System (Invitrogen, Carlsbad, CA, USA) and T7-$(dT)_{24}$-oligonucleotide primer (Affymetrix). After the cDNA was purified by cDNA Cleanup Module (Affymetrix), biotin-labeled cRNA was synthesized by using the BioArray High yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA). The cRNA was purified by an IVT cRNA Cleanup Spin Column (Affymetrix), and then fragmented. Twenty μg of the fragmented cRNA was hybridized to a RAE230A probe array for 18 hr at 45°C at 60 rpm. After hybridization, the array was washed and stained by streptavidin-phycoerythrin using the Fluidics Station 400 (Affymetrix). Finally, the array was scanned by Gene Array Scanner (Affymetrix). The digital image files were processed by Affymetrix Microarray Suite version 5.0.

Statistical analysis

The data from biochemistry was expressed as the mean value of the 5 animals in each group. In the present experiments, the data of blood biochemistry showed large variations and we thus employed Mann-Whitney's U-test (Snedecor and Cochran, 1989) for analyzing the drug effects.

Gene expression data were analyzed by using GeneSpring® version 6.1 (Silicon Genetics, Santa Clara, CA, USA). Expression data was normalized by the mean value (global normalization; adjusted to an arbitrary value of 500). After filtering the genes by flags (present or marginal call at least 12 of the 24 samples in each experiment), the fold change determination to the concurrent control samples was performed in order to extract drug-related changes. In some cases, Student's *t*-test (Snedecor and Cochran, 1989) with Benjamin's adjustment ($p<0.05$) was also used for the age-difference comparison using the control samples.

RESULTS

Phenotype change by APAP, INH and CCl4

Time-course changes in plasma AST, ALT and LDH after administrations of APAP, INH, and CCl4 are shown in Table 1. In APAP-treated rats, significant increases of ALT and AST were noted in adult rats at 24 hr after treatment of 1000 mg/kg. Young rats treated with the same dosage showed a tendency of increment of these enzyme activities, but it was not statistically significant. In the cases of INH and CCl4, no obvious increase of these enzymes was detected. Although a significant statistical difference in young rats was detected 9 hr after INH-treatment, it was a decrease in the measure and thus considered not to be toxic.

Histopathological findings of the liver are shown in Table 2. At 24 hr post-dose of APAP, necrosis, increased eosinophility and vacuolation of hepatocyte were observed with inflammatory cells infiltration at 1000 mg/kg in both ages. Of these, hepatocyte necrosis in adult rats was more evident than that in young rats. In the young rats, minimal necrosis (grade +/–) was only noted in 2 out of 5 animals (Photo 1A), whereas all of the adult rats had necrosis: 2 of them were grade + (Photo 1B) and the remaining 3 were grade +/–.

No obvious changes were found in the liver with this dose range of INH. Although CCl4 caused some pathological changes in the liver, no obvious age-related differences were noted. Both biochemistry and histopathology indicated that hepatotoxicity of APAP was evident, i.e., increases in the leak of the enzymes and centrilobular necrosis of hepatocytes, and adult rats appeared to be more susceptible to hepatotoxicity than the young ones.

Age-dependent gene expression

Based on the biochemical and histopathological data, the age-related difference appeared to be specific for APAP among the three chemicals. This suggested that there should be some difference in the factors specifically involved in APAP-induced hepatotoxicity. In order to elucidate the cause of the difference, we tried to extract genes with different expression by age from control animals.

Nine control animals in each time point were employed for both young and adult rats from the TGP database, and the genes that changed more than 2-fold between the ages with statistical significance ($p < 0.05$) in all time points (3, 6, 9, and 24 hr) were extracted (Table 3). Of the total 11 genes extracted, 7 genes were lower and 4 genes were higher in adult rats than in young ones. They contained "hemoglobin beta chain complex" and "aminolevulinic acid synthase 2" (the rate-limiting enzyme in hepatic porphyrin-heme biosynthesis), both of which appeared to reflect the biosynthesis of red blood cells in the liver, and this appears to be reasonable, because extramedullary hematopoiesis decreases with age. As for changes related to APAP toxicity, it was noteworthy that the lower value of glutathione *S*-transferase Yc2 (GSTYc2) and the higher value of Cyp3A13 in adult rats were clearly indicated. Fig.1 shows the time- and dose-dependent effects of APAP on the expression of these two genes. GSTYc2 (Fig. 1A) was found to be always higher in young than in adult rats. Nine hr after

Table 1. Changes in Blood biochemistry after treatment with 1000 mg/kg of Acetaminophen (APAP), 200 mg/kg of Isoniazid (INH) and 300 mg/kg carbon tetrachloride (CCl4).

Compound	Age (week)	Dose (mg/kg)	Time (hr)	Number of Amimals	AST (IU/L)	ALT (IU/L)	LDH (IU/L)
APAP	6	0	3	5	66.2 ± 9.6	35.0 ± 8.7	111.2 ± 47.3
			6	5	63.4 ± 7.8	32.2 ± 4.1	105.2 ± 32.0
			9	5	53.4 ± 4.2	28.6 ± 3.4	81.6 ± 24.0
			24	5	59.0 ± 6.3	37.0 ± 3.1	105.2 ± 33.8
		1000	3	5	66.2 ± 7.3	36.8 ± 5.2	88.2 ± 14.2
			6	5	61.8 ± 4.2	31.2 ± 1.8	119.2 ± 21.4
			9	5	60.2 ± 4.1	30.8 ± 5.9	107.2 ± 30.1
			24	5	76.4 ± 14.1	51.0 ± 17.2	134.4 ± 31.9
	12	0	3	5	68.4 ± 17.1	29.2 ± 5.4	115.6 ± 29.8
			6	5	56.6 ± 7.3	25.0 ± 2.5	122.6 ± 44.9
			9	5	52.6 ± 2.2	25.2 ± 2.2	100.8 ± 54.6
			24	5	65.6 ± 12.5	29.8 ± 5.3	122.8 ± 62.1
		1000	3	5	63.4 ± 11.1	30.8 ± 7.7	109.2 ± 24.7
			6	5	58.6 ± 3.0	26.6 ± 3.8	93.0 ± 15.0
			9	5	52.0 ± 3.7	26.4 ± 4.7	121.8 ± 62.2
			24	5	287.8 ± 221.0 *	78.4 ± 62.3 *	315.4 ± 208.1
INAH	6	0	3	5	59.8 ± 7.2	34.0 ± 4.5	92.0 ± 19.8
			6	5	55.2 ± 7.2	26.8 ± 4.9	114.2 ± 36.1
			9	5	55.4 ± 5.0 *	27.4 ± 2.3	82.6 ± 29.5
			24	5	59.6 ± 8.2	33.2 ± 4.7	109.4 ± 46.5
		200	3	5	58.2 ± 5.8	31.4 ± 3.6	86.6 ± 22.9
			6	5	50.0 ± 2.2	24.0 ± 2.0	92.0 ± 16.2
			9	5	39.2 ± 6.7	24.6 ± 1.3	94.6 ± 26.1
			24	5	47.6 ± 6.7	21.2 ± 6.6	96.0 ± 25.6
	12	0	3	5	54.8 ± 10.5	25.0 ± 7.6	93.4 ± 44.9
			6	5	49.8 ± 11.7	24.2 ± 3.6	87.0 ± 38.6
			9	5	46.0 ± 16.2	22.6 ± 4.4	112.0 ± 85.9
			24	5	50.8 ± 9.1	26.6 ± 9.7	99.0 ± 30.6
		200	3	5	51.2 ± 10.8	27.2 ± 4.0	85.2 ± 34.6
			6	5	42.4 ± 3.6	22.4 ± 3.9	77.0 ± 29.2
			9	5	29.0 ± 7.1	19.2 ± 4.9	83.0 ± 30.4
			24	5	41.4 ± 11.0	14.2 ± 4.1	89.8 ± 41.2
CCl4	6	0	3	5	66.6 ± 2.7	37.2 ± 7.2	209.0 ± 109.8
			6	5	62.8 ± 5.1	31.4 ± 2.1	189.2 ± 78.5
			9	5	62.6 ± 9.9	30.0 ± 4.2	163.0 ± 55.4
			24	5	66.6 ± 4.0	37.2 ± 2.7	186.2 ± 67.2
		300	3	5	67.8 ± 4.3	39.4 ± 4.5	201.4 ± 74.9
			6	5	65.2 ± 5.7	29.8 ± 4.6	205.8 ± 48.3
			9	5	63.2 ± 5.8	28.4 ± 1.5	200.2 ± 59.1
			24	5	75.0 ± 13.3	42.2 ± 4.6	192.6 ± 38.2
	12	0	3	5	73.0 ± 18.8	37.8 ± 7.6	156.6 ± 30.4
			6	5	90.6 ± 38.4	47.6 ± 25.2	181.2 ± 32.5
			9	5	131.8 ± 162.9	123.2 ± 206.7	239.6 ± 100.7
			24	5	63.4 ± 10.6	34.6 ± 4.0	171.6 ± 51.0
		300	3	5	65.6 ± 9.9	35.6 ± 5.7	213.2 ± 58.6
			6	5	75.4 ± 21.1	37.0 ± 16.5	179.2 ± 71.4
			9	5	72.6 ± 17.8	31.4 ± 10.7	235.8 ± 110.0
			24	5	68.8 ± 1.5	36.6 ± 4.1	201.8 ± 60.6

Data are expressed as mean ± SD of 5 measurements. * $p<0.05$; significantly different from control by Mann-Whitney's U-test.

dosing, APAP clearly up-regulated the expression of the enzyme and the values after the induction were still higher in young than adult rats. In the case of CYP3A13, its expression was always lower in young than in adult rats and was not affected by APAP at all (Fig. 1B).

In order not to miss other molecular species, the expressions of CYP and GST were checked. There are

Table 2. Histopathological changes of liver by 1000 mg/kg of acetaminophen (APAP), 200 mg/kg of isoniazid (INH) and 300 mg/kg of carbon tetrachloride (CCl4).

Compound	Age (Week)	Time (hr)	Findings	Number of animals							
				Control				High dose			
			Grade	–	±	+	++	–	±	+	++
APAP	6	3	No abnormal findings								
		6	No abnormal findings								
		9	Increased eosinophility of hepatocyte	5				2	3		
		24	Necrosis of hepatocyte	5				3	2		
			Increased eosinophility of hepatocyte	5					1	2	2
			Vacuolar change of hepatocyte	5				4		1	
			Inflammatory infiltration	5					1	4	
	12	3	No abnormal findings								
		6	No abnormal findings								
		9	No abnormal findings								
		24	Necrosis of hepatocyte	5					3	2	
			Increased eosinophility of hepatocyte	5						2	3
			Vacuolar change of hepatocyte	5				4	1		
			Inflammatory infiltration	5					2	3	
INAH	6	3	No abnormal findings								
		6	No abnormal findings								
		9	No abnormal findings								
		24	No abnormal findings								
	12	3	No abnormal findings								
		6	No abnormal findings								
		9	No abnormal findings								
		24	No abnormal findings								
CCl4	6	3	Degeneration, hydropic	5				2	2	1	
		6	Degeneration, hydropic	5				1	2	2	
			Cellular infiltration	5				4	1		
		9	Degeneration, hydropic	5				1	4		
			Cellular infiltration	5				4	1		
		24	Degeneration, hydropic	5				1	4		
			Cellular infiltration	5				2	3		
			Degeneration, fatty	5					1	4	
	12	3	Degeneration, hydropic	5				2	2	1	
		6	Degeneration, hydropic	5				2	3		
			Cellular infiltration	5					5		
		9	Degeneration, hydropic	5				2	3		
			Cellular infiltration	5				1	4		
		24	Degeneration, hydropic	5				3	2		
			Cellular infiltration	5					5		
			Degeneration, fatty	5					2	3	

Grade indication: no change (–), minimal (±), slight (+), moderate (++).

Table 3. Differentially expressed genes in control animals between 6 and 12 week-old rats.

Probe Set ID	Gene ID	Gene Symbol	Fold change v.s. 6W				P-value				Gene Title
			3H	6H	9H	24H	3H	6H	9H	24H	
1387123_at	NM_012753	Cyp17a1	0.23	0.34	0.26	0.24	3.9E-04	4.1E-02	1.6E-03	1.9E-03	cytochrome P450, family 17, subfamily a, polypeptide 1
1371089_at	AA945082	Yc2	0.21	0.28	0.15	0.30	2.4E-04	7.0E-03	3.5E-03	8.6E-03	glutathione *S*-transferase Yc2 subunit
1368160_at	NM_013144	Igfbp1	0.23	0.14	0.23	0.33	1.4E-03	2.0E-03	4.7E-02	2.8E-02	insulin-like growth factor binding protein 1
1371102_x_at	X05080	Hbb	0.42	0.43	0.27	0.35	9.4E-03	2.0E-03	7.9E-05	3.1E-03	hemoglobin beta chain complex
1371245_a_at	BI287300	---	0.45	0.39	0.24	0.38	1.4E-02	9.4E-04	3.4E-05	2.5E-03	---
1367985_at	NM_013197	Alas2	0.42	0.41	0.32	0.42	7.0E-04	4.6E-03	2.5E-05	1.4E-03	aminolevulinic acid synthase 2
1387022_at	NM_022407	Aldh1a1	0.42	0.41	0.35	0.44	3.5E-03	1.2E-02	3.5E-03	1.1E-02	aldehyde dehydrogenase family 1, member A1
1369465_at	NM_012584	Hsd3b	2.6	2.4	2.7	2.0	1.9E-03	2.9E-02	1.7E-03	3.1E-02	steroid delta-isomerase, 3 beta
1390146_at	BF414998	RGD1306105_predicted	2.5	3.4	3.1	2.9	2.0E-03	1.6E-03	1.7E-03	1.1E-03	similar to RIKEN cDNA 2610318G18 (predicted)
1370387_at	U46118	Cyp3a13	6.4	5.1	4.7	3.4	1.4E-03	1.3E-02	3.0E-04	1.4E-03	cytochrome P450, family 3, subfamily a, polypeptide 13
1368171_at	NM_017061	Lox	2.7	5.6	7.4	3.6	1.9E-02	2.5E-02	7.9E-05	2.7E-02	lysyl oxidase

Nine control animals for each time point were employed for both young and adult rats from the TGP database, and the genes that changed more than 2-fold between the ages with statistical significance ($p < 0.05$, Student's *t*-test with Benjamin's adjustment) in all time points (3, 6, 9, and 24 hr) were extracted. Fold changes of 12-week vs. 6-week and their p values at each time point are also shown.

19 probe sets annotated with GST, while GSTYc2 was the only one with a difference between young and adult. A comparison between young and adult rats was made at 24 hr after the treatment with 1000 mg/kg of APAP and significant differences were detected in three GSTs, i.e., GST mu 2, microsomal GST 3 (predicted), and GST omega, all of which showed lower values in adult than in young rats (data not shown). There are 63 probe sets annotated with CYP, and only CYP17A1 and CYP2C40 (in addition to CYP3A13) were found to be differentially expressed between young and adult rats, and the former two had much lower values in adult than in young rats. No probe sets with different sensitivity to APAP treatment between ages were detected in CYPs. As CYP17A1 and CYP2C40 are involved in steroid biosynthesis and arachidonic acid metabolism, respectively, CYP3A13 was found to be the only candidate for CYP with age-related difference that produces active metabolites of APAP.

The above results could explain the observation that adult rats showed high sensitivity to APAP. In order to explain the specificity of the chemicals, we checked the expressions of CYP2E1 and *N*-acetyltransferase, which are considered to be involved in production of active metabolites of INH and/or CCl4. As described above, CYP2E1 had no age-related difference, and this was the same for the INH- or CCl4-treated group. There are 6 probe sets annotated with *N*-acetyltransferase, and none of them showed significant age-related differences regardless of treatment with INH (data not shown).

Measurement of GSH and SH

It is a consensus that APAP is converted into an active metabolite that is detoxified by GSH conjugation and hepatotoxicity emerges when intracellular GSH is depleted. From the present results, it was suggested that adult rats had a high ability to produce the active metabolite but low capacity of detoxification and that subsequently this caused GSH-depletion more easily as compared with young rats. This was confirmed by direct measurements of hepatic GSH and SH contents by the DTNB method. The results are shown in Fig. 2. Both GSH and free SH contents per wet weight of liver in adult rats showed a value twice as high as that in young rats. Twenty-four hr after treatment with 1000 mg/kg of APAP, these measures did not change in young rats, whereas they showed a remarkable decrease in adults with a statistical significance in free SH contents. This supported our assumption that the high sensitivity to APAP in adult rats is attributed to the high degree of GSH-depletion.

Gene expression changes by APAP

Numbers of altered genes with 2-fold or more of

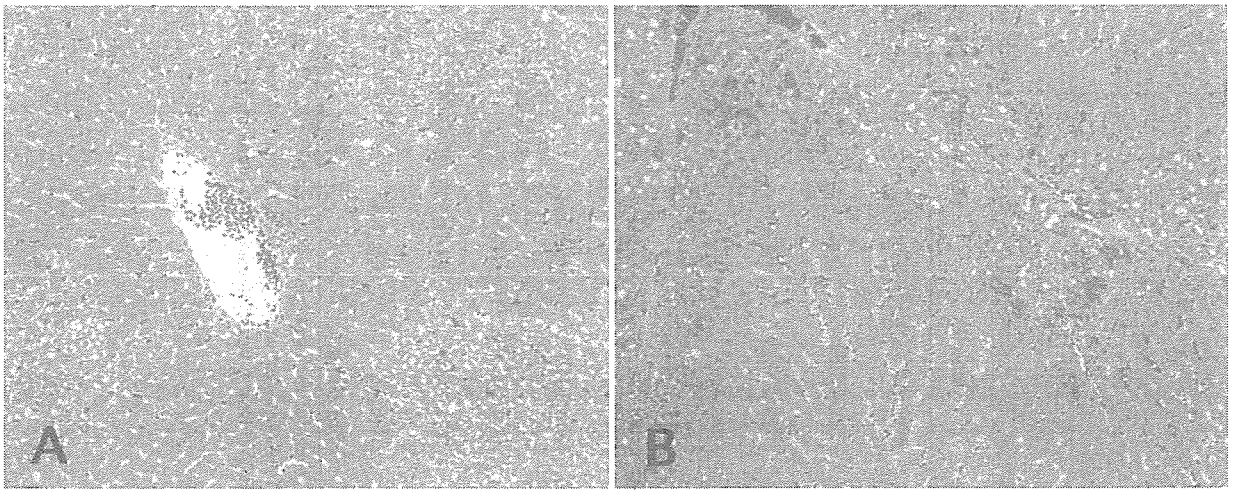

Photo 1. H&E-stained sections of rat liver 24 hr after oral administration of 1000 mg/kg APAP. A: 6-week-old rat; centrilobular necrosis +/−, eosinophility ++, inflammatory infiltration +. B: 12-week-old rat; centrilobular necrosis +, eosinophility ++, inflammatory infiltration +.

the corresponding control in the highest doses of APAP, INH, and CCl4 are shown in Table 4. In this analysis, we did not use statistical filtering because we considered that it was favorable to overview the drug-related changes in order to compare the age difference between young and adult rats. The numbers of genes affected by APAP were greater in adult rats than those in young rats for all time points. In contrast, INH and CCl4 did not show any clear tendency in terms of the age-related difference in the numbers of mobilized genes.

The altered genes with more than 3-fold change vs. control at 24 hr post-dose of APAP in adult rats are listed in Table 5 (up-regulated genes), and those with less than 1/3 vs. control at 24 hr in adult rats are listed in Table 6 (down-regulated genes). The fold change of

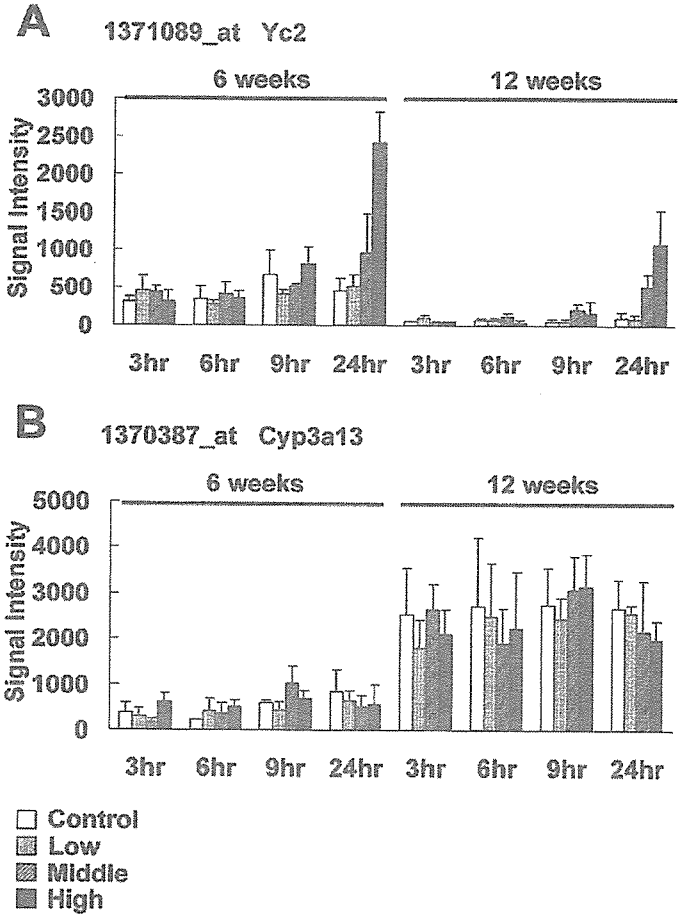

Fig. 1. Time- and dose-dependent effects of APAP on the expression of glutathione *S*-transferase Yc2 (1371089_at) and Cyp3a13 (1370387_at). Rats 6- or 12-week-old were treated with 50 (low), 300 (middle) and 1000 (high) mg/kg of APAP and sacrificed at 3, 6, 9, and 24 hr after treatment. Gene expression in liver was analyzed by using GeneChip® RAE230A probe arrays and the signals obtained by Affymetrix Microarray Suite version 5.0 were normalized by using the mean value of each chip adjusted to an arbitrary value of 500 and expressed as mean ± SD (N=3).

each gene in young rats is also included in these tables. Response to oxidative stress (Hsp1a, Hsp1b, Hmox1, Txnrd1, Hspb8, Hspca, Hspb8), glutathione metabolism (Gss, Gstp, Gsr, Gclc), and response to DNA damage (Ddit4, Prp19, Apex1, Gadd45a) were up-regulated in adult rats. Cell proliferation (pcna, Nap1l1, Gnl3), part of glutathione metabolism (Gsr, Gclc), UDP-glucuronocyltransferase (RGD:708541, RGD:708417) were up-regulated in both young and adult rats. However, microsomal enzymes (Cyp7A1, Cyp3A11, Cyp17A1, Comt, Gulo, G6pc, Sec11, RGD:2467, G6pc, Fmo1, Gulo) and mitochondrial enzymes (Glul, Oat, Ehhadh, Abcc8, Otc, Abat, Ivd, Abcc9, Aadat, Ca5a, Acsl1) were dominantly down-regulated in adult rats. Of these, some mitochondrial enzymes (Oat, Ehhadh and Ivd) were also down-regulated in young rats. These results indicated that the gene expression changes in adult rats were also greater than those in young ones, reflecting the degree of liver damage.

Changes of some representative genes are depicted as Fig. 3 and Fig. 4, which show time- and dose-dependency. In genes such as Egr1, Dnajb1 (predicted), Ddit3, and Hspca (Fig. 3A-D), their expression was highly up-regulated 24 hr after treatment of the highest dose and the extent of induction was remarkably different between ages. On the other hand, in the genes such as Hsp a1a, Hmox-1, Gadd45, and Txnrd (Fig. 4A-D), their induction was larger in adult than young 24 hr after treatment, whereas induction in the young ones occurred at an earlier time point than the adults, i.e., the extent of induction of these genes were much higher in the young than in the adult at early time points. Namely, part of the protective response against APAP started earlier in young rats.

By analyzing the time- and dose-dependent manner, it was revealed that some of stress-related genes responded faster in young than adult rats by APAP. Since expression of these genes was not affected by INH or CCl4, it was not a feature of young rats but rather specific for APAP among these chemicals. Furthermore, reviewing the histopathology in Table 2, cell infiltration with minimal extent occurred at 9 hr only in the young rats, supporting the result of the above gene expression analysis.

DISCUSSION

The mechanism of hepatotoxicity by APAP has been proposed in some papers (Cohen and Khairallah, 1997; Parkinson, 2001; Bessems and Vermeulen, 2001; Irwin *et al.*, 2004). The most widely accepted mecha-

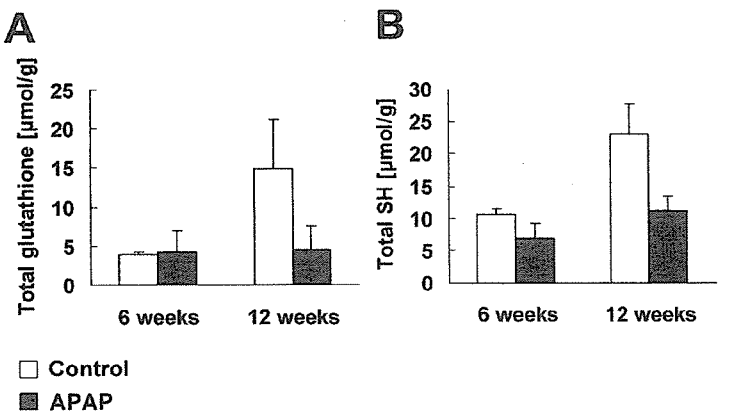

Fig. 2. Effects of APAP on hepatic content of total glutathione and SH of 6- and 12 week-old rats.
Both 6- or 12-week-old rats received 1000 mg/kg APAP orally and liver samples obtained 24 hr later. A: total glutathione contents expressed as μmol glutathione per g wet weight, B: total SH contents equivalent to μmol glutathione per g wet weight. Values are mean ± SD (N=3). A statistically significant reduction of total SH was observed in 12-week rats ($p<0.05$).

nism is that APAP is biotransformed to a reactive intermediate (*N*-acetyl-*p*-benzoquinone imine) by cytochrome P450s (Cyp2E1 and Cyp3A) in liver. The main detoxification pathway is considered to be GSH-conjugation. Finally, overflow of intermediate and/or depletion of GSH cause protein arylation and/or oxidation, and subsequently leads to necrosis of the liver. Expression changes by APAP in the present study were generally in agreement with the above mechanism of action, i.e., response to oxidative stress, glutathione metabolism, response to DNA damage, cell proliferation, and conjugation enzymes were up-regulated and several microsomal enzymes and mitochondrial enzymes were down-regulated. Of these, down-regulation of several microsomal enzymes and mitochondrial enzymes could be explained by the organelle function, because the microsome was the place where the reactive metabolite was produced and cell death was closely related to mitochondrial damage (Jaeschke and Bajt, 2006). These changes by APAP were also greater in adult rats than in young rats at 24 hr after dosing.

Table 4. Number of genes whose expression was changed more than 2-fold by 1000 mg/kg of APAP, 200 mg/kg of INH or 300 mg/kg of CCl4.

Compound	Age (week)	Time (hr)	Number of Genes	
			Increased	Decreased
APAP	6	3	27	67
		6	14	32
		9	19	39
		24	137	149
	12	3	32	88
		6	33	198
		9	34	67
		24	220	193
INAH	6	3	15	17
		6	6	13
		9	5	25
		24	3	34
	12	3	4	15
		6	1	20
		9	18	19
		24	6	62
CCl4	6	3	15	25
		6	10	73
		9	5	31
		24	21	39
	12	3	34	24
		6	4	24
		9	14	12
		24	9	64

Expression data was normalized by mean value (global normalization; adjusted to an arbitrary value of 500) and filtering of the genes by flags (present or marginal call at least 12 of the 24 samples in each experiment) was performed. The fold change of each of the probe sets by the drug was calculated and the numbers of the genes showing more than 2-fold or less than half of control value were counted for each age.

Although there have been several studies where hepatotoxicity of APAP in rodent was analyzed by gene expression (Reilly *et al.*, 2001; Ruepp *et al.*, 2002; Irwin *et al.*, 2004; Heinloth *et al.*, 2004), there is a limited number of papers regarding the age difference in the susceptibility of hepatotoxicity. A few papers described age-related differences such as weanling vs. mature (Allameh *et al.*, 1997), 11-day vs. 33-day-old (Green *et al.*, 1984), or 4-month vs. 25-month-old rats (Rikans and Moore, 1988), but the cause of the differences was not well clarified (Rikans, 1989; Tarloff *et al.*, 1996). There was no information about the difference in susceptibility concerning young to mature age, either. As 6 week-old rats are generally used for toxicological tests (and thus we employ this age for creating our toxicogenomics database), we are interested in the sensitivity of rats of this age against hepatotoxicants compared with matured rats, such as 12-week-olds.

In the present study, a single oral dose of APAP at 1000 mg/kg caused marginal hepatotoxicity in young rats, such as a tendency for an increase in plasma enzymes and minimal hepatocyte necrosis. Under the same condition, adult rats showed more prominent toxicity. This age-related difference should be based on APAP-specific mechanism(s), since no age-related differences were noted in INH or CCl4-induced hepatotoxicity. From the present results and literatures, the main mechanism was considered to be as follows: a) the expression of CYP3A13 that produces the active metabolite of APAP is higher in adult than in young rats, b) among the GST species that detoxify the active metabolite of APAP, the expression of GSTYc2 is lower in adult than in young, and c) the expression of GST, mu 2, microsomal GST 3 (predicted), GST omega 1 are inhibited by APAP.

The modification of APAP toxicity by modifying Cyp3A has been reported in rodents. Inhibition of Cyp3A prevented APAP hepatotoxicity (Kostrubsky *et al.*, 1997), whereas caffeine, dexamethazone, troglitazone or pregnenolone 16 alpha-carbonitrile, all of

Table 5. List of genes that showed expression of more than 3-fold of control by APAP at 24 hr at 12-week-old rats.

Probe Set ID	Gene Symbol	Gene ID	Fold change 6W	Fold change 12W	Gene Title
1368247_at	Hspa1a /// Hspa1b_mapped	NM_031971		37.3	heat shock 70kD protein 1A /// heat shock 70kD protein 1B (mapped)
1370912_at	Hspa1b_mapped	BI278231		30.0	heat shock 70kD protein 1B (mapped)
1368321_at	Egr1	NM_012551		17.3	early growth response 1
1372510_at	Srxn1	AI172302	2.7	17.0	Sulfiredoxin 1 homolog (S. cerevisiae)
1383302_at	Dnajb1_predicted	BM384926		12.4	DnaJ (Hsp40) homolog, subfamily B, member 1 (predicted)
1387060_at	Klf6	NM_031642		10.1	Kruppel-like factor 6
1370080_at	Hmox1	NM_012580		9.3	heme oxygenase (decycling) 1
1368160_at	Igfbp1	NM_013144	4.0	8.8	insulin-like growth factor binding protein 1
1370436_at	LOC246263	AF062389	3.1	8.4	kidney-specific protein (KS)
1386321_s_at	Trib3	H31287	3.9	7.9	tribbles homolog 3 (Drosophila)
1388986_at	---	AI598339		7.8	Transcribed locus
1367856_at	G6pdx	NM_017006	2.9	7.8	glucose-6-phosphate dehydrogenase X-linked
1389528_s_at	Jun	BI288619		7.7	Jun oncogene
1367795_at	Ifrd1	NM_019242		7.4	interferon-related developmental regulator 1
1388271_at	---	BM383531	4.4	7.3	---
1374404_at	Jun	BI288619		6.9	Jun oncogene
1388722_at	Dnajb1_predicted	AA945704		6.8	DnaJ (Hsp40) homolog, subfamily B, member 1 (predicted)
1368308_at	Myc	NM_012603		6.4	myelocytomatosis viral oncogene homolog (avian)
1368947_at	Gadd45a	NM_024127		6.4	growth arrest and DNA-damage-inducible 45 alpha
1386958_at	Txnrd1	U63923		6.4	thioredoxin reductase 1
1368121_at	Akr7a3	NM_013215	5.2	6.3	aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)
1368718_at	Aldh1a4	M23995		6.2	aldehyde dehydrogenase family 1, subfamily A4
1370688_at	Gclc	J05181	2.7	5.9	glutamate-cysteine ligase, catalytic subunit
1375852_at	Hmgcr	BM390399		5.9	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
1371785_at	Tnfrsf12a	BI303379		5.3	tumor necrosis factor receptor superfamily, member 12a
1368124_at	Dusp5	NM_133578		5.3	dual specificity phosphatase 5
1398343_at	Dnaja4	AI104324		5.0	DnaJ (Hsp40) homolog, subfamily A, member 4
1374433_at	RGD1563902_predicted	BI301532		4.9	Similar to Ferritin light chain (Ferritin L subunit) (predicted)
1369590_a_at	Ddit3	NM_024134		4.7	DNA-damage inducible transcript 3
1398791_at	Txnrd1	NM_031614		4.6	thioredoxin reductase 1
1387282_at	Hspb8	NM_053612		4.5	heat shock 22kDa protein 8
1387599_a_at	Nqo1	J02679	4.2	4.5	NAD(P)H dehydrogenase, quinone 1
1387022_at	Aldh1a1	NM_022407	2.2	4.5	aldehyde dehydrogenase family 1, member A1
1390026_at	Bag3	AI231792		4.3	Bcl2-associated athanogene 3
1370030_at	Gclm	NM_017305	4.4	4.3	glutamate cysteine ligase, modifier subunit
1388850_at	Hspca	BG671521		4.3	heat shock protein 1, alpha
1388898_at	Hsph1	AI236601		4.2	heat shock 105kDa/110kDa protein 1
1384427_at	Mdm2_predicted	BI296301		4.2	transformed mouse 3T3 cell double minute 2 homolog (mouse) (predicted)

Table 5. Continued.

Probe Set ID	Gene Symbol	Gene ID	Fold change 6W	Fold change 12W	Gene Title
1370583_s_at	Abcb1 /// Abcb1a	AY082609	3.6	4.1	ATP-binding cassette, sub-family B (MDR/TAP), member 1 /// ATP-binding cassette, sub-family B (MDR/TAP), member 1A
1368147_at	Dusp1	BE110108		4.1	dual specificity phosphatase 1
1388721_at	Hspb8	BG380282		4.1	heat shock 22kDa protein 8
1368213_at	Por	AI407454		4.1	P450 (cytochrome) oxidoreductase
1372523_at	Gclc	AA892770	2.2	4.0	glutamate-cysteine ligase, catalytic subunit
1386995_at	Btg2	BI288701		4.0	B-cell translocation gene 2, anti-proliferative
1386922_at	Ca2	AI408948	4.5	4.0	carbonic anhydrase 2
1387283_at	Mx2	NM_134350	2.0	3.9	myxovirus (influenza virus) resistance 2
1372389_at	Ier2	BF420059		3.6	immediate early response 2
1371237_a_at	Mt1a	AF411318	4.5	3.6	metallothionein 1a
1372261_at	---	AI409067	2.7	3.5	Transcribed locus
1373473_a_at	Napl1l	BM386384	2.1	3.5	nucleosome assembly protein 1-like 1
1367733_at	Ca2	NM_019291	3.2	3.5	carbonic anhydrase 2
1373810_at	Pla2g12a_predicted	BF284175	3.6	3.5	phospholipase A2, group XIIA (predicted)
1377092_at	---	BF389682		3.4	Transcribed locus
1388622_at	Nol5a	AW535890		3.4	nucleolar protein 5A
1389573_at	Chac1_predicted	AI170665		3.4	ChaC, cation transport regulator-like 1 (E. coli) (predicted)
1375895_at	---	BI275908		3.3	CDNA clone IMAGE:7302535, with apparent retained intron
1368332_at	Gbp2	NM_133624		3.3	guanylate nucleotide binding protein 2
1369061_at	Gsr	NM_053906	2.2	3.3	glutathione reductase

Fold changes in 6-week-old rats are also shown for values higher than 2.

which are Cyp3A inducers, increased APAP hepatotoxicity (Jaw and Jeffery, 1993; Madhu *et al.*, 1993; Jue *et al.*, 2002; Guo *et al.*, 2004). Therefore, it would be reasonable to assume that the difference in basal expression levels of Cyp3A13 between young and adult rats accounts for the difference in susceptibility to APAP toxicity. Cyp2E1 is another enzyme responsible for biotransformation, i.e., inhibition of Cyp2E1 decreased the production of a reactive metabolite in the liver microsome from humans (Hazai *et al.*, 2002), although this enzyme did not show an age-difference. Based on the present results, participation of CYP3A is considered to be larger than that of CYP2E in the rat.

GSTYc2 was one isoform of glutathione *S*-transferase, and was known to play an important role in aflatoxin detoxification (Buetler and Eaton, 1992; Hayes *et al.*, 1992 and 1994). Although the role of GSTYc2 in APAP detoxification has not been investigated, it could have some role as GST conjugation, a key detoxification pathway of the reactive intermediate of APAP.

When the total of the GST and SH-contents in the liver were measured, they were found to be higher in adult than in young under the control condition, while their reduction by APAP-treatment was larger in adult than in young. This could be explained by our assumption from gene expression analysis that the production of active metabolite is higher and the rate of the supply of the detoxification enzyme is lower in adult rats.

The age-related difference in the number of mobilized genes at 24 hr after APAP-treatment was considered to be correlated with the phenotype. Namely, most of these changes in the expression reflected the results of pathological changes. When the precise analysis of the genes showing age-related difference was made in terms of time- and dose-related responses, another view emerged. Among the genes with age-related difference at 24 hr after APAP-treatment, there were not only genes with differing levels of expression but also differences in the response time.

Table 6. List of genes that showed expression of less than 0.3-fold of control by APAP at 24 hr in 12-week-old rats.

Probe Set ID	Gene Symbol	Gene ID	Fold change		Gene Title
			6W	12W	
1371412_a_at	Nrep	BE107450	0.25	0.06	Neuronal regeneration related protein
1386977_at	Ca3	NM_019292	0.20	0.08	carbonic anhydrase 3
1374251_at	---	AA893192		0.11	Transcribed locus
1367896_at	Ca3	AB030829		0.13	carbonic anhydrase 3
1369864_a_at	Sds	NM_053962	0.47	0.17	serine dehydratase
1387665_at	Bhmt	U96133	0.40	0.18	betaine-homocysteine methyltransferase
1374244_at	LOC501038	AI411141	0.35	0.18	Ab2-060
1376976_at	Sectm1	AI009823		0.18	secreted and transmembrane 1
1373686_at	Serpina6_mapped	AA893495	0.42	0.19	serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 6 (mapped)
1389681_at	Pvrl2	BI296388	0.24	0.21	Poliovirus receptor-related 2 (herpesvirus entry mediator B)
1368171_at	Lox	NM_017061	0.33	0.21	lysyl oxidase
1374531_at	---	AA926305		0.22	Transcribed locus
1368826_at	Comt	NM_012531		0.22	catechol-O-methyltransferase
1393221_at	RGD1564865_predicted	AA866264		0.23	similar to 20-alpha-hydroxysteroid dehydrogenase (predicted)
1368458_at	Cyp7a1	NM_012942	0.45	0.23	cytochrome P450, family 7, subfamily a, polypeptide 1
1368172_a_at	Lox	BI304009		0.25	lysyl oxidase
1373778_at	---	BE349670	0.32	0.26	Transcribed locus
1368270_at	Apobec1	NM_012907		0.26	apolipoprotein B editing complex 1
1373814_at	RGD1310066	BI291270		0.26	similar to mKIAA1002 protein
1398759_at	Tgfb1i4	NM_013043	0.34	0.27	transforming growth factor beta 1 induced transcript 4
1388583_at	Cxcl12	BF283398	0.42	0.27	chemokine (C-X-C motif) ligand 12
1368778_at	Slc6a6	NM_017206	0.34	0.27	solute carrier family 6 (neurotransmitter transporter, taurine), member 6
1368543_at	Nox4	NM_053524		0.27	NADPH oxidase 4
1375560_at	RGD1310475_predicted	AA945579		0.28	similar to RIKEN cDNA 0610010D20 (predicted)
1388426_at	Srebf1	BF398848	0.50	0.28	sterol regulatory element binding factor 1
1387053_at	Fmo1	NM_012792		0.28	flavin containing monooxygenase 1
1375216_at	Pvrl2	AA850909	0.31	0.28	poliovirus receptor-related 2 (herpesvirus entry mediator B)
1387263_at	Pklr	NM_012624		0.28	pyruvate kinase, liver and red blood cell
1374493_at	---	H35017		0.29	Transcribed locus
1369664_at	Avpr1a	NM_053019	0.36	0.30	arginine vasopressin receptor 1A
1377015_at	Me3_predicted	BF395080	0.43	0.30	Malic enzyme 3, NADP(+)-dependent, mitochondrial (predicted)
1371137_at	Acox2	X95189		0.30	acyl-Coenzyme A oxidase 2, branched chain
1372684_at	Smtn	AW435036		0.30	smoothelin
1387123_at	Cyp17a1	NM_012753	0.30	0.30	cytochrome P450, family 17, subfamily a, polypeptide 1
1367729_at	Oat	NM_022521	0.30	0.30	ornithine aminotransferase
1374625_at	Hes6	AI176616	0.47	0.30	hairy and enhancer of split 6 (Drosophila)
1373975_at	LOC368066	AI232716		0.30	similar to thioether *S*-methyltransferase

Fold changes in 6-week-old rats are also shown for values lower than 0.5.

Especially, genes related to defensive responses to stress, e.g., heat shock protein, Hmox-1, Gadd45, and thioredoxin reductase were markedly induced by APAP in young rats at 6 or 9 hr after treatment, whereas adult rats did not respond as yet. This is considered to be due to the age-related difference in the toxicokinetics, i.e., young rats responded to APAP in the early stage of exposure because of the low SH level but recovered at 24 hr because of the high detoxification capacity.

In the case of CCl4, its active metabolite is reported to be produced by CYP2E1 (Parkinson, 2001) and this fits the present observation that neither the expression of CYP2E1 nor hepatotoxicity of CCl4 showed a difference between the ages. Although GSH conjugation should play an important role in their detoxification, the participation of GSTYc2 is considered to be less important for CCl4 in rats. It has been reported that both *N*-acetylation and CYP2E1 are involved in hepatotoxicity of INH (Parkinson, 2001). In the present study, no age-related difference was found in the expression of *N*-acetylating enzymes and CYP2E1, in accordance with the same extent of hepatotoxicity of INH for both ages. However, one should be careful in these conclusions, since phenotypical changes by INH and CCl4 were minimal in the present

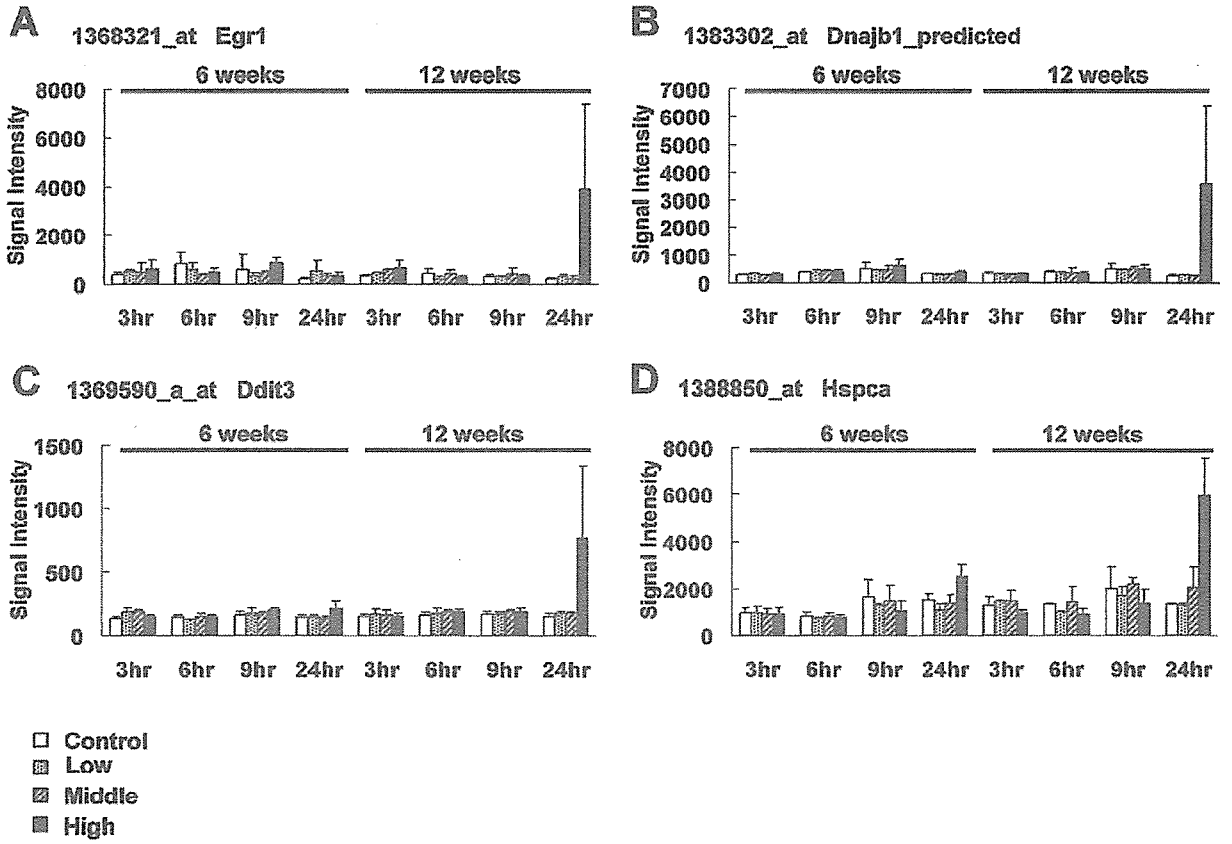

Fig. 3. Time- and dose-dependent effects of APAP on expression of Egr1 (A), Dnajb1 (predicted) (B), Ddit3 (C), and Hspca (D). Rats 6- or 12-week-old were treated with 50 (low), 300 (middle) and 1000 (high) mg/kg of APAP and sacrificed at 3, 6, 9, and 24 hr after treatment. Gene expression in liver was analyzed by using GeneChip® RAE230A probe arrays and the signals obtained by Affymetrix Microarray Suite version 5.0 were normalized by using the mean value of each chip adjusted to an arbitrary value of 500 and then expressed as mean ± SD (N=3). Note that the expression of these genes was markedly increased by APAP-treatment at 24 hr in adult (12-week-old) rats.

condition, which was optimized for their repeated administration for up to 28 days in our standard protocol.

The TGP database has huge gene expression data as well as the traditional toxicity study parameters, currently for over 100 chemicals. Although the main purpose of the project is to predict toxicity in the early stage of drug development, it is also useful for drawing some mechanistic insight in observed toxicity. In the present study, it would have been impossible to pick up such genes responding in a different time course with age, unless a multi-time, multi-dose protocol was employed. In general, dose or time points tend to be limited in the collection of toxicological data by microarray due to the cost. The present study has clearly showed the advantage of our protocol with enough numbers of data points. This promises a good use for our database now being created in the project.

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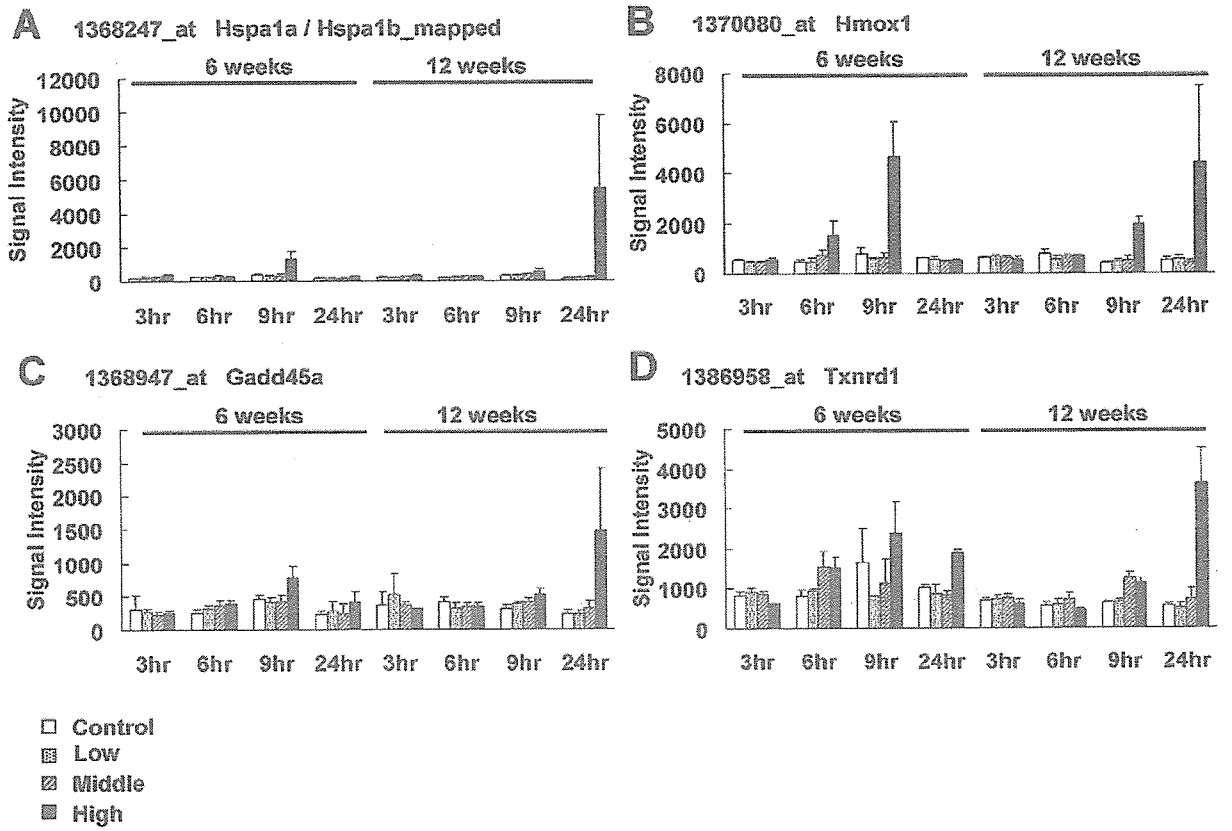

Fig. 4. Time- and dose-dependent effects of APAP on expression of Hsp a1a/a1b (A), Hmox-1 (B), Gadd45 (C), and Txnrd (D). Rats 6- or 12-week-old were treated with 50 (low), 300 (middle) and 1000 (high) mg/kg ofAPAP and sacrificed at 3, 6, 9, and 24 hr after treatment. Gene expression in liver was analyzed by using GeneChip® RAE230A probe arrays and the signals obtained by Affymetrix Microarray Suite version 5.0 were normalized by using the mean value of each chip adjusted to an arbitrary value of 500 and then expressed as mean ± SD (N=3). Note that the induction of these genes by APAP at 24 hr was larger in adult (12-week-old) than young (6-week-old) rats, whereas the young rat responded to APAP earlier.

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EVALUATION OF METHODS FOR DURATION OF PRESERVATION OF RNA QUALITY IN RAT LIVER USED FOR TRANSCRIPTOME ANALYSIS

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ABSTRACT — In The Toxicogenomics Project, about 150 chemicals are administered to rats, and gene expression in the liver analyzed by Affymetrix GeneChip and stored in the database. As the quality of RNA greatly influences the accuracy of gene expression data, conditions of the storage of the sample are very important. Recently, an RNA stabilization solution, RNAlater®, has become commercially available. In this study, the new storage method was compared with the traditional storage method (stored in freezer or liquid nitrogen) under various conditions by looking at the degradation of RNA assessed by its total yield, OD260/280 ratio, 28S/18S ratio, and quantity of β-actin. It was confirmed that RNAlater® preserved the liver tissue sample by maintaining the quality of RNA for one year (in liquid N_2 or −80°C), for 3 days (4°C), or for 2 hr (room temperature) without degradation of RNA. Quality of RNA samples dissolved in buffer RLT and stored at −20°C tended to decrease, but samples stored at −80°C were almost equivalent to those stored in liquid nitrogen. In conclusion, we recommend the following procedure for preservation of liver tissue for extraction of RNA: 1) tissues removed should be put into chilled RNAlater® as soon as possible; 2) samples in RNAlater® must be stored overnight or longer at 4°C and can be left for as long as 2 weeks without freezing; 3) samples in RNAlater® can be stored for at least one year under less than −20°C and 4) samples dissolved in buffer RLT can be preserved at least for one year under −80°C.

KEY WORDS: mRNA stability, Rat, Liver

INTRODUCTION

The Toxicogenomics Project is a 5-year collaborative project conducted by the National Institute of Health Sciences (NIHS) and 17 pharmaceutical companies in Japan that started in 2002 (Urushidani and Nagao, 2005). In April 2005, some rearrangements were made and the project is now conducted by NIHS, the National Institute of Biomedical Innovation, and 15 pharmaceutical companies. Its aim is to construct a large-scale toxicology database of transcriptome for prediction of toxicity of new chemical entities in the early stage of drug development. About 150 chemicals, mainly medical compounds, have been selected, and administered to rats to obtain gene expression profile in the liver (also in kidney in some cases) using Affymetrix GeneChip in addition to traditional toxicological measures, including body/organ weight, blood biochemistry, hematology and histopathology. In order to overcome the species difference, bridging experiments are also performed using primary cultures of human and rat hepatocytes. Needless to say, the quality of the data should be good in order to create a drug safety predicting system with high accuracy. Since RNA is quite labile, it is quite important to preserve tissues, stabilizing the RNA for experiments of gene expression profiling. So far, isolated tissues had been promptly frozen in liquid nitrogen and then kept as such or in a deep

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freezer. However, this is costly and may be risky if the freezer is in trouble, and prompt melting is required to ensure the quality of RNA. Recently, a new RNA stabilization solution, RNAlater® (Ambion, Austin, TX, USA) has become commercially available and is used in many laboratories (Grotzer *et al.*, 2000; Wang *et al.*, 2001; Rodrigo *et al.*, 2002; Mutter *et al.*, 2004). According to the manufacturer's notes, RNAlater® makes it possible to preserve tissues at a relatively high temperature and to repeat freezing-thawing, keeping the RNA stabilized. This is quite convenient, especially in our project, where vast numbers of samples are processed. To assess the usefulness of this product, we checked the following points:

1) Effects of the conditions (temperature and period) of fixing before freezing the sample.
2) Long-term preservation of samples fixed with RNAlater® at 4°C overnight compared with traditional procedures (liquid N_2 or freezers).
3) Effects of freezing-thawing on the RNA quality in the sample.
4) Stability of RNA in the sample dissolved in Buffer RLT.

The quality of RNA was estimated by the amount of total RNA, the OD260/280 ratio, 28S/18S ratio on agarose electrophoresis, and the amount β-actin mRNA quantified by RT-PCR. Based on the results of these assessments, we determined the standard protocol of sample preservation for our project.

MATERIALS AND METHODS

Preparation of total RNA

Male Sprague-Dawley rats were purchased from Charles River Japan Inc., (Atsugi, Japan) and used at 10 weeks of age. The animals were killed by exsanguination under anesthesia, and the livers removed quickly and provided for each experiment. The weights of microtubes with 0.5 mL RNAlater® were measured in advance. A piece of liver tissue (~50 mg) was cut out by using a disposable biopsy punch (ϕ=5 mm, Kai Industries, Gifu, Japan) and put into the pre-weighed microtube with or without RNAlater®. Total RNA was isolated using the RNeasy kit. The liver tissue was homogenized in 0.5 mL RLT with a Mill Mixer (Qiagen) and zirconium beads. The lysate corresponding to ca. 20 mg tissue was transferred to a microtube with an equivalent volume of chloroform, and 3 volumes of TRIzol LS Reagent (Invitrogen life technologies, Carlsbad, CA, USA) were added to the homogenate. The mixture was then shaken vigorously for 30 s, let stand at room temperature for 5 min and centrifuged thereafter at 12,000 *g* for 15 min. The resultant aqueous layer was transferred to a new tube. To the aqueous solution, an equivalent volume of 50% ethanol was added and mixed by pipetting. The whole mixture was transferred to an RNeasy mini column. The following procedure was performed according to the user's manual. Finally, absorbance of the purified RNA fraction was measured spectrophotometorically at 260 and 280 nm.

Electrophoresis of total RNA

To confirm the quality of total RNA, the total RNA solution (1μl) was applied to 1% agarose gel-electrophoresis, and we then visualized the bands of 28S and 18S with ethidium bromide under UV light. Band density was measured by light capture (Atto Co., Tokyo, Japan).

Preparation of cDNA and Real-time PCR

Synthesis of complementary DNA (cDNA) from total liver RNA (2 μg) was performed by SuperScript II First-strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was performed by using "ABI PRISM 7900 Sequence Detection System" (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol in which 5 μl of each cDNA preparation (1/10 dilution) or appropriately diluted standard DNA was added to a 20 μl reaction mixture containing 10 μl of "SYBR Green PCR Master Mix" (Applied Biosystems), 100 nM of each primer and DEPC-water in 0.2 ml MicroAmp optical tubes (Applied Biosystems). The thermal cycling condition comprised initial denaturation (at 95°C for 10 min) and extension (40 cycles at 95°C for 15 sec and 60°C for 1 min). Copies of β-actin mRNA were quantified and presented as copy number per total RNA (μg). Standard DNA and primer sets for β-actin were prepared according to Kasahara *et al.* (2005).

Statistical analysis

Parametric data were analyzed by the F test followed by the Student's *t*-test (Snedecor and Cochran, 1989). A value of $p<0.01$ or 0.05 was considered to be statistically significant.

RESULTS

Effects of conditions (temperature and period) of fixing before freezing the sample

According to the manufacturer's protocol, the tis-

sues were to be fixed in RNAlater® solution before storage. We varied the time and temperature, i.e., rat liver tissues were soaked in RNAlater® and kept for 1, 3, 8, and 15 days under room temperature or in a refrigerator (4°C), and the quality of RNA was checked (Fig. 1). The total RNA yield and OD260/280 ratio of the samples soaked in RNAlater® and kept under room temperature or in a refrigerator were unchanged for the entire period from the first day, whereas the 28S/18S ratio of the sample kept under room temperature was

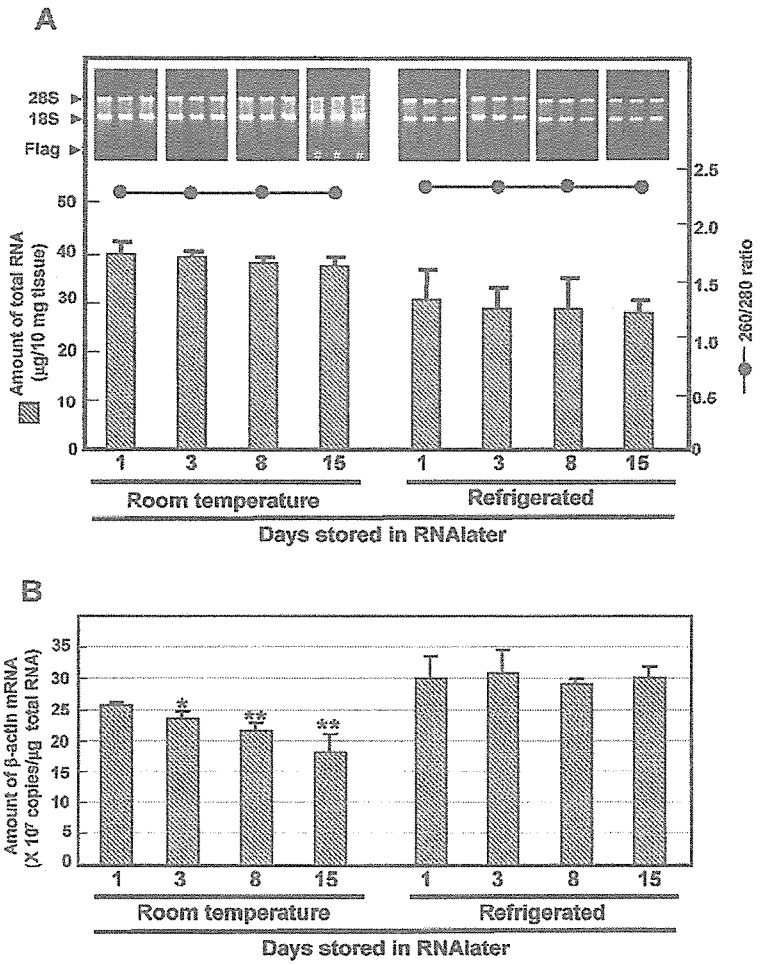

Fig. 1. Influence of temperature and time on RNA quality of rat liver in RNAlater®.
A: Rat liver tissue was soaked in RNAlater® for 1, 3, 8 or 15 days under room temperature or in a refrigerator (at 4°C). Total RNA was extracted from each tissue, and the amount of total RNA (bar braph) and the 260/280 ratio (line graph) were calculated and expressed as the mean ± SD (n=3). Total RNA was subjected to agarose gel-electrophoresis (upper photos), band density of 28S and 18S was measured by densitometer, and the ratio of the 28S/18S band density was calculated. The Flags indicate the degradation of RNA with the following; #; 0.8 ~ 1, ##; ~ 0.8. B: Real-time PCR of β-actin was performed using cDNA synthesized from the total RNA. The amount of expression of β-actin mRNA was expressed as copy number per 1 μg of total RNA. *, **Significantly different from the sample at 1 day by Student's t-test: *$p<0.05$;**$p<0.01$.

found to be abnormal at the 15th day (Fig. 1A). The amount of β-actin mRNA in samples kept in the refrigerator was unchanged throughout the experimental period, whereas this parameter significantly decreased in samples kept for 3 days or more at room temperature (Fig. 1B).

Long-term preservation of samples fixed with RNAlater® 4°C overnight compared with the traditional procedure (liquid N_2 or freezers)

In the next experiment, isolated liver tissues were soaked in RNAlater®, stored overnight in the refrigerator, and then kept at −80 or −20°C, or at −80°C after removing RNAlater®. As a comparison, isolated tissues were frozen in liquid N_2 and kept in liquid N_2 or in a freezer at −20 or −80°C. Their RNA quality was checked at 1, 7 days and 1, 3, 6, and 12 months later. A comparison was made against the sample kept at −80°C with RNAlater® for each time point. These protocols and the results are summarized in Tables 1 - 4.

At one day, the only detectable change was found in the total RNA yield of fresh sample stored at−20°C, and the other parameters were unchanged. At the 7-day stock, the total yield of RNA from the fresh sample stored in liquid N_2 and the sample stored in RNAlater® at −20°C was significantly reduced (Fig. 2-II, C, D; Table 1). Abnormal S28/S18 ratios were observed in one out of 4 fresh samples stored at −80°C and in all 4 samples stored at −20°C. Obvious degradation of RNA was observed in all the fresh samples stored for 7 days at −20°C (Fig. 2-II, F; Table 3), so further preservation was ceased at that point.

At one-month stock, fresh samples stored in liquid N_2 or at −80°C showed significant reduction in the RNA yield with normal S28/S18 ratio (Fig. 2-III, D, E; Table 1, 3). The amount of β-actin mRNA was significantly decreased in the fresh sample stored at −80°C, whereas it increased in the sample RNAlater®-pretreated, stored at −80°C, soaked in RNAlater® and stored at −80°C (Fig. 2-III, B, C, E; Table 4).

Table 1. Evaluation of the methods with and without RNAlater® for preservation of the total RNA in the tissue.

Total RNA yield	Storage period					
Storage method	1-day	7-days	1-month	3-months	6-months	12-months
(a) RNA*later*®, −80℃	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.
(b) RNA*later*® (removed), −80℃	-----	-----	-----	-----	-----	-----
(c) RNA*later*®, −20℃	-----	↓	-----	-----	-----	-----
(d) Raw tissue in liq. N_2	-----	↓	↓	↓	↓	↓
(e) Raw tissue, −80℃	-----	-----	↓	↓	↓	↓
(f) Raw tissue, −20℃	↓	-----	N.P.	N.P.	N.P.	N.P.

N.E.: not evaluated, -----: no change, ↓: decreased ($p<0.05$) vs. method (a), N.P.: not performed. Storage method (a): Isolated liver tissues were soaked in RNAlater® and stored overnight in the refrigerator, and then kept at −80℃ (in a deep freezer). (b) Liver tissues were treated as (a) but stored at −80℃ after removing RNAlater®. (c) Liver tissues treated as (a) and kept at −20℃ (in a freezer) in RNAlater®. (d) Liver tissues were immediately frozen in liquid N_2 and kept in liquid N_2. (e) Frozen tissues were kept at −20℃ (in a freezer). (f) Frozen tissues were kept at −80℃ (in a deep freezer). On the assumption that method (a) was the best, comparisons were made against (a) at each time point.

Table 2. Evaluation of methods with and without RNAlater® for preservation of the quality of RNA (OD 260/280 ratio) in the tissue.

O.D. 260/280 ratio	Storage period					
Storage method	1-day	7-days	1-month	3-months	6-months	12-months
(a) RNA*later*® −80℃	-----	-----	-----	-----	-----	-----
(b) RNA*later*® (removed), −80℃	-----	-----	-----	-----	-----	-----
(c) RNA*later*® −20℃	-----	-----	-----	-----	-----	-----
(d) Raw tissue in liq. N_2	-----	-----	-----	-----	-----	-----
(e) Raw tissue −80℃	-----	-----	-----	-----	-----	-----
(f) Raw tissue −20℃	-----	-----	N.P.	N.P.	N.P.	N.P.

-----: no change N.P.: not performed. Storage methods (a) – (f) are described in Table 1.

For storage for 3 months, there were significant reductions of total RNA in fresh samples stored in liquid N_2 or at −80°C, and abnormal S28/S18 ratio and significant reduction of β-actin mRNA were observed in fresh samples stored at −80°C as well (Fig. 2-IV, D, E; Table 1, 3, 4). For 6-month storage, both fresh samples stored in liquid N_2 and at −80°C showed significant reduction of total RNA, abnormal S28/S18 ratio, and significant reduction of β-actin mRNA (Fig. 2-V, D, E, Table 1, 3, 4). After 12 months of storage, although obvious degradation of RNA was observed in fresh samples stored in liquid N_2 or at −80°C, the extent was less than that at 6 months (Fig. 2-VI, D, E; Table 1, 3, 4).

Influence of time to processing on stability of RNA in frozen samples

When RNA extraction is performed for vast numbers of frozen samples at once, the stability of RNA may be affected since the thawing time is long and varied. In this experiment, fresh samples frozen immediately in liquid N_2 or samples treated with RNAlater® and then frozen were returned to room temperature for various times, and the quality of extracted RNA was checked. In fresh samples, no abnormality in total RNA and OD260/280 ratio was observed at any time point, whereas abnormal 28S/18S ratios were observed at 20 min or later, and significant reduction of β-actin was observed at 10 min or later (Fig. 3, upper panels). On the other hand, samples treated with RNAlater® at 4°C overnight and stored at −80°C did not show any signs of RNA degradation throughout the period of the 2 hr experiments (Fig. 3, lower panels).

Preservation of RNA in RLT reagent

In our project, an RNeasy mini kit (Qiagen) is used for RNA extraction. The stability of RNA in tissue dissolved by Buffer RLT (contained in the kit) was examined under various conditions (Fig. 4). At one-day storage, no significant changes of total RNA and OD260/280 ratio were observed for any conditions, whereas abnormal 28S/18S ratio and significant reduction of β-actin mRNA was observed in samples stored at room temperature (Fig. 4-I, E). For the 7-day stor-

Table 3. Evaluation of methods with and without RNAlater® for preservation of the quality of RNA (28S/18S ratio on RNA electrophoresis) in the tissue.

28S/18S ratio	Storage period					
Storage method	1-day	7-days	1-month	3-months	6-months	12-months
(a) RNA*later*®, −80℃	-----	-----	-----	-----	-----	-----
(b) RNA*later*® (removed), −80℃	-----	-----	-----	-----	-----	-----
(c) RNA*later*®, −20℃	-----	-----	-----	-----	-----	-----
(d) Raw tissue in liq. N_2	-----	-----	-----	-----	*	-----
(e) Raw tissue, −80℃	-----	*	-----	*	*	-----
(f) Raw tissue, −20℃	-----	**	N.P.	N.P.	N.P.	N.P.

-----: no change, *: 28S/18S<1 in a few samples, **: 28S/18S<1 in all samples, N.P.: not performed. Storage methods (a) – (f) are described in Table 1.

Table 4. Evaluation of methods with and without RNAlater® for preservation of β-actin mRNA quantified by Real-time PCR.

Amount of β-actin mRNA	Storage period					
Storage method	1-day	7-day	1-month	3-month	6-month	12-month
(a) RNA*later*®, −80℃	N.E.	N.E.	N.E.	N.E.	N.E.	N.E.
(b) RNA*later*® (removed), −80℃	-----	-----	-----	-----	-----	-----
(c) RNA*later*®, −20℃	-----	-----	-----	-----	-----	-----
(d) Raw tissue in liq. N_2	-----	-----	-----	-----	↓	↓
(e) Raw tissue, −80℃	-----	↓	↓	↓	↓	-----
(f) Raw tissue, −20℃	-----	↓	N.P.	N.P.	N.P.	N.P.

N.E.: not evaluated, -----: no change, ↓: decreased ($p<0.05$) vs. method (a), N.P.: not performed. Storage methods (a) – (f) are described in Table 1. On the assumption that the method (a) was the best, comparisons were made against (a) at each time point.