age, an abnormal 28S/18S ratio was observed both in samples stored at room temperature and at 4°C (Fig. 4-II, D,E). Samples stored at room temperature also showed significant reduction in β-actin mRNA (Fig. 4-II, E). Obvious signs of RNA degradation were observed at 7 days in samples stored at room temperature and 4°C, so further examinations were not performed under these conditions.

In samples stored at −20 and −80°C for one month, a significant increase in RNA yield was observed, whereas no abnormalities were noted in the OD260/280 or 20S/18S ratios (Fig. 4-III, B, C). In samples stored for 3 months, no significant changes or abnormalities were detected for any conditions (Fig. 4-IV). In samples stored for 6 months, no changes were observed in any conditions except that the samples stored at −20°C showed a significant decrease in β-actin mRNA (Fig. 4-V, C). For samples stored for 12 months, the samples stored at −20°C showed an abnormal 28S/18S ratio and significant decrease in β-actin mRNA (Fig.4-VI, C).

DISCUSSION

The aim of our project is to create a database of transcriptome that is appropriate for prediction of drug toxicity in the early stage of drug development. It is therefore quite important to obtain gene expression data with high accuracy, and subsequently the method of preservation of the samples and the RNA extraction procedure are highly important. In the present study, the quality of RNA was checked in terms of the following 4 measures: amount of β-actin mRNA, 28S/18S ratio of RNA on agarose electrophoresis, yield of total RNA, and OD260/280 ratio. The amount of β-actin mRNA showed a good correlation with the 28S/18S ratio and appeared to be more sensitive than the latter: namely, β-actin was decreased earlier than when the

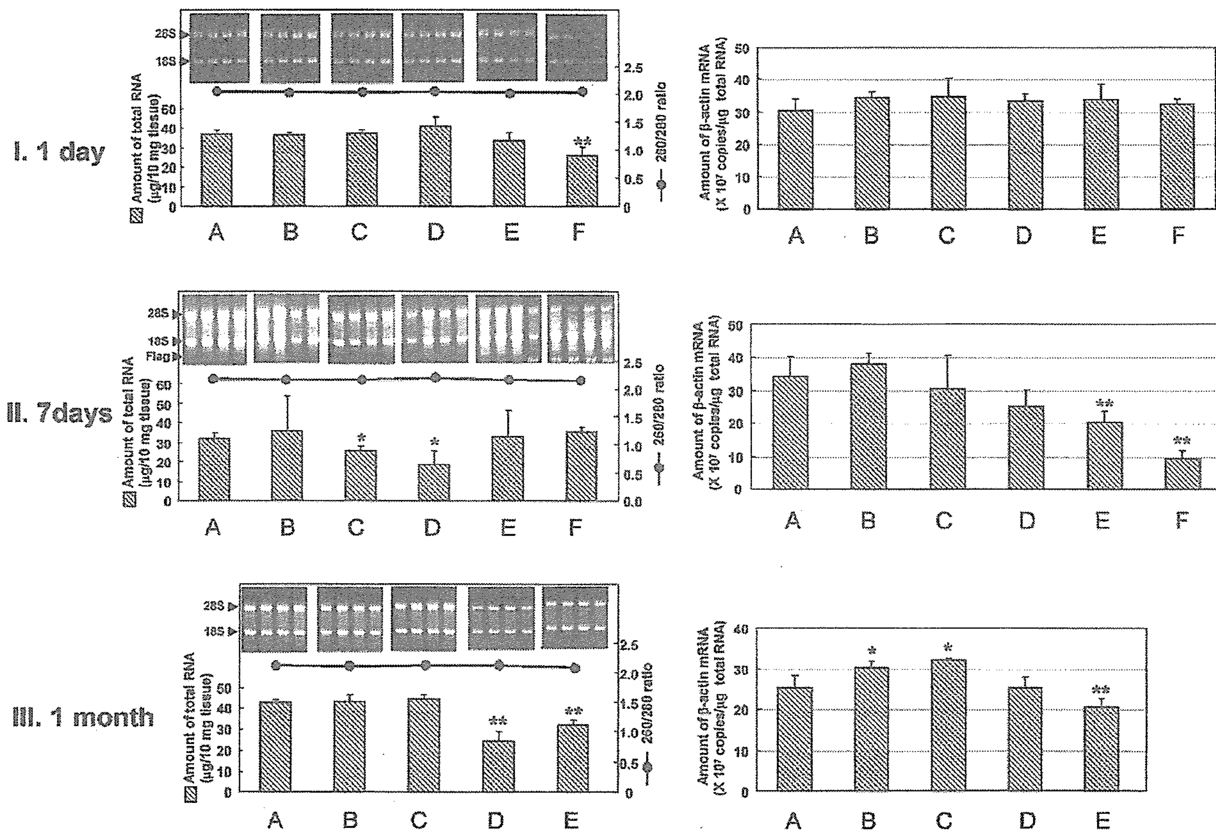


Fig. 2-1.

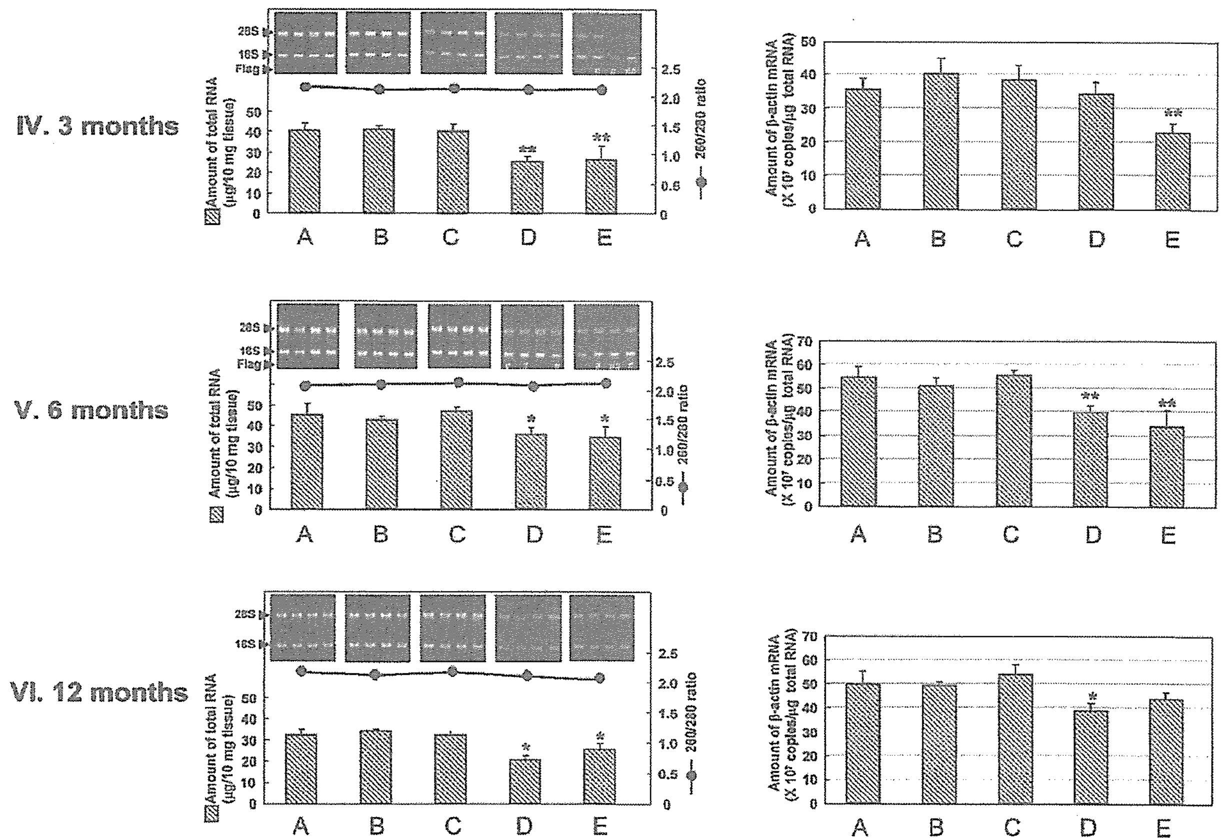


Fig. 2-2. Comparison of the methods with and without RNAlater® for preservation of tissue in terms of quality of RNA. Rat liver tissue was soaked in RNAlater® overnight at 4°C, and stored under the following conditions: A: at –80°C with RNAlater®, B: at –80°C after removing RNAlater®, C: at –20°C with RNAlater®. Raw tissues without treatment with RNAlater® were stored under the following conditions: D: in liquid nitrogen, E: at –80°C, F: at –20°C. Total RNA was extracted at 1 day, 7 days, one month, 3 months, 6 months and 12 months after storage. Left panels: Total RNA was extracted from each tissue, and the amount of total RNA (bar graph) and the 260/280 ratio (line graph) were calculated and expressed as the mean± SD (n=4). 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. Right panels: 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: *$p<0.05$;**$p<0.01$. Raw tissue stored at –20°C (F) was not examined later than 7 days, because it showed obvious signs of RNA degradation at the 7th day.

abnormal ratio of 28S/18S ratio was detected. The total yield of RNA and OD260/280 ratio were not correlated with the 28S/18S ratio. This could be due to employment of the column method for extraction of RNA where degraded short RNA chains were also harvested. The OD 260/280 ratio is an index of the contamination of proteins and thus showed no abnormalities in the case of RNA degradation.

Whether the normal range of these parameters assures the quality of RNA for GeneChip analysis is another question. It is well known that the stability of RNA varies largely between genes (Timofeeva *et al.*, 2000), and degradation of RNA differentially affects each probe set for a gene (Thach *et al.*, 2003). Therefore, the present study does not necessarily assure quantification of each gene expression. However, it is not practical to perform GeneChip analyses for all the conditions in the present study in order to assess the

individual stability of all the genes. Here we offer the minimal requirement for construction of the database for Toxicogenomics.

According to the manufacturer's protocol, the samples are to be preserved by RNAlater® for 1 week under room temperature and for 4 weeks under 4°C. Similar results were obtained in our laboratory. As for the amount of β-actin mRNA in the sample stored with RNAlater® at room temperature, some reduction was observed at 3 days and obvious reduction was detected at 8 days, whereas no reduction of RNA quality was detected under 4°C as long as 15 days. Therefore, we concluded that the sample soaked in RNAlater® could be stored at room temperature for a few days, but preferentially should be stored in a refrigerator which would make it possible to keep it for at least 2 weeks.

The manufacturer's protocol recommends removing RNAlater® after overnight treatment when preserved in the freezer. In our project, however, we harvested 160 samples (N=5 for each of 4 time and 4 dose points in a duplicated manner) per experiment, but 48 of them (N=3 for each of 4 time and 4 dose points) were analyzed by GeneChip. This means only 30% of the tubes are homogenized and the remaining ones are stored for backup, so it is quite cumbersome if RNAlater® is to be removed from all the tubes. We compared RNA quality between samples frozen with and without RNAlater® and no difference was observed. It appears that RNAlater® can be left during storage in the frozen state, at least in the case of liver tissue.

For samples stored in liquid N_2 or at −80°C, some signs suggesting RNA degradation appeared until 6 months, whereas degradation tended to reduce by 12 months. This irrational result might be explained as follows. In the experiments shown in Fig. 2, the samples should have been thawed before homogenization, and this took 5 to 10 min because there were so many tubes. The total number of the samples was small when the 12-month samples were processed, and thus the time for processing was shorter than the other time points. This suggested that the time from thawing to

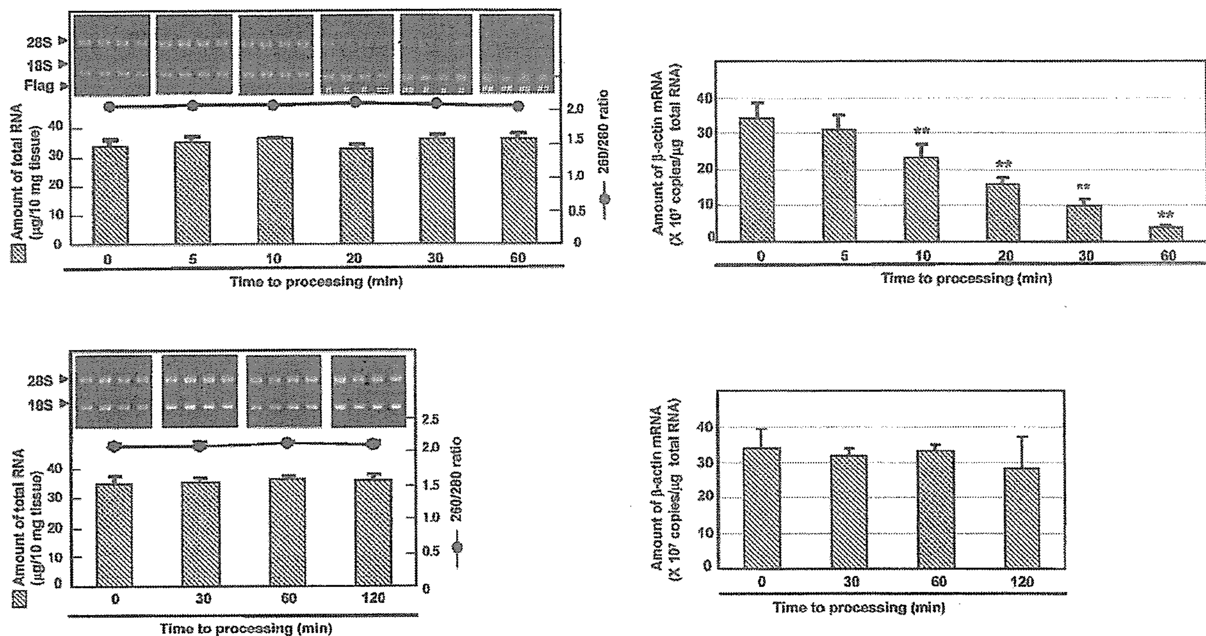


Fig. 3. Effects of freezing-thawing on the RNA quality in the sample.
Upper panels: Non-treated samples were taken out from liquid nitrogen and left for 0, 5, 10, 20, 30 or 60 min under room temperature and then lysed in buffer RLT.
Lower panels: RNAlater®-fixed samples were taken out from the deep freezer and left for 0, 30, 60, or 120 min under room temperature and then lysed in buffer RLT. Checking of RNA quality (left panels) and quantification of β-actin mRNA by real-time PCR (right panels) were performed in the same way as in Fig 2.

homogenization affected RNA quality. In order to confirm this, we compared these samples: frozen without preservative in liquid N_2 and frozen at −80°C after RNAlater®-treatment by leaving them to thaw. In the samples without RNAlater®, signs of RNA degradation appeared at 10 min or later, whereas RNA appeared to be stable in the RNAlater®-treated samples for at least 2 hrs. It also appeared that degradation of RNA was more dependent on the time of thawing than that of storage when stored without preservatives. This clearly means that storage in RNAlater® has a merit in processing many frozen samples at once without concern for RNA degradation. The manufacturer's note points out that RNAlater®-treated samples can be repeated in the freezing-thawing cycle up to 10 times. We also confirmed this description (data not shown), although freezing-thawing is restricted to once in our general protocol.

The manufacturer's note also describes that RNAlater® is useful in preserving cultured cells, i.e., cells are scraped from the plates, harvested by centrifugation and RNAlater® added to the pellet. However, this procedure is quite cumbersome and might affect gene expression in the cell. In our project, we directly dissolved the cells by Buffer RLT Reagent, a component of the RNA-extraction kit. It would be quite convenient if the RNA in the dissolved sample were stable under this condition. The present experiments using liver tissues demonstrated that the RNA in samples dissolved in the buffer appeared to be stable for at least one year at −80°C or in liquid N_2.

From the experiments described above, we offer

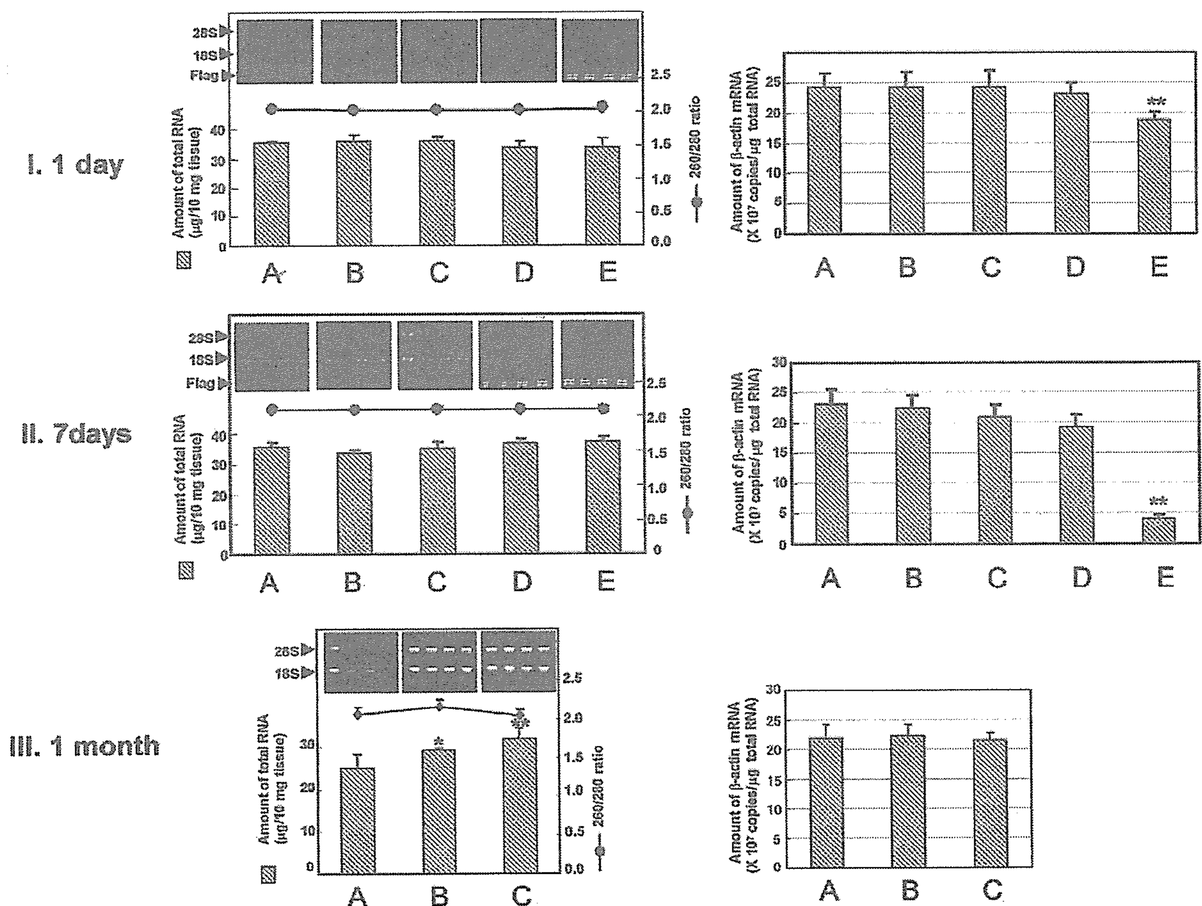
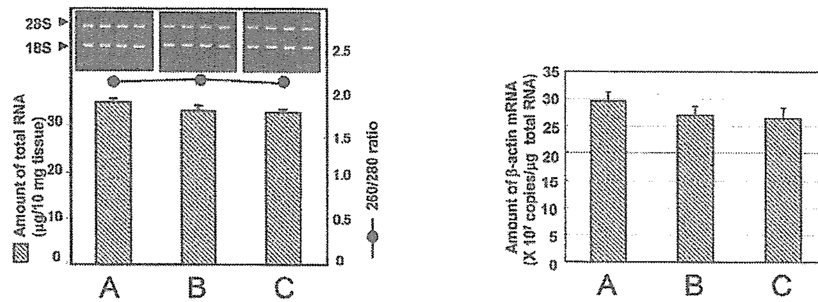
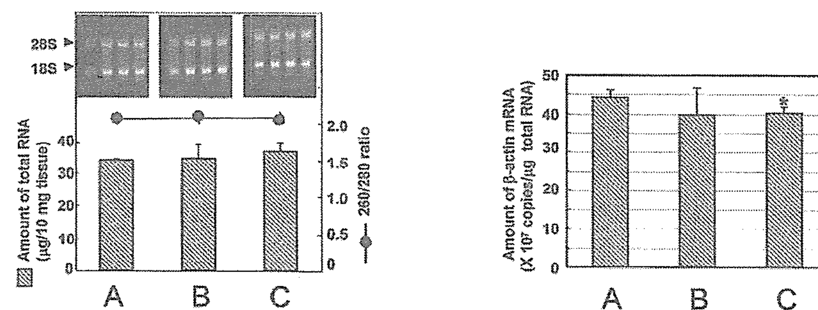


Fig. 4-1.

IV. 3 months



V. 6 months



VI. 12 months

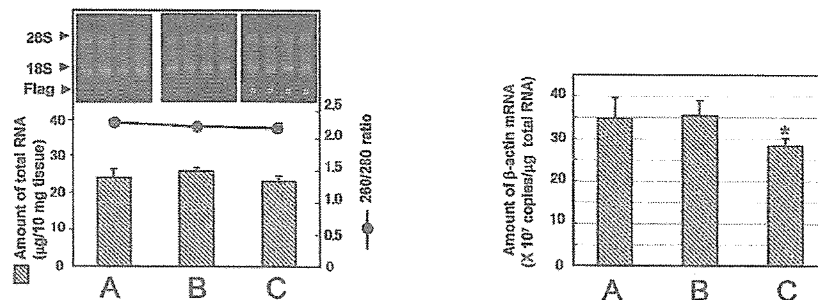


Fig. 4-2. Evaluation on stability of RNA in the sample dissolved in Buffer RLT. Rat liver tissue was dissolved in buffer RLT. The liver lysate, 100 μl each, was dispensed to microtubes and stored under the following conditions: A: in liquid nitrogen, B: −80°C, C: −20°C, D: 4°C, E: room temperature. Total RNA was extracted 1 day, 7 days, one month, 3 months, 6 months and 12 months after storage. Checking of RNA quality (left panels) and quantification of β-actin mRNA by real-time PCR (right panels) were performed in the same way as in Fig 2. The lysates stored at −20°C and room temperature (D, E) were not examined later than 7 days, because they showed obvious signs of RNA degradation at the 7th day.

the following protocol as a minimum requirement for RNA extraction from rat liver for GeneChip analysis.

1. Liver tissues are to be dissected into 5 mm cubic pieces on ice and put into a tube containing more than 10 volumes of chilled RNAlater® as soon as possible. A disposable biopsy punch is useful for this purpose.
2. The tissue samples are to be fixed in RNAlater® overnight at 4°C. It can be stored at least one day at room temperature, and 2 weeks at at 4°C, in the non-frozen state.
3. For longer storage of RNAlater®-fixed samples, they should be kept under −20°C and are stable for at least one year. For this purpose, RNAlater® is not necessarily removed.
4. When RNA is extracted from samples treated with RNAlater® and frozen for storage, they should be homogenized with Buffer RLT within 2 hr.
5. The samples dissolved in Buffer RLT can be stored for at least one year without RNA degrada-

tion at −80°C.

ACKNOWLEDGMENT

This study was supported in part by a grant from the Ministry of Health, Labor and Welfare (H14-Toxico-001).

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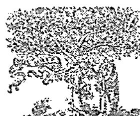

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Life Sciences xx (2005) xxx – xxx

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Effect of the difference in vehicles on gene expression in the rat liver—analysis of the control data in the Toxicogenomics Project Database

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Received 30 July 2005; accepted 1 November 2005

Abstract

The Toxicogenomics Project is a 5-year collaborative project by the Japanese government and pharmaceutical companies in 2002. Its aim is to construct a large-scale toxicology database of 150 compounds orally administered to rats. The test consists of a single administration test (3, 6, 9 and 24 h) and a repeated administration test (3, 7, 14 and 28 days), and the conventional toxicology data together with the gene expression data in liver as analyzed by using Affymetrix GeneChip are being accumulated. In the project, either methylcellulose or corn oil is employed as vehicle. We examined whether the vehicle itself affects the analysis of gene expression and found that corn oil alone affected the food consumption and biochemical parameters mainly related to lipid metabolism, and this accompanied typical changes in the gene expression. Most of the genes modulated by corn oil were related to cholesterol or fatty acid metabolism (e.g., CYP7A1, CYP8B1, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase, squalene epoxidase, angiopoietin-like protein 4, fatty acid synthase, fatty acid binding proteins), suggesting that the response was physiologic to the oil intake. Many of the lipid-related genes showed circadian rhythm within a day, but the expression pattern of general clock genes (e.g., period 2, arylhydrocarbon nuclear receptor translocator-like, D site albumin promoter binding protein) were unaffected by corn oil, suggesting that the effects are specific for lipid metabolism. These results would be useful for usage of the database especially when drugs with different vehicle control are compared.
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Keywords: Toxicogenomics; Vehicle control; Methylcellulose; Corn oil; Lipid metabolism; Rat; Liver

Introduction

The Toxicogenomics Project is a 5-year collaborative project by the National Institute of Health Sciences (NIHS) and 17 pharmaceutical companies in Japan which started in 2002 (Urushidani and Nagao, 2005). In April 2005, some rearrangements were made and now the project is conducted by NIHS, the National Institute of Biomedical Innovation, and 16 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 medicinal compounds, have been selected, and the following are examined for each. The in vivo test using rat consists of a single administration test (3, 6, 9 and 24 h with 4 dose levels including vehicle control) as well as a repeated administration test (3, 7, 14 and 28 days with 4 dose levels including vehicle control), and the data of body weight, general symptoms, histopathological examination of liver and kidney, and blood biochemistry are obtained from each animal. The gene expression in liver (and kidney in some cases) is comprehensively analyzed by using Affymetrix GeneChip. An in vitro test using rat and human hepatocytes is also carried out to accomplish the bridging between the species. By April 2005, more than 100 chemicals, covering wide medication categories, have been finished or are ongoing.

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0024-3205/$ - see front matter © 2005 Published by Elsevier Inc.
doi:10.1016/j.lfs.2005.11.010

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Along with the effects of the chemicals, a vast amount of control data is being accumulated.

The main purpose of the project is to predict toxicity in the early stage of drug development. The potential usefulness of microarray data for the estimation of toxicity of drugs makes it possible for this technology to be used in the late stage of development, i.e., application in the field of regulatory science. In this case, however, more strict and precise validation is needed in order to assure the reliability of the data. It is well known that a difference in the platform considerably effects a variation in the microarray data (Waring et al., 2004) and this is quite difficult to overcome. In our project, either methylcellulose or corn oil is employed as vehicle, according to the dispensability of the drug. It is quite possible that the difference in the vehicle control affects the analysis, as observed by multiple comparison of drug effects. In traditional toxicological study, comparison of the drug is exclusively made against its vehicle control. However, in the transcriptome database, it is usually necessary to make a comparison among various drugs by clustering or discriminant analysis. The history of this field is not old enough for collecting appropriate data regarding this issue. Our database enables us to make various comparisons among different vehicles, protocols, facilities, chip versions, etc. In this present report, we focus on the influence of vehicles on the control parameters including the gene expression profile in the rat liver as a basic study for future analysis.

Materials and methods

Animal treatment

Male Sprague-Dawley rats were purchased from Charles River Japan Inc., (Kanagawa, Japan) at 5-weeks of age. After a 7-day quarantine and acclimatization period, the animals were divided into groups of 5 animals using a computerized stratified random grouping method based on the body weight for each age. The animals were individually housed in stainless-steel cages in a room that was lighted for 12 h (7:00–19:00) daily, ventilated with an air-exchange rate of 15 times per hour, and maintained at 21–25 °C with a relative humidity of 40–70%. Each animal was allowed free access to water and pellet food (CRF-1, sterilized by radiation, Oriental Yeast Co., Japan).

According to the protocol in our project, rats in each group were orally administered with various drugs suspended or dissolved either in 0.5% methylcellulose solution or corn oil according to their dispersibility. Each drug had 4 different dose levels, including the vehicle control alone, which was exclusively analyzed in the present study. Drug treatment was performed between 9:00 and 11:30 a.m. For single-dose experiments, rats were sacrificed at 3, 6, 9, and 24 h after dosing. For repeated dose experiments, the animals were treated for 3, 7, 14 or 28 days, and they were sacrificed 24 h after the last dosing. Body weights were recorded every day while food consumption was recorded every 4 days during repeated dosing. Blood samples were collected upon sacrifice in tubes containing heparin lithium (blood biochemistry), EDTA-2K (hematology), or 1/9 vol of 3.8% citric acid (coagulation), and the following items were examined: hematology: the numbers of red blood cells, reticulocytes, white blood cells, eosinophils, monocytes, platelets, neutrophils, basophils, and lymphocytes, hemoglobin, mean red blood cell volume, mean hemoglobin contents, and mean hemoglobin concentration (Advia 120, Bayer); blood coagulation: prothorombin time, active partial prothrombin time, and fibrinogen (Sysmex CA-5000, Sysmex); and blood biochemistry: alkaline phosphatase, total cholesterol, triglyceride, phospholipid, total and direct bilirubin, glucose, blood urea nitrogen, creatinine, Na, K, Ca, Cl, inorganic phosphate, total protein, albumin, globulin/albumin ratio, aspartate aminoptansferase, alanine aminotransferase, lactate dehydrogenase, and γ-glutamyltranspeptidase, which were determined by an autoanalyzer (Hitachi 7080).

When the analysis was performed (April 2005), 65 compounds had been completed in 4 different contract research organizations. In order to eliminate the variations due to the difference in the facility, we selected a laboratory (Japan Bioassay Center, Kanagawa, Japan) where at least 7 experiments for each vehicle were completed. As 10 experiments were done with methylcellulose as the vehicle there, the latest 3 of them were excluded from the present analysis to match the numbers. Therefore, each time point consists of 35 (5 rats for 7 experiments) animals.

The experimental protocols were reviewed and approved by the Ethics Review Committee for Animal Experimentation of National Institute of Health Sciences.

Microarray analysis

After collecting the blood, the animals were euthanized by exsanguination from the abdominal aorta under ether anesthesia. An aliquot of the sample (about 30 mg) for RNA analysis was obtained from the left lateral lobe of the liver in each animal immediately after sacrifice, kept in RNA later® (Ambion, Austin, TX, USA) overnight at 4 °C, and then frozen to send to the facility in the National Institute of Health Sciences.

Total RNA was isolated using RNeasy kit by Bio Robot 3000 (Qiagen, Valencia, CA, USA). Homogenization was conducted by Mill Mixer (Qiagen) and zirconium beads. Purity of the RNA was checked by gel electrophoresis confirming the 260/280 nm ratio was between 2.0 and 2.2.

Microarray analysis was conducted on 3 out of 5 samples for each group by using GeneChip® RAE230A probe arrays (Affymetrix, Santa Clara, CA, USA), containing 15923 probe sets. The procedure was conducted basically according to the manufacturer's instructions using Superscript Choice System (Invitrogen, Carlsbad, CA, USA) and T7-$(dT)_{24}$-oligonucleotide primer (Affymetrix) for cDNA synthesis, cDNA Cleanup Module (Affymetrix) for purification, and BioArray High yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA) for synthesis of biotin-labeled cRNA. Ten micrograms of fragmented cRNA was hybridized to a RAE230A probe array for 18 h at 45 °C at 60 rpm, after which the array was washed and stained by streptavidin-phycoerythrin using

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Fluidics Station 400 (Affymetrix) and scanned by Gene Array Scanner (Affymetrix).

In the middle of the project (2004), Affymetrix released ver. 2.0 GeneChip and we switched from RAE230A to 230.2. Two out of seven experiments were performed using the new chips, and they were excluded from the present analysis in order to maintain consistency. Therefore, each time point consisted of 15 measures (3 rats for 5 experiments) in the case of gene expression analysis.

The digital image files were processed by Affymetrix Microarray Suite version 5.0 and the intensities were normalized for each chip by setting the mean intensity to 500 (per chip normalization). The results of the DNA microarray analysis are available upon request (e-mail to turushid@dwc.doshisha.ac.jp).

Statistical analysis

For conventional toxicological parameters, it is common that many unimportant changes with statistical significance are observed because of the large numbers of measurements. In the present study, more than 40 parameters were measured for 8 time points (3, 6, 9, 24 h for single and 3, 7, 14, 28 days for repeated administration). For comparison between methylcellulose and corn oil, we applied Student's t-test with Bonferroni's adjustment for each parameter, i.e., p value was multiplied by 8 and $p<0.01$ was considered to be statistically significant.

For gene expression data, it is problematic to use a standard t-test, because of too many comparisons, but it is also not good to use a too conservative adjustment, because of the small

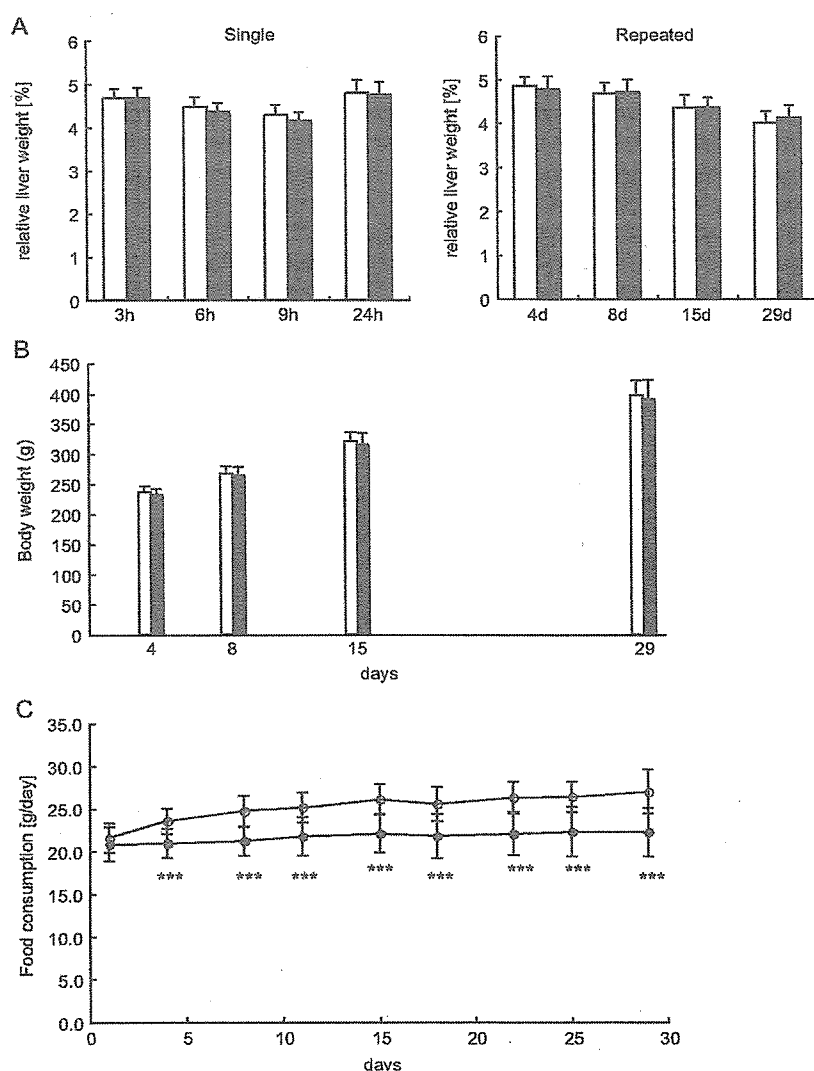

Fig. 1. Effects of different vehicles on relative liver weight, body weight, and food consumption of rats. Liver weight/body weight within 24 h after administration of vehicle and 24 h after the repeated administrations (for 3, 7, 14 and 28 days) of vehicle were measured at autopsy (A). Open and filled columns represent methylcellulose and corn oil, respectively. Body weight 24 h after the repeated administrations (for 3, 7, 14 and 28 days) of vehicle (B) and food consumption measured every 4 days and expressed as g/day (C) are plotted. Again, open and filled symbols represent methylcellulose and corn oil, respectively. Values are expressed as mean± SD of 35 rats each for each time point. Food consumption data were obtained from rats that received either vehicle for 28 days. ***Statistically significant between methylcellulose and corn oil by Student's t-test with Bonferroni's adjustment, at $p<0.001$.

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numbers of samples compared with the numbers of genes. In the present study, we considered that the β-error should be small, since our purpose was to pick up the possible vehicle effects on gene expression. Before comparison, the genes that showed less than 20 of the expression value after per chip normalization in all the samples were excluded. Genes extracted were those showing at least 1.5 fold difference between two vehicles, with $p<0.01$ (uncorrected t-test).

Results

It is common that some statistically significant but unimportant differences are observed in toxicological tests where huge numbers of parameters are measured and compared. In the present analysis of the vehicle effect, there appeared to be some differences that could not be ignored. Fig. 1A depicts the relative weight of the liver (liver weight/body weight). As is widely known, this parameter showed a clear circadian rhythm, i.e., it decreases toward the evening (9 h after dosing) and goes back in the next morning (Fig. 1A, left). In the case of rats receiving corn oil, this parameter tended to be lower than that in methylcellulose group at 6 and 9 h after dosing ($p=0.03$ and $p=0.005$, respectively, by standard t-test, but $p=0.24$ and $p=0.04$, respectively, by Bonferroni's adjustment and not significant at $p<0.01$), whereas the values returned to the same level at 24 h after administration. There was no difference in this parameter in the repeated administration, suggesting that the tendency of the decrease in the liver weight by corn oil was not accumulated during repeated dosing (Fig. 1A, right). Fig. 1 shows the body weight change (B)

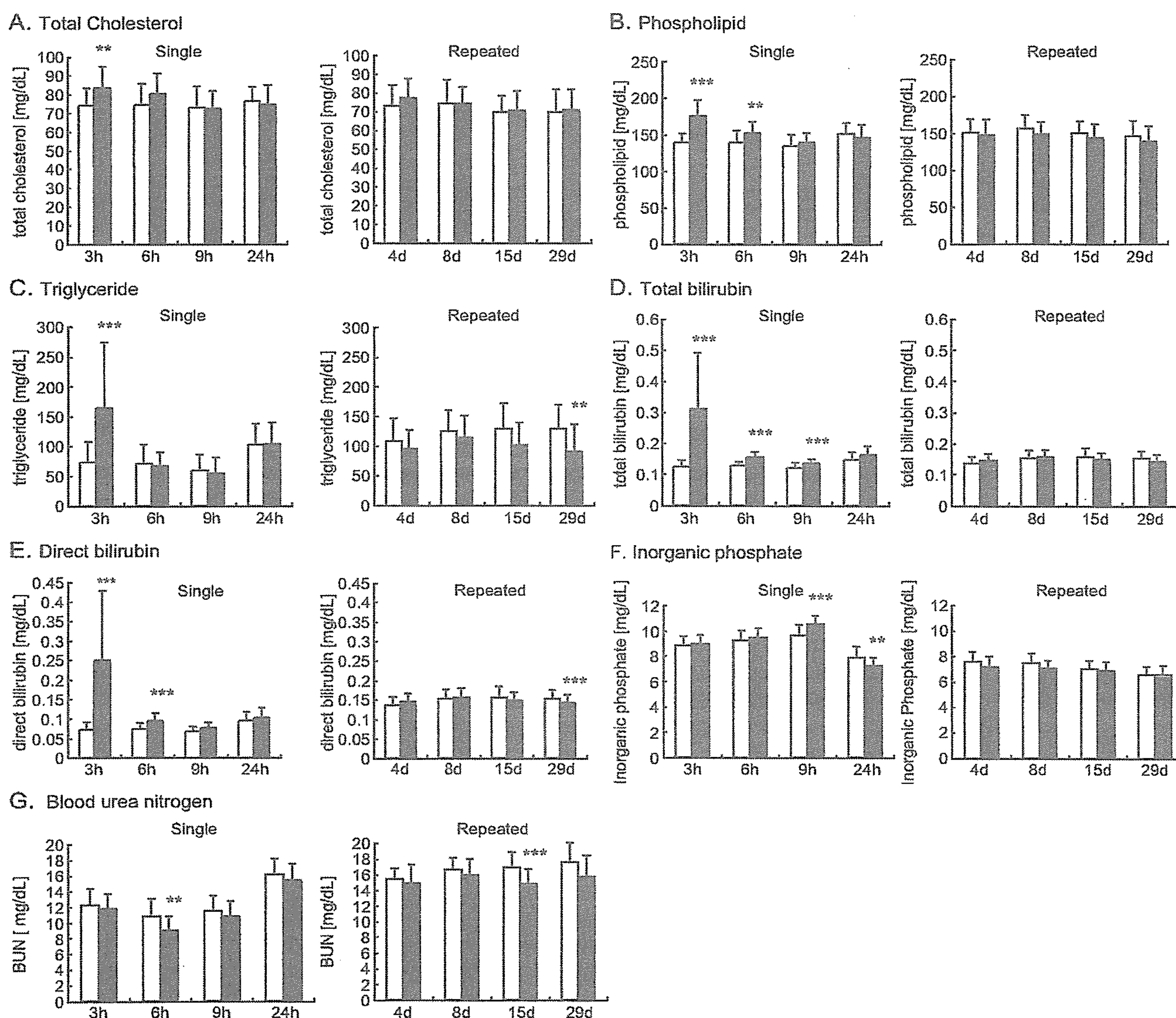

Fig. 2. Blood biochemical parameters in rats receiving methylcellulose or corn oil. Total cholesterol (A), triglyceride (B), phospholipid (C), total bilirubin (D) and direct bilirubin (E), inorganic phosphate (F), and blood urea nitrogen (G) showed a significant difference between methylcellulose (open columns) and corn oil (closed columns) among 36 parameters. Values are expressed as mean±SD of 35 rats for each time point. Statistically significant between methylcellulose and corn oil by Student's t-test with Bonferroni's adjustment, at $^{**}p<0.01$, $^{***}p<0.001$.

Table 1
List of genes showing at least a 1.5 fold difference with $p < 0.01$ (uncorrected t-test) between methylcellulose and corn oil at any point during 24 h after single dose or at 29th day of repeated dose

Probe set ID	Gene title	Gene symbol	3 h	6 h	9 h	24 h	29 d
1367707_at	fatty acid synthase	Fasn	0.995	1.738	1.300	0.722	0.596
1367708_a_at	fatty acid synthase	Fasn	0.982	1.500	1.182	0.826	0.678
1367729_at	ornithine aminotransferase	Oat	1.223	1.140	1.066	0.792	0.653
1367836_at	carnitine palmitoyltransferase 1, liver	Cpt1a	1.325	1.483	1.535	0.888	1.305
1367854_at	ATP citrate lyase	Acly	1.081	1.741	1.295	0.882	0.760
1367946_at	PDZ and LIM domain 1 (elfin)	Pdlim1	0.762	0.661	0.777	0.914	0.977
1367959_a_at	sodium channel, voltage-gated, type I, beta polypeptide	Scn1b	1.042	1.013	1.567	1.613	1.278
1368035_a_at	protein tyrosine phosphatase, receptor type, F	Ptprf	0.935	1.009	0.649	0.920	0.985
1368160_at	insulin-like growth factor binding protein 1	Igfbp1	1.239	0.485	1.238	0.631	1.143
1368272_at	glutamate oxaloacetate transaminase 1	Got1	1.623	0.989	0.965	0.687	0.813
1368275_at	sterol-C4-methyl oxidase-like	Sc4mol	0.902	1.224	1.552	0.929	0.857
1368428_at	X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound	Xpnpep2	0.839	0.896	0.668	0.783	0.916
1368435_at	cytochrome P450, family 8, subfamily b, polypeptide 1	Cyp8b1	1.534	1.860	1.570	0.711	0.798
1368458_at	cytochrome P450, family 7, subfamily a, polypeptide 1	Cyp7a1	0.471	1.768	1.310	0.586	0.607
1368569_at	aldo-keto reductase family 1, member B7	Akr1b7	0.197	0.971	0.190	2.782	5.398
1369073_at	nuclear receptor subfamily 1, group H, member	Nr1h4	1.165	1.594	1.190	0.903	1.047
1369195_at	fatty acid binding protein 2, intestinal	Fabp2	0.901	1.135	1.022	1.753	1.753
1369238_at	inhibin beta E	Inhbe	1.441	1.553	1.067	1.034	0.934
1369415_at	basic helix-loop-helix domain containing, class B2	Bhlhb2	1.019	1.814	1.449	0.983	0.862
1369440_at	ATP-binding cassette, sub-family G (WHITE), member 8	Abcg8	0.676	0.538	0.701	0.893	1.440
1369493_at	prolactin receptor	Prlr	0.609	0.958	0.717	1.147	1.975
1369663_at	epoxide hydrolase 2, cytoplasmic	Ephx2	1.073	1.346	1.616	1.196	1.844
1369674_at	purinergic receptor P2X, ligand-gated ion channel, 5	P2rx5	1.930	0.838	0.893	0.811	1.065
1369790_at	tyrosine aminotransferase	Tat	0.771	0.624	1.094	0.735	0.837
1369864_a_at	serine dehydratase	Sds	1.509	0.429	0.872	0.482	0.538
1370024_at	Fatty acid binding protein 7, brain	Fabp7	1.044	0.964	1.057	1.312	1.524
1370336_at	pregnancy-induced growth inhibitor	Okl38	0.615	0.694	0.735	1.091	1.139
1370355_at	stearoyl-Coenzyme A desaturase 1	Scd1	1.206	1.197	1.065	0.904	0.604
1370427_at	platelet derived growth factor, alpha	Pdgfa	1.066	0.912	1.155	0.559	1.097
1371127_at	bone morphogenetic protein 1 (procollagen C-proctinase)	RGD:620739	0.815	1.175	0.950	0.991	1.557
1371234_at	fibrinogen, B beta polypeptide	Fgb	1.165	0.990	0.896	0.894	0.639
1371279_at	histone 2a /// similar to Histone H2A.1	RGD:621437	1.093	0.910	0.798	0.604	0.843
1371595_at	Transcribed locus, weakly similar to XP346694.1 Rattus norvegicus LOC360381 gene	---	0.738	0.639	0.607	0.824	0.938
1371754_at	solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25	Slc25a25	0.939	0.891	1.187	0.912	0.660
1372188_at	Endothelial cell growth factor 1 (platelet-derived) (predicted)	---	1.043	1.548	1.053	1.187	1.051
1372276_at	Transcribed locus	---	0.941	1.673	1.209	1.340	1.714
1372536_at	Chaperone, ABC1 activity of bc1 complex like (S. pombe) (predicted)	---	0.953	0.973	0.659	0.904	1.082
1374265_at	Similar to arylacetamide deacetylase (esterase) (predicted)	---	0.964	1.221	1.511	0.872	0.853
1374932_at	---	---	0.818	1.705	0.745	1.055	0.897
1375367_at	PDZ and LIM domain 2	Pdlim2	1.283	1.623	1.002	0.870	0.896
1375422_at	---	---	1.094	0.936	1.875	1.039	1.025
1375552_at	---	---	0.889	1.176	0.927	0.932	1.584

Data are expressed as the ratio of gene expression (methylcellulose = 1) and columns with significant change are shaded ($N = 15$ for each group).

together with food consumption (C). Although both methylcellulose and corn oil groups got weight in the same rate during repeated dosing, food consumption in the corn oil group was significantly lower than that in methylcellulose group by about 15% throughout the period of repeated administration.

Among the hematological and blood biochemical parameters, mean corpuscular hemoglobin concentration (at 15th day), platelets (at 9 h), monocytes (at 3 h), prothrombin time (at 29th day), activated partial thromboplastin time (at 24 h), fibrinogen (at 3 h), chloride (at 3 h) showed statistically significant differences between corn oil and methylcellurose. However, these changes were not considered to be important, since their changes were small and no changes in related parameters were associated. On the other hand, total cholesterol, phospholipids, triglyceride, and bilirubin (both total and direct) were found to be significantly different between vehicle controls (Fig. 2A–E). All of them showed significantly higher values in the corn oil group at 3 h after dosing, and the differences abated or disappeared at 6 h or later. In the repeated administration, the corn oil group showed rather lower values of triglyceride, total and direct bilirubin. Inorganic phosphate showed a significantly higher value in corn oil at 9 h and went down to a lower value than methylcellulose (Fig. 2F). Blood urea nitrogen (Fig. 2G) showed a lower value in corn oil at 6 h and 15th day.

Scatter plots of gene expression between vehicle controls at each time point revealed that most of the genes distributed within a 2-fold range of their 45° line, meaning that few

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Table 1 (*continued*)

Probe set ID	Gene title	Gene symbol	3 h	6 h	9 h	24 h	29 d
1375619_at	---	---	1.171	0.667	0.789	0.971	0.756
1375796_at	interferon gamma induced GTPase (predicted)	Igtp_predicted	1.005	0.883	1.021	1.787	0.847
1376313_at	two pore segment channel 2 (predicted)	RGD:1311779	1.040	0.800	0.871	0.991	1.956
1376657_at	immunoglobulin superfamily, member 4A (predicted)	Igsf4a_predicted	1.060	1.749	1.510	0.868	1.022
1376704_a_at	necdin-like 2 (predicted)	Ndnl2_predicted	1.048	0.886	1.061	0.995	0.660
1376892_at	---	---	1.109	0.949	0.854	0.654	0.953
1376958_at	Similar to serine (or cysteine) proteinase inhibitor, clade B, member 9	---	0.456	0.824	1.105	1.206	1.039
1377361_at	---	---	1.004	0.667	0.794	0.890	1.020
1379252_at	Immunoglobulin superfamily, member 4A (predicted)	---	1.083	1.505	1.476	0.861	0.970
1383075_at	cyclin D1	Ccnd1	0.636	0.693	0.945	1.029	1.211
1384178_at	Leucine rich repeat containing 4B (predicted)	---	0.906	0.656	0.890	0.802	0.884
1384288_at	Transcribed locus	---	1.119	0.596	1.136	0.835	1.054
1386041_a_at	Kruppel-like factor	Klf2	1.986	0.850	1.345	1.004	1.053
1386789_at	---	---	1.305	0.611	1.196	1.134	1.003
1387017_at	squalene epoxidase	Sqle	0.927	1.279	1.832	1.058	0.980
1387022_at	aldehyde dehydrogenase family 1, member A1	Aldh1a1	0.769	1.099	0.913	1.039	1.804
1387123_at	cytochrome P450, family 17, subfamily a, polypeptide 1	Cyp17a1	1.003	0.982	1.106	1.309	1.613
1387183_at	carnitine O-octanoyltransferase	Crot	1.058	1.116	1.142	0.914	1.569
1387283_at	myxovirus (influenza virus) resistance 2	Mx2	0.721	0.777	0.503	1.565	1.280
1387307_at	histidine ammonia lyase	Hal	0.903	0.826	0.665	0.819	1.072
1387312_a_at	glucokinase	Gck	1.180	1.610	0.865	0.941	1.077
1387391_at	cyclin-dependent kinase inhibitor 1A	Cdkn1a	1.576	1.373	0.734	0.684	0.892
1387396_at	hepcidin antimicrobial peptide	Hamp	0.731	0.642	1.232	1.145	1.051
1387643_at	fibroblast growth factor 21	Fgf21	1.385	1.919	1.138	1.191	1.157
1387665_at	betaine-homocysteine methyltransferase	Bhmt	1.234	1.064	1.083	0.655	0.850
1387670_at	glycerol-3-phosphate dehydrogenase 2	Gpd2	1.169	1.650	1.430	0.872	0.864
1387730_at	paired box gene 8	Pax8	1.527	1.114	0.991	0.822	0.827
1387809_at	mitogen-activated protein kinase kinase 6	Map2k6	1.017	1.443	1.139	0.622	0.742
1387848_at	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	Hmgcr	0.810	1.306	1.963	1.057	0.876
1388210_at	mitochondrial acyl-CoA thioesterase 1	Mte1	1.085	1.245	1.928	1.099	1.120
1388395_at	G0/G1 switch gene 2 (predicted)	G0s2_predicted	2.129	1.930	1.041	1.204	1.036
1388426_at	sterol regulatory element binding factor 1	Srebf1	0.648	0.787	0.694	0.855	0.885
1388531_at	progesterone receptor membrane component 2 (predicted)	Pgrmc2 predicted	1.032	1.518	1.149	0.938	1.036
1388679_at	TBC1 domain family, member 14 (predicted)	Tbc1d14 predicted	0.815	1.582	1.111	0.883	1.038
1388792_at	growth arrest and DNA-damage-inducible 45 gamma (predicted)	Gadd45 gpredicted	0.340	1.095	0.994	0.669	0.595
1388872_at	Isopentenyl-diphosphate delta isomerase	Idi1	1.029	1.077	1.772	1.249	0.870
1388924_at	angiopoietin-like protein 4	Angptl4	2.172	1.589	1.105	0.673	0.990
1389161_at	Transcribed locus	---	1.547	1.566	1.153	0.819	1.014
1389253_at	vanin 1 (predicted)	Vnn1 predicted	1.127	1.614	1.846	1.232	1.650
1389430_at	Transcribed locus	---	0.917	1.198	1.517	0.863	1.008
1390383_at	adipose differentiation-related protein	ADRP	1.296	1.784	0.870	0.952	0.913
1390607_at	nNOS-intereacting DHHC-containing Dem protein-L	RGD:1303254	0.936	1.463	1.509	0.691	0.878
1390662_at	Ab2-427	---	0.594	0.996	0.819	1.114	0.980
1392607_at	Transcribed locus	---	0.756	0.855	0.725	0.441	1.298

genes were affected by the vehicle (data not shown). Table 1 shows the list of genes that showed at least a 1.5-fold difference between vehicles with $p<0.01$ either in single dose experiment or in the 29th day of repeated dosing. Many of the genes listed are related to lipid metabolism. They were usually up-regulated by corn oil between 3 and 9 h after dosing and returned to the same level or lower at 24 h and at the 29th day. However, there were some exceptional cases, such as aldo-keto reductase 1B7 (down-regulated at 3 and 9 h but up-regulated at 24 h and 29th day), or aldehyde dehydrogenase 1A1 (only up-regulated after repeated administration). Among the genes in Table 1, there are interesting ones, i.e., CYP7A1, CYP8B1, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase, fatty acid synthase, squalene epoxidase, angiopoietin-like protein 4, which are selected and shown as graphs in Fig. 3A–F. The former 5 genes all showed a circadian rhythm in that their expression in the afternoon to the evening was higher than that in the morning to noon. The administration of corn oil appeared to increase this peak. On the other hand, angiopoietin-like protein 4 showed constant expression during the day in methylcellulose, whereas corn oil markedly increased the expression of this gene at 3 and 6 h of administration.

As is obvious from Figs. 1A and 3, there exists a circadian rhythm in rat liver. In order to examine whether the observed changes were due to a disturbance in the basic rhythm, expression of various clock genes were checked and we found that the rhythm (other than that related to lipid metabolism) was relatively unaffected. As typically representative of clock genes, the expression patterns of period 2, D site albumin

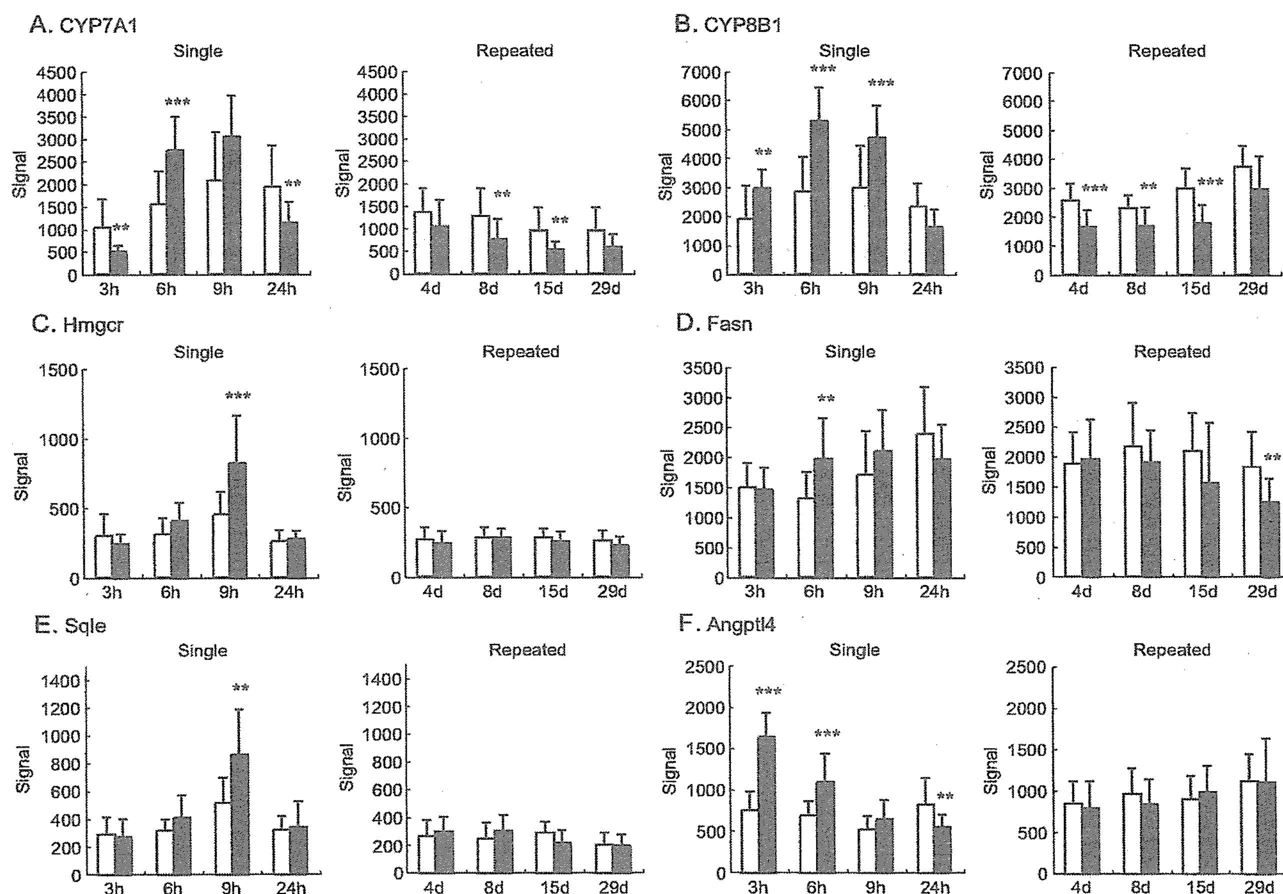

Fig. 3. Expression changes of CYP7A1 (A, Affymetrix ID 1368458_at), CYP8B1(B, Affymetrix ID 368435_at), 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (C, Affymetrix ID 1387848_at), fatty acid synthase (D, Affymetrix ID 1367708_a_at), squalene opixidase (E, Affymetrix ID 1387017_at) and angiopoietin-like protein 4 (F, Affymetrix ID 1388924_at) are shown. Open and filled symbols represent methylcellulose and corn oil, respectively. Values are expressed as mean±SD of 15 rats of each for each time point. Statistically significant between methylcellulose and corn oil by uncorrected Student's t-test at $^{**}p<0.01$, $^{***}p<0.001$.

promoter binding protein, and arylhydrocarbon receptor nuclear translocator-like are shown in Fig. 4A–C.

Discussion

The ultimate goal of our project is to create a gene expression database for prediction of hepatotoxicity in the early stage of drug development. For this purpose, it was desirable that the vehicle for suspending drugs was unified to be methylcellulose. However, there are many test compounds with poor dispersibility, and strong detergents or organic solvents are undesirable because of their potent bioactivity, so we inevitably chose corn oil as a vehicle for the highly hydrophobic compounds.

Corn oil contains 9.2 kcal/g and supplies 10.1 kcal/day for 7-week-old rats (5 ml/kg corresponds to 1.1 g for 250 g body weight). Rats around this age consume about 25 g diet per day in the present study (Fig. 1C), which corresponds to about 90 kcal/day (CRF-1 carries 3.6 kcal/g), meaning that the administered corn oil is equal to about 11% of the total calories. Moreover, this is administered once in the morning when the feeding behavior of the rat is normally inactive. Then we were concerned that the difference between corn oil and methylcellulose cannot be ignored. In fact, the food intake of the rats in the corn oil group was significantly decreased by about 15% compared with methylcellulose group without any changes in body weight. This suggests that the rats self-controlled their total calorie intake to a constant level and so corresponding gene expression changes should have occurred.

In the acute phase, total cholesterol, triglyceride, phospholipids, and bilirubin were elevated 3 h after the administration of corn oil, which were considered to be due to rapid absorption of oil. We are not sure why plasma bilirubin was increased; it might reflect an increase in the absorption of bile components when large amounts of lipid were absorbed in the form of micelle. These parameters all returned to the same level as in the methylcellulose group 6–9 h after administration. In the repeated dose experiments, which correspond to 24 h after dosing, triglyceride and bilirubin were decreased in the corn oil group, suggesting that some adapting system lowering plasma lipid was induced during a continuous elevation of lipid component in the diet.

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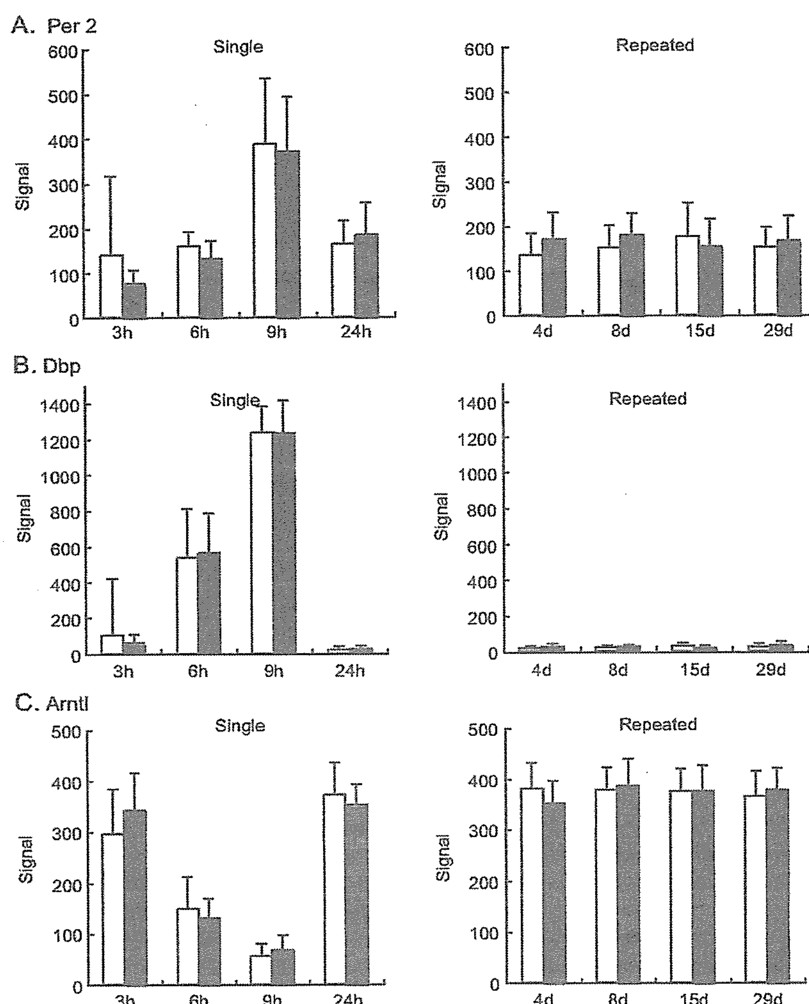

Fig. 4. Expression patterns of representative clock genes, period 2 (A, Affymetrix ID 1368303_at), D site albumin promoter binding protein (B, Affymetrix ID 1387874_at), and arylhydrocarbon receptor nuclear translocator-like (C, Affymetrix ID 1370510_a_at) are shown. Open and filled symbols represent methylcellulose and corn oil, respectively. Values are expressed as mean±SD of 15 rats for each time point. No statistically significant difference was observed between methylcellulose and corn oil at any time point.

The change in gene expression in the liver was more complex. Although the numbers of differentially expressed genes were small in respect of the total 16,000 probe sets, there were still considerable numbers of genes showing different patterns between vehicles, most of which were related to lipid metabolism. In the corn oil-treated group, the expression of CYP7A1 (cholesterol 7α hydroxylase), the rate-limiting enzyme of bile acid synthesis or elimination of cholesterol (Mast et al., 2005), showed a clear circadian rhythm as reported (Kai et al., 1995; Ishida et al., 2000), and it was lower at 3 h but higher at 6 and 9 h than that in the methylcellulose group. The expression was then lowered again at 24 h after dosing, and this pattern appeared to continue during repeated administration, i.e., the expression value in the corn oil group stayed about 60% of those in the methylcellulose group until the 29th day. On the other hand, CYP 8B1 (cholesterol 12α hydroxylase), which catalyzes the synthesis of cholic acid and controls the ratio of cholic acid over chenodeoxycholic acid in the bile, showed a less marked but obvious circadian pattern as reported (Ishida et al., 2000). It showed a continuously higher expression from 3 to 9 h than methylcellulose, returned to the same level at 24 h, and no difference was observed in the repeated dosing. These changes were considered to be the reflection of the transiently high consumption of bile due to the bolus injection of corn oil.

The rate-limiting enzyme of cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase, also showed a circadian rhythm and the administration of corn oil markedly increased its expression at 9 h. At this point, squalene epoxidase and sterol-C4-methyloxidase were also increased. The former is a microsomal enzyme that catalyzes the oxidation of squalene to 2,3-oxidosqualene, the last reaction of non-sterol metabolites in the cholesterol biosynthesis pathway (Hidaka et al., 1990). The latter is known as one of the components essential for sterol biosynthesis in yeast (Darnet and Rahier, 2003), and it used to be termed neurorep1 and was discussed in relation to the repair

process of damaged neurons (Uwabe et al., 1997). Looking at the high expression level in liver and induction by corn oil alone, we considered that the induction of sterol-C4-methyloxidase was a general phenomenon related to lipid metabolism rather than neurophysiology.

One noticeable gene is angiopoietin-like protein 4, which was recently shown to be involved not only in lipid metabolism via inhibition of lipoprotein lipase activity (Yoshida et al., 2002) but also in various diseases (Xu et al., 2005). It was reported that its expression in adipose tissue and liver was affected by the nutrient status, e.g., induced by fasting (Ge et al., 2005). In the present study, this gene was markedly up-regulated at 3–6 h after corn oil treatment and returned to the same level as methylcellulose at 9 h or later. It is of interest to elucidate why oil intake resembles fasting in case of angiopoietin-like protein 4 expression.

Fatty acid synthase was up-regulated by corn oil at 6 h after dosing and then returned to the same level as methylcellulose, whereas it was down-regulated (about 60% of methylcellulose) after repeated administration. In contrast, fatty acid binding protein family members, involved in lipid uptake, were up-regulated 24 h after single and repeated administrations of corn oil. These reactions in the repeated phase are considered to be adaptive responses suitable for lipid intake. In addition to these two enzymes, there were genes showing significantly different expression in the corn oil group at 29th day, i.e., serine dehydratase, ornithine aminotransferase, stearoyl CoA desaturase, aldehyde dehydrogenase 1A1, and epoxide hydroxylase 2. Of these, the latter two were up-regulated whereas the others were down-regulated.

The increase of aldehyde dehydrogenase and epoxide hydroxylase is considered to be favorable for the condition of high lipid diet, since both enzymes are reported to be involved in the detoxication of the metabolites associated with lipid metabolism (Choudhary et al., 2005; Newman et al., 2005). As for the down-regulated genes, the decrease of fatty acid synthase and stearoyl CoA desaturase, both are in the pathway of fatty acid synthesis, which might reflect a decrease in the need of fatty acid. Serine dehydratase and ornithine aminotransferase are known to be induced by high protein diet, glucagon, or glucocorticoid (Hunter and Harper, 1977; Bourdel et al., 1983). Based on the present data, it is difficult to conclude whether the change was due to the relative reduction of protein in the diet, or to the secondary change in endocrinological status. Moreover, it should be noted that the circadian pattern could not be obtained in the present experiments of repeated administration. Although most of the observed changes could be interpreted as an adaptation for the rapid absorption of oil from the gut in the acute phase and for the continuously elevated composition of lipid in the food in the chronic phase, it is difficult to map all the changes to various metabolic pathways, and to give reasonable explanations. Further confirmation is obviously needed, but the present study has supplied many valuable suggestions.

Many of the genes affected by corn oil treatment exhibit their own circadian rhythm generally with low expression at 3 h (around noon), increasing from 6 h (late afternoon) to 9 h (evening), and returning to low expression at 24 h (morning) of dosing. There is a long blank period between 9 and 24 h after dosing, as it was practically impossible to perform measurements after midnight in the present project. It was therefore possible that the actual peak of some genes occurred between 9 and 24 h after dosing, or midnight. We were concerned that the compulsory administration of oil in the morning disturbs not only feeding behavior but also the circadian rhythm itself. However, it was confirmed that the expression patterns of representative clock genes were unaffected, suggesting that changes in gene expression were not due to the disturbance of the original circadian rhythm. Moreover, the expression levels of clock genes in repeated dosing (corresponding to the 24 h value) were also unchanged, suggesting that disturbance of the circadian rhythm during repeated administration of oil was unlikely.

The present analysis of the data in our database would provide useful information for future experiments to elucidate the detailed mechanism of lipid metabolism. It also provides valuable information for the analysis of the activity of compounds when a comparison of chemicals dosed with different vehicles is made. Since the data accumulated in our database appeared to be of high quality and reproducibility, at least in terms of the effect of vehicles, we expect that drug actions, especially related to toxicity, may be sensitively detected using our database.

Acknowledgements

This study was supported in part by a grant from the Ministry of Health, Labour and Welfare (H14-Toxico-001).

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Microarray analysis on CYPs expression in pregnant rats after treatment with pregnenolone-16α-carbonitrile and phenobarbital

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Received 23 August 2004
Available online 2 November 2004

Abstract

We previously reported the protein expression profiles of nine cytochrome P450 isozymes (CYPs) in pregnant rat's liver, fetal liver, and placenta after treatment with pregnenolone-16α-carbonitrile (PCN), dexamethasone (DEX), or phenobarbital (PB). In this study, the gene expression of 40 CYPs and 2 orphan nuclear receptors for CYP inducers, that is, Nr1i2 (CYP3A subfamily inducible by PCN) and Nr1i3 (CYP2B subfamily inducible by PB), in pregnant rat's liver, fetal liver, and placenta was investigated at one time. Fischer 344 (F344) pregnant rats were daily treated intraperitoneally with 50 mg/kg of PCN or 80 mg/kg of PB from 13 to 16 days of gestation (DG). They were sacrificed on 17 DG, and microarray analysis using Affymetrix Rat Expression Array 230A was performed. Ten genes expression significantly increased in dam's liver in PCN group, and seven genes expression in PB group. On the other hand, four genes expression increased in fetal liver in PCN group, and three genes expression increased in PB group. Being common to dam's and fetal livers, the gene expression of Cyp3A1 (CYP3A subfamily) and cytochrome P-450e (CYP2B subfamily) increased in both PCN and PB groups. In placenta, the expression of Cyp3A1 gene was significantly induced in PB group, and it also showed a tendency to increase in PCN group. The expression of Nr1i2 gene was significantly elevated only in dam's liver of PCN group, while the expression of Nr1i3 gene showed no changes in all groups. The results of the present study of 40 CYPs gene expression mostly corresponded to our previous reports on 9 CYPs protein expression.
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Keywords: CYPs; Microarray analysis; Pregnenolone-16α-carbonitrile; Phenobarbital; Pregnant rat

Introduction

Cytochrome P450 isozymes (CYPs) are important in metabolizing xenobiotics. CYPs are divided into several groups based on their amino acid sequences, and foreign chemicals are metabolized mainly by families CYP 1–4 (Nelson et al., 1993). The majority of CYPs are found in the liver, and some CYPs are also found in extrahepatic tissues such as kidneys, adrenal glands, lungs, small intestines, brain, skin, and placenta (Black and Coon, 1987; Simmons and Kasper, 1989). Recently, the existence of the orphan nuclear receptors, which induce CYPs by some inducers such as pregnenolone-16α-carbonitrile (PCN) or phenobarbital (PB), is attracting researchers' attention (Masuyama et al., 2000; Mikamo et al., 2003; Xiong et al., 2002; Zhang et al., 1999).

Previously, we reported that CYP3A1 was detected in rat placenta through pregnancy (Ejiri et al., 2001). After that, we examined the induction of CYPs proteins in pregnant rat liver, placenta, and fetal liver by Western blot analysis and immunohistochemistry using commercially available antibodies against nine CYPs after treatment with PCN, dexamethasone (DEX), and PB. As a result, CYP3A1 protein was induced by PCN and DEX in dam's and fetal livers, with no prominent induction in placenta

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0014-4800/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexmp.2004.08.009

(Ejiri et al., 2003). In addition, after PB injection, CYP3A1 protein was significantly induced, CYP2B1 protein was detected, and CYP2D1 protein was reduced in dam's liver, and CYP3A1 and CYP2C6 proteins were induced in fetal liver (Ejiri et al., in press). In placenta, no significant induction of CYPs was observed after PB treatment.

Recently, DNA microarray technology has been developed, and an enormous amount of gene expression data can be examined at one time (Kiyosawa et al., 2003; Meneses-Lorente et al., 2003; Sehata et al., 2004). This study was carried out to examine the gene expression profiles focusing on 40 CYPs and 2 orphan nuclear receptors for CYP inducers, pregnane X receptor (Nr1i2) and constitutive androstane receptor (Nr1i3), in pregnant rat liver and placenta and fetal liver using DNA microarray technology. The protocol of this study was approved by the Animal Care and Use Committee of Graduate School of Agricultural and Life Science, the University of Tokyo.

Materials and methods

Animals

Twelve pregnant rats of the Fischer 344 (F344) strain were purchased from Saitama Experimental Animal Co. (Saitama, Japan). The animals were individually housed in plastic cages in an animal room under controlled conditions (temperature: 23 ± 2°C; relative humidity: 55 ± 5%; light/dark cycle: 14/10 h), and fed commercial pellets (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum. The day of a vaginal plug being recognized was designated as 0 day of gestation (0 DG).

Chemicals

Pregnenolone-16α-carbonitrile (PCN) was purchased from Sigma Co. (St. Louis, MO), and phenobarbital sodium

Table 1
Drug metabolizing CYP isozymes examined

CYP family	CYP subfamily	CYP	Probe ID	Accession no.
1	1A	3-Methylcholanthrene-induced rat cytochrome P-450MC	1370269_at	X00469
		Cytochrome P-450 ISF/BNF-G mRNA	1387243_at	K02422
	1B	Cytochrome P450, subfamily 1B, polypeptide 1	1368990_at	NM_012940
2	2A	Cytochrome P450 IIA1	1387511_at	NM_012692
		Cytochrome P450 IIA1	1369275_s_at	NM_012692
		Cytochrome P450, subfamily 2A, polypeptide 1 (Cyp2a2)	1369424_at	NM_012693
		Cytochrome P450IIB3	1369136_at	NM_012542
	2B	Cytochrome P450-e (phenobarbital-inducible) gene, 3′ end	1371076_at	AI454613
		Cytochrome P450, 2b19 (Cyp2b15)	1387722_at	NM_017156
		Cytochrome P450 CYP2B21	1387993_at	AF159245
		Cytochrome P450IIB3	1370475_at	M20406
	2C	Cytochrome P450 PB1 (PB1-2 allele)	1370580_a_at	M18336
		Cytochrome P450, 2c39	1370241_at	AA800502
		Cytochrome P450 2c13	1370495_s_at	J02861
		Cytochrome P450 15-beta gene (Cyp2c12)	1368155_at	NM_031572
		Cytochrome P450, subfamily IIC	1387328_at	NM_019184
		p450Md mRNA for cytochrome P-450	1387949_at	M58041
	2D	Cytochrome P450 2d18 (Cyp2d18), mRNA	1370329_at	U48220
		emb|X52029.1|RSIID4G Rattus norvegicus P450IID4 gene	1387913_at	U48220
		Cytochrome P450, subfamily IID3	1370496_at	AB008424
		Cytochrome P450, subfamily IID2	1367917_at	NM_012730
		Cytochrome P450CMF1b (Cyp2d5), mRNA	1370377_at	M25143
	2E	Cytochrome P450, subfamily 2E, polypeptide 1	1367871_at	NM_031543
	2F	Cytochrome P450, subfamily 2F, polypeptide 1	1368608_at	NM_019303
	2G	Cytochrome P450, subfamily 2G, polypeptide 1	1371142_at	M33296
	2S	Cytochrome P450, family 2, subfamily, polypeptide 1	1390282_at	BI274639
	2T	Cytochrome P450 monooxygenase CYP2T1	1368265_at	NM_134369
3	3A	Cytochrome P-450PCN (PNCN-inducible) (Cyp3A1)	1387118_at	NM_013105
		Pregnenolone 16-alpha-carbonitrile-inducible CYP	1370593_at	U09742
		Cytochrome P450, 3a18 (Cyp3a18), mRNA	1398307_at	D38381
		Cytochrome P450, 3A9 (CYP3A9), mRNA	1370387_at	U46118
4	4A	Cytochrome P450, 4a12 (Cyp4a12), mRNA	1368607_at	NM_031605
		Similar to cytochrome P450 4A3	1370397_at	M33936
		Cytochrome P450, 4A1 (Cyp4a1), mRNA	1368934_at	NM_016999
	4B	Cytochrome P450, subfamily 4B, polypeptide 1	1370399_at	M29853
	4F	Cytochrome P450 4F5 (CYP4F5), mRNA	1392720_at	BG376949
		Cytochrome P450 4F6 (CYP4F6), mRNA	1387916_at	U39208
		Cytochrome P450 4F5 (CYP4F5)	1388055_at	U39207
		Cytochrome P450, subfamily IVF, polypeptide 14	1368467_at	NM_019623
		Cytochrome P450 4F4 (CYP4F4), mRNA	1387973_at	U39206

Table 2
Primer sequences, cycle numbers, and annealing temperature

Gene	Sequence		Cycle number			Annealing temperature (°C)
			Liver	Fetal liver	Placenta	
CYP3A1	Sense	GAGGAGTAATTTGCTGACAGACCTGC	24	26	34	67
	Antisense	CCAGGAATCCCCTGTTTCTTGAA				
GAPDH	Sense	GAGTATGTCGTGGAGTCTACTG	22	22	22	58
	Antisense	GCTTCACCACCTTCTTGATGTC				

(PB) and corn oil from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). PCN was dissolved in corn oil, and PB was dissolved in physiological saline immediately before used; the concentration was adjusted to 50 mg/ml for PCN and 80 mg/ml for PB.

Treatments

From 13 to 16 DG, three dams were daily treated intraperitoneally with 50 mg/kg of PCN (PCN group), three dams with 80 mg/kg of PB (PB group), three dams with 0.1 ml/kg of corn oil alone (CO group) as control for PCN group, and three dams with 0.1 ml/kg of saline alone (Sa group) as control for PB group, respectively. All animals were sacrificed by exsanguination under ether anesthesia on 17 DG.

Histopathological examination

At necropsy, halves of dam's liver, fetal liver, and placenta were fixed in 10% neutral-buffered formalin. Paraffin sections (4 μm) were stained with hematoxylin and eosin (HE) for histopathological examination.

RNA extraction and microarray analysis

The remaining halves of dam's liver, fetal liver, and placenta were cut into slices less than 5mm thick as soon as possible. Then, the slices were submerged in the RNA*later* RNA Stabilization Reagent. After incubation at 4°C for overnight, samples were stored at −80°C until used. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN Inc., CA, USA) from frozen tissues

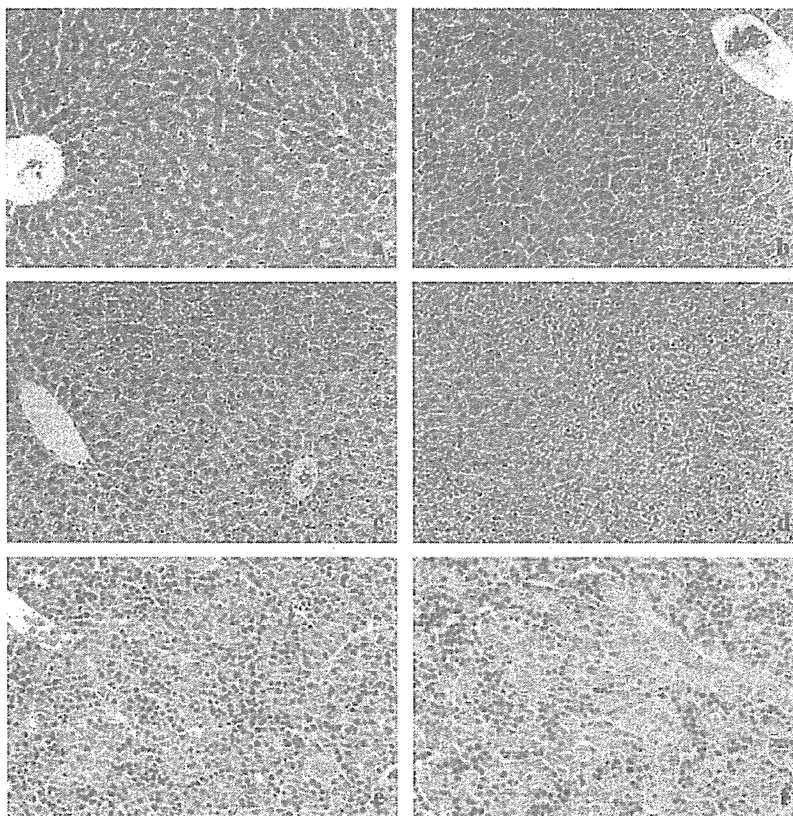

Fig. 1. Histology of dam's liver of CO group (a), PCN group (b), Sa group (c), and PB group (d), and histology of fetal liver of CO group (e) and PCN group (f). In the dam's liver, slight swelling of hepatocytes is observed in b, and moderate vacuolization of hepatocytes is observed in d. In the fetal liver, slight swelling of hepatocytes is observed in f. HE, ×100 (a, b, c, and d) or ×200 (e and f).

Table 3
Changes in CYPs genes expression in dam's liver, fetal liver, and placenta of pregnant rats treated with PCN

		Genes	Fold changes	*t* test	Accession no.
Dam's liver					
Up-regulated	2A	Cytochrome P450 IIA1 (hepatic steroid hydroxylase IIA1) gene (Cyp2a1)	1.573	0.000	NM_012692
		Cytochrome P450 IIA1 (hepatic steroid hydroxylase IIA1) gene (Cyp2a1)	1.676	0.005	NM_012692
	2B	Cytochrome P-450e (phenobarbital-inducible) gene, 3' end and flank	5.608	0.000	AI454613
		Cytochrome P450IIB3 (Cyp2b3)	1.075	0.010	M20406
	2C	p450Md mRNA for cytochrome P-450	1.332	0.028	M58041
	2F	Cytochrome P450, subfamily 2F, polypeptide 1 (Cyp2f1)	1.453	0.023	NM_019303
	3A	Cytochrome P-450PCN (PNCN-inducible) (Cyp3A1)	2.019	0.012	NM_013105
		Cytochrome P450, 3a18 (Cyp3a18)	16.276	0.000	D38381
		Cytochrome P450 3A9 (CYP3A9)	1.210	0.024	U46118
	4F	Cytochrome P450 4F4 (CYP4F4)	1.352	0.031	U39206
Down-regulated	1A	Cytochrome P-450 ISF/BNF-G	0.308	0.005	K02422
	4A	Cytochrome P450, 4A1 (Cyp4a1)	0.504	0.014	NM_016999
Fetal liver					
Up-regulated	2A	Cytochrome P450 IIA1 (hepatic steroid hydroxylase IIA1) gene (Cyp2a1)	1.433	0.029	NM_012692
	2C	p450Md mRNA for cytochrome P-450	3.893	0.006	M58041
	3A	Pregnenolone 16-alpha-carbonitrile-inducible cytochrome P450	21.486	0.047	U09742
		Cytochrome P-450PCN (PNCN-inducible) (Cyp3A1)	21.361	0.001	NM_013105
Placenta					
Up-regulated	3A	Cytochrome P-450PCN (PNCN-inducible) (Cyp3A1)	2.786	0.166	NM_013105

according to the manufacturer's instructions. Microarray analysis was performed according to the Affymetrix protocol. Briefly, of total RNA, 10 μg was used for cDNA synthesis using the T7-$(dT)_{24}$ primer [primer sequence: 5′-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG -$(dT)_{24}$-3′]. Following this, biotin-labeled cRNA was synthesized from the cDNA using the Enzo High Yield RNA Transcription Labeling Kit (Enzo Diagnostics, NY, USA). Then 25 μg of biotin-labeled cRNA was fragmented and stored at −20°C until ready to perform hybridization. The hybridization solution was prepared using GeneChip Eukaryotic Hybridization Control Kit (Affymetrix) and was hybridized to the Affymetrix Rat Expression Array 230A at 45°C for 16 hours in GeneChip Hybridization Oven 640 (Affymetrix). The chips were washed and stained using the Fluidics Station (Affymetrix), and scanned with GeneArray Scanner.

Data analysis

The microarray imaging data were analyzed using the Microarray Suite 4.0 (Affymetrix) and Spotfire Pro Version 4.2 program (Spotfire Inc., MA, USA). After global

Table 4
Changes in CYPs gene expression in dam's liver, fetal liver, and placenta of pregnant rats treated with PB

		Genes	Fold changes	*t* test	Accession no.
Dam's liver					
Up-regulated	2A	Cytochrome P450 IIA1 (hepatic steroid hydroxylase IIA1) gene (Cyp2a1)	1.344	0.008	NM_012692
		Cytochrome P450 IIA1 (hepatic steroid hydroxylase IIA1) gene (Cyp2a1)	1.347	0.023	NM_012692
	2B	Cytochrome P-450e (phenobarbital-inducible) gene, 3′ end and flank	12.975	0.005	AI454613
	2C	Cytochrome P450 PB1 (PB1-2 allele)	1.086	0.037	M18336
	2D	Cytochrome P450, subfamily IID2 (Cyp2d2)	1.235	0.012	NM_012730
	2F	Cytochrome P450, subfamily 2F, polypeptide 1 (Cyp2f1)	1.403	0.021	NM_019303
	3A	Cytochrome P-450PCN (PNCN-inducible) (Cyp3A1)	2.006	0.000	NM_013105
Down-regulated	1A	Cytochrome P-450 ISF/BNF-G	0.651	0.002	K02422
	2E	Cytochrome P450, subfamily 2E, polypeptide (Cyp2e1)	0.802	0.042	NM_031543
Fetal liver					
Up-regulated	2A	Cytochrome P450 IIA1 (hepatic steroid hydroxylase IIA1) gene (Cyp2a1)	1.146	0.038	NM_012692
	2B	Cytochrome P-450e (phenobarbital-inducible) gene, 3′ end and flank	6.703	0.000	AI454613
	3A	Cytochrome P-450PCN (PNCN-inducible) (Cyp3A1)	1.664	0.000	NM_013105
Down-regulated	4A	Cytochrome P450 4A3 (CYP IVA3)	0.542	0.047	M33936
Placenta					
Up-regulated	3A	Cytochrome P-450PCN (PNCN-inducible) (Cyp3A1)	24.784	0.047	NM_013105