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Gene Expression Profiling of Acetaminophen Induced Hepatotoxicity in Mice

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Abstract

Microarray analysis of gene expression has become a powerful approach for exploring the biological effects of drugs, particularly at the stage of toxicology and safety assessment. Acetaminophen (APAP) has been known to induce necrosis in liver, but the molecular mechanism involved has not been fully understood. In this study, we investigated gene expression changes of APAP using microarray technology. APAP was orally administered with a single dose of 50 mg/kg or 500 mg/kg into ICR mice and the animals were sacrificed at 6, 24 and 72 h of APAP administration. Serum biochemical markers for liver toxicity were measured to estimate the maximal toxic time and hepatic gene expression was assessed using high-density oligonucleotide microarrays capable of determining the expression profile of >30,000 well-substantiated mouse genes. Significant alterations in gene expression were noted in the liver of APAP-administered mice. The most notable changes in APAP-administered mice were the expression of genes involved in apoptosis, cell cycle, and calcium signaling pathway, cystein metabolism, glutatione metabolism, and MAPK pathway. The majority of the genes upregulated included insulin-like growth factor binding protein 1, heme oxygenase 1, metallothionein 1, S100 calcium binding protein, caspase 4, and P21. The upregulation of apoptosis and cell cycle-related genes were paralleled to response to APAP. Most of the affected gene expressions were returned to control levels after 72 hr. In conclusion, we identified potential hepatotoxicity makers, and these expressions profiling lead to a better understanding of the molecular basis of APAP-induced hapatotoxicity.

Keywords: Toxicogenomics, Acetaminophen, Microarray, Hetotoxicity

Toxicogenomics is a new interdisciplinary area of research being developed to monitor the expression of multiple genes, proteins, and metabolites simultaneously. It combines new technologies in genomics, proteomics, and metabolomics with traditional tools of pathology and toxicology to study biological response to drugs and other environmental xenobiotics. The development of the field of toxicogenomics will provide powerful new tool that may show gene and protein changes earlier and at treatment levels below the limits of detection of traditional measures of toxicity[1].

Microarray technology can be applied as a powerful tool to identify and characterize changes in gene expression associated with chemical-induced toxicity. In addition, microarrays can provide highly sensitive and predictive biomarkers for toxicity and new information on mechanisms of action through ac characteristic toxic signatures[2-4].

Hepatotoxicity is one of the major causes of drug withdrawal from the market. Although many of the clinical hepatotoxicity events cannot be predicted in the preclinical trials, some mechanisms of hepatotoxicity caused by drug treatment have been well investigated[5-6].

The liver is one of the primary targets for various types of chemical-induced toxicity. Chemical concentrations in the liver are often much higher than the peak plasma concentration. The liver is also the major site for metabolizing xenobiotics and these chemicals can lead to the formation of active metabolites. Therefore, to investigate the gene expression changes and compare the gene expression profiles to the extent of liver toxicity are useful for the assessment of liver toxicity.

APAP is a common over-the-counter medication used for its analgesic and antipyretic properties; however, it is also one of the leading causes of drug-induced liver failure[7-8]. At pharmacological doses, APAP is metabolized by sulfation and glucuronida-

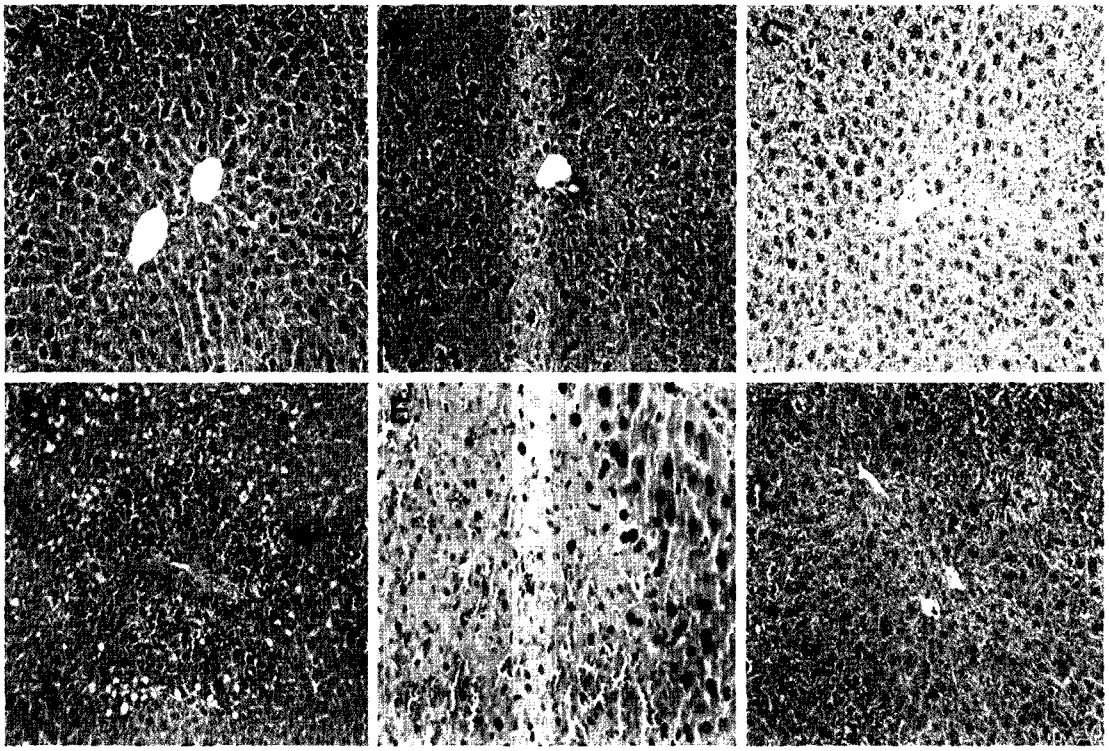

Fig. 1. Centrilobular necrosis in liver sectic ıs of animals that administered acet minophen. Representative H & E stained to histological liver sections derived from ICR mice treated with 50 mg/kg (A, B, C) and 500 mg/kg (D, E, F) at 6, 24, and 72 hr, respectively.

tion, and to a lesser extent, by cytochrome CYP2E1 that produces a reactive metabolite, NAPQI, which is detoxified by conjugation with GSH[9-10].

The purpose of the present study is to determine the correlations between biochemical markers for hepatotoxicity and hepatic gene expression profiles at various doses of APAP-administration in rats, and then to extrapolate the DNA microarray data for predicting hepatotoxic chemicals.

Histopathology and Clinical Chemistry

No significant histopathological alterations were observed in livers of 50 mg/kg AP treated mice at any time points compared to controls. At the 500 mg/kg dose, typical necrotic changes in centrilobular areas were identified through histopathological analysis (Fig. 1). Biochemical parameters for hepatotoxicity were not significantly altered at any time point in 50 mg/kg APAP. After the mice were treated with 500 mg/kg of APAP, serum ALT and AST activity were markedly increased at 6 hr and decreased to level of controls at 24 hr (Fig. 2). We concluded that 50 mg/kg doses of APAP produced no conventional signs of toxicity and thus would be considered subtoxic according to our criteria, while the 500 mg/kg dose was clearly hepatotoxic.

Gene Expression Changes

To analyze gene expression profiles of APAP-treated mice, we compared APAP treated group versus vehicle control group at each time points using two way ANOVA methods and identified 2,284 genes out of total 32,381 probe sets. By statistical criteria of >1.5 fold changes at $P<0.05$ (False discovery rate <0.05), a total of 854 genes were significantly changed in at least one of treatment conditions.

Hierarchical clustering analysis was performed with 854 differentially expressed genes according to their similarity in expression pattern by dose and time of APAP administration (Fig. 3). There were the greatest similarities in pattern between the high dose groups at 6 hr. These data showed a strong dependency on dose and time of treatment.

Principal component analysis (PCA) using the 854 genes was conducted to visualize, in three dimensional space, the distribution of the samples derived from livers of mice treated with APAP at different doses and for different times (Fig. 4). PCA revealed a

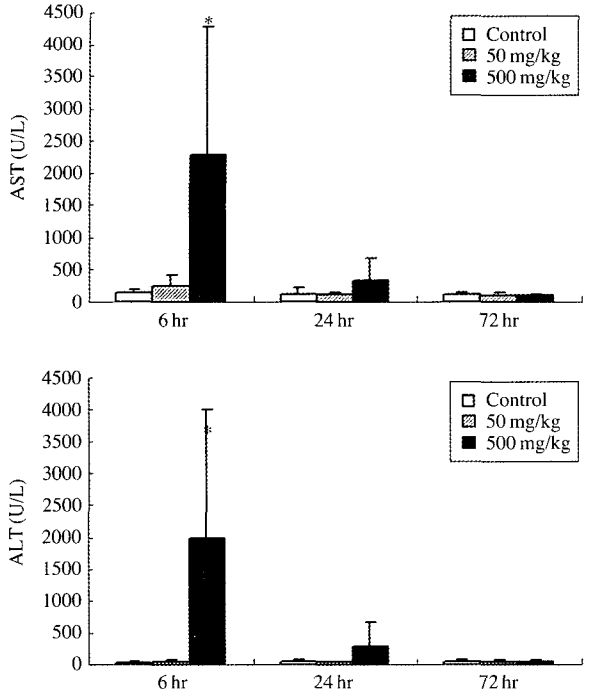

Fig. 2. Changes of AST and ALT in serum of APAP administered mice. Blood samples are collected at 6, 24, 72 hr after administration. Data are expressed as mean ± S.E. from 3 mice. Significantly different from control group (*, $P < 0.05$).

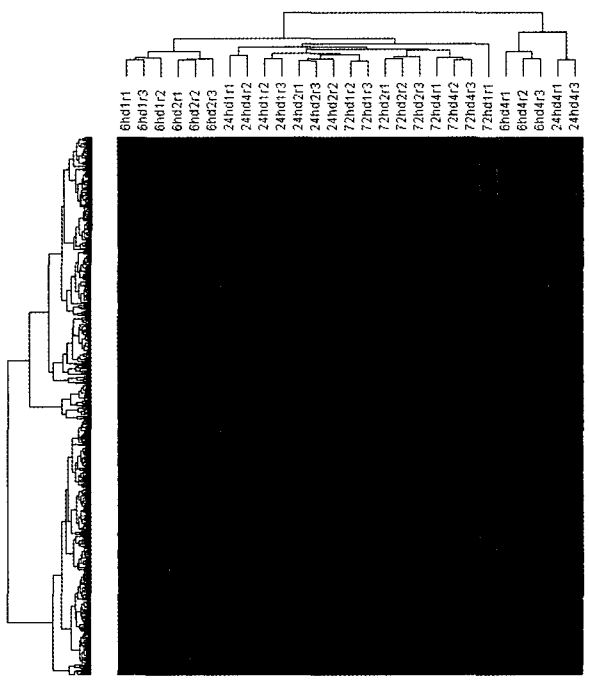

Fig. 3. Hierarchical clustering analysis from > 1.5 fold differentially changed genes (854 genes) in liver of acetaminophen administration. ($P < 0.05$)

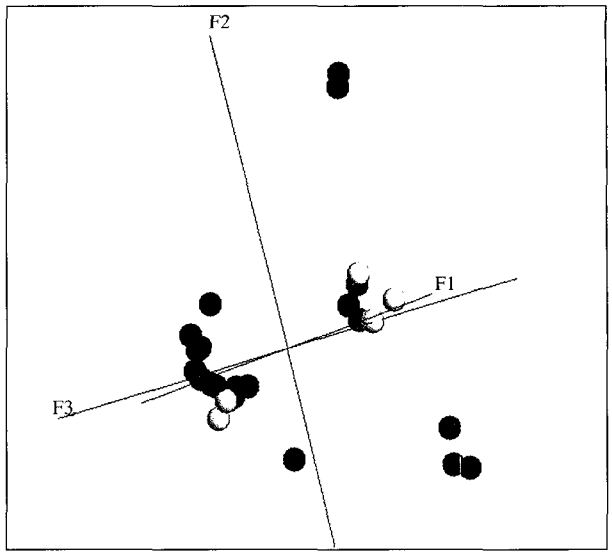

Fig. 4. Principal component analysis (PCA) for > 1.5 fold differentially changed genes after acetaminophen administration. The colored circles represent experimental groups; Yellow, 6 h control; Red, 24 h control; Black, 72 h control; Margenta, 6 h 50 mg/kg; Cyan, 6 h 500 mg/kg; Blue, 24 h 50 mg/kg; Green, 24 h 500 mg/kg; Grey 72 h 50 mg/kg; Brown, 72 h 500 mg/kg.

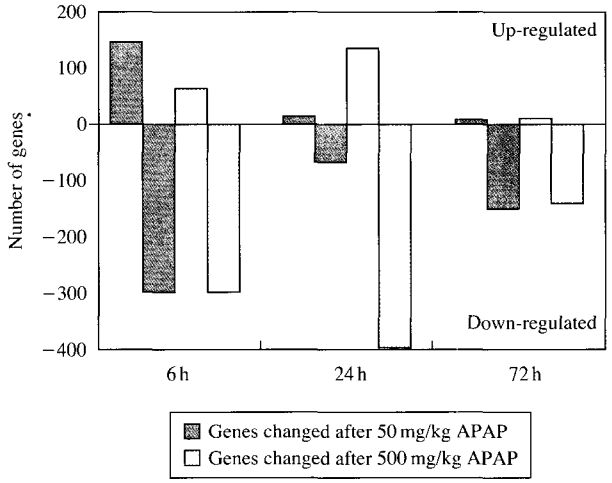

Fig. 5. Direction of gene regulation at each time points and dose after acetaminophen administration.

distinct separation between the low and high dose group. PCA result was consistent with the hierarchical clustering analysis and correlated with the biochemical and histopathological alterations observed in the livers of APAP-treated mice.

The number of the genes affected increased with the increased amount of APAP administration. Some

Table 1. Differentially expressed genes after APAP administration. (fold change > 5)

Gene bank accession No.	Gene name	Fold change		
		6 h	24 h	72 h
BC013448	glucose-6-phosphatase, catalytic	2.3	−17.2	−1.4
BC012715	hydroxysteroid dehydrogenase-5, delta < 5 > -3-beta	−9.0	−33.1	1.2
BC010796	camello-like 1	−1.4	−5.3	1.6
BC030937	chaperone, ABC1 activity of bc1 complex like (S. pombe)	1.5	−9.5	1.0
BC019908	cytochrome P450, family 2, subfamily c, polypeptide 29	−2.2	−6.3	−1.2
BC011129	carbonic anhydrase 3	−5.0	−18.7	1.8
AJ428064	transmembrane protein 7	−9.3	−1.3	1.7
BC011486	muscle, intestine and stomach expression 1	−6.8	1.4	−2.0
AF128423	macrophage receptor with collagenous structure	1.6	26.8	1.3
X53798	chemokine (C-X-C motif) ligand 2	591.3	13.2	1.3
M22326	early growth response 1	372.8	6.7	2.2
AF071180	formyl peptide receptor-like 1; formyl peptide receptor, related sequence 2; formyl peptide receptor, related sequence 1	14.1	9.8	1.6
BC012965	chemokine (C-X-C motif) ligand 13	15.6	53.9	1.2
BC057889	CD14 antigen	62.8	63.8	1.6
BC023815	growth arrest and DNA-damage-inducible 45 beta	12.2	22.2	1.6
AF009246	RAS, dexamethasone-induced 1	46.0	23.6	−1.3
BC013644	replication initiator 1	15.5	2.1	1.1
BC006950	immediate early response 3	68.7	11.1	−1.2
L28177	growth arrest and DNA-damage-inducible 45 alpha	39.6	2.8	−1.8
BC055885	serum amyloid A 3	5.9	26.7	1.4
BC013345	insulin-like growth factor binding protein 1	222.1	1.5	1.0
X14607	lipocalin 2	12.3	51.5	2.9
BC055885	serum amyloid A 3	7.2	40.0	1.3
BC052031	suppressor of cytokine signaling 3	26.2	12.7	−2.6
BC037997	chemokine (C-X-C motif) ligand 1	60.5	19.0	−1.0
BC027635	S100 calcium binding protein A9 (calgranulin B)	15.2	10.6	3.0
BC010757	heme oxygenase (decycling) 1	23.0	2.8	1.1
AK011040	metallothionein 1	16.1	36.5	−1.0
BC043723	interferon-related developmental regulator 1	13.5	3.0	−1.4
BC021815	mutL homolog 1 (E. coli)	−5.4	−1.2	−1.7
D63764	pyruvate kinase liver and red blood cell	−5.6	−5.6	2.1
BC022665	sulfotransferase family, cytosolic, 1C, member 1	−3.9	−5.5	−1.1
BC056643	aldo-keto reductase family 1, member C6	−4.4	−9.0	1.1
AX376573	G protein-coupled receptor 91	−6.9	−18.8	1.1
M90397	B-cell leukemia/lymphoma 3	5.1	1.5	−1.7
BC030067	chemokine (C-X-C motif) ligand 10	11.4	1.6	−1.2
BC057985	orosomucoid 2	2.2	40.1	3.4
AF257475	general transcription factor II I repeat domain-containing 1	−1.1	5.3	2.1
AJ536019	resistin like gamma	22.1	4.6	1.7
BC022952	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	138.5	6.4	−1.2
BC019946	activating transcription factor 3	172.8	7.5	−2.1
AK080161	neuregulin 4	5.3	12.3	1.4
L76381	S100 calcium binding protein A8 (calgranulin A)	20.3	30.6	2.8
BC032962	interleukin 1 receptor, type II	13.5	1.4	−1.0
BC061154	chitinase 3-like 3; chitinase 3-like 4	6.3	5.0	−1.1
AK004844	tumor necrosis factor receptor superfamily, member 1b	1.8	4.0	−1.1
AF222928	SAM domain, SH3 domain and nuclear localisation signals, 1	6.3	5.4	1.8
BC023349	arginase type II	7.3	18.1	1.3
BC053918	DNA segment, Chr X, Immunex 50, expressed	7.4	3.5	−1.4
	serine (or cysteine) proteinase inhibitor, clade A, member 3C	4.1	11.2	−1.1
AK020067	DNA segment, Chr 10, Wayne State University 102, expressed	4.9	1.2	1.6
BC031744	hypothetical protein MGC30332; U2 small nuclear RNA auxiliary factor 1-like 4	7.0	1.3	−1.7
AB025278	A kinase (PRKA) anchor protein (gravin) 12	17.3	4.0	−1.6
AY169235	stefin A1	8.7	5.9	−1.0
BC004770	nuclear receptor subfamily 4, group A, member 1	6.2	2.0	1.1
BC052833	histidine decarboxylase	7.5	1.5	1.1
BC028634	regulator of G-protein signaling 1	5.8	4.5	1.5
BC061255	caspase 4, apoptosis-related cysteine protease	8.0	3.0	−1.1

Table 1. Continued.

Gene bank accession No.	Gene name	Fold change		
		6 h	24 h	72 h
AK007808	chemokine (C-C motif) receptor-like 2	29.0	1.2	−1.1
AK012702	calcium/calmodulin-dependent protein kinase II, delta	4.6	2.1	1.1
BC029234	ADP-ribosylation factor-like 4	11.4	2.2	−1.3
AF064448	feminization 1 homolog b (C. elegans)	6.4	5.2	−1.6
BC039698	lipin 2	18.6	−2.1	1.5
AF359385	calcium-binding tyrosine-(Y)-phosphorylation regulated (fibrousheathin 2)	12.6	−2.7	−1.2
BC052705	dual specificity phosphatase 8	12.4	1.0	−2.0
BC003735	A kinase (PRKA) anchor protein 2; paralemmin 2	11.7	1.3	1.1
BC019154	thrombomodulin	44.3	5.2	−1.4
BC006591	glycoprotein 49 B	6.4	3.7	−1.6
BC025169	RIKEN cDNA 1810008K03 gene	16.7	2.0	2.6
BC021530	solute carrier family 39 (zinc transporter), member 14	3.1	4.8	1.5
BC012955	tribbles homolog 3 (Drosophila)	9.5	2.4	1.3
AK007630	cyclin-dependent kinase inhibitor 1A (P21)	17.5	4.4	−1.4
BC064038	Spi-C transcription factor (Spi-1/PU.1 related)	6.3	2.8	−1.1
BC053450	inositol 1,4,5-trisphosphate 3-kinase C	5.4	1.5	1.4
BC067248	growth differentiation factor 15	15.2	2.0	2.8
BC027536	B-cell leukemia/lymphoma 2 related protein A1d; B-cell leukemia/lymphoma 2 related protein A1b; B-cell leukemia/lymphoma 2 related protein A1a	5.4	5.6	1.2
BC006728	myelocytomatosis oncogene	20.2	2.2	−2.3
AJ344065	testis derived transcript	1.4	6.1	1.3
AF159159	cyclin L1	12.3	1.5	−1.2
AK053190	CCAAT/enhancer binding protein (C/EBP), delta	6.4	7.5	−1.3
BC021391	zinc finger protein 36	21.9	1.9	−1.1
BC013551	Down syndrome critical region homolog 1 (human)	5.7	1.6	−1.5
BC054739	protein phosphatase 1, regulatory (inhibitor) subunit 3C	−1.2	−6.5	−2.9
BC061207	GDP-mannose pyrophosphorylase B	−7.4	1.4	−1.5
BC053425	stromal cell-derived factor 2-like 1	−10.3	2.9	−1.1

genes appeared to be transiently increased at 6 and 24 hr and decreased to control level at 72 hr, while others showed a persistent alteration dependent on the dose and time of administration (Fig. 5).

Gene ontology biological process, molecular function and pathway mapping were performed using KEGG to evaluate the expression pattern on the basis of gene function and pathway. The result showed significantly changed genes were related to gluconeogenesis, glycolysis, TCA cycle, steroids synthesis, urea cycle, calcium signaling pathway, MAPK signaling pathway, cytokine-cytokine receptor interaction, glutathione metabolism, apoptosis and porphyrin metabolism (Table 1).

We were particularly interested in discovering gene expression changes at early time point of APAP-treated group that might be indicative of biological responses predictive of the observed toxic effects genes in the late phase. Review of differentially expressed genes after low and high dose of APAP at 6 hr revealed twenty genes were differentially expressed at 6 hr and 24 hr. These genes could be potential toxicity markers for evaluating a hepatotoxicant in the drug development process (Table 2).

Table 2. Differentially expressed genes at maximal toxic time, 6 hr after acetaminophen administration of 50 and 500 mg/kg.

* Heme oxygenase 1
* Solute carrier family 16 (monocarboxylic acid transporters)
* DNA segment, Chr 16, ERATO Doi 472, expressed
* DNA-damage inducible transcript 3
* Resistin like gamma
* Choline kinase alpha
* Claudin 2
* RIKEN cDNA 4930488L10 gene
* Early growth response 1
* Malic enzyme, supernatant
* Activating transcription factor 3
* Brain expressed myelocytomatosis oncogene
* Jun oncogene
* S100 calcium binding protein A9 (calgranulin B)
* RIKEN cDNA 1810013B01 gene
* Chitinase 3-like 3; chitinase 3-like 4
* BCL2-like 11 (apoptosis facilitator)
* Interferon-related developmental regulator 1
* Transmembrane protein 7
* human immunodeficiency virus type I enhancer binding protein 3

Discussion

Toxicogenomics provides the ability to examine in greater detail the underlying molecular events that precede and accompany toxicity, thus allowing prediction of adverse events at much earlier times compared to classical toxicological end points. In order for gene expression profiling to be a valuable tool in toxicology, it should be characterized for its ability to reflect the results derived from classical toxicology assays in a dose and time dependent manner Such phenotypic anchoring removes subjectivity from interpretation of expression data by distinguishing between the toxicological effect signals from other gene expression changes that may be unrelated to toxicity, such as the therapeutic effects of a compound[11-13].

To test our hypothesis that gene expression profiling can be indicators of subtle injury to the liver induced by a low dose of a substance that does not because overt toxicology, we exposed mice to acetaminophen (APAP) ranging from doses expected to have no hepatotoxic effect to doses known to cause liver toxicity. APAP was selected as a model hapatotoxicant for our studies because considerable information exists concerning its adverse effects in the liver.

In this study, we chose a toxic dose as any dose that causes either one or more of the following changes in the target organ: release of intracellular enzymes, histologically apparent cell death, or biochemically measurable organ dysfunction. And we chose as subtoxic a dose that causes none of these classic toxicologic effects, but induces changes in vital cell pathways, as manifested in expression changes of genes involved in such pathways. The threshold between subtoxic adverse effects and toxic tissue insult would be marked by additional gene expression alterations representing pathways such as apoptosis, necrosis, or inflammation[14].

The observation that doses of chemicals considered to be nontoxic on the basis of traditional measures of toxicity cause changes in gene expression indicative of adverse cellular effects changes in gene expression has potentially important implications for safety assessment of pharmaceuticals[15]. All alterations in gene expression should not be viewed as harmful. Thus, elaborated analysis of the types of changes is a requisite for distinguishing between responses to potentially harmful effects and responses representing compensatory homeostatic adjustments[16].

Hepatotoxicity of APAP has been a long-term focus of toxicological studies, and the pathological changes, along with alterations in biochemical pathways have been well documented. Recent development in genomics technologies has led to new investigation into gene expression alterations caused by acute treatment of APAP. Acute administration of APAP to rats caused significant changes in gene expression profiles[17-19].

Acetaminophen is a widely used non prescription drug with analgesic and antipyretic properties. However, acetaminophen intoxication is also one of the leading causes of drug-induced liver failure. APAP is predominantly metabolized by conjugation reactions to form sulfate and glucuronide metaboltes, which are excreted in the urine. A lesser amount of the drug is metabolized by cytochrome P450 2E1 to form the highly reactive metabolite N-acetyl-p-benzoquinoneimine (NAPQI), which is rapidly bound to intracellular glutathione and excreted in the urine as mercapturic acid. When excessive amounts of acetaminophen are ingested, the ability to conjugate is overwhelmed and metabolism by cytochrome P450 2E1 becomes much greater importance. In these situations, the capacity of glutathione to serve as an effective hepatoprotectant may be overwhelmed, and the hepatocyte becomes relatively defenseless against attack by the reactive metabolites.

In this study, we confirmed that the hapatotoxicity models of APAP administered mice were successfully conducted, and the toxic times point of APAP was estimated as 6 h. AST and ALT activities were elevated significantly at 6 h. Biochemical markers reflected the major gene expression profiles. With hierarchical clustering, KNN, PCA, KEGG pathway analysis, we identified changes in gene expression that may have the potential to serve as sensitive and predictive indicators of hepatotoxicty following exposure to APAP. The number of the genes affected increased with the increased amount of APAP administration. Some genes appeared transiently changed, while others showed a persistent alteration dependent on the dose and time of administration. The most notable changes in APAP-administered mice were the expression of genes involved in apoptosis[20], cell cycle[19], and calcium signaling pathway, cystein metabolism, glutatione metabolism, and MAPK pathway. The majority of the genes upregulated included insulin-like growth factor binding protein 1, heme oxygenase 1[15,21], metallothionein 1[15], S100 calcium binding protein, caspase 4, P21. The upregulation of apoptosis and cell cycle-related genes were paralleled in response to APAP. Most of the affected gene expressions were returned to control levels after 72 hr.

Microarray technology is widely used to elucidate the mechanism of chemical-induced toxicity, by profiling gene expression patterns provoked by drugs.

Therefore, this technology also have the possibility of being used as a tool for predicting the adverse effect of drug candidates and improving the process of risk assessment and safety evaluation. Gene expression profiling provides an early indication of toxicity because toxicants-mediated changes in gene expression are often detectable before clinical chemistry, histopathology, or clinical observations suggest a toxic effect.

Methods

Animals and Chemical Treatment

Male ICR mice (6-weeks old) were obtained from National Institute of Toxicological research (Seoul, Korea). Mice were maintained in a controlled environment in accordance with the guidelines prepared by AAALAC. The mice were randomly divided into three groups: vehicle control and two experimental groups. High dose used in the present study was based on the results of preliminary studies as the minimal dose that yielded histopathologic hepatotoxicity at 24 h after drug treatment (data not shown). In high dose group, a total of 15 mice were injected orally a single dose of 500 mg/kg body weight of APAP (Sigma-Aldrich, St. Louis, MO) in corn oil and 5 animals were subsequently killed at time point of 6, 24, and 72 h after drug treatment. In low dose group, 15 mice were treated with a $1/10^{th}$ dose (50 mg/kg body weight) of high dose group. In vehicle-control group, 15 mice were administered with corn oil. Five animals were neither fasted nor treated with APAP (non-treated).

Biochemical Analysis

Serum samples were prepared from blood withdrawn by heart puncture. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured by standard clinical chemistry assays with Automated Chemistry Analyzer.

Histopathological Analysis

The liver tissues collected in formalin were dehydrated in paraffin, sectioned at 5 μm, and stained with H&E. Histopathologic examinations of the liver sections were conducted by a pathologist and peer-reviewed.

RNA Preparation

Frozen tissues were pulverized in liquid nitrogen-cooled and pestle apparatus. The powdered tissue was then processed with trizol (Invitrogen, Carlsbad, CA) for isolation of total RNA, followed by Rneasy (Qiagen, Valencia, CA) purification. Purified total RNAs were analyzed with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, US) and confirmed that 28S/18S ratio was between 1.8 and 2.0 and 260/280 nm ratio between 2.0 and 2.2, respectively.

Microarray Analysis Using Applied Biosystems Array System

The Applied Biosystems Mouse Genome Survey Microarray contains 32,996 probes, representing 32,381 curated genes targeting 44, 498 transcripts. Three animals out of 5 per each group were selected as representative animals from each group on the basis biochemical and histopathological review, and total RNA from the selected 3 animals were further processed. Digoxigenin-UTP Labeled cRNA was generated and linearly amplified from 1 μg of pooled-total RNA from each group using Applied Biosystems Chemiluminescent RT-IVT Labeling Kit according to the manufacturer's protocol. Array hybridization, chemiluminescence detection, image acqusition and analysis were performed using Applied Biosystems Chemiluminescene Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer.

Differentially Expressed Genes Analysis

Microarray data were analyzed using the software Avadis 3.3 prophetic (Strand Genomics Pvt Ltd.). The local background was subtracted from the raw expression values for all spots. The ratios were then log-transformed (base 2) and normalized so that median log-transformed ratio equaled to zero. The gene expression ratios were median-centered across all samples. The expression ratio of each gene was made by dividing the normalized values of a gene in chemical treatment group by gene expression mean value in vehicle control group at each given time point. The genes indicating more than 2-fold change were usually taken into consideration for data analysis. We used the supervised analysis method for differentially expressed genes between control and treatment group at each given time point. Analysis of gene expression was performed using the Avadis software program. The permutation-based modified t-test was used to provide further confidence in these results. Differential gene expression was analyzed using a two-sample Welch Banjamin Bochberg t-statistic. Thus, differential gene expression associated with each group was tested using significance analysis of microarray. Gene expression values were manipulated and visualized with the R-packages (Free Software under the terms of the Free Software

Foundation's GNU General Public License). For analysis of data correlation, correspondence analysis (COA) was also performed using the AB1700 package in R. Hierarchical cluster analysis partitions the data into discrete hierarchical groups based on the trends of the data. The resulting gene lists were basically limited to the genes with the ratio changing values of fold over 1.5 and P-value < 0.05.

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