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Development of Luciferase Reporter Gene-based Cell Bioassay for the Aromatic Hydrocarbon Receptor Agonists

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The aromatic hydrocarbon receptor (AhR) is a ligand-activated transcription factor that mediates many of the biological and toxicological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related chemicals. The application of recombinant reporter plasmid such as the firefly luciferase gene has proven to be a very effective method to detect these chemicals. The bioassay system, CALUX, is sensitive in directly detecting AhR-agonists from a variety of environmental and biologic materials. However, responses of the AhR-dependent bioassays are dependent on the cell types used. Thus, we developed a sensitive bioassay using the recombinant mouse hepatoma cell (Hepa1c1c7) for the determination of dioxins. The recombinant cell line was stably transfected with firefly luciferase reporter gene (pGudLuc1.1). The transfected cells showed the highest induction of luciferase activity at 4.5 hr and a decrease beyond this time point. The system showed the highest sensitivity of detection ever reported. Upon TCDD exposure cells showed 2 fold increase at 10 pM and 7 fold increase at 100 pM, respectively. The passage number after the transfection played an important role in the sensitivity. The increase of passage number tended to increase the sensitivity of the cells up to 15. The media without phenol red showed a higher induction rate than with phenol red, suggesting the preferable use of phenol red-free media for the bioassay. Since each of the assays has unique characteristics that make them suitable for some screening applications and not others, development of sensitive bioanalytical methods based on a variety of cellular systems in a key to the successful determination of dioxins. The bioassay system developed in this study will contribute to further development of successful screening the AhR agonists among the environmental mixture. In addition, the rapid and sensitive nature of this cellular system can be applied as a valuable tool to screen the dioxin-like moieties among the prodrugs at the initial stage, thereby expediting the new drug discovery.

Key Words: AhR agonist, TCDD, Bioassay, Reporter gene, Screening

INTRODUCTION

The aryl hydrocarbon receptor (AhR) is a soluble, ligand-dependent transcriptional factor that regulates the induction of gene expression by its ability to bind ligands (Poland & Knutson, 1982; Denison et al, 1998a). Ligands for AhR include a wide range of chemical structures. High affinity ligands for the AhR include a variety of HAHs such as polychlorinated dibenzo-*p*-dioxins, dibenzofurans, and biphenyls, as well as numerous polycyclic aromatic hydrocarbons (PAHs) such as benzo(a)pyrene, 3-methylchlolanthrene, and other chemicals (Safe, 1990). Among these ligands, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) is the most potent AhR ligand ever known and it activates the receptor at the extremely low concentrations (Safe et al, 1989). The AhR is known to function in a manner similar to that of the steroid hormone receptors, although they are clearly members of distinct families of transcription factors (Holmes & Pollenz, 1997). Following ligand binding, the cytosolic ligand : AhR complex translocates into the nucleus, and dimerizes with the Ah receptor nuclear translocator (ARNT) protein (Denison et al, 1988; Probst et al, 1993). The ligand : AhR : ARNT complex binds with the dioxin responsive element (DRE) and stimulates transcription of an adjacent promoter and gene (Schmidt & Bradfield, 1996). The lack of TCDD toxicity in AhR knockout mice suggests that the AhR mediates most of the toxic and biologic effects of TCDD (Fernandez-Salguero et al, 1996).

To assess biological responses of such compounds in complex system, many bioanalytical techniques have been developed utilizing several aspects of the AhR-dependent mechanism of action (Garisson et al, 1996; Han et al, 2004; Jeong et al, 2005). These bioassay systems are known to be rapid, low-cost and sensitive screening methods for the detection and relative quantification of dioxin-like compounds in sample extracts. The application of recombinant reporter plasmid such as the firefly luciferase gene has

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; DRE, dioxin responsive element; TEF, toxic equivalent factor; CYP1A1, cytochrome P450 1A1.

proven to be a very effective method to detect these chemicals (Ziccardi et al, 2000). Cell bioassay based on a reporter gene system uses genetically modified cells that respond to chemicals that activate AhR. A common response resulting from activation of the AhR signaling pathway in cells is the induction of gene expression. This forms the basis of this type of bioassay. The recombinant cells used in the bioassay contain a stably transfected AhR-responsive firefly luciferase reporter gene that responds to dioxins and other chemicals that can bind the AhR and induce luciferase gene expression (El-Fouly et al, 1994). Induction of luciferase gene expression in the recombinant cells occurs in a time- and dose-dependent manner. The amount of induced luciferase activity is directly propotional to the amount and potency of the inducing chemicals. The measured luminescence resulting from exposure to a chemical is converted into a bioassay toxic equivalent factor (TEF) by the direct comparison of response for a given sample to a dose-response curve obtained from TCDD. Most of reporter gene assays used for dioxin detection target a highly inducible gene, cytochrome P450 1A1 (CYP1A1) (Garrison et al, 1996). For the DR CALUX system, CYP1A1 promoter region responsible for the TCDD exposure was coupled to the firefly luciferase reporter gene and this DNA construct was stably transfected in the recipient rat H4IIe hepatoma cells (Gizzi et al, 2005). Exposure of TCDD to this system is now widely applied for a quantitative measurement of dioxin.

Although various cell lines are used as a basis for the development of cell bioassays, each system has a different response pattern depending on the cell type used. Thus, development of a variety of bioanalytical systems with different cellular characteristics has been required to meet the various characteristics of chemical moieties and environmental exposure settings.

In this study, we attempted to develop a dioxin bioassay with the hepa1c1c7 mouse hepatoma cells and generate the optimal bioassay conditions for the improved detection of the AhR agonists.

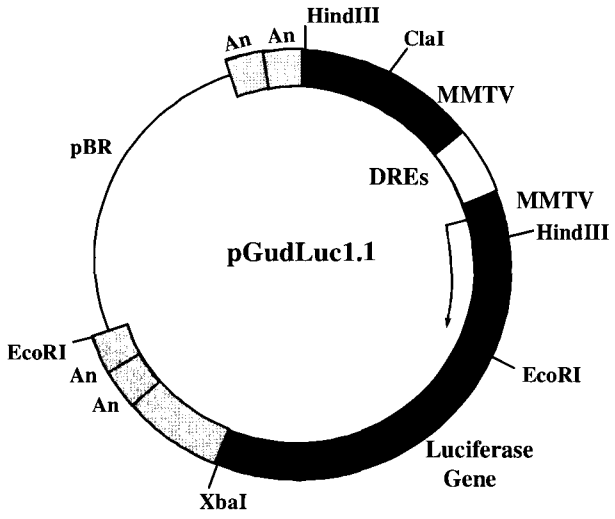

Fig. 1. The pGudLuc1.1 reporter vector is based on pGL2-Basic and has the firefly luciferase gene under control of a 484-bp fragment of the mouse CYP1A1 enhancer, that contains 4 DREs and the murine mammary tumor virus promoter.

METHODS

Chemicals

TCDD obtained from KOR Biomedical (Cambridge, MA). The purity of this compound was >99% as assayed by analytical high pressure liquid chromatography. TCDD was dissolved in DMSO, and aliquot (100 μM) were stored at −70°C. Various concentrations of TCDD were prepared by direct dilution of 100 μM aliquots into appropriate media. The tissue culture media were purchased from Hyclone and the antibiotics in media were from Sigma (St. Louis, MO, USA). The transfection reagent, lipofectamine was obtained from Invitrogen. The antibiotic G418 and substances of luciferase activity measurement were from Promega.

Description of plasmid

The pGudLuc1.1 is a kind gift from Dr. Mike Denison, UC Davis, U.S.A. The plasmid is 7.4 kb vector based on pGL2-Basic and has the firefly luciferase gene under control of a 484-bp fragment of the mouse CYP1A1 enhancer that contains 4 DREs and the murine mammary tumor virus promoter (Fig. 1). The pGudLuc1.1 vector without selectable marker gene was cotransfected with pSV2neo vector. The neomycin resistance gene of pSV2neo was used as a dominant selectable marker to select for stably transfected mammalian cells using neomycin (G418) (Fig. 2).

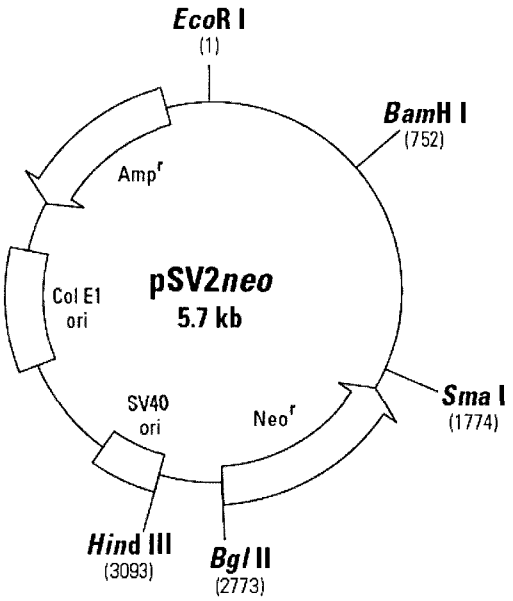

Fig. 2. Selection vector that confers neomycin resistance to eukaryotic cells. The neomycin resistance gene of pSV2neo can be used as a dominant selectable marker to select for stably transfected mammalian cells using neomycin (G418). pSV2neo contains a Col E1 origin of replication and ampicillin resistance for propagation and selection, respectively, in *E.coli*.

Cell culture and transfection

The hepa1c1c7 cells were cultured at 37°C in 5% CO_2 humidified incubator. Growth and maintenance medium consisted of Dulbeco's modified minimal essential medium (GIBCO, Gaithersburg, MD, USA) with 10% fetal bovine serum, penicillin G (50 units/ml) and streptomycin (50 μg/ml). Cells were subcultured at the confluency. Cells were cotransfected with 10 μg of the pGudLuc1.1 vector and 2 μg of pSV2neo using lipofectamine reagent. After 6 hr, the media were exchanged into the media with 20% FBS. Cells were allowed to grow for 2 days, after which the media was replaced with DMEM containing 0.3 mg/ml geneticin (G418). Cells were maintained in selective media for 2 weeks until visible colonies of surviving cells could be cloned.

Colony selection

After the transfection, the colonies were selected with G418. The luciferase activities among the surviving colonies were measured and the colonies highly expressing luciferase activity were isolated with the cloning rings.

Chemical treatment and measurement of luciferase activity

The selected colonies were cultured at final concentration 1.5×10^4 cells/ml in 24 well culture plate. The cells at 80% confluency were treated with DMSO (0.1%) or TCDD at the various exposure concentrations and time points. The plates were then transferred onto ice, the media were removed and the plates were rinsed twice with cold PBS. Cells were lysed with 100 μl of luciferase lysis buffer (Promega, Madison, WI, USA) and the lysed cell samples were collected and transferred into a microfuge tube wherein cell debris was pelleted by centrifugation. The supernatants were collected into 96 well plate. Luciferase activity in an aliquot of the cell lysis supernatant (25 μl) was determined by mixing it with 50 μl of luciferase assay reagent (Promega) and measuring the resulting luminescence in Micro-Lumat Plus LB 96V luminometer (Han et al, 2004).

Data analysis

Data are presented as the mean±SD. The differences between the groups were examined using Student's t-test and the significance was set at $p<0.05$.

RESULTS

Selection of sensitive colonies

After the geneticin selection, 5 out of 25 initially selected colonies showed the high sensitivity of luciferase activity. These colonies were designated as H-1, H-2, H-3, H-4 and H-5, respectively. Among these colonies, H-3 showed the highest activity at 1 nM TCDD after 5 hr exposure (Fig. 3). Clonal cells were continuously subcultured for the development of bioassay optimal condition.

Effects of TCDD on luciferase activity over time

Cells were exposed with 1 nM TCDD for the duration of 2 hr to 48 hr to optimize the exposure time. Cells transfected with pGudLuc1.1 showed time-dependent increase of luciferase activity with the maximum exposure time point of 4 and half hrs. After this time point, the activity was decreased (Fig. 4). The result indicate that 4.5 hr is the optimal exposure time to detect the luciferase response to TCDD exposure with a maximum capacity. Determination of the optimal exposure time point is essential to develop more efficient and time-saving bioassay conditions.

Dose-response relationship

Cells were treated over the range of 10^{-13} to 10^{-10} M TCDD for 4.5 hr. TCDD treatment showed a dose depen-

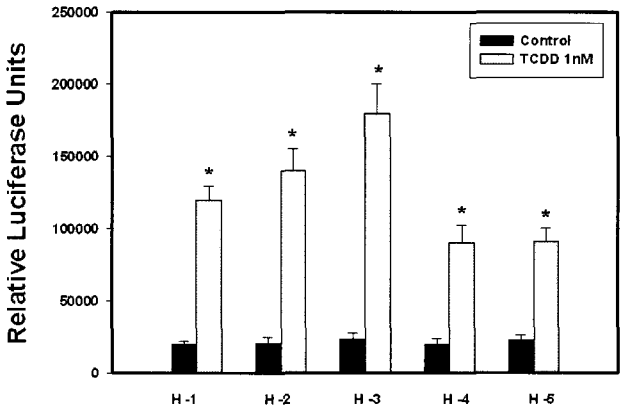

Fig. 3. Induction of luciferase activity by TCDD in stably transfected clones. Neomycin-resistant colonies of recombinant mouse hepatoma cell that had been stably transfected with pGudLuc1.1 were treated with 1 nM TCDD for 5 hr at 37°C. The luciferase activity of the lysates was measured as described in METHODS. *$p<0.05$.

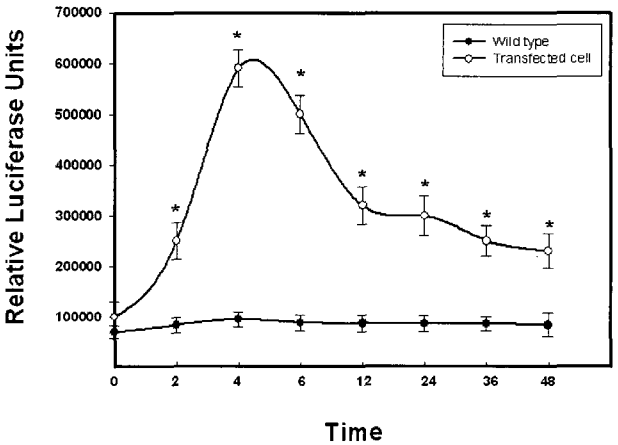

Fig. 4. Time dependent induction of luciferase activity by TCDD. Several 96 well microtiter plates containing recombinant mouse hepatoma cell at approximately 80% confluent were incubated with 1 nM TCDD for the indicated time points after which luciferase activity was determined as descried in METHODS. *$p<0.05$.

dent increase with 7 fold increase at 100 pM (Fig. 5). The minimal detection limit was as low as 10 pM. But, the minimal detection limit and the maximum efficacy were dependent upon the passage number. The rest of clonal cells also showed a similar pattern of increase.

Effects of passage number and dose on luciferase activity

After the transfection, cells were continuously subcultured to optimize the cell conditions for the best detection sensitivity. Cells showed the different response sensitivity upon subculture numbers (Fig. 6). This pattern was also dependent on the clonal cell types. At H-3 clonal cells, induction sensitivity was slightly increased as the number of passage increased up to 15. Cells at higher passage showed rather a higher sensitivity. Cells at passage 15 showed 4 fold increase at 0.1 nM and 7 fold increase at 1 nM, respectively. The result showed the highest sensitivity among the published data. While such high sensitivity may be transient, the study indicates that it is very important to identify the optimal passage number to maximize the bioassay sensitivity.

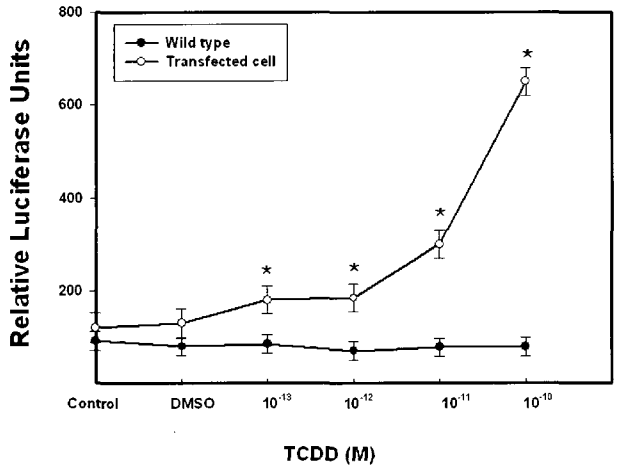

Fig. 5. Dose-response relationship for the induction of luciferase activity. Cells were treated with the indicated concentrations of TCDD for 4.5hr. Triplicates of each experiment were performed. *$p < 0.05$.

Optimization of treatment media conditions

To obtain the successful conditions for the bioassay, it is important to optimize the composite of culture medium. Cells were grown in the culture medium as described in material and method section. It is important to optimize the culture media composite at the time of treatment and measurement. In particular, phenol red, one of components in the culture media, is suggested to interfere with the AhR transduction pathway. Thus, we attempted to test the media with or without phenol red to optimize the cell culture media condition. The result showed that the media with phenol red increased the basal level of luciferase in the intact hepa1c1c7, which resulted in decrease of fold induction in the treated cells. The media without phenol red showed higher induction rate than with phenol red (Fig. 7).

Stability of cell system for the repeated applications

Although cells are successfully transfected with plasmids, it is not guaranteed that the phenotypic characteristics of the transfected cells may last generation to generation. Unless the genetic information carried into nucleus is fixed in the host genome, it is highly likely that the cellular phenotypic characteristics disappear as the subculture passage continues under in vitro systems. Our results demonstrated a stable induction of luciferase activities up to 15 passages (Fig. 6), which is not sufficient number of passage to consider as a successfully established cellular system. While the results obtained in the present study may be a transient phenomenon, it has demonstrated the importance of optimal cellular divisions to increase the sensitivity of this bioassay upon the AhR agonist exposure.

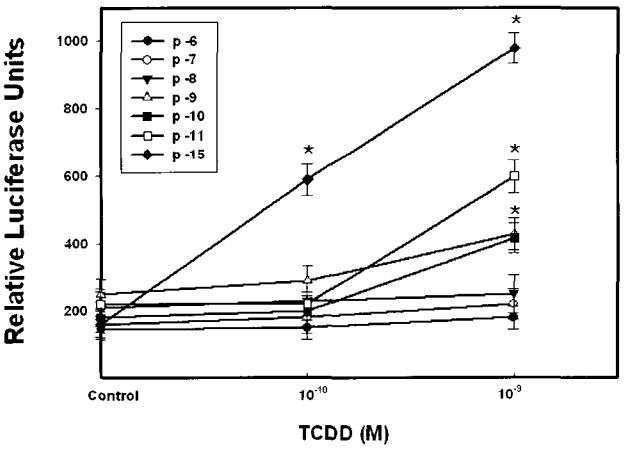

Fig. 6. Effects of passage numbers and dose on luciferase activity. Sensitivity with exposure to TCDD was dependent on the passage number. Cells at higher passage number showed a sensitive response at the low dose. The triplication of each experiments were performed. *$p < 0.05$.

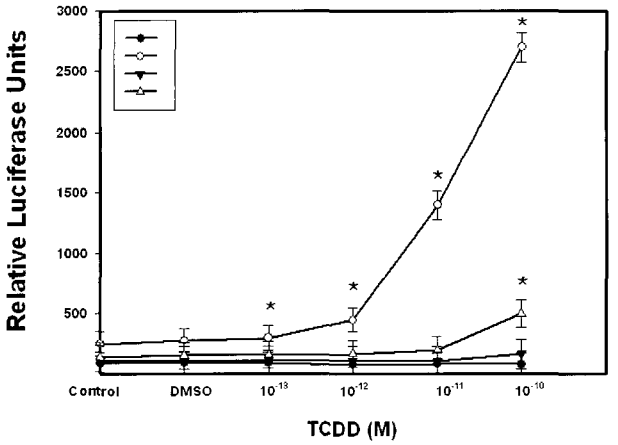

Fig. 7. Dose-response relationship under the different media component; Wild type (-●-) and transfected cells (-○-) with phenol-free medium, wild type (-▼-) and trasfected cells (-△-) with normal medium. Medium without phenol-red showed a higher induction fold than with normal medium. *$p < 0.05$.

DISCUSSION

The chemically activated luciferase gene expression *in vitro* cell bioassay, namely "CALUX", is a bioanalytical tool that is increasingly being used by research and commercial laboratories for the screening of dioxins and related compounds in a variety of samples. This screening tool is cheaper and more rapid than the conventional instrumental chemical analysis of dioxins. In addition, this type of bioanaytical method is actually necessary for the high number of samples required for food safety or large scale environmental monitoring.

Many bioassay systems based on the AhR-response mechanism have been developed and a wide spread application of this method for detection of the AhR agonists is now gaining a momentum (Garrison et al, 1996; Denison et al, 1998b; Jeong et al, 2005). The CALUX bioassay system has shown a significant improvement over the past several years with respects to its increased rapidity, sensitivity and lack of reporter gene inhibition by the inducing chemicals. However, differences in the response of these bioassays to TCDD exposure remain as a major obstacle for the further improvement. Since the basis of these bioassays is a recombinant cell lines, characteristics of the cell lines used are critical factors for the output of the bioassay system (Han et al, 2004). It is known that each cell line has specific advantages that can be used when identifying and characterizing AhR ligands. Thus, we attempted to develop the bioassay system for the AhR agonists, based on hepa1c1c7 hepatoma cell line. While the bioassays based on the mouse hepatoma cell line are developed, these assays utilize a transient-transfection protocol, which allows the use of the early stage of cells following the transfection. In addition, the media conditions that may interfere with the AhR transduction pathway were not critically considered. Therefore, in this study, we attempted to come up with the improved cellular bioassay system with its respect to its sensitivity and cellular stability. To begin with, the bioassay system developed in the present study revealed the incubation time-dependent increase of luciferase activity following TCDD exposure. The optimal exposure time was 4.5 hr. This time point is consistent with other studies which used the same plasmid construct (Nagy et al, 2002; Han et al, 2004). This system showed a very sensitive dose-response relationship upon TCDD exposure. A significant induction of luciferase activity was detectable as low as 10 pM and the induction fold was 2 fold at 10 pM and 7 fold at 100 pM, respectively, as compared with the untreated cells. This magnificent induction rate was observed with the cells of 15 subcultures. Considering the induction fold and the minimal detectable concentration, this is the most sensitive output among the bioassays ever reported, utilizing the similar mechanism of action. The most significant finding in this study is that such sensitivity of cellular system was closely associated with the passage numbers. In particular, the sensitivity difference by passage number was observed when cells were exposed below 10 pM (Fig. 7). The results indicate that there is a certain cellular division point following the transfection, which facilitates the luciferase reporter gene system with its maximum capacity. Therefore, it is necessary to identify the maximum induction points of cellular passage following the transfection to secure the optimal sensitivity. At this point, it is hard to determine whether the method described in this study is better than the transient method. However, the study demonstrated the importance of subculture numbers for the cellular bioassay and the necessity of the various approaches for the improvement of the CALUX type bioassays.

Phenol red, a component of the culture medium, has a tryptophan-like structure, which has an affinity with the AhR (Helferich & Denison, 1991; Heath-Pagliuso et al, 1998). Our result demonstrated that the media with phenol red has a higher basal activity of luciferase than without phenol red at the untreated cells. In addition, the induction fold upon TCDD exposure was higher in the cells cultured with the phenol red-free media. The present study provides a evidence that the medium components play an essential role for the optimization of the bioassay. Our cellular system showed a maximum sensitivity within 4.5 hr, which is considered relatively short reaction time. Thus, this system may be more appropriate for detecting AhR agonists that might normally be inactivated or converted to non-AhR binding forms by metabolism in longer incubation time. It has recently been reported that several prostagladins induce luciferase gene expression in H1L1.1c2 cells at 4 hr (Seidel et al, 2001), while no induction was observed in H1L6.1c2 or H1G1.1c3 cells at 24 hr (Han et al, 2004). In addition, synergistic effects of protein kinase C activators or luciferase inducers were observed in H1L1.1c2 cells at 4 hr, with little or no effects observed at 24 hr (Khan & Denison, 2000). Thus, our cellular system is suitable for identification and characterization of rapid AhR induction responses that would not be able to be observed in the cell systems with the longer incubation time. A major problem with use of the recombinant cells is that the phenotypic characteristics of the transfected cells disappear as the passage continues. This phenomenon is observed because the failure of the transfected gene is not fixed on the host cell genome (Yang & Rhim, 1995) Thus, it is quite possible that a high sensitivity observed in this study may be a transient effect and this sensitivity may be diminished after a sufficient number of passages. Considering the fact that the passage number above 50 is generally accepted as an established permanent cell line, the study needs more time to determine whether this particular clonal cells, H-3, would be able to become an established cell line for the continuous use. While the results obtained in the present study may be a transient phenomenon, it has demonstrated the importance of optimal cellular divisions to increase the sensitivity of this bioassay upon the AhR agonist exposure.

Overall, this study is believed to contribute to the improvement of AhR-dependent cell bioassay by providing the optimal conditions of subculture number, reaction time and culture media component. It is suggested that the cellular system described in this study is one of the most sensitive bioassay system ever reported for identification of the AhR agonists. However, it warrants further studies to determine whether such high sensitivity is sustainable throughout the series of future subcultures and this cellular system can be applied for the in-field screening system which requires the high stability of gene expression. Since each of the assays has unique characteristics that make them suitable for some screening applications and not others (Garrison et al, 1996), the bioassay system developed in this study will contribute to a further development of screening the AhR agonists among the environmental mixture. In addition, the rapid and sensitive nature of this cellular system can be applied as a valuable tool to screen the dioxin-like

moieties among the prodrugs at the initial stage, thereby expediting the new drug discovery.

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