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Toxicogenomics Study on α-Naphthylisothiocyanate (ANIT) Induced Hepatotoxictiy in Mice

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Abstract

α-Naphthylisothiocyanate (ANIT) induces intrahepatic cholestasis, involving damage to biliary epitheial cells. This study investigates hepatic gene expression and histopathological alterations in response to ANIT treatment in order to elucidate early time response of ANIT-induced hepatotoxicity. ANIT was treated with single dose (3, 6, and 60 mg/kg) in corn oil by oral gavage. Serum biochemical and histopathological observation were performed for evaluation of hepatotoxicity level. Affymetrix oligo DNA chips were used for gene expression profile by ANIT-induced hetpatoxicity. Hepatic enzyme levels (ALT, AST, and ALP) were increased in 24 hr high dose group. In microscopic observations, moderate hepatocellular necrosis, were confirmed 24 hr high dose groups. We found that gene expression patterns were dependent on time and dose. Our selected genes were related inflammation and immunomodulation. In this study, ANIT-induced hepatotoxicity was involved in acute phase responses and provides evidence for role of neutrophil could be mechanism associated with ANIT-mediated hepatotoxicity.

Keywords: Microarray, ANIT, Hepatotoxicity, Phenotype anchoring, Histopathology

A single administration of α-naphthylisothiocyanate (ANIT) produces cholangiolitic hepatitis, characterized by intrahepatic cholestasis, hepatocellular and biliary epithelial cell necrosis, and bile duct obstruction[1-3]. A pronounced infiltration of neutrophils into liver tissues occurs before the onset of overt liver damage in rats intoxicated once with ANIT[4]. However, the precise cholestatic mechanism of ANIT at a cellular level remains unclear. Various studies have implicated glutathione (GSH) in ANIT toxicity through the formation of an unstable thiocarbamoyl-GSH conjugate (GS-ANIT) within hepatocytes[5,6,13]. Dahm *et al.* protects against liver injury in rats treated once with ANIT, while co-administration of superoxide dismutase and catalase, oxygen free radical scavengers, do not protect against this liver injury, and have suggested the contribution of neutrophils to the development of ANIT-induced liver injury through a mechanism independent of the production of oxygen free radicals[7,12].

Microarray technology is a powerful tool that can be used to gain insight into species differences in toxicological responses, and is currently being employed to elucidate molecular mechanisms and identify markers of toxicology in rodents and humans. In addition, gene expression patterns have recently been shown to successfully predict molecular sequelae and clinical outcomes, with notable success in the field of cancer research[8-11].

In this study, we investigated the changes in hepatic gene expression during the ANIT-induced hepatotoxicity. Moreover, we elucidated relation between the microarray results and phenotype. Hepatoxicity levels were determined to measure blood biochemical and histopathological data.

Blood Biochemical and Histopathological Evaluation

Hepatoxicity was determined by measuring serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). ALT, AST level was significantly increased compare to control in ANIT 30 mg/kg and 60 mg/kg treated groups at 24 hr. However 2-8 hr groups were not changed in all doses (Fig. 1). In histopathological results (PAS stain), we found liver damage at 24 hr in 60 mg/kg treated group. The liver sections were shown moderate necrosis, degradation of connective tissue. Low and middle dose groups were normal at 2-8 hr, but minimal necrosis was observed at 24 hr group (Fig. 2). According to these toxicological parameters, we confirmed that hepatotoxicity induced by ANIT.

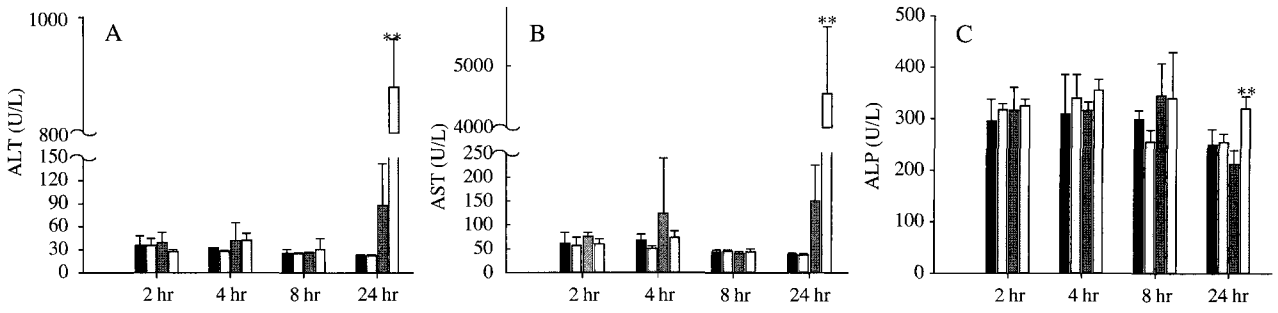

Fig. 1. Serum hepatic enzymes levels after ANIT administration. Values are means±SD. A: serum alanine animotransferase (ALT) level, B: aspartate aminotransferase (AST) levels, C: alkaline phosphatase (ALP). ** Significantly different from vehicle control ($P<0.01$).

Table 1. Significantly up- and down-regulated genes from 24hr group of ANIT treated mice liver.

Gene symbol	Description	Fold change
Up-regulation		
Lcn2	lipocalin 2	67.32
Saa2	serum amyloid A 2	39.41
Cxcl1	chemokine (C-X-C motif) ligand 1	19.33
Saa1	serum amyloid A 1	14.11
Tnfrsf12a	tumor necrosis factor receptor superfamily, member 12a	13.17
Orm2	orosomucoid 2	11.91
Tnfrsf12a	tumor necrosis factor receptor superfamily, member 12a	11.14
Mt2	metallothionein 2	7.87
Steap4	STEAP family member 4	6.35
Mt1	metallothionein 1	5.50
Sqle	squalene epoxidase	5.30
1300007C21Rik	RIKEN cDNA 1300007C21 gene	4.91
2010003K11Rik	RIKEN cDNA 2010003K11 gene	4.75
Hmox1	heme oxygenase (decycling) 1	4.44
Mt1	metallothionein 1	4.35
Osmr	oncostatin M receptor	4.11
1300007C21Rik	RIKEN cDNA 1300007C21 gene	3.28
Dhcr24	24-dehydrocholesterol reductase	3.28
Gnat1	guanine nucleotide binding protein, alpha transducing 1	2.09
Cebpd	CCAAT/enhancer binding protein (C/EBP), delta	1.95
Ccrn4l	CCR4 carbon catabolite repression 4-like (S. cerevisiae)	1.11
Down-regulation		
Pcaf	p300/CBP-associated factor	0.43
9030611N15Rik	RIKEN cDNA 9030611N15 gene	0.16
Ank3	ankyrin 3, epithelial	0.08
Upp2	uridine phosphorylase 2	0.03

Twenty-five genes with P value of 0.05 were shown to be regulated±2 fold or more by at least an individual of 24 hr group.

Gene Expression Analysis

RNA was isolated from livers of ANIT treated or control mice. The RNA quality was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, U.S.A.) and NanoDrop (Nanodrop, U.S.A.). In results, RNA quality and purity levels were acceptable for further experiment. Each synthesis stages (cDNA, cRNA, and cRNA fragmentation) were confirmed by agarose gel-electrophoresis. Microarray analysis was done to determine differences in hepatic gene expression between ANIT treated groups and vehicle control groups. Gene expression profiles of interest were significantly up- and down- regulated in ANIT treated mice at 24 hr when compared to control. Following ANIT treated group at 24 hr, there appear to be statistically significant changes ($P<0.05$) in gene expres-

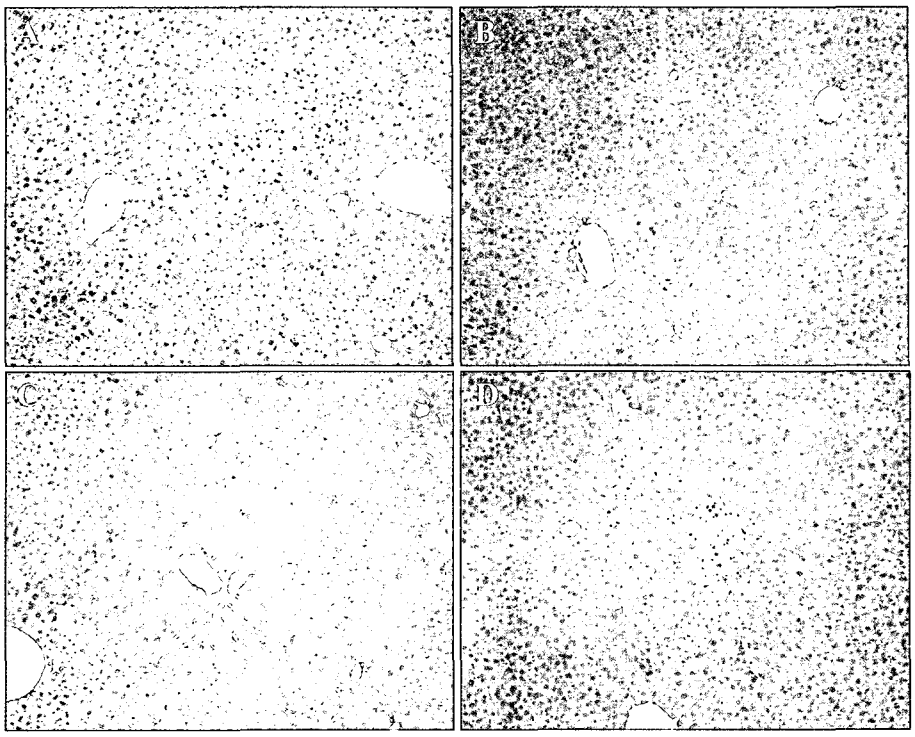

Fig. 2. Light photomicrographs of livers from ANIT-high dose treated mice. A: 2 hr group, B: 4 hr group, C: 8 hr group, D: 24 hr group. PAS stain 100X

Table 2. Commonly expressed genes from dose-dependent and time-dependent analysis. (P value < 0.05)

Gene symbol	Description	Fold change
Tnfrsf12a	tumor necrosis factor receptor superfamily, member 12a	62.03
Lcn2	lipocalin 2	28.93
Saa2	serum amyloid A 2	9.50
Orm2	orosomucoid 2	6.76
Saa1	serum amyloid A 1	4.77
Pcaf	p300/CBP-associated factor	0.40
9030611-N15Rik	RIKEN cDNA 9030611N15 gene	0.15
Upp2	uridine phosphorylase 2	0.04

sion when compared to the vehicle control. It also clustered with dose dependent manner (Fig. 3). Gene expression profiles showed that genes were 2 fold up- or down-regulated in mice treated middle and high dose of ANIT (Table 1); e.g. tumor necrosis factor receptor, serum amyloid A, metallothionein, uridine phophoryase 2. Time course analysis in high dose groups of ANIT showed that the few genes up- or down-regulated at 24 hr, and clustered in compliance with time (Fig. 4). Especially, 24 hr high dose group changed significantly when compared to other times. Several genes were selected common genes in results of dose-dependent analysis and time-dependent analysis (Table 2) ; e.g. tumor necrosis factor receptor subfamily, RIKEN cDNA 9030611N15 gene, serum amyloid A1, serum amyloid A2, orosomucoid 2, uridine phosphorylase 2 and lipocalin 2.

Discussion

The purpose of this study was to elucidate whether generation of hepatotoxicant associated gene expression with phenotype dependent doses and time, using microarray technology. We examined biochemical test and histopathological analysis for evaluation of ANIT-induced hepatotoxicity. In result, hepatic enzymes, AST, ALT, and ALP, increased in high dose at 24 hr, and also in histopathological data, 24 hr group of high dose treatment was shown necrotic lesion. In the event, we found that 24 hr high dose group was set off hepatotoxicity. For gene expression profile, we carried out microarray assay (Mouse 430A 2.0 array, Affymetrix, U.S.A). Hepatic gene expression was analyzed by two ways, dose-dependent analysis (24hr groups) and time-dependent analysis (High dose groups). In both analysis results, gene expression was significantly increased or decreased in 24 hr high dose group (Table 1, 2). Gene expression profiles were related to inflammation and immune response (lipocalin2, serum amyloid 2, tumor necrosis factor receptor superfamily etc.). Lipocalin 2 is an acute phase

Fig. 3. Hierarchical clustering of hepatic gene expression in 24hr group of ANIT treated mice.

protein in the liver. Lipocalin 2 coincides with high degree of apoptosis in an involuting tissue; lipocalin 2 may promote the cell death of invading neutrophils[14]. Serum amyloid A (SAA) has been linked to functions related to inflammation, pathogen defense. SAA is known best for its role during the acute phase response to an inflammatory stimulus such as infection, tissue injury, and trauma. Orosomucoid 2 is immunomodulating effects, the ability to bind basic drugs and many other molecules[15,16]. As above, ANIT -induced hepatotoxicity experiments were involved in acute phase responses and provides evidence for role

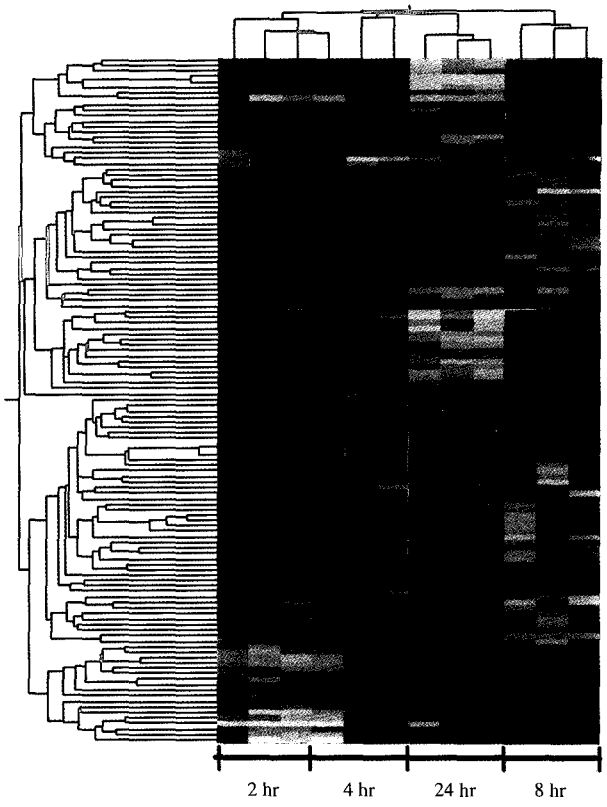

Fig. 4. Hierarchical cluster image showing the gene changes occurring in liver from mice treated high dose ANIT.

of neutrophil could be mechanism associated with ANIT-mediated hepatotoxicity. We confirmed our hypothesis that gene expression profile was related with phenotype of hepatotoxicity. Toxicological evaluation through gene expression profiling, blood chemistry and histopathological can provide a useful tool for risk assessment

Methods

Animals

Thirty male C57BL/6 mice (Japan SLC, Japan) weighing 20-26 g were used. These were housed in a controlled environment and provided rodent chow (Purina, Korea) and water ad labitum. All animals care was accredited by AAALAC (American Association of Laboratory Animal Care) and IACUC.

Chemicals

Trizol (Molecular research, U.S.A.), RNeasy MinElute Cleanup kit (QIAGEN, Germany), ANIT (Sigma, U.S.A), Chloroform (Sigma, U.S.A), Isopropanol (Sigma, U.S.A.), One-cycle cDNA synthesis kit

(Affymetrix, U.S.A.), IVT labeling kit (Affymetrix, U.S.A.), sample cleanup module (Affymetrix, U.S.A.) were used for this study.

Animal Treatment and Sample Preparation

Animals received orally doses of vehicle (corn oil), ANIT 6, 30, and 60 mg/kg, respectively. Mice were sacrificed under diethyl ether anesthesia at 2, 4, 8, and 24 hr after administration. Blood for clinical chemistry evaluation was collected from post vena cava. Livers were rapidly removed. Left lobe of liver put into liquid nitrogen for genomics study and the remaining liver was fixed in 10% neutralized buffered formalin.

RNA Isolation

Liver tissues were isolated using Trizol and further purified with RNeasy kit. Briefly, 2 mL homogenized tissue with Trizol mixed with 0.4 mL chloroform. Following centrifugation, aqueous phase collected and mixed with isopropanol for precipitation. Isolated mRNA was eluted RNase free water and purified RNeasy kit. Total RNA quality and quantity were check by NanoDrop and Agilent Bioanalyzer 2100.

Serum Biochemistry and Histopathological Analysis

ALT, AST, and ALP levels were measured using a clinical chemistry analyzer (Shimadzu, Japan). Section for histopathological analysis were processed and trimmed, embedded in paraffin, sectioned at 4 μm, and stained with PAS stain for microscopic examination.

cDNA and cRNA Synthesis

We performed with the Affymetrix standard protocol. Briefly, starting 10 μg of total RNA from every sample, we generated double strand cDNA using a 24mer primer with T7 RNA polymerase promoter site added to the 3′ end (Affymetrix One-cylcle cDNA synthesis kit). After cDNA synthesis, in vitro transcription was performed with the Affymetrix IVT labeling kit to produce biotin-labeled cRNA. We fragmented 15 μg of the cRNA product and hybridized it for 16 hr into Mouse 430A 2.0 array containing 22,600 transcripts. Each microarray was washed and stained with streptavidin-phycoerythrin and was scanned by Affymetrix GeneChip scanner 3000 according to the GeneChip expression analysis technical manual procedure.

Data Analysis

We were using the GeneSpring 7.2 (Silicon Genetics, U.S.A.) for gene expression analysis. A two step filtering algorithm was implemented to select highly expressed genes. First, genes were normalized by specific samples (control group signal). Data were filtered with 'present' flag in at least 3 of 48. Significantly expressed genes were determined using t-test or ANOVA. To find DEG (Differentially Expressed Gene), we selected up or down regulated genes (>2 folds) at 3 replicates. Cluster analysis and tree view were allied to identify similarly regulated group and to determine treatmemt-related effects.

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