MOLECULAR & CELLULAR TOXICOLOGY, Vol. 3, No. 3, 177-184, September 2007

Assessment of Feasibility for Developing Toxicogenomics Biomarkers by comparing *in vitro* and *in vivo* Genomic Profiles Specific to Liver Toxicity Induced by Acetaminophen

Jin Seok Kang[1], **Youn Kyoung Jeong**[1], **Soo Kyung Suh**[1], **Joo Hwan Kim**[1], **Woo Sun Lee**[1], **Eun Mi Lee**[1], **Ji He Shin**[1], **Hai Kwan Jung**[1], **Seung Hee Kim**[1] **& Sue Nie Park**[1]

[1]Department of Toxicological Researches, National Institute of Toxicological Research, Korea Food and Drug Administration, 194 Tongil-ro, Eunpyeong-gu, Seoul 122-704, Korea
Correspondence and requests for materials should be addressed to S. N. Park (suenie@kfda.go.kr)

Accepted 3 August 2007

Abstract

As a possible feasibility of the extrapolation between *in vivo* and *in vitro* systems, we investigated the global gene expression from both mouse liver and mouse hepatic cell line treated with hepatotoxic chemical, acetaminophen (APAP), and compared between *in vivo* and *in vitro* genomic profiles. For *in vivo* study, mice were orally treated with APAP and sacrificed at 6 and 24 h. For *in vitro* study, APAP were administered to a mouse hepatic cell line, BNL CL.2 and sampling was carried out at 6 and 24 h. Hepatotoxicity was assessed by analyzing hepatic enzymes and histopathological examination (*in vivo*) or lactate dehydrogenase (LDH) assay and morphological examination (*in vitro*). Global gene expression was assessed using microarray. In high dose APAP-treated group, there was centrilobular necrosis (*in vivo*) and cellular toxicity with the elevation of LDH (*in vitro*) at 24 h. Statistical analysis of global gene expression identified that there were similar numbers of altered genes found between *in vivo* and *in vitro* at each time points. Pathway analysis identified glutathione metabolism pathway as common pathways for hepatotoxicty caused by APAP. Our results suggest it may be feasible to develop toxicogenomics biomarkers or profiles by comparing *in vivo* and *in vitro* genomic profiles specific to this hepatotoxic chemical for application to prediction of liver toxicity.

Keywords: Acetaminophen, *in vivo, in vitro*, Toxicogenomics, Hepatotoxicity, Microarray

In general, evaluation of toxicity for a certain chemical is based on conventional toxicity tests, which presents effective screening ways, when toxic changes are evident. However, conventional toxicity tests have some shortages that it is not effective when lesions are mild or moderate, and it takes more time to find out the toxicities.

As developments of many foods, drugs and chemicals with new technologies, there have been occurred demands for new technologies asking to evaluate them more quickly and accurately not only for safety concern but for cost-effectiveness. Among newly developed technologies, toxicogenomic approaches employing microarray technology allows to investigate expressions of thousands of genes affected simultaneously in biological experiment such as chemical-induced toxicity test, and may serve as a valuable tool to evaluate new food and drugs.

Toxicogenomics combines transcript, protein and metabolite profiling with conventional toxicology[1], and its approach should help not only to discover highly sensitive and predictive biomarkers for toxicity but also to understand molecular cellular mechanism of toxicity, including fields of hepatotoxicity, nephrotoxicity, and genotoxicity[2]. It seems that toxicogenomics will provide powerful tool that may show gene and protein changes earlier, even at treatment levels below the limits of detection of traditional measures of toxicity, and it may be possible to apply toxicogenomics data into regulatory decision making[3] after biologic validation of toxicogenomics-based test methods[4] and reviewing and analyzing toxicogenomics data[5].

As REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) policy for improving chemical hazard management implemented, there will be limitations in animal testing, it looks necessary to develop a new *in vitro* method for toxicity testing, such as high content screening (HCS) model[6] or combining cell-based assays[7].

Liver is one of the primary targets affected by various toxicants and also a major site for metabolizing xenobiotics. Acetaminophen (APAP) is a common over-the-counter medication used for its analgesic and antipyretic properties, however, its use is often avoided in patients with chronic liver disease owing

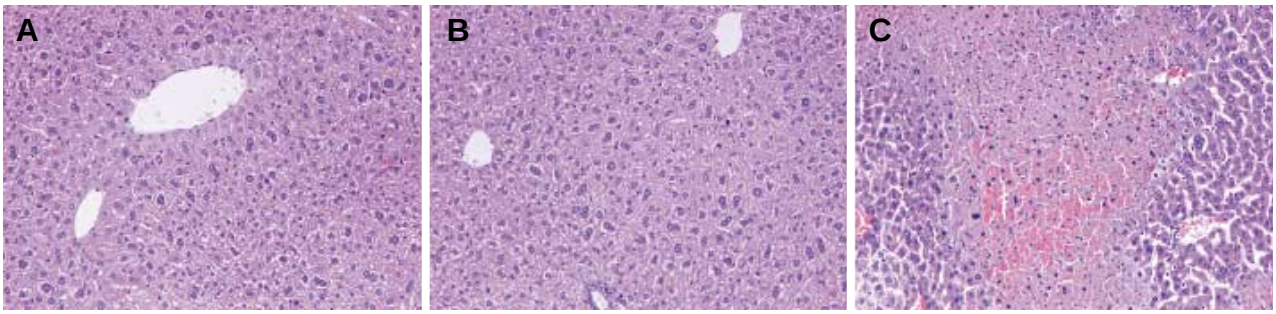

Figure 1. Histopathological findings of the livers of the mice. The mice were sacrificed at 24 h after following treatment. A: control; B: vehicle; C: APAP 800 mg/kg.

to hepatic toxicity[8].

Even though it seems that compounds with similar toxic mechanisms produce similar changes in gene expression *in vivo* and *in vitro* system[9], there was also discrepancy for gene expressions between animal and cell line system[10]. Therefore it may be worthwhile to compare transcriptional responses in livers of animals and hepatocyte cells line after exposure to chemicals to determine how faithfully the *in vitro* model system reflects *in vivo* responses using microarray since cell lines are more easily manipulated with consistency compared to primary hepatocytes.

In this study, we investigated the global gene expression from both mouse liver and mouse hepatic cell line treated with hepatotoxic chemicals, APAP, to gain a better understanding of molecular mechanisms of APAP-induced hepatotoxicity. And we further compared between *in vivo* and *in vitro* profiles, and assessed the feasibility of the extrapolation between two systems to compare of *in vitro* gene expression profiles to *in vivo* system.

Body and Liver Weight in Mice

During experiment, there was no death, and were no differences of body weight and absolute and relative liver weights between control and APAP-treated groups (data not shown).

Histopathological Examination of Liver in Mice

In high dose APAP treatment group, there were centrilobular necrosis and hemorrhage at 24 h after treatment (Figure 1). However, there were no histopatholgical changes and serum biochemistry at low doses at 6 and 24 h.

Serum Biochemistry in Mice

Data for serum biochemistry are shown in Figure 2. There were significant increases of AST and LDH in high dose treatment of APAP at 6 h ($P < 0.01$, $P <$

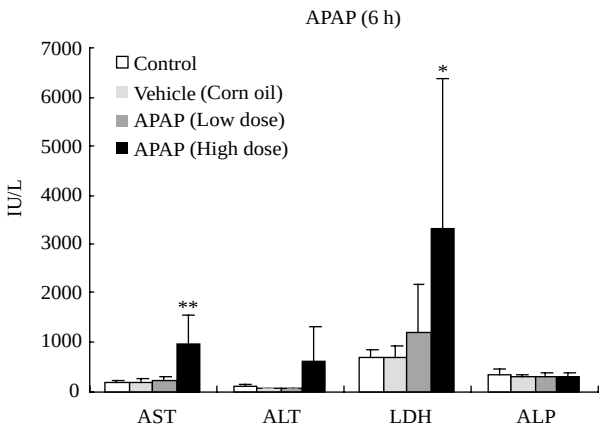

Figure 2. Changes of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), alkaline phosphatase (ALP) in serum of APAP-treated mice. Blood samples are collected at 6 h after administration. Data are expressed as mean ± SD from three or four mice. *,**Significantly different from control group ($P <$ 0.05, $P < 0.01$, respectively)

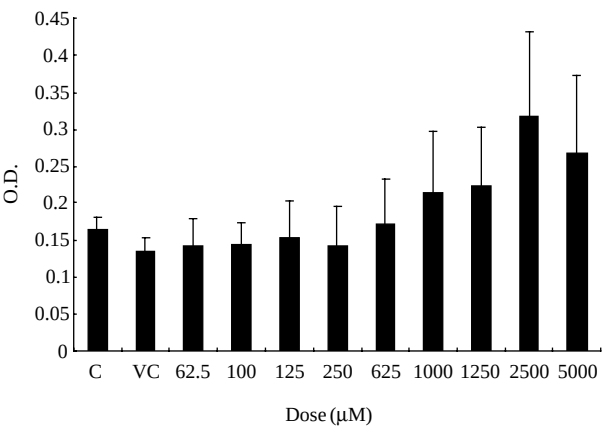

Figure 3. LDH assay in APAP-treated mouse cell line. Samples are collected at 24 h after APAP treatment. Data are expressed as mean ± SD.

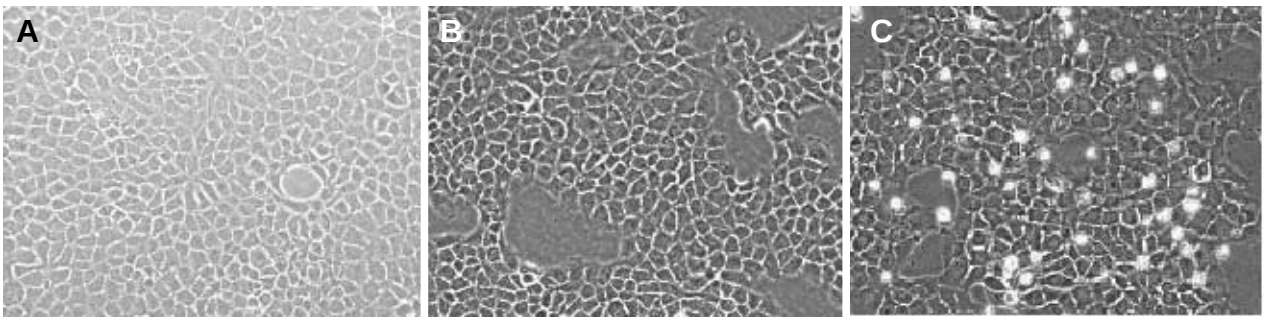

Figure 4. Morphological aspect of mouse hepatic cell line, BNL CL.2 Cells were treated with following materials and examined at 24 h after the treatment. A: control; B: vehicle; C: APAP 2,500 μM.

0.05, respectively). There were no alterations of these parameters at 24 h in APAP treatment (data not shown).

Cytotoxicity and Morphological Examination on Hepatic Cells, BNL CL.2

In vitro toxicity test showed that APAP treatment induced cellular toxicity over 1,000 μM at 24 h (Figure 3). Morphological examination of chemical-treated cell lines, cellular toxicity was evident at 24 h after treatment (Figure 4).

Microarray Analysis of Gene Expression Patterns in BNL CL.2 after Treatment of APAP

Global gene expression showing a significance over 0.05 by One-Way ANOVA showed that there were 1,206 differentially expressed genes (*in vivo*) and 671 genes (*in vitro*) at 6 h and 839 (*in vivo*) and 972 (*in vitro*) at 24 h by APAP. And there were commonly altered genes of 128 and 118 at 6 and 24 h, respectively, between *in vivo* and *in vitro*.

By time-dependent analysis, numbers of commonly up- or down-regulated genes between *in vivo* and in vitro caused by APAP treatment were four and three at 6 h and three and four at 24 h, respectively (Table 1). Hierarchical analysis showed that there were similar patterns at 6 h, not at 24 h between *in vivo* and *in vitro* (Figure 5). PCA analysis showed groups of APAP treatment were located at a different position compared to control group (Figure 6). High dose treatment group represented a clear distinction at 6 h both *in vivo* and *in vitro* system. On the while, *in vitro*, low and high dose treatment groups showed similar location at 24 h. Pathway analysis for differentially expressed genes by DAVID Bioinformatic Resources (NIAID/NIH) identified that there were 4 (*in vivo*) and 11 (*in vitro*) pathways caused by APAP treatment, and indicated glutathione metabolism pathway as common pathway between two systems (Table 2).

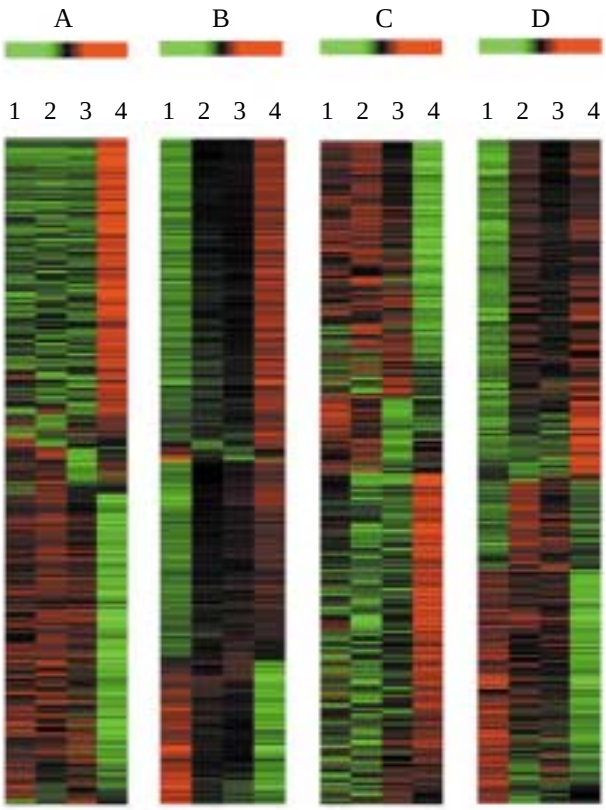

Figure 5. Hierarchical clustering analysis of genes altered by APAP treatment. A: *in vivo* at 6 h; B: *in vitro* at 6 h; C: *in vivo* at 24 h; D: *in vitro* at 24 h; Lane 1: control; Lane 2: Vehicle; Lane 3: Low dose treatment; Lane 4: High dose treatment.

Discussion

Hierarchical and *k*-means clustering analysis show-

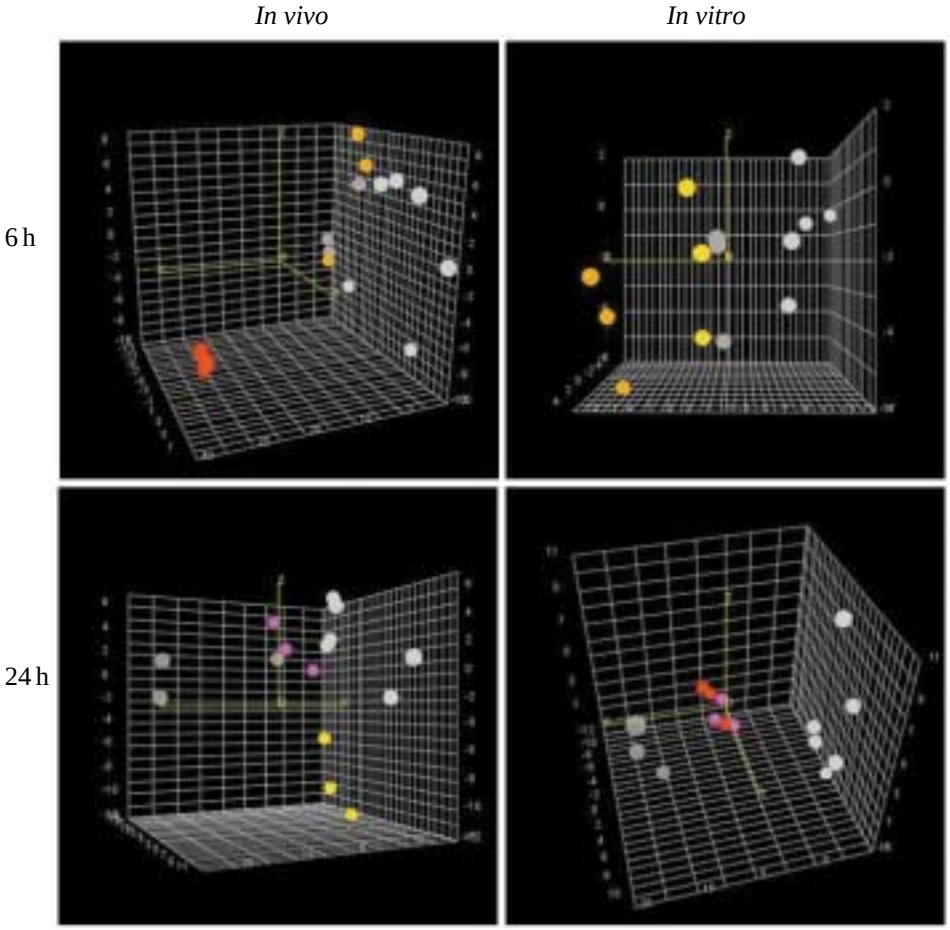

Figure 6. Principal Component Analysis (PCA) for altered genes by APAP treatment. *In vivo* at 6 h: Control as grey; Vehicle as dark grey; Low dose treatment as orange color; High dose treatment as red; *In vitro* at 6 h: Control as grey; Vehicle as dark grey; Low dose treatment as yellow; High dose treatment as orange color; *In vivo* at 24 h: Control as grey; Vehicle as dark grey; Low dose treatment as yellow; High dose treatment as pink; *In vitro* at 24 h: Control as grey; Vehicle as dark grey; Low dose treatment as pink; High dose treatment as red.

ed that there were generally similar patterns between *in vivo* and *in vitro*. Our PCA data provided a clear distinction between control or vehicle treatment group and APAP-treated groups. It suggests that gene expression data can be used to discern different hepatotoxic agents and toxicity endpoints[11]. Our previous data also showed gene expression profiles may provide useful methods of eliciting underlying molecular mechanism of drug susceptibility and of evaluating drug sensitivity *in vitro* correlated to *in vivo* (Jeong *et al*., Unpublished data).

Pathway analysis for differentially expressed genes identified that there were 4 (*in vivo*) and 11 (*in vitro*) pathways by APAP, representing *in vitro* system has more biological pathways than *in vivo* system. It seems there may be more defense mechanism existed in *in vivo* more than *in vitro*. And our experiments also showed there were many gene alterations relating to oxidative stress both *in vivo* and *in vitro*. Pathway analysis represented that glutathione metabolism pathway as common pathway in APAP-induced hepatotoxicity. In general, glutathione metabolism is related to detoxification of xenobiotics and maintenance of the redox state. However, elevated levels of glutathione have been also found in tumor cells[12,13]. In experimental models, expression of glutathione *S*-transferases is upregulated in foci and nodules, although some carcinomas showed down-regulation at end stages[14].

Statistical analysis of global gene expression identified that there were 1,206 differentially expressed genes (*in vivo*) and 671 genes (*in vitro*) at 6 h and 839 and 972 at 24 h after APAP treatment. It represented more numbers of genes found in *in vivo* than *in vitro* at 6 h after APAP treatment.

Our attempt to identify commonly altered genes between *in vivo* and *in vitro* showed DNA-damage related genes were involved in APAP-induced toxicity. APAP is metabolized by sulfation and glucuronidation and by CYP2E1 that produces a reactive metabolite, *N*-acetyl-*p*-benzoquinoneimine (NAPQI), which is detoxified by conjugation with GSH, and the analgesic acetaminophen causes a potentially fatal, hepatic centrilobular necrosis when taken in over-

Table 1. Commonly altered genes between *in vivo* and *in vitro* caused by APAP treatment.

Time & state	Gene symbol	Gene title	mRNA Accession No	*In vivo* (mg/kg)			*In vitro* (μM)		
				Vehicle	80	800	DMSO	250	2500
6 hr									
Up-regulated	Dscr1	Down syndrome critical region homolog 1 (human)	NM_019466	−0.3613	−0.278	2.5656	0.35531	0.49915	1.03363
	2210011G09Rik	RIKEN cDNA 2210011G09	–	0.4024	0.30505	2.02463	0.23868	0.47652	1.2026
	6330564D18Rik	RIKEN cDNA 6330564D18	–	−0.6333	−0.1584	1.12585	0.89169	0.89162	1.19227
	1810030N24Rik	RIKEN cDNA 1810030N24	NM_025471	0.33941	0.45263	1.00004	0.52673	0.64276	1.17899
Down-regulated	2810474O19Rik	RIKEN cDNA 2810474O19	NM_026054	0.50837	0.30708	−1.1103	−0.7528	−0.8772	−1.3159
	Nphp3	nephronophthisis 3 (adolescent)	XM_975214; XM_975243; XM_975277; NM_028721	−0.3702	−0.7755	−1.3747	−0.966	−0.8512	−1.4289
	Mtif2	mitochondrial translational initiation factor 2	NM_133767	−0.5158	−0.7746	−1.4928	−0.8039	−0.8179	−1.0722
24 h									
Up-regulated	Zfp688	zinc finger protein 688	NM_026999	−0.0064	0.0397	1.93217	1.05764	0.66443	1.14192
	Tm2d3; Tarsl2	TM2 domain containing 3; threonyl-tRNA synthetase like 2	NM_026795; NM_178056; NM_172310	−1.3787	−0.1962	1.43682	0.93338	0.90533	1.37433
	Eme1	essential meiotic endonuclease 1 homolog 1 (S. pombe)	NM_177752	−0.4857	−0.2002	1.30488	0.54343	0.68131	1.28013
Down-regualted	Mina	myc induced nuclear antigen	NM_025910	1.89002	0.64878	−1.0955	−0.3187	−0.4	−1.2085
	1700061D13Rik	RIKEN cDNA 1700061D13	–	−0.0989	−0.3566	−1.3666	−0.9212	−0.8292	−1.5352
	Sec22b	SEC22 vesicle trafficking protein homolog B (S. cerevisiae)	NM_011342	−1.953	−1.2857	−2.2999	−0.2328	0.05506	−1.0878
	Nat13	N-acetyltransferase 13	NM_028108	0.20891	−0.6208	−2.362	−0.3635	−0.5686	−1.0865

Table 2. Pathway analysis altered by APAP treatment (KEGG).

In vivo pathway	Number of related genes
Glutathione metabolism	5
Tight junction 7	
Arginine and proline metabolism	4
Urea cycle and metabolism of amino groups	4
***In vitro* pathway**	**Number of related genes**
Glutathione metabolism	3
Terpenoid biosynthesis	3
Propanoate metabolism	1
Butanoate metabolism	6
Abc transporters-general	6
Citrate cycle (tca cycle)	2
Biosynthesis of steroids	3
Ascorbate and aldarate metabolism	2
Antigen processing and presentation	6
Toll-like receptor signaling pathway	6
B cell receptor signaling pathway	5

dose, and these findings indicated that acetaminophen was metabolically activated by cytochrome P450 enzymes to a reactive metabolite that depleted glutathione (GSH) and covalently bound to protein[15]. Furthermore, the progression of APAP toxicity was dependent on DNA damage caused by activation of DNase in mice[16], associated with mitochondrial oxidant stress and peroxynitrite formation[17]. APAP-induced toxicity affected numerous aspects of liver physiology such as growth arrest and cell cycle regulatory proteins, stress-induced proteins[18], and altered levels of gene expression relating to lipid and energy metabolism[11,19].

APAP is metabolized via CYP450, especially CYP-2E1. CYP2E1 induction is associated with elevated hepatotoxicity[20,21]. And CYP2E1-null mice showed no toxicity when treated with APAP[22], suggesting that CYP2E1 is the principal enzyme responsible for the metabolic conversion of this chemical to their active hepatotoxic metabolite. However, our experiment showed that the expression of CYP2E1 was not picked up as shown in previous report[23].

Generally, it seemed that there were similar patterns between *in vivo* and *in vitro* in our experiments. However, there were somewhat discrepancies of expression patterns by time. In APAP-treated mice,

there were alterations of serum biochemical parameters at 6 h, in contrast to evident histopathological lesions were found at 24 h. Microarray data represented APAP treatment induced altered genes at 6 h more than at 24 h.

It was reported that there were good correlation between the histopathology, clinical chemistry, and gene expression profiles induced by hepatotoxicants[24]. However, our data suggest there may be time-dependent alterations of genes. Therefore, it is very important to set a relevant exposure time for toxicants, with an awareness of relations between early time and later time profiles. As there has been a trend recently in focusing on toxicogenomic profiles after short-term treatment of some chemicals *in vivo*[25-27], further studies are thus warranted to analyze detailed alterations from early to late stages of hepatoxic process, so that any hepatopreventive strategy can be based on a firm foundation.

As expected, there was not an exact correlation between two systems. It should be reminded that liver tissue and primary cells express different suites of genes and suggest they have fundamental differences in their cell physiology, and also indicating that care must be taken in extrapolating from primary cells to whole animal organ toxicity effects[10]. Further multi-dimensional data set for liver toxicity provides an informatics challenge requiring appropriate computational methods for integrating various toxicological data into profiles and models predicting toxicity[28].

In conclusion, our results suggest it may be feasible to develop toxicogenomics biomarkers or profiles by comparing *in vivo* and *in vitro* genomic profiles specific to these hepatotoxic chemicals for application to prediction of liver toxicity.

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit was obtained from Promega Co. (Madison, WI) while cytotoxicity detection kit was from Roche Molecular Biochemicals (Indianapolis, IN). Mouse Genome Survey Microarray gene chips were supplied by Applied Biosystems (Foster City, CA). This microarray platform has 33,012 probes, which are 60-mers, lie mostly within 1,500 base pairs of the 3′ end of the source transcript. APAP was obtained from Sigma (St. Louis, MO).

Animals and Chemicals Treatment

In vivo experiment, male 5-weeks old ICR mice were supplied by the Department of Laboratory Animal Resources, the National Institute of Toxicological Research, Food and Drug Administration, Seoul, Korea. The animals were housed in polycarbonated cages with hardwood chips in a room with 12/12 h light/dark cycles and controlled humidity and temperature. They were allowed free access to pellet chow during the experiment. All procedures were approved by the Institutional Animal Care and Use Committee of National Institute of Toxicological Research.

In preliminary study, we found that APAP treatment over a dose of 500 mg/kg induced hepatic lesions at 24 h. Six-week-old mice (n=32) were randomly allocated to four groups as follow: groups 1 as control, group 2 as vehicle (corn oil), group 3 and 4 as APAP low and high dose treatment (80, 800 mg/kg, respectively, dissolved in corn oil, oral). Mice were sacrificed at 6 and 24 h after the treatment.

Initial and final body weights were measured. At the end of the experiment, all animals were fasted overnight and euthanized by exsanguination under ether anesthesia. Blood was taken from the abdominal aorta, and serum biochemistry was performed for the following parameters: aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) using Prestige 24i (Tokyo Boeki Medical System, Japan).

At necropsy, half of livers were fixed in 10% phosphate-buffered formalin, and routinely processed for embedding in paraffin, and staining of 4 μm sections with hematoxylin and eosin for histopathological examination. And the remaining samples from all the animals were snap-frozen in liquid nitrogen for RNA extraction and subsequent analysis.

Cell line, Cell Culture and Chemicals Treatment

In vitro experiment, murine embryonic normal hepatic cell line, BNL CL.2 cells (ATCC TIB-73) was purchased from American Type Culture Collection (ATCC, Manassas, VA). The cells were cultured in DMEM medium supplemented with 100 units of penicillin-streptomycin/mL, 2 mM L-glutamine, and 10% FBS at 37°C in a 5% CO_2 atmosphere.

APAP was dissolved in dimethyl sulfoxide (DMSO) and were freshly diluted in culture media for each experiment. Vehicle concentrations were less than 0.5% in all experiments.

Cytotoxicity Assay

Cytotoxicity was assessed using Cytotoxicity De-

tection Kit according to manufacture's instruction (Roche, Germany). In brief, BNL CL.2 (2×10^5 cells/mL) was treated with APAP (0-5000 μM) or 0.5% DMSO (as vehicle control) and was incubated for 6 or 24 h, and supernatant was mixed with dye solution and catalyst for 30 min. The absorbance at 490 nm of the solution was measured using a spectrophotometer (Benchmark Plus™, Bio-Rad Laboratories, Hercules, CA).

We set a high dose as 2,500 μM as apparent cellular toxicity-inducing dose, and one-tenth dose as low dose as not showing apparent cellular toxicity. At 6 or 24 h after treatment, cells were harvested for RNA extraction.

RNA Isolation and Microarray Gene Expression Profiling

Total RNAs were extracted for gene expression analysis using the RNeasy Mini kit (Qiagene, Valencia, CA). The yield of RNA was determined spectrophotometrically by measuring the optical density at 260 nm. Total mRNA was converted into double-stranded cDNA using a Chemiluminescent RT-IVT labeling Kit (Applied Biosystems) and an oligo $(dT)_{24}$ primer. Digoxigenin-labeled cRNA was generated from the double-stranded cDNA using a Chemiluminescent RT-IVT labeling kit. Labeled cRNA was purified using cRNA purification kit (Applied Biosystems). The quality and quantity of RNA and cRNA was evaluated using spectrometry and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Each cRNA sample was fragmented by incubation for 30 min at 60°C in fragmentation buffer. The Mouse Genome Survey Microarray gene chip (Applied Biosystems) was hybridized with the fragmented digoxigenin-labeled cRNAs at 55°C for 16 h and then washed. After washing procedure, the chemiluminescent detection, image acquisition and analysis were performed using the Chemiluminescent Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer following the manufacturer's protocols. The chemiluminescent signals from the scanned images were quantified, corrected for background, and spot- and spatially-normalized using the 1700 Chemiluminescent Microarray Analyzer (Applied Biosystems). Microarray analyses were performed for each RNA sample (3 samples/group).

Data Analysis

For comparison of gene expression profiles after treatment of APAP, control, vehicle control, 80 and 800 mg/kg APAP (*in vivo*), and 0 (vehicle control), 250 and 2,500 μM APAP (*in vitro*) were tested. Two time points for cell harvest of 6 and 24 h after the treatment were chosen to investigate the time-relating pattern in animals or cells exposed to APAP. Data were analyzed from three independent experiments with samples at 6 and 24 h after APAP treatment in mice and cell line.

Gene expression data from microarray were input to GenPlex (Istech Co. Ltd., Korea). The signal log ratio values, which represent ratios of hybridization signals between control and treated cells, were calculated after quantile normalization. Significantly altered genes induced by APAP were extracted by One-Way ANOVA ($P < 0.05$).

K-means clustering was performed to identify genes that have a similar differential expression profile across conditions using AVADIS (Strand Life Sciences, Redwood city, CA). Pathway analyses were conducted using DAVID (The Database for Annotation, Visualization and Integrated Discovery) (http://david.abcc.ncifcrf.gov/) and the PANTHER (Protein ANalysis THrough Evolutionary Relationships) (http://www.pantherdb.org/). The extracted genes were categorized based on location, cellular components, and reported or suggested biochemical, biologic, and molecular functions.

Statistical Analysis

Statistical analyses for body weights, liver weights and serum biochemical parameters were performed with the Tukey-Kramer method using the JMP program (SAS Institute, Cary, NC). For all comparisons, probability values less than 5% ($P < 0.05$) were considered to be statistically significant.

Acknowledgements

This research was supported by a research grant, 06131Omics401 (2006) to Dr. Sue Nie Park from the National Institute of Toxicological Research, Korea Food and Drug Administration of Korea.

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