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Hepatic Gene Expression Analysis of 1, 1-Dichloroethylene Treated Mice

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Abstract

1, 1-dichloroethylene (DCE) is well known hepatotoxicant as a model acute hepatotoxicity and selectively injure the bile canalicular membrane of centrilobular hepatocytes. In this study, we investigated hepatic gene expression and histopathological changes in response to DCE treatment. DCE was administered once daily at 20 mg/kg up to 14 days via intraperitoneal injection. Five mice were used in each test group and were sacrificed at 1, 7, and 14 days. Serum biochemical and histopathological analysis were performed for evaluation of hepatotoxicity level. Direct bilirubin and total bilirubin activities were slightly elevated in treated group at 7 days. DCE treatment for 7 days resulted in centrilobular hepatocyte hypertrophy and hepatocyte vacuolation, and mild hepatocyte vacuolation and high hepatocyte basophilia were observed in 14 days treated group. One hundred twenty three up-regulated genes and 445 down-regulated genes with over 2-fold changes between treated and control group at each time point were used for pathway analysis. These data may contribute in understanding the molecular mechanism DCE-induced hepatotoxicity.

Keywords: DCE, Gene expression, Microarray, Liver, Mouse

1, 1-Dichloriethylene (DCE) is used in the production of polyvinylidene chloride copolymers. DCE containing copolymers include other compounds such as acrylonitrile, vinyl chloride, methacrylonitrile, and methacrylate. These copolymers are used in flexible packaging materials: as flame retardant coating for fiber, carpet backing, and piping: as coating for steel pipes; and in adhesive applications[1].

DCE is a well known hepatotoxicant as a model acute hepatotoxicity since low doses level rapidly and selectively injure the bile canalicular membrane of centrilobular hepatocytes[2,3]. DCE rapidly and selectively injures the bile canalicular membrane of hepatocytes. Thus DCE is of value as a tool to elucidate the consequences of changes in canalicular membrane integrity on bile formation. Hepatocyte necrosis and thrombosis were observed in rats and mice exposed to DCE[4-6]. Species differences in the response to DCE-induced toxicity have been observed in rats and mice, and greater susceptibility of the latter has been attributed to enhanced production of metabolites of DCE[7,8]. Furthermore, the hepatotoxicity in mice has been reported to require the bioactivation of DCE by cytochrome P450 system into several reactive species, which covanletly bind to the cellular matrix and presumably induce cell injury[9,10]. This toxicant provides a new tool to study structure-function associations because it selectively injures zone 3 bile canaliculi of fed rats. Ultrastructural changes included loss of microvilli from the canalicular membrane and membrane fragments within the dilated canalicular lumens[11]. However, the precise cholestatic mechanism of DCE at a cellular and molecular level remains unclear.

Using oligonucleotide DNA chip, the gene expression profile at different time-points has been analyzed after administration of DCE. Genes that are differentially expressed according to the time-points were analyzed using hierarchical and *k*-means clustering and the pathway analysis of deregulated genes were also analyzed. In this paper, we investigated the effect of the DCE in the gene expression and histopathological change in mouse liver.

Blood Biochemical and Histopathological Analysis

As shown Figure 1, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), Cholesterol, direct bilirubin (DBIL), total bilirubin (TBIL) activity was analyzed in all dose of DCE at 1, 7, and 14 days. No significant changes were observed in AST, ALP, and cholesterol activities (Figure 1A, C, D). ALT activity was significantly increased in all DCE-treated groups (Figure 1B). DBIL and TBIL activities were slightly elevated

in treated group at 7 days (Figure 1E, F). Histopathological analysis showed that no significant histopathological alterations were observed in the liver of DCE-treated mice for 1 day (Figure 2B). However, DCE treatment for 7 days resulted in centrilobular hepatocyte hypertrophy and hepatocyte vacuolation, and mild hepatocyte vacuolation and high hepatocyte basophilia were observed in 14 days treated group

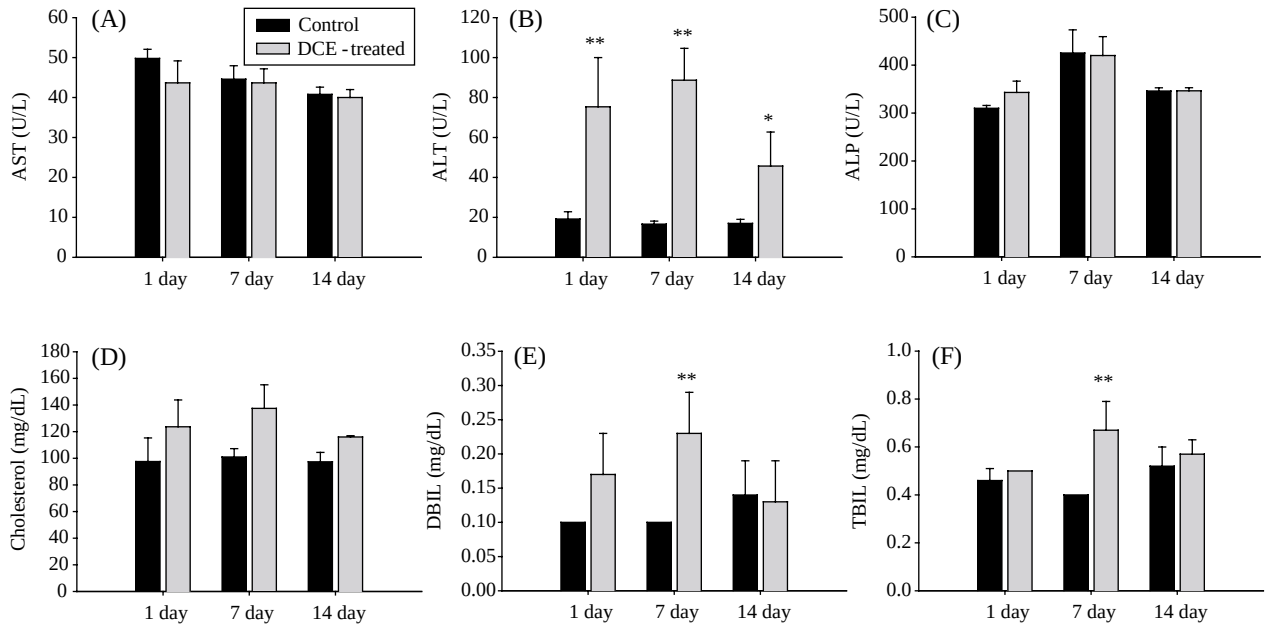

Figure 1. Blood biochemical data in the liver of DCE-treated mice. (A), AST (aspartate aminotransferase); (B), ALT (alanine aminotransferase); (C), ALP (alkaline phosphatase); (D), Cholesterol; (E) DBIL (direct bilirubin); (F) TBIL (total bilirubin). Values given are means ± SD. Significant difference was represented as * ($P < 0.05$); ** ($P < 0.01$).

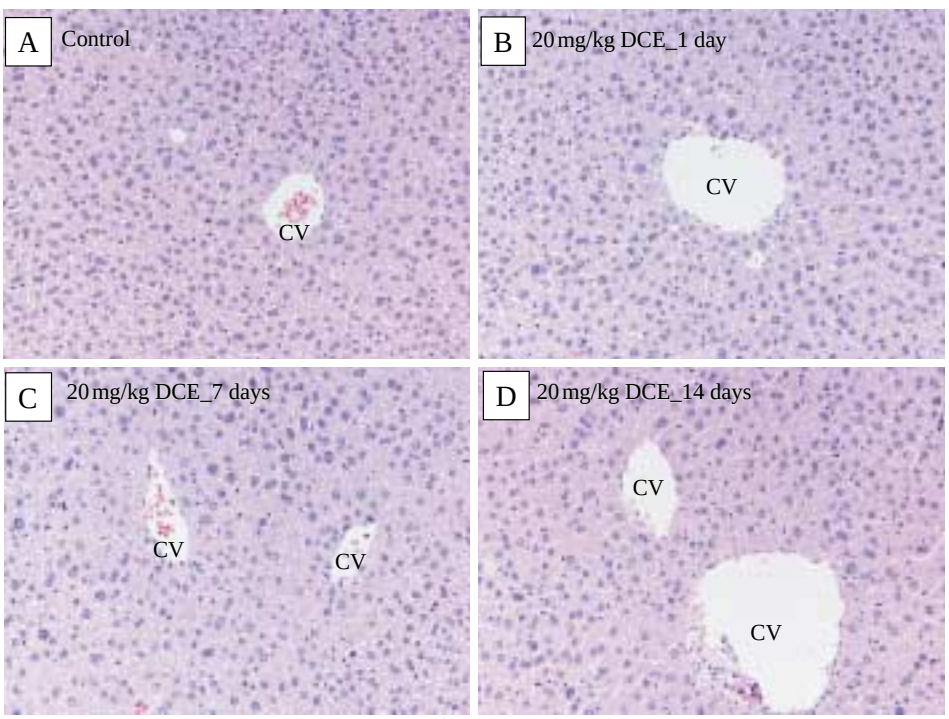

Figure 2. Histopathology of liver from mice administered with 20 mg/kg of DCE for 1, 7, and 14 days. (A) Control; (B) 1 day, similar to normal; (C) 7 days, mild centrilobular hepatocyte hypertrophy and mild hepatocyte vacuolation; (D) 14 days, mild hepatocyte vacuolation and high hepatocyte basophilia.

(Figure 2C, D).

Gene Expression Analysis

RNA was isolated from the livers of DCE-treated mice. Gene expression profiling of DCE in the liver was analyzed using DNA chip. DNA chip analysis was done to determine differences in hepatic gene expression between DCE- and vehicle-treated mice at each time points. Treatment with hepatotoxic doses of DCE caused the statistically significant, at least 2-fold up- or down-regulation of many probe set on the DNA chips. As shown in Table 1, deregulated genes through the DCE treatment (1, 7, and 14 days) were selected. For all time points, 111 genes were differentially expressed and were analyzed using hierarchical clustering (Figure 3). Hierarchical clustering showed that all treated groups were clustered by time dependently. This result represents that a repeated (7 and 14 days) administration of DCE has an influence in the liver in transcription level, along with histopathological changes in the same time points.

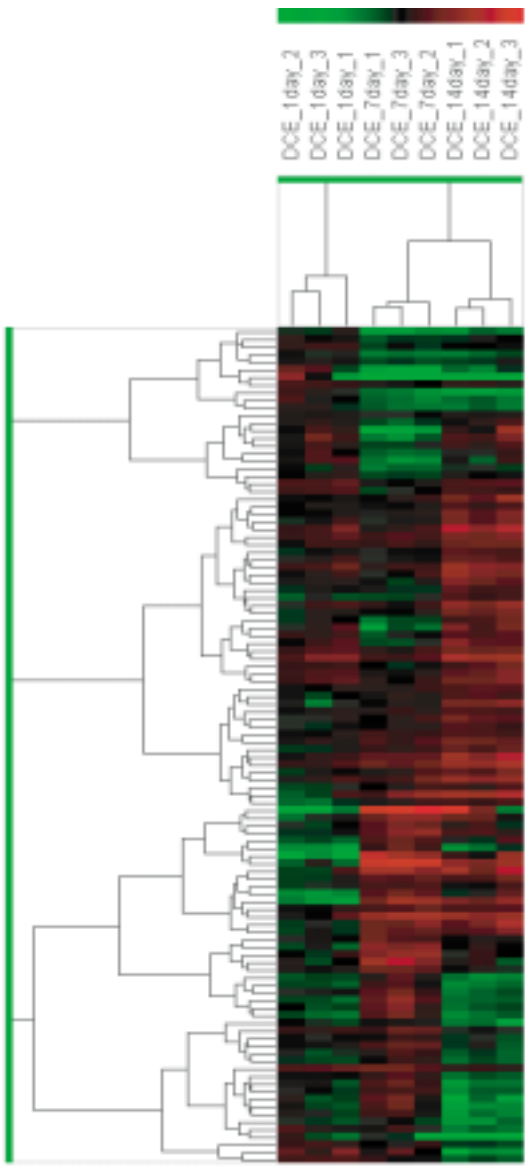

Figure 3. Hierarchical clustering of differentially expressed genes in the liver of DCE-treated mice. One Hundred and eleven deregulated genes were analyzed based on statistical significance as described in the Methods section. Heat map was represented with expression value of each individual array.

Differentially Expressed Genes in the Liver of DCE-treated Mice

Among 111 deregulated genes as mentioned above, we focused on the gene sets at 7 or 14 days showing the significant and gradual gene expression changes. *k*-means clustering of 111 deregulated genes at 7 or 14 days were performed, as shown in Figure 4. Based on *k*-means clustering (Figure 4), 27 up-regulated (Up-cluster 1 and 2) and 29 down-regulated (Down-cluster 1 and 2) genes were selected. As shown in Table 1, the up-regulated genes at DCE-treated groups included known genes involved in transport (*Slc35b1, Spcs3*), mRNA metabolism (*Lsm6, Snrpe*), protein biosynthesis (*Mrpl16*), protein metabolism (*Coq7*), cell cycle (*Clasp2, Anapc10*), intracellular protein transport (*Rab27b*), intracellular signaling cascade (*Cnih4*), and phosphate transport (*Col1a2*). On the other hand, gene representing a down-regulated expression included those involved in signal transduction (*Ms4a6b, Gpsm2*), carbohydrate metabolism (*Gbe1, Man1a*), transport (*Abca8b, Cse1l, Yipf5, Clpx, Mcoln1*), nucleoside metabolism (*Upp2*), cell adhesion (*Scarb2*), glutathione biosynthesis (*Gclm*), and Rac protein signal transduction (*Eps8l2*) (Table 2).

Key Pathway Analysis of Differentially Expressed Genes in the Liver of DCE-Treated Mice

The classification of molecular pathway for differentially expressed genes in DCE-treated mice was performed using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. One hundred twenty three up-regulated genes and 445 down-regulated genes with over 2-fold changes between treated and control group at each time point were used for pathway analysis. In KEGG pathway, most of up-regulated genes were differentially expressed in folate synthesis, Atrazine degradation, TGF-β signaling pathway, starch and sucrose metabolism, ubiquinone biosynthesis, cytokine-cytokine receptor interac-

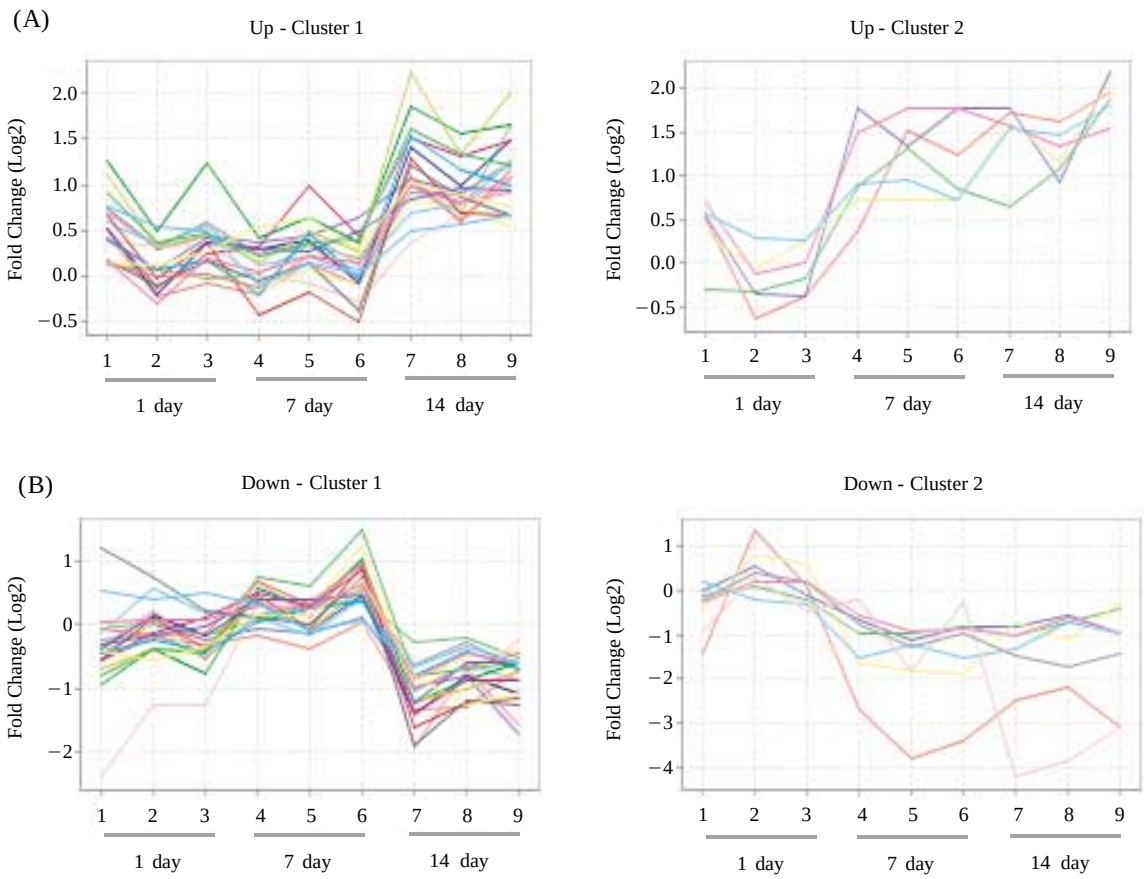

Figure 4. *k*-means clustering of 111 differentially expressed genes in the liver of DCE-treated mice. Among *k*-means clusters (*k*=8), up-/down expression profiles were selected and represented. (A) Up-regulated clusters, Up-Cluster 1 contains 21 DEGs significantly up-regulated at day 14 and Up-Cluster 2 contains 6 DEGs early up-regulated from 7 days. (B) Down-regulated clusters, Down-Cluster 1 contains 21 DEGs significantly down-regulated at day 14 and Down-Cluster 2 contains 8 DEGs gradually down-regulated.

tion. In the case of down-regulated genes, most of genes were differentially expressed in neuroactive ligand-receptor interaction, C21-steroid hormone metabolism, fatty acid metabolism, ascorbate and aldarate metabolism, tetrachloroethen degradation, bisphenol A degradation, nucleotide sugars metabolism, sphingolipid metabolism, T cell receptor signaling pathway (Table 3).

Discussion

The purpose of this study was to elucidate whether generation of hepatotoxicity associated gene expression with phenotype dependent time points, using microarray technology, would get information of hepatotoxic mechanism of DCE. DCE is frequently used as an experimental model of cholestasis, which causes selective bile canalicular injury[12]. This toxicant provides a tool to study structure-function associations because it selectively injures zone 3 bile canaliculi. Hepatocytes in zone 1 of the hepatic lobule play a role in the uptake and biliary excretion of bile acids and organic anions under physiological conditions, and hepatocytes in zone 3 may play a role only when there is a high-dose load. In our previous study, the gene expression profiles after cholestatic agent treatment were analyzed[13,14]. Along with this study, we were concerned with seeing how genes are regulated in the liver. In the blood biochemical analysis showed that DBIL and TBIL value was elevated in DCE-treated mice at 7 days. The major outcome determinant was direct bilirubin in cholestatic phenomenon. Also, pathological observation was confirmed hepatotoxic effects in mice liver. In microarray analysis showed that gene expression change in the liver of DCE-treated mice at all time points. Among the deregulated genes in DCE-treated group, genes

Table 1. Up-regulated genes in the liver of DCE-treated mice.

Gene symbol	Gene title	Acc No.	GO (BP)
Slc35b1	solute carrier family 35, member B1	NM_016752	transport
Blvra	biliverdin reductase A	AK010847	electron transport
Tmem147	transmembrane protein 147	NM_008085	
Lsm6	LSM6 homolog, U6 small nuclear RNA associated (S. cerevisiae)	AK019126	mRNA metabolism
Snrpe	small nuclear ribonucleoprotein E	AK019462	mRNA metabolism
1190005F20Rik	RIKEN cDNA 1190005F20 gene	AK004493	tRNA processing
Spcs3	signal peptidase complex subunit 3 homolog (S. cerevisiae)	BC019390	transport
1500003O03Rik	RIKEN cDNA 1500003O03 gene	NM_019769	biological process unknown
1700007K09Rik	RIKEN cDNA 1700007K09 gene	AK005730	
Ppp1r11	protein phosphatase 1, regulatory (inhibitor) subunit 11	NM_023162	
Clasp2	CLIP associating protein 2	AK005146	cell cycle
Ubap1	ubiquitin-associated protein 1	NM_023305	
Gtpbp3	GTP binding protein 3	NM_032544	
Rab27b	RAB27b, member RAS oncogene family	NM_030554	intracellular protein transport
Anapc10	anaphase promoting complex subunit 10	AK005303	cell cycle
Gcs1	glucosidase 1	NM_020619	carbohydrate metabolism
Zfp313	zinc finger protein 313	AK003584	development
Tmem85	transmembrane protein 85	NM_026519	
Mrpl16	mitochondrial ribosomal protein L16	NM_025606	protein biosynthesis
Cnih4	cornichon homolog 4 (Drosophila)	AK021301	intracellular signaling cascade
Zbtb8os	zinc finger and BTB domain containing 8 opposite strand	AK007377	biological process unknown
Col6a2	procollagen, type VI, alpha 2	Z18272	cell adhesion
Tgfbr2	transforming growth factor, beta receptor II	AK003861	protein amino acid phosphorylation
Coq7	demethyl-Q 7	AF080580	protein metabolism
1810048J11Rik	RIKEN cDNA 1810048J11 gene	AK007829	
Col1a2	procollagen, type I, alpha 2	NM_007743	phosphate transport; cell adhesion
Rfc4	replication factor C (activator 1) 4	BC003335	DNA replication

Table 2. Down-regulated genes in the liver of DCE-treated mice.

Gene symbol	Gene title	Probe ID	GO (BP)
D4Bwg0951e	DNA segment, Chr 4, Brigham & Women's Genetics 0951 expressed	AK003950	
Ms4a6b	membrane-spanning 4-domains, subfamily A, member 6B	NM_025658	signal transduction
Pbef1	pre-B-cell colony-enhancing factor 1	NM_021524	pyridine nucleotide biosynthesis
Abca8b	ATP-binding cassette, sub-family A (ABC1), member 8b	AF213393	transport
Chac1	ChaC, cation transport regulator-like 1 (E. coli)	BC025169	
Vnn1	vanin 1	NM_011704	nitrogen compound metabolism
Cd164l2	D164 sialomucin-like 2	AK009888	
Hnrpr	heterogeneous nuclear ribonucleoprotein R	AF441128	
Gbe1	glucan (1,4-alpha-), branching enzyme 1	NM_028803	carbohydrate metabolism
Gclm	glutamate-cysteine ligase , modifier subunit	NM_008129	glutathione biosynthesis
Nipsnap3a	nipsnap homolog 3A (C. elegans)	NM_025623	
Ppp1r2	protein phosphatase 1, regulatory (inhibitor) subunit 2	AK015349	
Upp2	uridine phosphorylase 2	BC027189	nucleoside metabolism
Man1a	mannosidase 1, alpha	NM_008548	carbohydrate metabolism
Eps8l2	EPS8-like 2	BC005492	Rac protein signal transduction
Cse1l	chromosome segregation 1-like (S. cerevisiae)	NM_023565	transport; apoptosis
Cdc40	cell division cycle 40 homolog (yeast)	AK004569	mRNA processing
Yipf5	Yip1 domain family, member 5	NM_023311	transport
Gpsm2	G-protein signalling modulator 2 (AGS3-like, C. elegans)	BC021308	signal transduction
Gtf3c2	general transcription factor IIIC, polypeptide 2, beta	AK012095	transcription
Wsb1	WD repeat and SOCS box-containing 1	NM_019653	intracellular signaling cascade
Clpx	caseinolytic peptidase X (E. coli)	NM_011802	transport
Scarb2	scavenger receptor class B, member 2	NM_007644	cell adhesion
Dcps	decapping enzyme, scavenger	AK005584	mRNA catabolism
Ccl27	chemokine (C-C motif) ligand 27	NM_011336	immune response
Magel2	Mus musculus melanoma antigen, family L, 2	NM_013779	
Mcoln1	mucolipin 1	NM_053177	transport
Mapk1ip1	mitogen activated protein kinase 1 interacting protein 1	AK009250	development
–	–	AK0024808	–

– indicates that information of gene annotation were not available in current public database.

Table 3. Key pathway of differentially expressed genes in DCE-treated mice.

KEGG pathway	Gene symbol	*P*-value
Up-regulated genes		
Folate biosynthesis	*Ddx56; Mov10l1*	0.005
Atrazine degradation	*Apobec3*	0.010
TGF-beta signaling pathway	*Bmp8a; Tgfbr2*	0.023
Starch and sucrose metabolism	*Ddx56; Mov10l1*	0.028
Ubiquinone biosynthesis	*Coq7*	0.029
Cytokine-cytokine receptor interaction	*Il9;Tgfbr2*	0.047
Down-regulated genes		
Neuroactive ligand-receptor interaction	*Gpr50; Gabrb2; Htr1f; Defcr-rs7; Glrb; Gzma;Trhr; Gabrq; Ptger3; Ptgdr; Gabra3*	9.17E-^{07}
Bile acid biosynthesis	*Acadsb; Adh4; Hsd3b7; Hsd17b12; Aldh9a1*	0.003
C21-Steroid hormone metabolism	*Akr1c18; Hsd3b4*	0.003
Fatty acid metabolism	*Acox1; Adh4; Aldh9a1; Ehhadh*	0.011
Ascorbate and aldarate metabolism	*Hsd3b7; Hsd17b12; Aldh9a1*	0.012
Tetrachloroethene degradation	*Hsd3b7; Hsd17b12*	0.019
Bisphenol A degradation	*Hsd3b7; Hsd17b12*	0.028
Nucleotide sugars metabolism	*Hsd3b7; Hsd17b12*	0.039
Sphingolipid metabolism	*Sgpp1; Ugt8a; Neu2*	0.045
T cell receptor signaling pathway	*Rasgrp1; Vav2; Fos; Cbl; Chuk*	0.049

involved in TGF-β signaling pathway such as *Bmp8a, Tgfbr2* were over-expressed. *Bmp8a* and *Tgfbr2* play a role in TGF-β signaling pathway. In cholestasis, bile acids induce hepatocyte apoptosis, while activation of hepatic stellate cells results in fibrosis. Since transforming growth factor-β (TGF-β) is a critical mediator in this process[15]. Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that orchestrates response to injury via ubiquitous cell surface receptors. The biological activity of TGF-β is restrained by its secretion as a latent complex (LTGF β) such that activation determines the extent of TGF-β activity during physiological and pathological events[16]. Flisiak and Prokopowicz reported that plasma TGF-β1 showed a significant positive correlation with the degree of liver insufficiency. This indicates the possible use of plasma TGF-β1 measurement as a good marker of liver function impairment[17].

On the other hand, genes involved in bile acid biosynthesis such as *Acadsb, Adh4, Hsd3b7, Hsd17b12, Aldh9a1, Akr1c18, Hsd3b4* were down-regulated in DCE treatment.

Bile acids are important physiological agents that subserve a number of functions, including absorption, solubilization, transport, and secretion of lipids. In the liver, they participate in the generation of bile flow and the secretion of cholesterol and phospholipids, such as phosphatidylcholine. When released into the intestine, they facilitate the uptake of cholesterol, fat-soluble vitamins, and other lipids. Moreover, the biosynthesis of bile acids from cholesterol is the most significant pathway for the elimination of cholesterol from the body. Moreover, bile acids may also act as signaling molecules by activating several nuclear receptors and regulating many pathways resulting in bile acids and cholesterol homeostasis. However, because of their inherent detergent and membrane disruptive properties, bile acids are intrinsically toxic and their intracellular accumulation may result in liver cell death. In fact, during conditions such as cholestasis bile acids-induced hepatotoxicity participates in both pathogenesis and perpetuation of liver injury. Therefore, hepatocellular bile acids levels are tightly maintained within a narrow concentration range[18]. During the cholestasis, down-regulation of bile acids synthesis pathway seems to protection mechanism from hepatocytes injury.

Using the microarray technology, we investigated the difference in gene expression profiles in the liver of DCE-treated mice. The information on differentially expressed genes in the liver could help to understand the molecular events in the cholestasis induced by cholestatic agents including DCE.

Methods

Chemicals and Animals

1, 1-dichloroethylene (DCE) was purchased from Sigma, and approximately 9-week-old BALB/c male mice (SLC, Inc., Japan) were kept in a 12-h light/dark cycle, under controlled temperature and humidity for 2 weeks prior to experiment in the animal room. The mice were fed standard food pellets. DCE was dissolved in corn oil and was administered once daily

at 20 mg/kg up to 14 days via intraperitoneal injection and control animals was administrated with corn oil. Five mice were used in each test group and necropsies were then performed at 1, 7, and 14 days.

Biochemical Analyses

Blood was collected from the inferior vena cava. Serum was separated by centrifugation. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) activities, and cholesterol, direct bilirubin (DBIL), and total bilirubin (TBIL) were determined using an automated clinical chemistry analyzer (Fuji film Co., Japan), at each time point. Data are expressed as the mean ± S.D. of 5 samples. The statistical significance between various experimental groups was tested using one-way ANOVA followed by the Dunnett test. *P*-values less than 0.05 were considered to be significant.

Histopathology

Liver samples collected from treated and control mice were fixed in 10% neutral buffered formalin, and embedded in paraffin. The sections (4 μm thick) were cut using RM2165 microtome (Leica, Germany), stained with hematoxylin and eosin (H&E), and examined under a light microscope (Nikon, Japan).

Isolation of RNA

The left lateral lobe of the liver was removed and processed for RNA extraction. For the microarray analysis, total RNA was extracted using the Trizol reagent (InVitrogen, USA) according to the instructions of the manufacturer and purified using RNeasy total RNA isolation kit (Qiagen, Germany). Total RNA was quantified by NanoDrop ND-1000 (NanoDrop, USA) and its integrity was assessed by 2100 Bioanalyzer (Agilent, Germany).

Microarray Experiment

For microarray analysis, fluorescent-labeled cDNA was prepared by reverse-transcription of total RNA in the presence of the coupled Cy3-dUTP or Cy5-dUTP (NEN) using Superscript II (InVitrogen, USA). Single -stranded cDNA probes were purified using a PCR purification kit (Qiagen, Germany). Probes were resuspended in hybridization solution (50% formamide, 5X SSC, 0.1% SDS). The Mouse oligo chip 10K (GenomicTree, Korea) was hybridized with the fluorescent-labeled cDNAs at 42˚C in a humid chamber for 16 hours.

Microarray Analysis

After proper washing, the slides were scanned using GenePix 4000B (Axon Instrument Inc., USA). Scanned images were calculated with GenePix 3.0 software (Axon Instrument Inc., USA) and analyzed with Genplex 2.0 software (Istech Inc., Korea). Background of spot intensity was corrected and miscellaneous spot were removed. The log gene expression ratios were normalized by Block-wise centering (Print-tip Lowess normalization) method. Totally, 111 differentially expressed genes on each time point (1, 7, and 14 days) were selected based on statistical one way ANOVA test ($P < 0.01$). The selected deregulated genes were analyzed by hierarchical clustering algorithm based on Pearson correlation and Complete Linkage. *k*-means partitioning clustering were performed based on Euclidean distance. Selected genes were annotated based on Probe ID with NCBI Genbank accession number.

KEGG Pathway Analysis

The classification of molecular pathway for differentially expressed genes in DCE-treated mice was performed using KEGG pathway database. Significant *P* value was calculated by the formula using hypergeometric distribution function: *P*-value=1−hypergeometric (i, j, k, l); where *i* is the number of Probe ID in common pathway, *j* is the number of Probe ID with pathway term; *k* is number of known genes with corresponding pathway term, *l* is the number of total known genes with pathway term. *P*-values less than 0.05 were considered to be significant.

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