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Gene Expression Analysis of Rat Liver Epithelial Cells in Response to Thioacetamide

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Abstract

Thioacetamide (TA) is potent haptotoxincant that requires metabolic activation by mixed-function oxidases. Microarray technology, which is massive parallel gene expression profiling in a single hybridization experiment, has provided as a powerful molecular genetic tool for biological system related toxicant. In this study we focus on the use of toxicogenomics for the determination of gene expression analysis associated with hepatotoxicity in rat liver epithelial cell line WB-F344 (WB). The WB cells was used to assess the toxic effects of TA. WB cells were exposed to two concentrations of TA-doses which caused 20% and 50% cell death were chosen and the cells exposed for periods of 2 and 24 h. Our data revealed that following the 2-h exposure at the both of doses and 24-h exposure at the low doses, few changes in gene expression were detected. However, after 24-h exposure of the cells to the high concentration, multiple changes in gene expression were observed. TA treatment gave rise predominantly to up-regulation of genes involved in cell cycle and cell death, but down-regulation of genes involves in cell adhesion and calcium ion binding. Exposure of WB cells to higher doses of the TA gave rise to more changes in gene expression at lower exposure times. These results show that TA regulates expression of numerous genes via direct molecular signaling mechanisms in liver cells.

Keywords: Thioacetamide, toxicogenomics, gene expression, rat liver epithelial cells

We are gaining information of numerous candidate genes that have been known and unknown their function in biological system through many projects has been done and are processing. Many techniques that are able to analyze many genes and proteins simultaneously in once are used to interpret the information. Microarray technology, one of them, permits the comparison of thousands of genes in different biological systems. Lately, microarray system has been used for the prediction of toxicity through gene expression induced toxicant[19,20] and has shown that compounds with similar toxic mechanisms produce similar changes in gene expression *in vivo*[8] and *in vitro* system[4]. Toxicogenomics is the newest study of the structure and output of the entire genome as it relates and responds to adverse xenobiotic exposure. It is defined as the application of the knowledge of genes associated with disease states to the study of the toxicology of chemical and physical agents. Comparing the transcriptional response profile of a new compound with a database of compounds with established hazards allows determination of the degree of similarity, if any, using various clustering algorithms using cDNA microarray techniques.

Thioacetamide (TA) is a hepatotoxic and hepatocarcinogenic agent, which is largely used as an inducer of liver necrosis, cirrhosis and carcinoma in animal model studies. At the cellular level, TA promotes the enlargement of the nucleoli[1] and accumulation of RNA in the cell nucleus[17], increases the amount and specific activity of total mRNA and disproportionately increases the amount of albumin mRNA in hepatocytes[5,15,18]. TA also diminishes the content of hepatic cytochrome P450 and inhibits the enzyme activity of the hepatic mixed function oxidase[2]. TA also binds to several subunits of glutathione-s-transferase (GST) and inhibits the expression of class α GST[16].

The aim of this study was to apply genomics technology to predictive toxicology using in vitro hepatotoxicity as a model. The cell line WB was chosen as a model system since it share some phenotypic properties of hepatocytes and bile duct cells[7,21]. A total of 148 genes exhibited differential expression in response to TA. These genes are mainly involved

in cell death, cell cycle, cell adhesion, calcium ion binding.

Cytotoxicity Assay

WB cells were exposed concentration of TA ranging from 0 to 100 mM. As shown in Fig. 1, cell survival decreases with increasing doses of TA. Following 24 h of treatment with TA, 20% cell death was observed with 5 mM TA and 50% cell death was observed with 10 mM TA (Fig. 1).

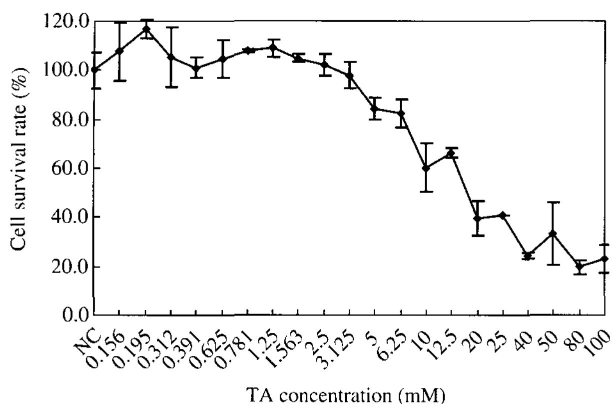

Fig. 1. Cytotoxicity assay data from TA treatment over a period of 24 h. Doses representative of approximately 20 and 50% cell death were chosen for this study.

Microarray Analysis

In order to identify genes that are transcriptionally regulated by TA in the rat liver epithelial cell line, WB, mRNA expression was analysed using DNA microarray analysis. For the purpose of this study, only those genes which displayed either greater than or equal to a twofold up- or down-regulation have been considered. In our present study, duplicate experiments were conducted on a single pooled RNA preparation from the cells. When the identical 2 RNA samples (vehicle control and TA treated) were labeled and hybridized repeatedly, a high linear correlation was obtained for replicates. Fig. 1 shows the scatter plots of relative gene expression levels of TA-treated cells *vs.* control cells.

Following 2 h treatment with 5 mM and 10 mM TA, there appere to be very few changes in gene expression when compared to the untreated control (Fig. 3 and 4). It also follows that 24 h of exposure to 5 mM TA also results in few changes in down-regulated gene expression. However, following 2 h of exposure to 10 mM TA, a increased genes in number of differentially expressed genes is observed, while 24 h exposure to 10 mM TA treatment results in both up- and down-regulation of genes (Fig. 4).

Table 1 shows a selection of genes, the expressions of which have been altered by 24 h of exposure to 10 mM TA treatment. Genes that displayed altered gene

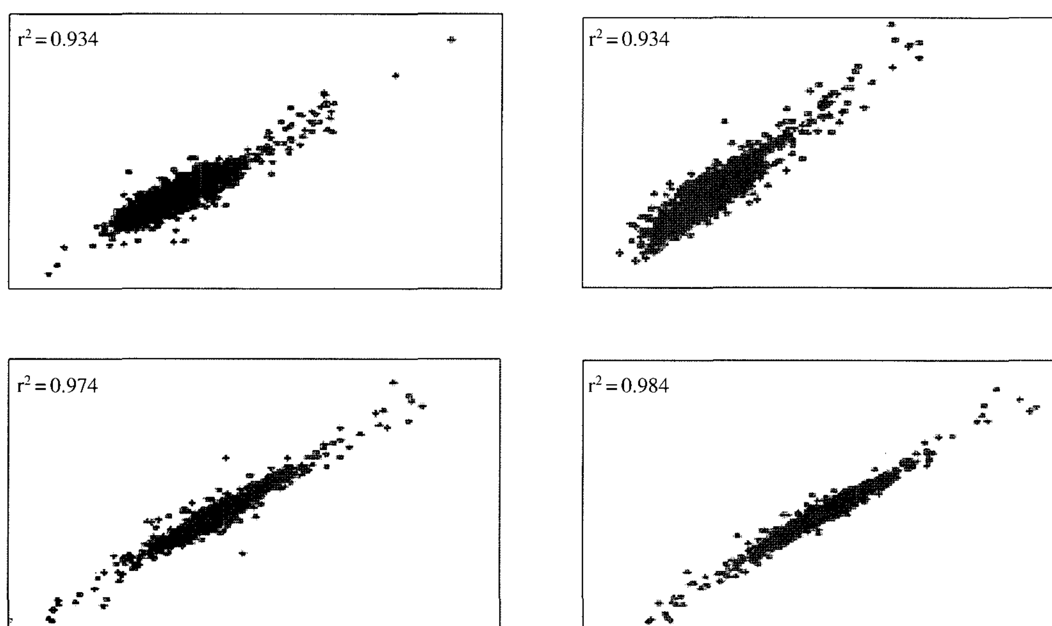

Fig. 2. A DNA microarray data set of two hybridizations with the same sample were used to analyze R^2. The scatter plots compare the spot intensities of 3,213 genes that are represented on each microarray. A. 2 h of exposure to 5 mM TA, B. 2 h of exposure to 10 mM TA, C. 24 h of exposure to 5 mM TA, D. 24 h of exposure to 10 mM.

Fig. 3. Hierarchical clustering of expressed genes following 2 h exposure of 5 and 10 mM TA treatment.

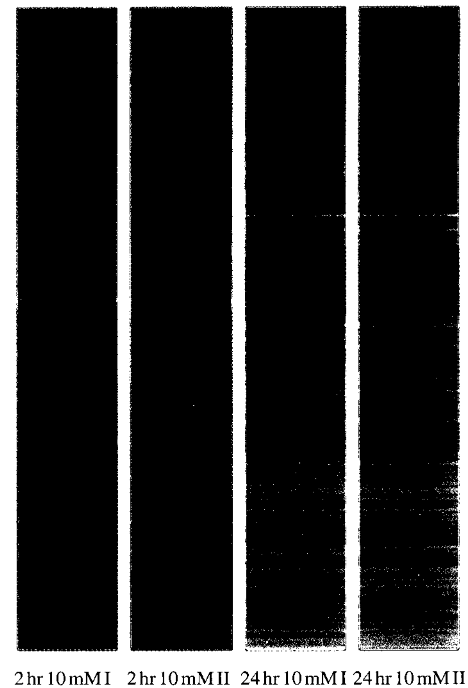

Fig. 4. Effects of genes expression following 2 h and 4 h treatment with 10 mM TA.

expression as a result of TA treatment were involved in processes such as cell death, cell cycle, cell adhesion and metabolism. These genes in Table 1 were members of the death response family.

Treatment of the WB cells with concentration of TA of 50% cell death was seen to give rise to increased alteration in gene expression, with more genes being affected even at the early 2 h time point.

Discussion

Gene expression changes have been used routinely to obtain specific mechanistic information concerning the type of action of a toxicant. Toxicogenomics is an approach that applies microarray technology to toxicological evaluation paradigms.

TA is a thiono-sulfur containing compound that is necrogenic[12] and carcinogenic[11]. It is commonly used for inducing fulminant hepatic failure[3] and liver cirrhosis in animal models[10,13,14].

The principal aim of this study was to whether it was possible to obtain gene expression profiles that were specific to exposure to TA. In order to establish this, it was necessary to investigate endpoints of toxicity that have been relatively well characterized. Liver toxicity is a well-established toxic endpoint and has been the subject of many investigations.

This study shows that treatment of WB cells with TA produces dose-dependent cell damage (based on the results obtained from the MTT cytotoxicity assays). Based on the results obtained from the MTT cytotoxicity assays, we chosed low (20% cell death) and high (50% cell death) dose points. Furthermore 2 h and 24 h time points were chosen at which to assess the gene expression and identify patterns of differential gene expression representative of liver toxicity. mRNA extracted from treated WB cells was used to probe rat toxicology arrays containing 4,800 genes of toxicological relevance.

Simple linear correlation coefficient (R^2) is a measure of linear association between two variables. The R^2 was calculated for each scatter plot. The R^2 can be calculated for two samples at the same time and thus allows comparison for each scatter plot separately. In our present study, the $R^2 \geq 0.93$ illustrates an overall robust performance of the platform and hybridization.

Low concentrations are not feasible for expression profiling studies, since at subtoxic doses the number and magnitude of gene expression changes are drastically reduced. But, treatment of WB cells with high doses of TA resulted in multiple differential gene expression following not 2 h but 24 h exposure time. Some genes up-regulated by only 2 h exposure at high

Table 1. Discriminator genes related to death response after 24 h treatment of WB cells with 10 mM TA

Gene ID/Cluster	Description	Tendency of gene regulation
AA965232	Serine (or cysteine) proteinase inhibitor, member 1	↑
AA963225	Cathepsin B	↑
AA925894	Ephrin A1	↓
AA963231	High mobility group box 1	↓
AA957593	Tissue inhibitor of metalloproteinase 1	↑
AI137043	Nerve growth factor, beta	↑
AA955679	Thioredoxin reductase 1	↑
AA874884	Heme oxygenase (decycling) 1	↑
AA875417	Cyclin D1	↑
AA924772	Metallothionein 3	↑
AA955755	Survival of motor neuron 1, telomeric	↑
AA817957	Presenilin-2	↓
AA818840	Interferon regulatory factor 3	↓
AA859385	Vimentin	↑
AA965073	Golgi reassembly stacking protein 2	↓
AA900933	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptide	↑
AA818247	Polypyrimidine tract binding protein 1	↓
AA899537	Lectin, galactose binding, soluble 3	↓
AI059291	Protein kinase, cAMP-dependent, regulatory, type 2, alpha	↓
AI044732	Fumarylacetoacetate hydrolase	↓
AA957593	Tissue inhibitor of metalloproteinase 1	↑
AA955881	Fatty acid synthase	↑
AI136637	Growth factor, erv1 -like	↑
AI146192	B-cell translocation gene 2, anti-proliferative	↑
AI030084	Gelsolin	↑
AA899537	Lectin, galactose binding, soluble 3	↑
AA957593	Tissue inhibitor of metalloproteinase 1	↑
AA996434	Phosphoinositide-3-kinase, class 3	↑
AA924247	Protein kinase, AMP-activated, beta 1 non-catalytic subunit	↓
AA901280	Programmed cell death 8 (apoptosis-inducing factor)	↓
AA818383	Phosphatidylinositol 3-kinase, regulatory subunit, polypeptide 2	↓
AA900628	Nischarin	↓
AA817957	Presenilin-2	↓
AI137969	Mismatch repair protein	↓
AA818144	C-reactive protein, petaxin related	↓
AA956371	Scavenger receptor class B, member 1	↑
AI145654	Protein kinase, cAMP dependent regulatory, type I, alpha	↑
AI059907	Chemokine (C-X-C motif) ligand 10	↑
AA858888	Tubulin, beta 5	↑
AA859203	Transporter protein; system N1 Na+ and H+-coupled glutamine transporter	↑
AI030632	TNFRSF1A-associated via death domain	↓

dose of TA was normalized in 24 h exposure. These genes could be early marker of TA toxicity. Cluster analysis will also provide further insight into the differential gene expression observed in this study.

Although the results described within this study are by no means conclusive, they do provide the early beginnings of an accurate toxicity prediction test. The discovery of accurate predictors of early toxicity will help shorten the duration of many risk assessments and toxicity studies. Further experiments both in vivo and in vitro evaluating many more known toxicants are necessary to determine whether genomics can provide an accurate tool for predictive toxicology. Cross-species studies are also planned to allow a comparison of gene expression patterns to be made between human and rat, allowing us to extrapolate data from the rat to human.

Even though we have found many known genes for toxicity using DNA microarray expriments, we also found many ESTs which have interesting expression patterns. Our results demonstrated gene expression that has not been previously associated with the chemicals we used and suggested that such results will provide valuable informations for further studies of the classification of compound associated signature.

Methods

Chemicals

Thioacetamide (TA) and 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide were purchased from Sigma Chemical Co. (St. Louis, MO).

Culture of WB Cells

WB-F344 (WB) cell, a kind gift of Dr. J. Trosko of Michigan State University (East Lansing, MI, USA) was cultured in D-media (Formula No. 78-5470EF, Gibco BRL, Grand Island, NY, USA) containing 3 mL/L PSN mixture (Gibco BRL, Grand Island, NY, USA) in the presence of 5% fetal bovine serum (Gibco BRL, Grand Island, NY, USA). Cells were incubated in a 37°C humidified incubator containing 5% CO_2 and 95% air. Cells were grown in 175 cm^2 flask (Nunc, Paisley, UK) and the culture medium was changed every other day.

MTT Cytotoxicity Assay

The cytotoxic effects of TA on WB cells were measured by MTT assay, based on the ability of live cells to convert tetrazolium salt into purple formazan. In brief, the cells were seeded at a density of 2×10^5 cells/mL in 24-well microplates and incubated overnight at 37°C, 5% CO_2. Then the cells were treated with different concentrations of TA or its vehicle, deionized water for 24 h. Following treatment, the cells were rinsed three times with PBS and then incubated with addition of MTT stock solution (5 mg/mL) for one tenth the original culture for the last 4 hr of incubation. After media was removed, added 200 μL of DMSO to each well and then pipetted up and down to dissolve the crystals. We determined absorbance at 540 nm using an ELISA reader (EL800, Bio-Tek Instruments, Inc., Vermont, USA). All the measurements were performed in triplicate. Results are expressed as the percentages proliferation with respect to vehicle control group.

Dosing of WB Cells

WB cells were seed at a density of 2×10^5 cells/mL in 10 cm dishes and incubated overnight at 37°C, 5% CO_2. On reaching confluency, cells were then dosed with TA (5 and 10 mM) for period of 2 and 24 h. Doses of TA were chosen as being representative of 20% and 50% cell death as determined by MTT assay. Four dishes of WB cells were used per treatment.

RNA Isolation

Following the appropriate incubation period, WB cells were washed three times in PBS and the cells scraped into 1 mL TRIzol Reagent™ (Invitrogen, Carlsbad, CA) per dish. RNA was extracted from four individual culture incubations and equal amount of RNA from each conditions were pooled. The extracted RNA was dissolved in RNase-free water, and its concentration and purity was determined from absorbance measurements at 260 and 280 nm using a spectrophotometer. Quality of the RNA was checked by visualizaion of the 28S : 18S ribosomal RNA ratio on a 1% agarose gel.

Hybridization in DNA Microarray

A rat 4.8 K cDNA microarray, developed in-house at GenoCheck Co. Ltd., was used for gene expression profiling studies[9]. A complete listing of the genes on this microarray is available at the following Web site: http://www.genocheck.com. cDNA microarray chips were prepared according to[6]. cDNAs were amplified in 100 μL polymerase chain reaction (PCR) mixture. A sample of PCR products (5 μL) was separated on 1% agarose gels to ensure quality of the amplification. The remaining PCR product were purified by ethanol precipitation and resuspended in spotting buffer kit (Genocheck Co. Ltd, Ansan) and spotted onto CMT-GAPS II slides (Corning Inc., Corning NY). For microarray hybridization, total RNA in each condition was pooled and used for hybridization and each total RNA sample (50 μg) was labeled with Cy3 (vehicle control) or Cy5 (test sample)-conjugated dCTP (Amersharm, Piscataway, NJ) by a reverse transcription reaction using reverse transcriptase, SuperScript (Invitrogen, Carlsbad, California). The fluorescently labeled cDNAs were mixed and hybridized simultaneously to the cDNA microarray chip. The cDNA chips were scanned with an Axon 4000 B Scanner (Axon Instruments, Foster City CA) using laser excitation of the 2 fluors at 532 and 635 nm wavelength for the Cy3 and Cy5 labels. The resulting TIFF images were analyzed and the quantification of spot intensities, qualities, and local background was performed automatically by the GenePix Pro 3.0 software package (Axon Instruments, Inc., Foster City, CA) using variable spot diameter in the range 70-180 μm and a manual supervision for any inaccuracies in the automatic spot detection. The expression intensity information for all genes was exported into Microsoft Excel spreadsheet. The raw fluorescent signal intensity values were initially subjected to a spot quality filter to ensure the accuracy of the expression ratios. Spots marked with a "Flag" by the software, indicating the signal was too weak to make a call, were excluded.

Data Management

Gene expression values for each chip were normalized to the intensity-dependent (LOWESS) normalization, then was normalized by with-print-tip group normalization method for each print-tip, 8 tips were used for making 4.8 K cDNA microarray. We gathered fold changes from M, $\log_2R$-$\log_2G$ (R is Cy5 signal and G is Cy3 signal). We have chose gene expression of two fold changed, over 2 and down 0.5 ($M \geq 1$ and $M \leq -1$). Experiment normalized, cluster analysis and ontology were performed with GeneSpring software 5.0 (Agilent Technologies).

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