RECOMBINANT CELL LINES FOR THE DETECTION OF DIOXINS AND AH RECEPTOR LIGANDS - NOT ALL ASSAYS ARE CREATED EQUAL

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Introduction

Halogenated aromatic hydrocarbons (HAHs), such as the polyhalogenated dibenzo-p-dioxins, biphenyls and dibenzofurans, represent a large group of compounds which because of their ubiquitous distribution, resistance to biological and chemical degradation, high toxicity and potential for bioaccumulation/biomagnification, can have a significant impact on the health and well being of human and animals. The worldwide distribution of HAHs and their presence in a variety of wildlife, domestic and human tissues as well as in food, water, and soil samples, jas resulted in the development of numerous methods for their detection and quantitation. Although chemical extraction procedures coupled with high resolution gas chromatography-mass spectrometry is considered the gold standard for the identification and quantitation of individual HAH congeners, the high cost and time consuming nature of these methods has led to the development of alternative assay methodologies. Several rapid and inexpensive screening bioassays capable of detecting and estimating the relative potency of complex mixtures of HAHs have been developed by our lab and others[1]. These bioanalytical methods are based on the ability of the compounds to be specifically recognized and bound by antibodies (immunoassays) or their ability to transduce a specific biological response in vitro or in cells in culture (bioassays). These bioassays are based on the mechanism of action of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related HAHs and utilize the Ah receptor (AhR), a ligand-dependent factor which mediates the effects of these chemicals[2]. One cell bioassay (chemically activated Luciferase expression (CALUX)) utilizes a recombinant cell line (mouse or rat liver cells) that contain a stably integrated AhR-responsive luciferase reporter gene plasmid (pGudLuc1.1) and takes advantage of the ability of the AhR to activate gene expression. Exposure of these cells to extracts containing TCDD and/or related HAHs results in the induction of luciferase expression in a time-, dose- and chemical specific manner[1,3,4]. Although this particular CALUX bioassay system provides a avenue by which to detect dioxins and related AhR ligands, it has a limitation with regards to the transient nature of the measured induction response from this specific plasmid (garrison). The instability of the luciferase gene in this specific plasmid results from the targeting of the expressed luciferase to peroxisomes, where it is subsequently degraded. This results in a reduced reporter gene signal at all times later than 4-6 hours after chemical treatment and the maximal fold induction would be dramatically reduced if the induction response were examined at later times that are commonly used[3]. Here we have compared the time and dose response curves for TCDD using the pGudLuc1.1-CALUX cell bioassay and two new recombinant cell lines that contain other stably transfected reporter gene plasmids (i.e. they contain the genes for a mutated form of firefly luciferase or that of enhanced green fluorescent protein (EGFP)).

Materials and Methods

Construction of the pGudLuc6.1 Expression Vector and Stable Transfection

The DRE driven, TCDD responsive reporter plasmid pGudLuc6.1 was constructed by subcloning

the 1810 bp HindIII fragment excised from the plasmid pGudLuc1.1 into the HindIII site upstream of the luciferase gene in the plasmid pGL3-basic (Promega). This fragment contains four functional DREs from the murine CYP1A1 gene that confer TCDD responsiveness upon a MMTV promoter and adjacent luciferase gene. Mouse hepatoma (hepa1c1c7) cells were stably co transfected with this plasmid and pSV2neo using polybrene and responsive clones isolated as previously described[3]. This resulted in the isolation of a TCDD-responsive stable cell line (i.e. H1L6.1c3).

Cell Cultures and Chemical Treatment

Mouse hepatoma (Hepa1c1c7) cells that had been previously stably transfected with pGudLuc1.1 (designated as H1L1.1c2 cells) or pGreen1.1 (designated as H1G1.1c3 cells) and the H1L6.1c3 cells were grown as previously described[3,5]. For the luciferase induction studies, H1L1.1c2 and H1L6.1c3 cells were grown in 24 well plates and incubated with the indicated concentration of TCDD for the indicated time and luciferase activity in aliquots of cell lysates determined as previously described using an Anthos Lucy 2 microplate luminometer[3]. For GFP induction studies, H1G1.1c3 cells were plated into clear-bottomed black 96 well microplates and incubated with the indicated concentration of TCDD for the indicated time and GFP activity in intact cells was measured (without the removal of media) using a Fluostar microtiter plate fluorometer with excitation and emission wavelengths of 485 nm (25 nm bandwidth) 515 nm (10 nm bandwidth), respectively[5]. All samples were run in triplicate and the reporter gene activity present in wells containing DMSO only were subtracted from the reporter gene activity in all treated samples. Reporter gene activity was normalized to sample protein concentration, determined using the fluorescamine protein assay and bovine serum albumin as the standard[5]. Values are presented as the mean ± SD and are expressed as a percent of the maximal TCDD activity observed in each cell line.

Results and Discussion

The time course of induction of luciferase and EGFP by 1 nM TCDD in the three recombinant cell lines is shown in figure 1. Similar to what we have observed previously, the time course of induction with pGudLuc1.1-containing H1L1.1c2 cells is transient, with maximal activity observed at 4-6 hours after treatment with TCDD[3]. In contrast, expression of luciferase from luciferase from the pGudLuc6.1-containing H1L6.1c3 cells and the pGreen1.1-containing H1G1.1c3 cells progressively increases with time, with luciferase activity increasing at a significant faster than that of EGFP. The difference between the results obtained with the cell lines containing the two different luciferase genes was not unexpected. The relatively rapid loss of luciferase activity in H1L1.1c2 cells derives from the fact that the firefly luciferase gene that was used to create this responsive cell line (present in the pGL2 vector from Promega) results in the production of firefly luciferase that contains a peroxisomal targeting sequence. This sequence targets the final luciferase protein to the peroxisome where it is likely that peroxisomal enzymes are responsible for the subsequent loss in activity. The luciferase gene expressed in the H1L6.1c3 cells (from the pGudLuc6.1 vector) contains several mutated residues that result in and increase its stability in mammalian cells (this gene is contained in the pGL3 vector from Promega). These mutations have deleted the peroxisomal targeting sequence such that the resulting luciferase protein now accumulates in cytosol, and this and other minor amino acid mutations have significantly stablized the luciferase protein. This increased stability is readily apparent in figure 1. EGFP expression in H1G1.1c3 cells also progressively increases with time, although the induction occurs at a slower rate than that in H1L6.1c3 cells. This slow rate of induction has been previously observed and results both from a slow rate of formation of the final correctly folded fluorescent EGFP protein, but also the relatively lower sensitivity of a fluorescent assay as compared to a luminescent assay. >From an instrumental analysis point of view, luminescence is a significantly more sensitive

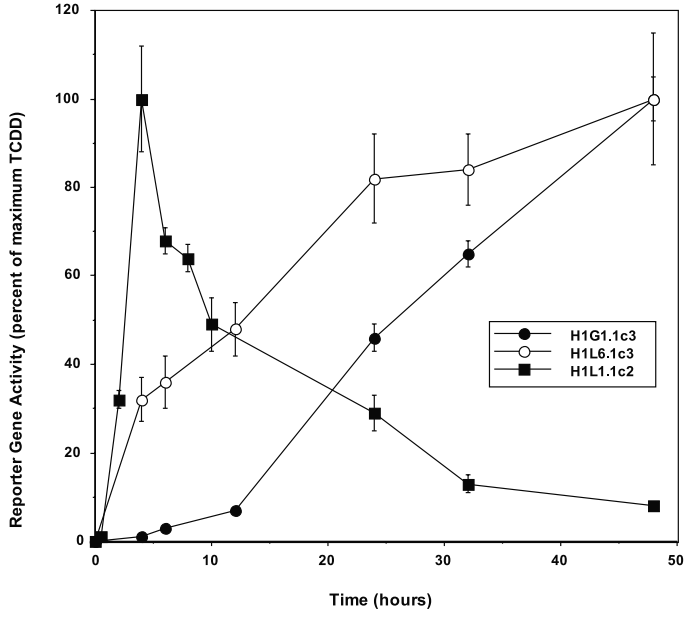

Figure 1. Time course of induction of reporter gene expression in three different stably transfected TCDD-responsive cells lines.

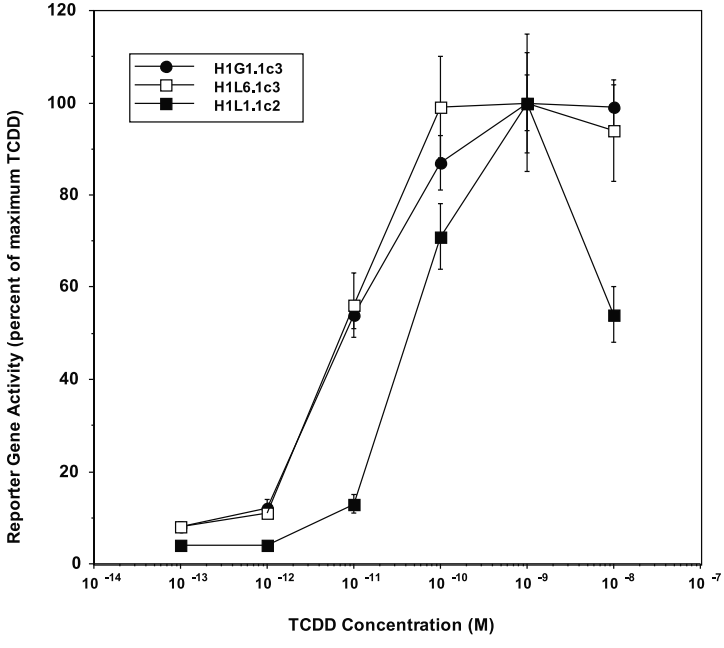

Figure 2. TCDD dose response curves for the induction of reporter gene expression in three different stably transfected TCDD-responsive cells lines.

technique than that of fluorescence and this could explain why luciferase activity is detected at a much earlier time than that of EGFP. Dose response analysis with each cell line at more optimal analysis times (4 hours for H1L1.1c2 cells and 24 hours for H1L6.1c3 and H1G1.1c3 cells) reveal similar relative dose response curves and EC_{50}s for H1L6.1c3 and H1G1.1c3 cells, while the EC_{50} for TCDD in the H1L1.1c2 cells was about 5-6-fold less potent (figure 2). The reason for the lower potency in the H1L1.1c2 cells is unclear at this time but may be related to the relatively greater instability of the luciferase protein in these cells and/or an effect resulting from elements at the specific site of genomic integration of the plasmid. Although our results demonstrate differences in the response of these cells to TCDD, each cell line has specific advantages that can be used when identifying and characterizing AhR ligands. The H1L1.1c2 cells would be most advantageous when examining ligands that induce AhR-dependent gene expression in a transient manner, such as that observed with prostaglandins[6], where the induction response is over by 6 hours. The other cell lines would be less appropriate for that type of response. We believe that the H1L6.1c3 and H1G1.1c3 cells would be more appropriate for screening of samples for HAHs or more stable AhR ligand screening analysis since the time of induction would be less critical. It has been reported that increasing the time of induction also allows metabolically less stable AhR ligands present in a sample extract (i.e. PAHs) to be degraded and thus not contribute to the overall induction response[7]. H1L1.1c2 cells would be less appropriate for HAH analysis since samples would optimally have to be examined within 4-6 hours after treatment, a period of time where metabolically less stable contaminants could also activate the AhR. This could result in false positives and/or overestimation of activity. H1G1.1c3 cells can also be used to examine increases in EGFP in real time in intact cells and as such, the same cells can be followed for extended periods to examine the relative potency of a given ligand or extract. Overall, although the recombinant cells bioassay systems we have developed can be used to identify and characterize AhR ligands, each of the assays have characteristics that make them appropriate for some application and not others. The choice of which cell line to use will certainly be dependent on the specific questions and issues being examined.

Acknowledgments.

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