrations of CYP1A1/1A2 inducers, induction via measurement of SEAP activity was not determined.

Induction of reporter activities compared between the stable cell line and transient expression system: The reporter activities of the stable cell line were significantly enhanced in response to TCDD. To assess the sensitivity of CYP1A1/1A2 induction screening, the fold induction of reporter activities in the stable cell line was compared with that in the transient expres-

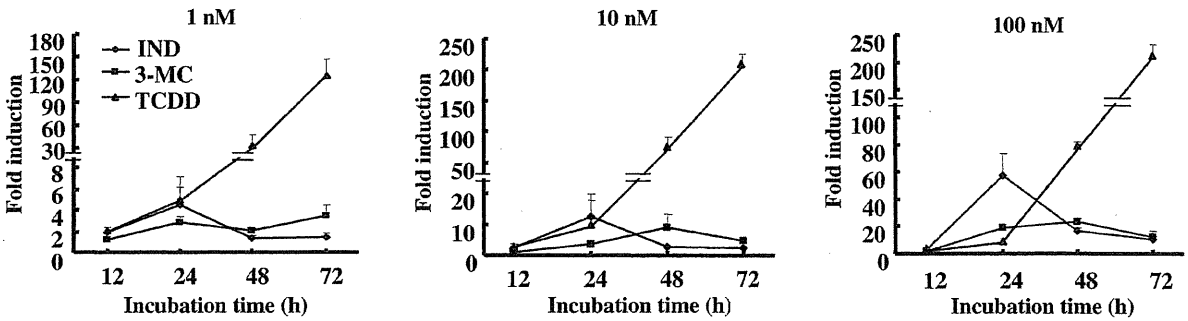

Fig. 3. Time- and dose-dependent changes of Luc activity induced by AhR ligands in the stable cell line
Cells were seeded at 5.0×10^4 cells in 24-well tissue culture plates and treated with IND (closed diamonds), 3-MC (closed squares), and TCDD (closed triangles) (1–100 nM). Cells were harvested at 12, 24, 48, and 72 h after compound treatment and the lysate was used for assays of Luc activity and protein concentration. Luc activity was normalized with protein concentration, and the values are each shown as the ratios of the average for the control treated with 0.1% DMSO. Each paint represents the means of 3 samples and error bars represent standard deviations.

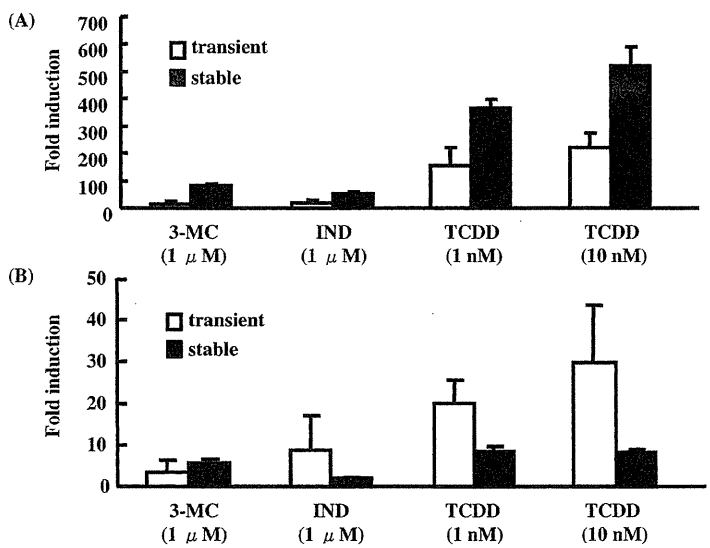

Fig. 4. Comparison of induction response for typical CYP1A1/1A2 inducers between the stable cell line and transient expression system
Cells were seeded 1.0×10^5 cells/well in 24-well tissue culture plates with 0.5 mL of DMEM for 24 h before compound treatment. HepG2 cells were seeded 1.0×10^5 cells/well in 24-well tissue culture plates with 0.5 mL of DMEM for 20 h before transfection. The pd-1A1/1A2 dual reporter gene plasmid (0.8 μg) was transiently transfected using Targefect F-1 according to the manufacturer's instructions and incubated for 4 h. Two cells (stable cell line; closed bars, transient expression system; open bars) were treated with TCDD (1 nM and 10 nM), 3-MC (1 μM), and IND (1 μM) for 48 h. Then, reporter activities were measured. Luc and SEAP activities were normalized by protein concentration, and the values are each shown as the ratio of the average for the control treated with 0.1% DMSO. The columns represent the means of 3 samples and the error bars represent standard deviations. (A) Luc activity, (B) SEAP activity.

sion system using HepG2 and pd-1A1/1A2 (**Fig. 4**). Two CYP1A1/1A2 induction screening systems were exposed to 3 strong inducers of CYP1A1/1A2: TCDD, 3-MC, and IND. As a result, the responsiveness of Luc activity in the stable cell line was observed as both more sensitive and more stable than that of the transient expression system (**Fig. 4A**). The Luc activity of the stable cell line was elevated 2-fold compared to the transient expression system. In contrast, the induction of SEAP activity in the stable cell line was less than that in the transient expression system (**Fig. 4B**). However, when the 2 experiments were treated with 3-MC, the responsiveness of SEAP activity in the stable cell line was equivalent to that in the transient expression system. These results show that the stable cell line is an adequately sensitive assay for assessing typical CYP1A1/1A2 inducers.

Transcriptional activation of *CYP1A1* and *CYP1A2* genes by various compounds: To demonstrate the utility of the stable cell line for typical CYP1A1/1A2 inducers, cells were treated with proton

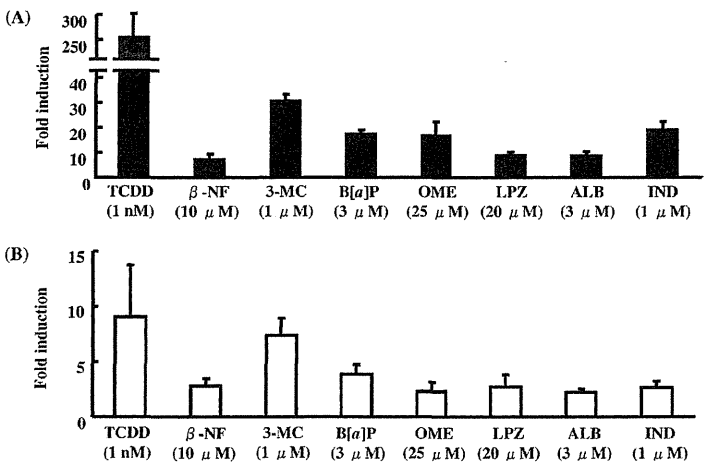

Fig. 5. Transcriptional activation of *CYP1A1* and *CYP1A2* genes by various CYP1A1/1A2 inducers in the stable cell line
Cells were seeded 1.0×10^5 cells/well in 24-well tissue culture plates with 0.5 mL of DMEM 24 h before treatment with various compounds. The cells were treated with TCDD (1 nM), β-NF (10 μM), 3-MC (1 μM), B[a]P (3 μM), OME (25 μM), LPZ (20 μM), ALB (3 μM), and IND (1 μM) for 48 h. Then, reporter activities were measured. Luc and SEAP activities were normalized by protein concentration, and the values are each shown as the ratio of the average for the control treated with 0.1% DMSO. The columns represent the means of 3 samples and the error bars represent standard deviations. (A) Luc activity, (B) SEAP activity.

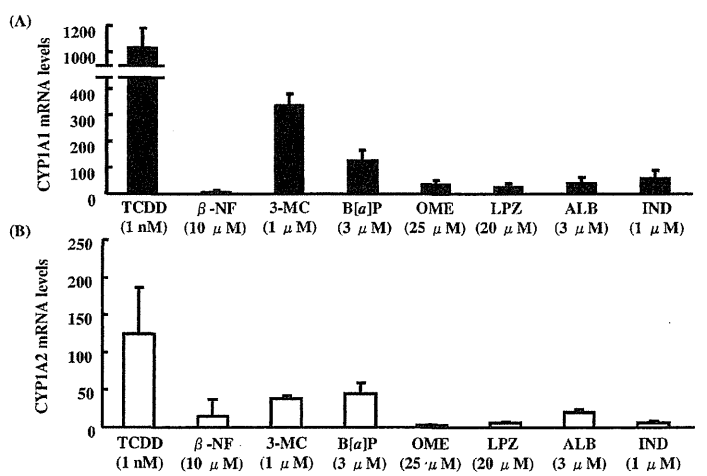

Fig. 6. Influence of various CYP1A1/1A2 inducers on CYP1A1 and CYP1A2 mRNA levels in the stable cell line.
Cells were seeded 2.0×10^5 cells/well in 12-well tissue culture plates with 1 mL of DMEM for 24 h before compound treatment. The cells were treated with TCDD (1 nM), β-NF (10 μM), 3-MC (1 μM), B[a]P (3 μM), OME (25 μM), LPZ (20 μM), ALB (3 μM), and IND (1 μM) for 48 h. After 48 h, total mRNA was extracted and subjected to quantitative reverse-transcriptase PCR. GAPDH was used as the internal standard. Each value is shown as the ratio of the average for the control treated with 0.1% DMSO. The columns represent the means of 3 samples and the error bars represent standard deviations. (A) CYP1A1 mRNA level, (B) CYP1A2 mRNA level.

pump inhibitors, ALB, IND, PAHs, and TCDD (**Fig. 5A** and **B**).[23-27] Among these compounds, TCDD (1 nM) produced the maximum increase in Luc activity, which was 263.6 ± 49.9-fold above DMSO-treated cells (**Fig. 5A**). Among the PAHs tested, 3-MC (1 μM) and B[a]P (3 μM) were also strong CYP1A1 inducers; they increased the Luc activity by 31.1 ± 2.2-fold and 17.7 ± 1.3-fold, respectively. When cells were treated with therapeutic agents such as proton pump inhibitors (OME 25 μM and LPZ 20 μM) and ALB (3 μM), Luc activity was elevated from 9.0 ± 1.0-fold to 17.1 ± 5.2-fold compared to that with DMSO control. IND (1 μM), an endogenous ligand for AhR isolated from normal human urine, also increased Luc activity (19.5 ± 2.9-fold). As with Luc activity, SEAP activity was also elevated 9.1 ± 4.7-fold upon TCDD treatment. Furthermore, 3-MC and B[a]P produced 7.4 ± 1.5-fold and 3.9 ± 0.9-fold increases in SEAP activity, respectively (**Fig. 5B**). Although induction of simultaneously measured SEAP activity was lower than that of Luc activity, the induction pattern among the test-

ed compounds was similar between the Luc and SEAP activities.

As shown in **Figures 6A** and **B**, intrinsic CYP1A1 and CYP1A2 mRNA levels substantially increased in the stable cell line treated with the various compounds described above. The profile of enhanced CYP1A1 and CYP1A2 mRNA levels was similar to that of the reporter gene assay (**Fig. 6A**). The level of CYP1A1 mRNA induced by TCDD was more than 1,000-fold above the DMSO-treated cells. 3-MC, B[*a*]P, proton pump inhibitors, ALB, and IND produced significant increases in CYP1A1 mRNA level that were more than 30-fold above the DMSO control, while β-NF moderately enhanced mRNA level compared to other compounds. The influence of various compounds on CYP1A2 mRNA level was also examined (**Fig. 6B**). Similar to the results observed with the reporter gene assay, TCDD produced the maximum increase of 124.6 ± 61.6-fold above the

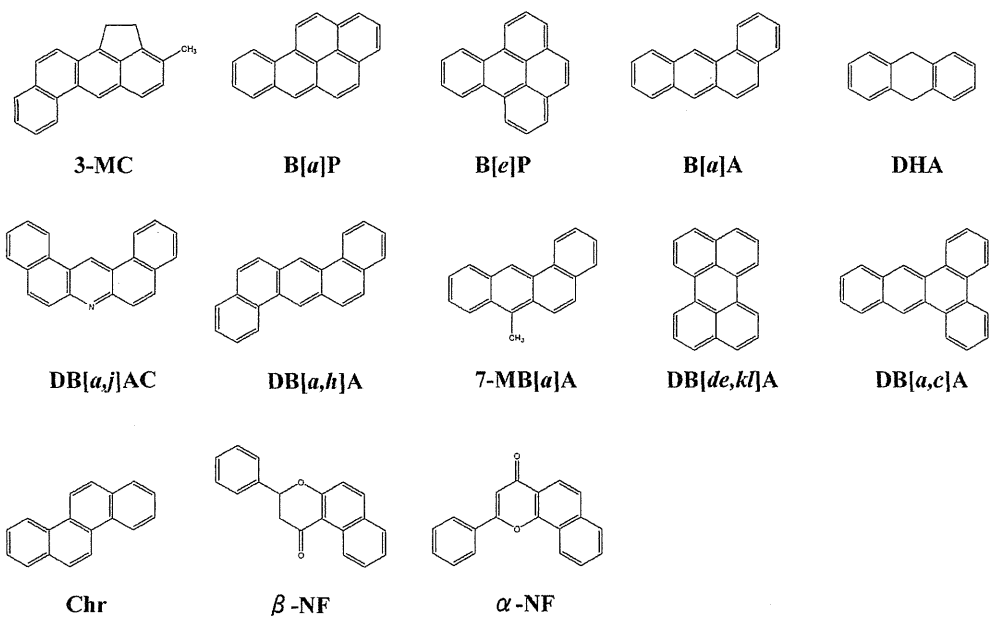

Fig. 7. Structures of PAHs used in this study

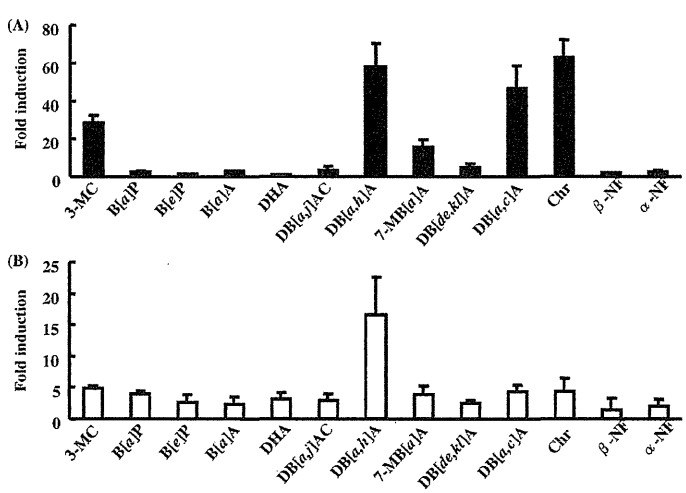

Fig. 8. Screening for assessment of CYP1A1/1A2 induction by 13 PAHs
Cells were seeded 1.0×10^5 cells/well in 24-well tissue culture plates with 0.5 mL of DMEM for 24 h before treatment with the various compounds. The cells were treated with 1 μM of 3-MC, B[*a*]P, B[*e*]P, B[*a*]A, DHA, DB[*a,j*]AC, DB[*a,h*]A, 7-MB[*a*]A, DB[*de,kl*]A, DB[*a,c*]A, Chr, β-NF, α-NF for 48 h. Then, reporter activities were measured. Luc and SEAP activities were normalized by protein concentration, and each value is shown as the ratio of the average for the control treated with 0.1% DMSO. The columns represent the means of 3 samples and the error bars represent standard deviations. (A) Luc activity, (B) SEAP activity.

DMSO control in CYP1A2 mRNA levels. 3-MC and B[*a*]P also proved to be strong inducers for CYP1A2 mRNA, resulting in 37.8 ± 4.5-fold and 44.2 ± 14.9-fold increases, respectively. These experiments demonstrate that induction profiles of the reporter activities in a stable cell line treated with typical CYP1A1/1A2 inducers are closely correlated to those that increase intrinsic CYP1A1 and CYP1A2 mRNA levels.

Screening assays for assessing transcriptional activation of *CYP1A1* and *CYP1A2* genes by PAHs: The extent of CYP1A1/1A2 induction activity differs for each PAH, although both are highly induced by PAHs. In order to further substantiate the ability of the stable cell line to be used to assess CYP1A1/1A2 inducers, cells were treated with 13 PAHs (1 μM, **Fig. 7**). As shown in **Figures 8A** and **B**, DB[*a,h*]A produced significant increases in Luc and SEAP activities that were 58.0 ± 12.1-fold and 16.6 ± 6.0-fold above the DMSO control, respectively. On the other hand, DB[*a,c*]A and Chr appeared to be strong inducers of Luc activity only, resulting in increases that were 46.5 ± 11.6-fold and 63.0 ± 9.3-fold above the DMSO control, respectively (**Fig. 8A**). Furthermore, Luc activity was also enhanced by 3-MC and 7-MB[*a*]A. The remaining PAHs did not induce Luc activity greater than 5-fold compared to the DMSO control. SEAP activity of the stable cell line treated with all tested PAHs (except for DB[*a,h*]A) exhibited modest induction and similar extents (**Fig. 8B**). These results show that the stable cell line has high reporter activities at relatively low concentration (1 μM) that were easily detectable on CYP1A1/1A2 induction.

Discussion

CYP1A1/1A2 induction has significant implications in the pharmacokinetics and toxicity of xenobiotics such as therapeutic agents and environmental chemicals. To date, information about CYP1A1/1A2 induction has been obtained via measurement of specific enzyme activity and mRNA expression level in cellular systems such as human hepatocytes and human hepatoma cell lines.[28-31] The most relevant information was derived from enzyme activity in human hepatocytes. However, methods for determining enzyme activity are time consuming and require cumbersome laboratory procedures and are therefore low-throughput. On the other hand, the reporter gene assay is well suited for high-throughput screening to assess induction of CYP1A1/1A2.[32]

To test the stable cell line created in this study, induction of reporter activities by strong CYP1A1/1A2 inducers was measured. TCDD induced activity in a time-, dose-, and cell density-dependent manner (**Figs. 1** and **2**). When the stable cell line was treated with 1 nM TCDD for 48 h, enhancement of both Luc (642.3 ± 72.5-fold) and SEAP (6.8 ± 1.0-fold) activities was significant enough to simultaneously assess the transcriptional activation of *CYP1A1* and *CYP1A2* genes under optimal conditions. Other researchers have established similar reporter cell lines—101L cells and DRE12–6 cells—for assessing CYP1A1 induction.[33] In 101L cells, 2 nM TCDD produced a 22-fold increase in Luc activity after 24 h of exposure.[34] DRE12–6 cells treated for 28 h with 10 nM TCDD exhibited a 20-fold increase in Luc activity.[10] However, 101L and DRE12–6 cells integrated only part of the *CYP1A1* promoter region and 3 copies of XRE, respectively. In addition, these reporter gene systems were not capable of assessing CYP1A2 induction. Although it is difficult to directly compare the results obtained under various conditions, the stable cell line generated in this study exhibited high sensitivity to TCDD in CYP1A1/1A2 induction.

To demonstrate the utility of the stable cell line for the majority of compounds previously identified as CYP1A1/1A2 inducers, cells were treated with proton pump inhibitors, ALB, IND, PAHs, and TCDD (**Figs. 5A** and **B**). The results clearly show that all compounds caused induction of reporter activity. The fold induction in the SEAP activity was lower than that of the Luc activity, while the induction pattern among tested compounds resulted in similar induction of Luc and SEAP activities. In addition to assessing transcriptional activation of *CYP1A1* and *CYP1A2* genes by measuring reporter activities, we determined that the same compounds altered intrinsic CYP1A1 and CYP1A2 mRNA levels (**Figs. 6A** and **B**). In general, most compounds exhibiting induction in the reporter assay also exhibited increased CYP1A1 and CYP1A2 mRNA levels. Other researchers have demonstrated pharmacologic profiling of CYP1A1/1A2 induction in HepG2 cells and human hepatocytes.[35] Use of mRNA expression to detect CYP1A1/1A2 induction helps predict the enhancement of these enzymatic activities and increases the throughput. In the present study, detection of CYP1A1 and CYP1A2 mRNA levels was more sensitive than the reporter activities derived from a dual reporter gene. One reason for this is that the reporter gene assays generally display high backgrounds. However, the reporter gene assays have the advantages of being readily available and reproducible, and they are well suited for high-throughput screening approaches. The stable cell line created in this study indicated a similar effect in the induction response of reporter activities and intrinsic CYP1A1 and CYP1A2 mRNA levels. Accordingly, the enhancement of reporter activities in the stable cell line sufficiently reflected responses of *in vivo* CYP1A1/1A2 induction at a level comparable to that of specific enzyme activity and mRNA expression level in human hepatocytes and human hepatoma cell lines.

In order to further substantiate the ability of the stable cell line to assess CYP1A1/1A2 inducers, 13 PAHs were used to treat the cells (**Figs. 8A** and **B**). The induction profile of the SEAP activity by PAHs was similar to that of

the Luc activity. Interestingly, DB[*a*,*h*]A appeared to be a potent inducer common to Luc and SEAP activities. On the other hand, DB[*a*,*c*]A and Chr appeared to be strong inducers, particularly of Luc activity. Until now, much research has been done to assess the inducibility of CYP1A1/1A2 after exposure to PAHs.[36] Of these, DB[*a*,*h*]A was classified as a strong CYP1A1/1A2 inducer. Exposure of HepG2 cells to these compounds produced marked induction in CYP1A1 and CYP1A2 mRNA levels and enzymatic activities.[37] In addition, the reporter gene system utilized a stable reporter cell line and also indicated high response to DB[*a*,*h*]A. DB[*a*,*c*]A also caused significant increase of CYP1A1 and CYP1A2 mRNA levels in HepG2 cells, but these levels were lower than the levels of induction by DB[*a*,*h*]A. In CYP1A2, DB[*a*,*c*]A was as high as the mRNA induction level by 3-MC. These observations are consistent with our dual reporter assay results. On the other hand, although Chr produced a maximum increase in the Luc activity, this result was different from previous investigations.[38,39] In general, the four-ring PAHs such as Chr have a weaker tendency for induction than five-ring PAHs such as DB[*a*,*h*]A and DB[*a*,*c*]A.[38,39] This discrepancy might be due to difference in experimental conditions and the ability to metabolize Chr. Overall, these results show that the stable cell line results in high reporter activities at relatively low concentration (1 μM) that were easily used to detect CYP1A1/1A2 induction. However, B[*a*]P, a typical CYP1A1/1A2 inducer, did not induce reporter activity at the concentration of 1 μM, so we treated the same compound at a concentration of 3 μM. We found that B[*a*]P induced reporter activity and that the pattern of reporter activity for other PAHs was similar to that at the concentration of 1 μM. Thus, the stable cell line might be available to assess interactions between environmental pollutants such as PAHs and therapeutic agents.

Corchero *et al.* reported that the *CYP1A1* and *CYP1A2* genes are separated by a 23-kb segment that contains no other open reading frame. The *CYP1A1* and *CYP1A2* genes are in head-to-head orientation, revealing that the 5′-flanking region is common between the 2 genes.[11] These genes have been reported to be simultaneously regulated through common regulatory elements.[14] To our knowledge, this is the first report in which the stable cell line contained the 23-kb full-length regulatory region and could be used to simultaneously assess transcriptional activation of the *CYP1A1* and *CYP1A2* genes. Results obtained from the stable cell line could be used to rapidly and accurately predict the CYP1A1/1A2 induction. In addition, although the molecular mechanism of AhR-mediated activation has been extensively studied in CYP1A1, the same in CYP1A2 remains unclear. In this regard, the 23-kb full-length regulatory region in this stable cell line may be valuable for elucidating the precise mechanism of CYP1A2 induction.

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Original Article

Malondialdehyde-modified low density lipoprotein (MDA-LDL)-induced cell growth was suppressed by polycyclic aromatic hydrocarbons (PAHs)

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ABSTRACT — Malondialdehyde-modified low-density lipoprotein (MDA-LDL) and oxidized LDL (Ox-LDL), which accelerate the pathogenesis of arteriosclerosis, are thought to be involved in parthenogenesis caused by smooth muscle cell proliferation. In this study, we investigated the suppression mechanism of polycyclic aromatic hydrocarbons (PAHs) on the growth of an MDA-LDL-induced human acute monocyte leukemia suspension cell line (THP-1 cells). We found that PAHs suppressed MDA-LDL-induced THP-1 cell growth. Cotreatment with benzo[*a*]pyrene (BaP) or 3-methylchoranthrene (3-MC) decreased MDA-LDL-induced THP-1 cell growth, whereas treatment with benzo[*e*]pyrene (BeP) or pyrene, which is not a ligand for the arylhydrocarbon receptor (AhR), did not decrease THP-1 cell growth. Our findings clearly demonstrated that THP-1 cell growth, which was suppressed by PAHs, was restored by the addition of α-naphtoflavone, which is a partial antagonist to AhR. Moreover, it was shown that cotreatment with MDA-LDL and BaP markedly induced the expression of human cytochrome P4501A1 (hCYP1A1) messenger ribonucleic acid (mRNA) and significantly induced the expressions of p53 and p21 mRNAs. In support of these findings, AhR small interfering RNA suppressed the induced level of p21 mRNA and by BaP and the overexpression of hCYP1A1 significantly induced levels of p21 mRNA. On the other hand, the uptake rate of [^{14}C]BaP into cells was increased more significantly by cotreatment with MDA-LDL than by treatment with [^{14}C]BaP alone. These results strongly suggest that the suppression of MDA-LDL-induced THP-1 cell growth is caused by the increased uptake of PAHs, which strongly activate the AhR signal pathway accompanying DNA damage.

Key words: AhR, hCYP1A1, MDA-LDL, PAHs, p21

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants; exposure to PAHs has been suspected to be associated with the occurrence of pulmonary diseases. Benzo[*a*]pyrene (BaP) is a well-studied PAHs found in many environmental complex mixtures, including cigarette smoke and air pollution. BaP is highly mutagenic and must be metabolically activated to exert its carcinogenic potential (Miller and Ramos, 2001). In addition, BaP induces metabolic enzymes such as human cytochrome p4501A1/2 (hCYP1A1/2) and hCYP1B1 via arylhydrocarbon receptor (AhR) (Harrigan *et al.*, 2006; Jonsson *et al.*, 2006; Willett *et al.*, 2006). Thus, these hCYPs are involved in BaP-induced carcinogenesis (Galván *et al.*, 2005; Kim *et al.*, 1998; Harrigan *et al.*, 2004).

The pathogenesis of arteriosclerosis, now generally considered to be an inflammatory disease, is well characterized, but the initiating event is unknown (Ross, 1999). Individuals who smoke are at increased risk for developing arteriosclerosis and associated clinical events (Chouraki *et al.*, 2008; Antoniades *et al.*, 2008; Iribarren *et al.*, 1999; LaCroix *et al.*, 1991). These findings, combined with data from clinical studies, indicate that the pathogenesis of arteriosclerosis is accelerated through a number of different mechanisms, which collectively contribute to increased risk of developing the disease. The subendothelial accumulation of foam cells, which are primarily derived from monocytes and macrophages through

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the uptake of oxidized LDL (Ox-LDL), plays a key role in the initiation of arteriosclerosis (Steinberg and Witztum, 1990). It is well known that the LDL receptor, which is down-regulated when intracellular cholesterol levels are increased, is not involved in the intracellular accumulation of cholesterol in foam cells. However, macrophages avidly accumulate Ox-LDL via scavenger receptors that are unresponsive to intracellular cholesterol levels (Goldstein and Brown, 1977; Ylä-Herttuala *et al.*, 1991). Furthermore, Ox-LDL induces proliferation of monocytes and macrophages, smooth muscle cells, and other cells (Heery *et al.*, 1995; Zhao *et al.*, 2005; Matsumura *et al.*, 1997; Martens *et al.*, 1998; Hamilton *et al.*, 1999).

Generally, the pathogenesis of arteriosclerosis is accelerated through MDA-LDL and Ox-LDL. Interestingly, MDA-LDL was reportedly involved in atherogenesis by causing smooth muscle cell proliferation (Ozer *et al.*, 1993), which was generated by the uptake of MDA-LDL in macrophages via scavenger receptors; PAHs have also been reported to induce arteriosclerosis (Iwano *et al.*, 2005). However, the interaction between PAHs and MDA-LDL has not been clarified. Therefore, in this study, we investigated the suppression mechanism of MDA-LDL-induced THP-1 cell growth by PAHs to clarify the association between PAHs and MDA-LDL.

MATERIALS AND METHODS

Chemicals

BaP, β-naphthoflavone (β-NF), benzo[*e*]pyrene (BeP), pyrene, and α-naphthoflavone (α-NF) were purchased from Tokyo Chemical Industry Corporation (Tokyo, Japan). 3-Methylcholanthrene (3-MC) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade and purchased from commercial sources.

Cell culture

THP-1 (human acute monocytic leukemia) cells were obtained from Japan Human Health Sciences Foundation (Tokyo, Japan) and HepG2 (human hepatoma cell lines) cells were obtained from Riken Bioresource Center (Ibaraki, Japan). THP-1 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium from Invitrogen (Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. HepG2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) from Wako (Osaka, Japan) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 0.1 mM nonessential amino acids. THP-1 cells were seeded onto 96-well plates (3×10^4 cells/well) and 24-well plates (1×10^6 cells/well) from Becton Dickinson (Heidelberg, Germany). THP-1 cells were incubated with 0.1 ml (96-well plate for cell proliferation) and 0.5 ml (24-well plate for cell proliferation and incorporation of [^{14}C] BaP) of the medium containing PAHs (BaP, 3-MC, BeP, and pyrene): β-NF, MDA-LDL, 0.1% DMSO, or cotreatment with PAHs and MDA-LDL. The HepG2 cells were seeded onto 24-well plates at 5×10^4 cells/well for 24 hr, and used for a virus infection experiment.

Recombinant adenovirus

Adenovirus-expressing human *CYP1A1* and *CYP1A2* (Ad-hCYP1A1 and Ad-hCYP1A2) were done with the AdEasy™ System (Quantam Biotechnologies, Montreal, Canada) according to a previous report (Aoyama *et al.*, 2009). AdCont (AxCALacZ), which expresses β-galactosidase, was provided by Dr. Izumi Saito (Tokyo University) and used as a control adenovirus. The titer of the recombinant adenovirus, 50% titer culture infection dose ($TCID_{50}$), and multiplicity of infection (MOI) were determined as reported previously (Kamiyama *et al.*, 2007).

Infection of recombinant adenovirus

The HepG2 cells were incubated with 0.1 ml of a medium containing a recombinant adenovirus for 1 hr followed by the addition of 0.9 ml of medium and further culturing for 96 hr. Seventy-two hr after the adenovirus infection, the HepG2 cells were treated with 0.01 μM and 0.1 μM BaP or 0.1% DMSO for 24 hr.

Modification of LDL by malondialdehyde

Malondialdehyde was generated at room temperature by rapid acid hydrolysis (0.2 ml of 12 M HCl) of tetramethoxypropane (Nacalai Tesque, Kyoto, Japan). Then, 4.6 ml of 0.1 M sodium phosphate buffer (pH 6) was added to this reaction medium and the solution was adjusted to pH 6 with 10 M NaOH. Synthesized MDA was subsequently added to freshly prepared LDL (Chemicon, Tokyo, Japan) (2 mg/ml) and incubated at a final concentration of 0.02 mM for 3 hr at 37°C. The reaction was stopped by dialysis against phosphate-buffered saline (PBS) (pH 7.6) for 24 hr at 4°C. The extent of modification was determined by a colorimetric thiobarbituric acid assay (Fogelman *et al.*, 1980; Patton and Kurtz, 1951).

Tritiated thymidine incorporation assay

Proliferation of THP-1 cells was determined by the incorporation of [^{3}H] thymidine into cellular DNA. Briefly, 10 μl of 10 μCi/ml methyl [^{3}H] thymidine (80 Ci/mmol, Amersham Bioscience) was added to each well of

a 24-well plate and incubated for the final 18 hr in each experiment. The medium was then aspirated, cells were washed with ice-cold 10% trichloroacetic acid to precipitate DNA and unincorporated labeled thymidine was removed. Cells were dissolved in 0.1 M NaOH to hydrolyze the acid-insoluble material. Radioactivity was measured using a liquid scintillation counter (Beckman Counter LS 6500 AT, Fullerton, CA, USA).

WST-8 growth assay

THP-1 proliferation was determined by WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-dinitrophenyl)-2H-tetrazolium, monosodium salt] assays using a Cell Counting Kit-8 (Donjindo, Kumamoto, Japan). Briefly, 10 μl of WST-8/1-methoxy-phenazine methosulfate solution was added to each well of a 96-well plate and incubated for 2 hr at 37°C. The absorbance at 450 nm was then measured with a multiwell spectrophotometer (MPR-A4i; Toso, Tokyo, Japan).

[^{14}C]BaP incorporation assay

BaP incorporation into cellular DNA was determined by incorporation of [^{14}C]BaP. Briefly, 2 μl of [^{14}C]BaP (60 mCi/mmol, Amersham Bioscience) was added to each well of a 24-well plate. The medium was then aspirated, cells were washed with ice-cold 10% trichloroacetic acid to precipitate DNA and unincorporated labeled BaP was removed. Cells were dissolved in 0.1 M NaOH to hydrolyze the acid-insoluble material. Radioactivity was measured using a liquid scintillation counter.

Quantitative real-time polymerase chain reaction (PCR)

Total ribonucleic acid (RNA) was extracted from the cells using TRIzol according to the manufacturer's protocol. The cDNA was synthesized from 1 μg of total RNA using an oligo dT-adaptor and avian myeloblastosis virus ExScript reverse transcriptase (Takara, Shiga, Japan). Quantitative real-time PCR was performed using a Thermal Cycler Dice™ TP800 (Takara). The primer sequences used in this study were as follows: hCYP1A1: sense (5′-GGCCACTTTGACCCTTACAA-3′) and anti-sense (5′-CAGGTAACGGAGGACAGGAA-3′); hCYP1A2: sense (5′-GTTCCTGCAGAAAACAGTCCA-3′) and anti-sense (5′-CTGTGCTTGAACAGGGCAC -3′); AhR: sense (5′-GCACGAGAGGCTCAGGTTATCA-3′) and anti-sense (5′-GTGCATTAGACTGGACCCAAGTC-3′); p53: sense (5′-CTGTCCCTTCCCACAAAACC-3′) and anti-sense (5′-CCACTCGGATAAGATGCT-3′); p21: sense (5′-CGACTGTGATGCGCTAATGG-3′) and anti-sense (5′-CCAGTGGTGTCTCGGTGACA-3′); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): sense (5′-TCGGAGTCAACGGATTTGGTCGTA-3′) and anti-sense (5′-ATGGACTGTGGTCATGAGTCCTTC-3′). PCR was performed using a SYBR® Premix Ex Taq™ (Takara). The reaction was carried out under the following conditions: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles at 95°C for 5 sec, and 60°C for 30 sec. To confirm the amplification specificity, the PCR products were subjected to a melting curve analysis. The mRNA expression levels of the hCYP1A1, hCYP1A2, AhR, p53, and p21 in each sample were normalized to that of GAPDH.

Small interfering RNA

Double-strand siRNAs (25-mer) that target the AhR and control siRNA that target estrogen receptor α (ERα) were designed and synthesized by Invitrogen. The corresponding target mRNA sequences for the siRNAs were as follows: AhR siRNA, UUAAGUCGGUCUCUAUGCCGCUUGG; ERα siRNA, UAGUCAUUGCACACUGCACAGUAGC. HepG2 cells were transfected with siRNA when cells reached 30-50% confluence (48-well plate), according to the manufacturer's protocol. Briefly, gene-specific siRNA oligomers (20 nM) and 0.4 μl/well lipofectamine RNAiMAX were diluted in 40 μl/well DMEM (Invitrogen). After 20-min incubation at room temperature, the complexes were added to the cells in a final volume of 240 μl medium. Forty-eight hr after the siRNA transfection, the HepG2 cells were treated with 0.01 μM and 0.1 μM BaP or 0.1% DMSO for 48 hr.

Preparation of S9 fraction

HepG2 cells were washed with ice-cold PBS (without $CaCl_2$ and $MgCl_2$) and then removed with a cell scraper. The cells were suspended in 0.25 M sucrose/1 mM EDTA/10 mM Tris HCl buffer (pH 7.4). The sonicated cells were then homogenized under ice-cold conditions. After centrifugation of the homogenated cells at 9,000 × *g* for 20 min at 4°C, the supernatant (S9) was collected. Protein content was determined by using a Protein Assay Kit (Bio-Rad, Tokyo, Japan) according to the manufacturer's protocol, with bovine serum albumin as the standard.

7-Ethoxyresorufin O-deethylase (EROD) assays

CYP1A activity was determined using an EROD assay according to the methods of Burke and Mayer (1974) with the following modifications. The reaction mixture contained 1 μM 7-ethoxyresorufin (Sigma), S9 fraction (200 μg of S9 protein), and 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 0.5 ml. After preincubation of the mixture at 37°C for 5 min, 0.5 mM NADPH

(Wako) was added to initiate the reaction, and the mixture was further incubated for 10 min. The reaction was terminated by the addition of 2 ml of ice-cold methanol, and the samples were then centrifuged at 9,000 × *g* for 10 min. The metabolite, resorufin, was measured using Powerscan®HT (Dainippon Sumitomo Pharma, Osaka, Japan) (excitation and emission wavelengths of 530 nm and 585 nm, respectively).

Statistical analysis

Data are expressed as the mean ± standard (S.D.) error from three determinations. Statistical comparison of two groups was performed using a two-tailed Student's *t*-test. *P*-values less than 0.05 were considered to be statistically significant.

RESULTS

Effects of PAHs on MDA-LDL-induced THP-1 cell growth

We examined the effects of MDA-LDL on THP-1 cells growth. THP-1 cell growth increased nearly 30% compared to the control by treatment with 0.4 mg/ml MDA-LDL. We further investigated whether PAHs had an effect on MDA-LDL-induced THP-1 cell growth. Cotreatment with BaP or 3-MC and MDA-LDL reduced MDA-LDL-induced THP-1 cell growth (Fig. 1-A). On the other hand, cotreatment with BeP or pyrene, which does not activate the AhR, and MDA-LDL did not reduce MDA-LDL-induced THP-1 cell growth (Fig. 1-B). Or, that the experiments with Ox-LDL yielded similar results to those observed in MDA-LDL; however, we examined the role of the MDA-LDL in this study because the results of MDA-LDL were clearer than the results of Ox-LDL.

Effects of α-NF or β-NF on suppression of MDA-LDL-induced THP-1 cell growth by BaP

Among the PAHs, BaP and 3-MC activate AhR-mediated signal transduction, but pyrene and BeP do not. To clarify whether AhR-mediated signal transduction is involved in the suppression of MDA-LDL-induced THP-1 cell growth by BaP, we examined the effect of α-NF, a partial antagonist to AhR, on [^{3}H] thymidine incorporation. Cotreatment with BaP and MDA-LDL reduced [^{3}H] thymidine incorporation. The suppression of [^{3}H] thymidine incorporation into THP-1 cells was clearly restored by the addition of 1 μM α-NF (Fig. 2-A). β-NF is a potent agonist to AhR, but not to PAHs, and does not cause cell damage. To clarify the involvement of AhR in suppression of MDA-LDL-induced THP-1 cell growth, we further examined whether β-NF, as well as BaP, suppresses MDA-LDL-induced THP-1 cell growth. Co-treatment with β-NF and MDA-LDL did not reduce the MDA-LDL-induced THP-1 cell growth (Fig. 2-B).

Effect of MDA-LDL and BaP on hCYP1A1 mRNA expression

To further clarify whether AhR-mediated signal transduction was activated by BaP, we examined the effect of cotreatment with MDA-LDL and BaP on the expression of hCYP1A1 mRNA. hCYP1A1 is a BaP-activating enzyme, and the expression was induced by activation of the AhR pathway. Cotreatment with MDA-LDL and BaP markedly induced the expression of hCYP1A1 mRNA (28-fold). In contrast, BaP treatment also induced the expression, but at a low level (9.8-fold) (Fig. 3).

The role of MDA-LDL in BaP activation in THP-1 cells

Together with the above results, cotreatment with BaP and MDA-LDL was predicted to cause strong BaP activation due to the acceleration of BaP incorporation into the cells. We next examined the incorporation of BaP into THP-1 cells. Incorporation of [^{14}C]BaP with MDA-LDL into THP-1 cells increased 5.6-fold compared to the results from treatment with [^{14}C]BaP alone (Fig. 4).

Analysis of the suppression mechanism of MDA-LDL-induced THP-1 cell growth by BaP

Together with these results, induced hCYP1A1 is predicted to transform BaP to an activated form causing DNA damage and, consequently, increased levels of tumor suppressor proteins such as p53 and p21, the proliferation inhibitor protein. Therefore, we next measured p53 and p21 mRNA levels by cotreatment with MDA-LDL and BaP. As expected, cotreatment with BaP and MDA-LDL significantly induced the expression of p53 (2.6-fold) and p21 (1.9-fold) mRNAs compared to that of the control. Treatment with BaP alone did not induce the expression of mRNAs (Fig. 5).

Effect of knockdown of AhR and overexpression of hCYP1A1 on the expression level of p21 mRNAs

To determine the influence of the AhR pathway or metabolic activation of BaP by hCYP1A1 on the expression level of p21 mRNA, we performed knockdown of the AhR using AhR siRNA and overexpression of hCYP1A1 using an hCYP1A1-expressing adenovirus (Ad-hCYP1A1). It was difficult to overexpress the recombinant protein in THP-1 cells through a cDNA expression vector. Therefore, in this study, we used HepG2 cells and a hCYP1A1-

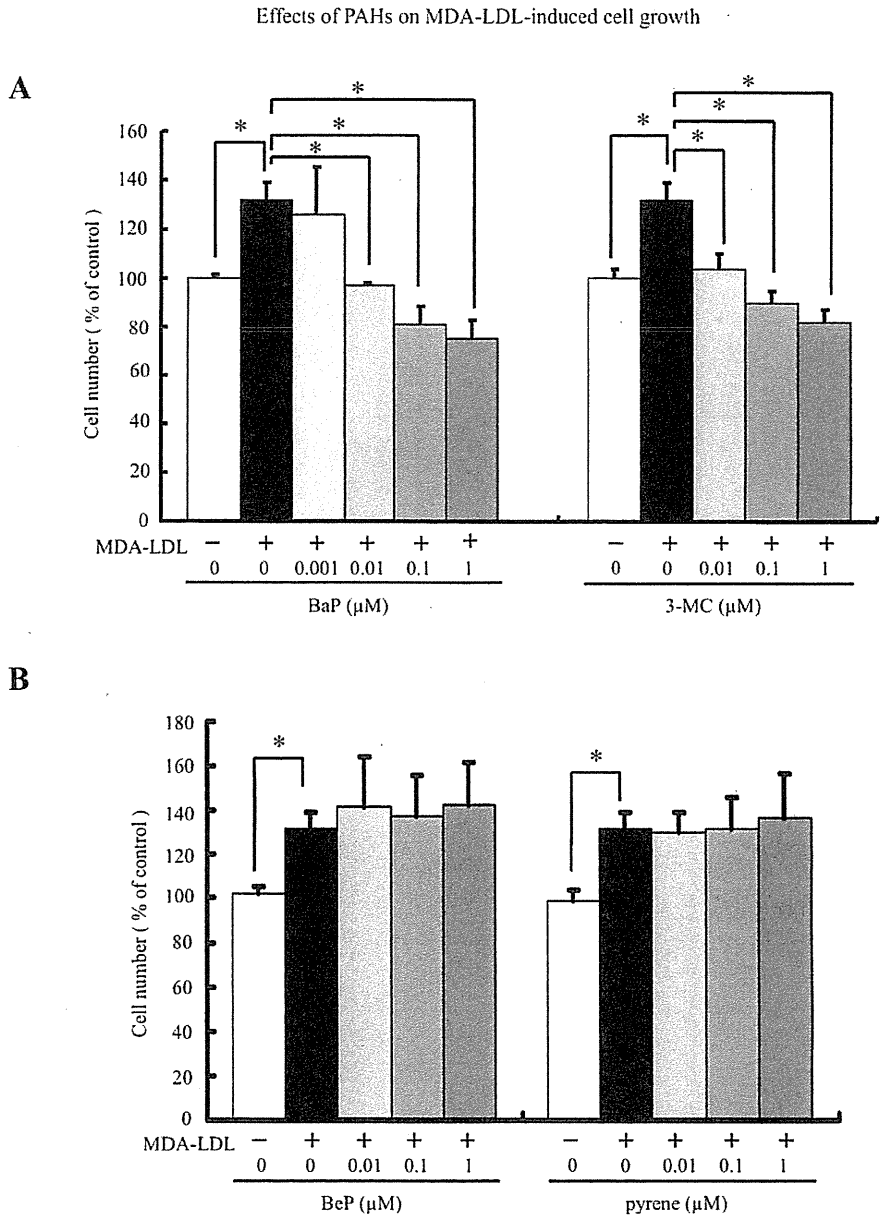

Fig. 1. Effects of BaP and 3-MC (A) and BeP and pyrene (B) on MDA-LDL-induced THP-1 cell growth. THP-1 cell proliferation was assessed by a WST-8 assay. Concentrations of MDA-LDL and PAHs were 0.4 mg/ml and 0.01-1 μM, respectively. Data show the mean ± S.D. of at three independent experiments. $^{*}p < 0.001$.

expressing adenovirus. As a result, the levels of AhR and CYP1A1 mRNA were downregulated by 73% and more than 50%, respectively, compared with the control (Fig. 6-A). The level of p21 mRNA was induced in a dose-dependent manner by the addition of BaP compared with the control (1.7- to 2.0-fold) in 48-hr cultured cells. However, when AhR siRNA was introduced into cells, p21 mRNA was not induced in 48-hr cultured cells (Fig. 6-

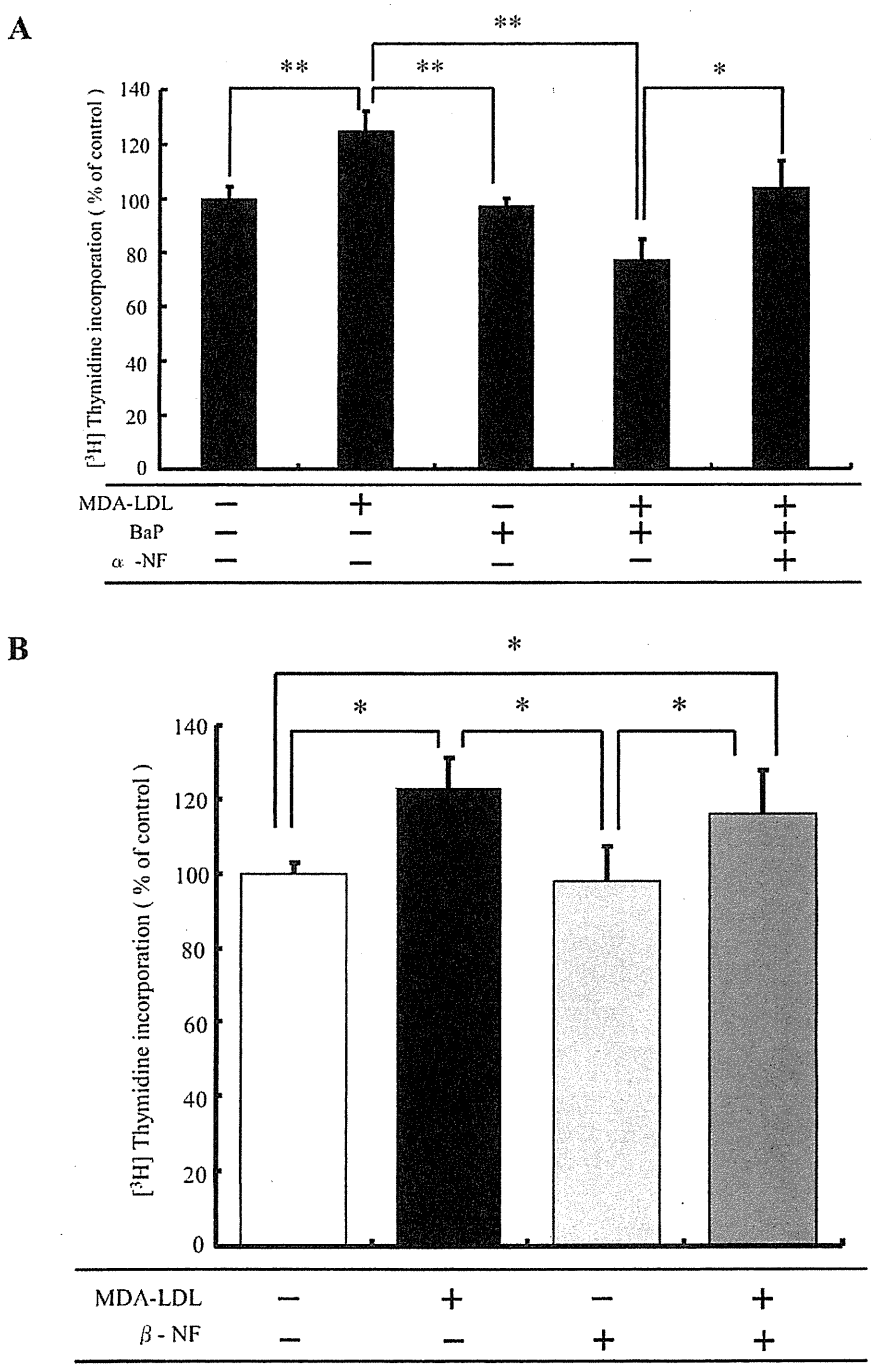

Fig. 2. Effects of α-NF (A) and β-NF (B) on BaP suppression of MDA-LDL-induced THP-1 cell growth. THP-1 cell proliferation was assessed by a [³H] thymidine incorporation assay. Concentrations of BaP, MDA-LDL, α-NF, and β-NF were 0.1 μM, 0.4 mg/ml, 0.1 μM, and 0.1 μM, respectively. Data show the mean ± S.D. of at three independent experiments. $^{*}p < 0.01$; $^{**}p < 0.001$.

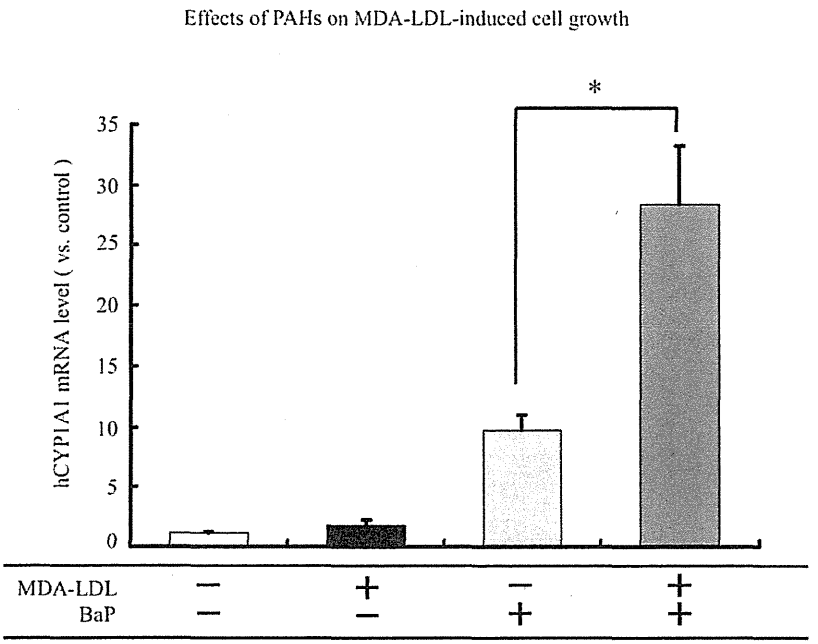

Fig. 3. Expression of hCYP1A1 mRNA in THP-1 cells. THP-1 cells were incubated with DMSO, BaP, MDA-LDL, or MDA-LDL along with BaP for 12 hr. Total RNA was prepared 12 hr later as described in the experimental procedures. The mRNA expression of the target genes was analyzed by real-time RT-PCR, and the mRNA levels of the target gene were normalized to that of GAPDH. Concentrations of MDA-LDL and BaP were 0.4 mg/ml and 0.1 μM, respectively. Data show the mean ± S.D. of at three independent experiments. $^{*}p < 0.01$.

B). Moreover, p21 mRNA was induced in a dose-dependent manner by treatment with Ad-hCYP1A1 and BaP in 24-hr cultured cells (Fig. 6-C) but not with Ad-hCYP1A2 and BaP. Typical catalylic activities for hCYP1A1 and hCYP1A2 were detected with EROD. EROD activities of overexpressed hCYP1A1 and hCYP1A2 were 2.0 and 2.39 times higher, respectively, than that of the control (Fig. 6-D).

DISCUSSION

Macrophage proliferation is recognized as an important factor of pathogenesis in the development of arteriosclerosis. Studies from several research groups have shown that Ox-LDL induced macrophage proliferation (Matsumura *et al.*, 1997; Martens *et al.*, 1998; Hamilton *et al.*, 1999). Interestingly, PAHs have been known to induce arteriosclerosis (Curfs *et al.*, 2005; van Grevenynghe *et al.*, 2004), and several observations support the hypothesis that the aggravation of arteriosclerosis is caused by PAHs coexiting with Ox-LDL such as MDA-LDL. However, it is unclear as to how arteriosclerosis is promoted by PAHs. Therefore, we expected that MDA-LDL-induced cell growth is supported by the presence of PAHs. In the present study, we used THP-1 cells to examine whether MDA-LDL-induced cell growth was caused by PAHs. Contrary to expectations, our results demonstrated that cotreatment of BaP or 3-MC with MDA-LDL decreased MDA-LDL-induced THP-1 cell growth (Fig. 1-A). Interestingly, we found that there were differences between the ligands (BaP or 3-MC) and nonligands (pyrene or BeP) of the AhR (Figs. 1-A and B). In addition, we conducted experiments using an AhR partial antagonist (α-NF) and agonist (β-NF) other than PAHs to determine whether depressed MDA-LDL-induced THP-1 cell growth in the AhR could be found under the same conditions. It is of interest that α-NF clearly restored the synergistic action by the combination of BaP and MDA-LDL (Fig. 2-A), but β-NF did not depress MDA-LDL-induced THP-1 cell growth. It seems that activation of the AhR pathway is involved in the depression of MDA-LDL-induced THP-1 cells growth; however, involvement of another mechanism after activation of the AhR pathway was predicted. Next, we designed an experiment to investigate the expression level of hCYP1A1 mRNA. Higher expression of mRNA was observed after cotreatment of