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Improvement of Chemically-activated Luciferase Gene Expression Bioassay for Detection of Dioxin-like Chemicals

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ZHANG ZHI-REN, XU SHUN-QING[1], ZHOU YI-KAI, XU YONG-JUN, LIU ZHI-WEI, CAI XIAO-KUN, AND TAN XIANG-LIN

Institute of Environmental Medicine, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

Objective To improve the chemically-activated luciferase expression (CALUX) bioassay for detection of dioxin-like chemicals (DLCs) based on the toxicity mechanisms of DLCs. **Method** A recombinant vector was constructed and used to transfect human hepatoma (HepG2). The expression of this vector was 10-100 folds higher than that of pGL2 used in previous experiments. The transfected cells showed aromatic hydrocarbon receptor (AhR)-meditated luciferase gene expression. The reliability of luciferase induction in this cell line as a reporter of AhR-mediated toxicity was evaluated, the optimal detection time was examined and a comparison was made by using the commonly used ethoxyresoufin-*O*-deethylase (EROD) activity induction assay. **Result** The results suggested that the luciferase activity in recombinant cells was peaked at about 4 h and then decreased to a stable activity by 14 h after TCDD treatment. The detection limit of this cell line was 0.11pmol/L, or 10-fold lower than in previous studies, with a linear range from 1 to 100 pmol/L, related coefficient of 0.997, and the coefficient of variability (CV) of 15-30%. **Conclusion** The luciferase induction is 30-fold more sensitive than EROD induction, the detection time is 68 h shorter and the detection procedure is also simpler.

Key words: Dioxin-like chemicals; Luciferase; Reporter gene; TCDD; CALUX

INTRODUCTION

Dioxin-like chemicals (DLCs) can combine with aromatic hydrocarbon receptor (AhR) and induce many biochemical changes in body. DLCs include three types of chemicals: polychlorinated dibenzo-*p*-dioxin (PCDD), polychlorinated dibenzofuran (PCDF) and coplanar polychlorinated biphenyl (PCB). 2, 3, 7, 8- tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototypical and most potent member of DLCs[1]. DLCs are ubiquitous environmental pollutants and are very stable in the natural environment. They are lipophilic and can bioaccumulate and biomagnify in the food chain. Exposure to and bioaccumulation of DLCs have been observed to produce a variety of species- and tissue-

[1]To whom correspondence should be addressed. E-mail:shunqing@public.wh.hb.cn

Biography of the first author: Zhang Zhi-Ren, male, born in September, 1974, ph. D candidate, majoring in technology on molecular biology.

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specific effects, such as tumor promotion, lethality, birth defect, hepatoxicity, wasting syndrome, epidermal effect, myelotoxicity, immunotoxicity, induction of numerous enzymes, including that of cytochrome P4501A1[2]. Consequently it is necessary to strengthen the monitoring of DLCs.

Detection of DLCs is difficult because they are presented as complex mixtures in low dose. Samples may theoretically contain up to 75 different PCDDs, 135 different PCDFs and 209 different PCBs isomers and congeners. Current techniques for detection and quantifying DLCs mainly involve gas chromatography (GC) associated with mass spectrometry (MS), immunoassay, and bioassay.

The immunological method is simple, but the antibody is still not commercial and the experimental results have no comparability among different laboratories[3]. So this method is not used wildly.

The GC-MS analysis is the international standard method but it is time-consuming, its cost and procedure is complex. There are many requirements to be met by this method, including representative sampling and appropriate storage, efficient extraction, high selectivity in the clean-up, high specificient in the gas chromatography, high selectivity in the detection, safe and reliable quantification, good reproducibility, useful confirmatory information. In addition, for hazard and risk assessment GC-MS analysis only provides information about presence and concentration of known DLCs, but limited information on the biology effects of the samples, particularly when there are many interaction among various DLCs[4,5].

Bioassay is based on the toxicity mechanisms of action of DLCs. The DLCs can bind to AhR with high affinity. After binding, the AhR undergoes a process termed transformation, during which two molecules of hsp90 dissociate from the DLCs:AhR complex, and, after forming a complex with a second protein (the Ah receptor nuclear translocator), the heterometric AhR complex is converted into its DNA binding. The high-affinity binding of heterometric AhR complex with a specific DNA sequences, the dioxin-responsive element (DRE), results in transcriptional activation of adjacent genes[6,7]. A common and rapid response that is under the control is the induction of cytochrom (P4501A) isoenzymes and the ethoxyresorufin-*O*-deethylase (EROD) activity. Currently, it is hypothesized that most of the biological/toxic effects of the DLCs are mediated by the AhR and the binding of DRE. Many studies have also proved the hypothesis. So the induction of P4501A and EROD activity has been wildly used as a bioassay to evaluate the relative toxicological potency of complex mixture containing the DLCs[8]. EROD activity assay was often used *in vitro* to detect DLCs. But EROD activity was inhibited when the concentration of the DLCs is high. And the detection limit is not low. So Aarts, J. M. M. J. G., Denison, M. S. and other persons developed the chemical-activated luciferase gene expression (CALUX) bioassay for detection of DLCs which is based on the pGL2 vector[9]. The CALUX bioassay is based on the luciferase reporter gene that is a prokaryotic gene and has no post-transcriptional control. And the luciferase activity is not influenced by compound tested.

The aim of this study is to improve the CALUX bioassay for DLCs detection which is based on the toxicity mechanisms of DLCs. A recombinant vector, based on the luciferase reporter vector pGL3 whose expression often increases 10- to 100-fold than pGL2 which was used in previous experiment, containing the luciferase reporter gene under transcriptional control of DRE was constructed and used to transfected the human hepatoma (HepG2). The cell line shows AhR-meditated luciferase gene expression. The reliability of luciferase induction in this cell line as a reporter of AhR-mediated toxicity

was evaluated and a comparison was made with the commonly used EROD induction assay.

MATERIAL AND METHODS

Chemicals

Molecular biological enzymes were purchased from Huamei Ltd. (China, Shanghai) or Promega Corporation. 2, 3, 7, 8- tetrachlorodibenzo-*p*-dioxin was purchased from Accustanfards, Inc. (New Haven, CT). Dimethylsulphoxide (DMSO) was provided by BIB Corporation.

Construction of Luciferase Expression Vector

To get the DREs, plasmid pHAV (from Dr. James P. Whitlock Jr. , Stanford University, Fig. 1) was cleaved with *Hin*dIII and a smaller *Hin*dIII fragment was isolated by agarose gel electrophoresis. Following digestion by *Bam*HI and *Eco*RV, the smaller *Hin*dIII fragment was cleaved into three fragments. Among the three fragments, the 630bp fragment was purified by agarose gel electrophoresis. Because restricting enzyme *Bam*HI and *Bgl*II was an isoschizomer and the producing end of *Eco*RV was blunt, a fragment with two *Bgl*II cohesive termini would be produced after a *Bgl*II linker was ligased and then cleaved with *Bgl*II.

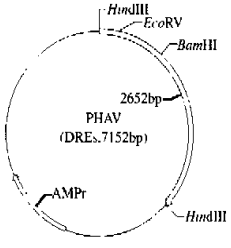

FIG. 1. Structure of plasmid pHAV.

To get the MMTV promoter, plasmid pMCat (from Dr. James P. Whitlock Jr. , Stanford University, Fig. 2) was cleaved with *Hin*dIII and the smaller *Hin*dIII fragment was purified by agarose gel electrophoresis. The purifed fragment was cleaved with *Bgl*II to produce cohesive 5′*Bgl*II termini and cohesive 3′*Hin*dIII termini.

The two fragment containing DREs and MMTV promoter were connected with T4DNA ligase to get a 1 020bp fragment containing DREs and MMTV promoter with cohesive 5′*Bgl*II termini and cohesive 3′*Hin*dIII termini. The expression vector was prepared by subcloning the 1 020bp fragment into the *Bgl*II- *Hin*dIII site immediately upstream of the luciferase reporter gene in the plasmid pGL3-promoter (Promega, Fig. 3). In this reporter plasmid, the luciferase's expression was under the control of DREs. The recombinant vector was transformated into competent cells (JM109) and grown up on a selective plate containing ampicillin. The recombinant vector was identified by restriction analysis with *Bgl*II and *Hin*dIII and compared with that of pGL3-promoter in gel electrophoresis[10,11].

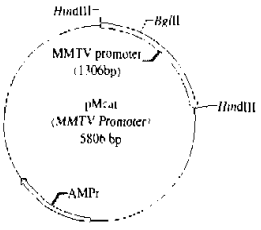

FIG. 2. Structure of plasmid pMcat.

Cell Culture, Tansient Transfection and Chemical Treatment

Human hepatoma (HepG2) and recombinant cell line were cultured in RPMI1640 supplemented with 10% heat-inactivated fetal calf serum and a 37℃ incubator with saturated humidity and 5% (v/v) CO_2 atmosphere[12].

The HepG2 cells were plated in 60mm culture dish at a density of 3×10^5 cells in 5 ml of medium. After cultured for 24 h, the cells were transiently transfected with the desired plasmid (15 μg) by using calcium phosphate mediated transfection method[13]. The transfected cells were allowed to grow for 48 h, followed by the addition of DMSO (0. 5%) or TCDD (1nmol/ L) dissolved in DMSO and further cultured for 24 h. The cell harvest and luciferase activity detection were described below.

Cell Culture, Stable Transfection and Chemical Treatment

To produce stable clonal cell lines, the desired cells were cotransfected with the vectors PTK-Hyg and recombinant by using calcium phosphate mediated transfection method. Following 24 h of growth in nonselective medium, the transfected cells were replated into selective medium containing hygromycin. After about 4 wk of growth in selected medium, TCDD-inducible luciferase activity of all clones was determined and the clone with the greatest ration inducible to constitutive luciferase activity was selected

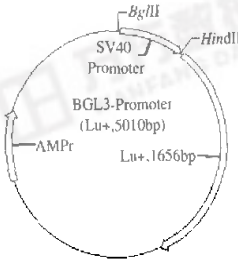

FIG. 3. Structure of plasmid pGL3-Promter

for further analysis[13]. The stable clonal cells were plated in 6-well dishes at a density of 2×10^5 cells in 3 ml of medium. After cultured for 4 h, the cells were exposed to the indicated concentration of TCDD in DMSO.

Cell Harvest and Luciferase Induction Assay

The cells were incubated, and followed by removal of the medium and three washes of the exposed cells with cold phosphate buffered saline (PBS). The washed cells were lysed by luciferase lysis reagent (Promege) for 15 min at room temperature. The cell debris was removed by centrifugation and luciferase activity of the cleared supernatant determined immediately or stored at - 80℃. To measure luciferase activity, 20 μl of cell lysate was added to 100 μl luciferase assay reagent(Promega) and the resulting bioluminescence was quantified by using Lumate LB 9570 luminometer over 3 s immediately. The concentration of protein was detected by using Bio-Rad method. Luciferase activity was expressed as relative light unit (RLU) per microgramme protein[15,16].

EROD Induction Assay

To detect EROD activity, the wild type cells were plated in 6-well dishes at a density of 2×10^5 cells in 3 ml of medium. After cultured for 24 h, the cells were exposed to the indicated concentration of TCDD in DMSO. The cells were incubated for 72 h, and followed by removal of the medium and three washes of the exposed cells with cold phosphate buffered saline (PBS). The washed cells were stored at - 80℃ to lyse cells. The EROD activity was determined by using fluorescent method. The concentration of protein was detected by using Bio-Rad method. EROD activity was expressed as pmol resorufin production per microgramme protein per min[17,18].

Data Statistical Analysis

The data were imported into a spreadsheet program (Excel) for further calculations. Each value represents the average of four replicates. The detection limit is the plain value plus three times standard deviations (SD). Coefficients of variation (CV) were calculated by (SD/mean × 100%). Correlation coefficients (r) were calculated by using least-squares linear regression analysis.

RESULTS AND DISCUSSION

Restriction Analysis of Recombinant Vector

The recombinant vector was identified by restriction analysis with *Bgl*II and *Hin*dIII and compared with that of pGL3-promoter in gel electrophoresis (Fig. 4). Theoretically, *Bgl*II and *Hin*dIII digest of the recombinant vector and pGL3-prometer was separated into two bands in gel (4 801bp and 1 020bp) and *Bgl*II and *Hin*dIII digest of pGL3-prometer was also separated into two bands in gel (4 801bp and 209bp) respectively. Fig. 4 shows that the recombinant plasmid is separated into two bands, the larger one was about 5 000bp and the smaller one was about 1 000bp, in gel. The results in Fig. 4 indicated that the structure of recombinant plasmid was correct.

Transient Transfection Experiment

Transient transfection of the recombinant into HepG2 and the result of subsequent exposure to DMSO or TCDD are shown in Fig. 5. TCDD-treatment of HepG2 cells transiently transfected with recombinant vector containing DREs produced a significant induction of luciferase activity (80 fold). The results demonstrated that the recombinant vector was constructed successfully and could confer TCDD- and AhR-dependent induction response. TCDD-dependent luciferase activity induction could also be observed in many other AhR-containing cell lines, such as H4IIE, Hepa1c1c-wt, MLE-BV, GPC16, MCF7 and so on[19,20].

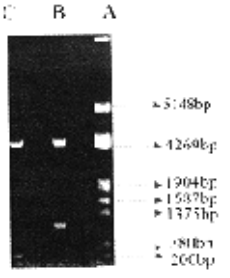

FIG. 4. Identification of recombinant plasmid by restriction analysis with *Bgl*II and *Hin*dIII and compared with that of pGL3-promoter in gel electrophoresis.
A: DNA marker; B: Digestion of recombinant plasmid by *Hin*dIII and *Bgl*II; C: Digestion of vector pGL3-promoter by *Hin*dIII and *Bgl*II.

The Time Course of Luciferase Activity Induction in Stable Transfection Cell Line

The time course of luciferase activity induction in stable transfection cell line was studied in order to choose the optimal detection time. The stable transfected cells were exposed to DMSO (0.5%) or TCDD (1 nmol/L) and then the luciferase activity was determined at every 2 h, after 2 h growing, and up to 28 h postexposure. TCDD-inducible luciferase activity in stable transfection cells peaked at about 4 h, and then decreased to a stable level at about 14 h after TCDD treatment (Fig. 6). The luciferase activity peaked at about 4 h because luciferase was susceptible to proteolysis and was moderately unstable when expressed within mammalian cells. The half-life of luciferase in HepG2 cells was about 3 h. The stability of luciferase in cell lysates was shown to be 6 weeks at 4℃. Detergent lysis extracts were not stable when freeze-thawed but were stable when stored at -80℃.

Luciferase and EROD Induction Responses of Stable Transfection HepG2 Cell to TCDD

TCDD induced EROD and luciferase activity were dose-dependenty in HepG2-wt and HepG2-luc cells, respectively (Fig. 7). The limit of detection of TCDD in HepG2-luc cells (0.11 pmol/L) was thirty times less than in HepG2-wt cells (3.5 pmol/L). These results suggest a thirty-fold improvement can be achieved in sensitivity of the HepG2-luc cells as compared with the HepG2-wt cells. The range of determination of TCDD in HepG2-

luc cells was 1~100 pmol/L and was wider than that of the HepG2-wt cells. Related coefficient was 0.997, and the coefficient of variability (CV) was 15%~30%. The induction time of luciferase was 68 h shorter than EROD induction, and the detection procedure was also simpler.

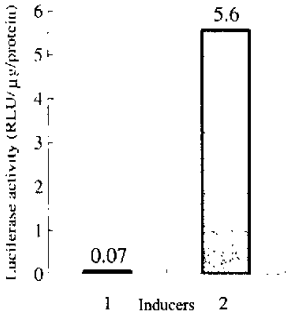

FIG. 5. Dimethylsulphoxide and 2, 3, 7, 8- tetrachlorodibenzo-*p*-dioxin-induction luciferase activity in transient transfectional cells.
1: Dimethylsulphoxide; 2: 2, 3, 7, 8- tetrachlorodibenzo-*p*-dioxin.

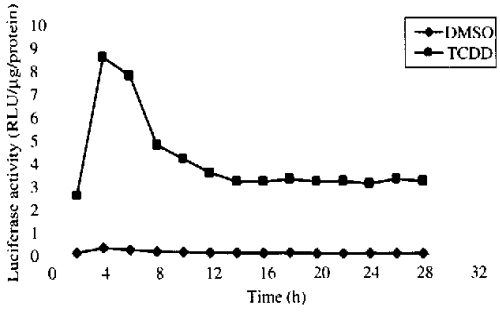

FIG. 6. The time course of luciferase induction by TCDD and DMSO.

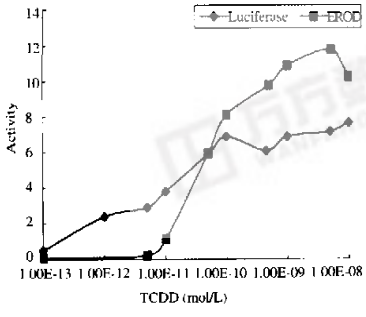

FIG. 7. Dose-effect curves for the induction by 2, 3, 7, 8-TCDD of EROD activity in HepG2-wt and luciferase content in HepG2-luc cells.

The vector used in our study, was pGL3 but was pGL2 in previous studies. Both were the products of Promega Corporation. Under the same regular elements, the expression efficiency of pGL3 was 10~100 times higher than that of pGL2. The limit of detection of TCDD in reported recombinant cells was about 1.2 pmol/L which was about ten times higher than that of our recombinant cells[9].

Since the luciferase induction assay was not based on the endogeneous genes, such as EROD, but on a reporter gene constructed *in vitro*, the nucleotide sequences of reporter gene and upstream regulating elements could be manipulated to improve sensitivity. In this study, an improved vector was used and a 10-fold sensitivity was achieved. In addition, luciferase reporter gene was a prokaryotic gene and had no post-transcriptional regulation so the linear relation was better than that of endogeneous gene.

In this study, a simple, sensitive and rapid method for detecting DLCs was developed based on the luciferase reporter gene that was under the control of dioxin responsive element. The transfected cell line could be used for rapid screening and semi-quantitation of DLCs. This method can be used alone or in combination with chromatography.

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