Pregnant F344/Jcl rats were obtained from Saitama Experimental Animal Company, Saitama, Japan. They were kept under controlled conditions (temperature, 23 ± 2°C; relative humidity, 55 ± 5%) using an isolator caging system and were fed commercial pellets (MF, Oriental Yeast Co., Tokyo, Japan) and normal tap water *ad libitum*.

Treatments

Pregnant rats were injected with 60 mg/kg of ENU (Sigma, St. Louis, MO) or an equivalent volume of buffer alone intraperitoneally on day 13 of gestation, and dams were euthanized and fetuses were collected at 3, 6, 12, 24 and 48 h after the treatment, respectively. Fetal telencephalon was excised from a fetus and subjected to the cell cycle and microarray analyses.

Cell cycle analysis by laser scanning cytometer

Two fetal telencephalons from a dam were pooled and cells were isolated by mechanical trituration. After being washed with PBS, cells were smeared onto a slide glass by CytoFuge2 (StatSpin, Norwood, MA) and fixed in 70% ethanol at 4°C. Cells were treated with RNase A (Sigma) and stained with propidium iodide (Sigma). Cell cycle phase analysis was carried out using a laser scanning cytometer (Olympus, Tokyo, Japan). Ten thousand cells were examined in each sample. Using the WinCyte analysis software (Olympus), doublets and debris were discarded and then percentages of cells in the various phases of the cell cycle were calculated.

Microarray analysis

Four to six fetal telencephalons from a dam were pooled and total RNA was isolated with RNeasy Protect Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. Microarray analysis was carried out according to the

Affymetrix Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Briefly, the second strand cDNA was prepared from total RNA using the SuperScript double-strand cDNA synthesis kit (Invitrogen, Carlsbad, CA) with T7-$(dT)_{24}$ primer (primer sequence: 5'-GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG-$(dT)_{24}$-3', Amersham Biosciences, Piscataway, NJ). Then, biotin-labeled cRNA was synthesized from the double-stranded cDNA using the Enzo High Yield RNA Transcription Labeling Kit (Enzo Life Sciences, Farmingdale, NY). The hybridization solution was prepared with GeneChip Eukaryotic Hybridization Control Kit (Affymetrix), and hybridized to the Affymetrix Rat Expression Array U34A for 16 h at 45°C in GeneChip Hybridization Oven 640 (Affymetrix). The chips were then washed and stained using the Fluidics Station (Affymetrix), and scanned with GeneArray Scanner (Hewlett Packard, Palo Alto, CA). Microarray analyses were performed twice in each time point using total RNA samples from independent pairs of ENU-treated and control dams.

Microarray data analysis

The microarray imaging data were analyzed using the MicroarraySuite (Affymetrix). After hybridization intensity data were captured, intensity values of each probe were automatically calculated. Data were compared between the ENU-treated and control groups. Prior to comparing any two measurements, scaling (target signal value = 500) and global normalizing procedure were performed. The fold change was derived by the ratio of average differences from one experimental array compared with a control array. ESTs and genes with low reliability (detection *p*-value

> 0.05) were discarded from the data. A greater than 1.5-fold increase or decrease in expression was used as criteria for a meaningful change in gene expression between the ENU-treated and control groups.

Results

Cell cycle analysis by laser scanning cytometer

An accumulation of cells in the S phase and an increase in the number of apoptotic cells (cells with sub-G1 DNA content) were observed immediately after the administration of ENU as also observed in a previous study (Katayama et al., 2005). The number of cells in the S phase peaked at 6 h and that of apoptotic cells peaked at 12 h after the treatment. Both apoptosis and cell cycle alteration returned to the control lever at 48 h after the treatment (Fig. 1). Some of the fetuses were also analyzed histopathologically using hematoxylin and eosin-stained sections and found an increase in the number of apoptotic cells in ENU-administered fetuses as also observed in a previous study (Katayama et al., 2001; data not shown).

Microarray analysis

From the cell cycle analysis, two time points were selected for microarray analysis. It was anticipated that genes involved in apoptosis and cell cycle arrest would be identified at 6 h after the treatment (injury phase) and genes involved in regeneration and carcinogeneis would be identified at 24 h after the treatment (recovery phase). Up-regulated and down-regulated genes at each time point are presented in tables 1 to 4.

At 6 h after the treatment (injury phase), up-regulation of p53 target genes (p21, cyclin G1, Mdm2 and Bax) was detected as also observed in a previous study (Table1;

Katayama et al., 2002). The tumor suppressor p53 has been implicated in the cellular responses to DNA-damaging agents. In response to DNA damage, p53 is up-regulated and transactivates a series of genes involved in the induction of apoptosis, cell cycle arrest and DNA repair (Ko and Prives, 1996). It has been shown that neuroepithelial cell apoptosis and cell cycle arrest induced by ENU are also mediated by the regulation of p53 and p53 target genes (Leonard et al., 2001; Katayama et al., 2002, 2005). The data from the present study further confirmed the involvement of p53 and its transcriptional target genes in ENU-induced neuroepithelial cell apoptosis and cell cycle arrest. These data also demonstrate the high reliability of the present microarray analysis. In addition, increased expression of other genes involved in cell cycle control (retinoblastoma protein and PCNA) and DNA repair (O^6-methylguanine-DNA methyltransferase) and decreased expression of cholesterol biosynthesis-related genes (liver stearoyl-coenzyme A desaturase, cytosolic 3-hydroxy 3-methylglutaryl coenzyme A synthase, squalene synthase, growth-response protein (CL-6)) were detected (Tables 1 and 2).

At 24 h after the treatment (recovery phase), up-regulation of genes involved in cell proliferation (cyclin D1, IgE binding protein, Id1.25 and Id3a) was detected (Table 3). Cyclin D1 plays a central role in the cell cycle transition through G1 to S phase (Sherr and Roberts, 1999). IgE binding protein, also called as galectin-3, is involved in the regulation of Wnt/β-catenin signaling pathway (Shimura et al., 2004). Id proteins are transcriptional factors and inhibit cell differentiation and promote proliferation (Ruzinova and Benezra, 2003). It is reasonable to consider that cell cycle progression is accelerated to compensate for the lost populations in the recovery phase. Though their precise roles have not been clarified yet, the expression of a few

transcriptional factors (NF1-X1 and LIM homeodomain protein (LH-2)) was decreased (Table 4).

Discussion

Studies have shown that from gestational day 12 to 13, about 70% of mouse telencephalic cells are neuroepihtelial cells (D'Sa-Eipper and Roth, 2000); therefore, the results from our cell cycle and microarray analyses are considered to mainly represent the changes in the cell cycle distribution and gene expression in neuroepithelial cells.

ENU alkylates mainly the O^6 position of guanine (Shibuya and Morimoto, 1993), and O^6-alkylguanine induces GC-AT transitions. O^6-methylguanine-DNA methyltransferase plays an important role in the elimination of DNA ethyl adducts (Bronstain et al., 1992), and its expression was elevated in the injury phase. However, the brain eliminates O^6-alkylguanine at a much lower rate than other organs, and the long-term retention of O^6-alkylguanine in the brain is thought to be the cause of brain neoplasms (Koestner, 1990). It is reported that both DNA excision repair and O^6-methylguanine-DNA methyltransferase are necessary for the removal of O^6-alkylguanine (Bronstain et al., 1992). Thus, it is possible that DNA excision repair dose not work well in the fetal CNS and requires further evaluation.

Cholesterol metabolism plays an essential role in neural development (Farese and Herz, 1998), and squalene synthase deficient mice exhibit defective neural tube closure (Tozawa et al., 1999). In injury phase, several enzymes required for cholesterol biosynthesis (liver stearoyl-coenzyme A desaturase, cytosolic 3-hydroxy 3-methylglutaryl coenzyme A synthase, squalene synthase) were down-regulated. In addition, the expression of growth-response protein (CL-6), which is also known to be

involved in cholesterol homeostasis (Janowski, 2002), was decreased. Decreased expression of these genes including squalene synthase may have some roles in the abnormal development of the CNS caused by the ENU administration (Katayama et al., 2000).

In the recovery phase, the expression of two Id genes was elevated. Id proteins act as dominant negative antagonists of the basic helix-loop-helix (bHLH) family of the transcription factors, which positively regulate differentiation in many cell lineages (Ruzinova and Benezra, 2003). bHLH factors are also known to have important roles in cell fate decisions during corticogenesis (Ross et al., 2003). In the developing CNS, Id proteins are expressed in neuroepithelial cells (Jen et al., 1997), and thought to maintain neuroepithelial cells in an undifferentiated state by inhibiting bHLH factors (Ross et al., 2003; Iavarone and Lasorella, 2004). Id proteins are also expressed in astrocytic tumors, and the degree of expression of Id proteins well correlates with the grade of tumor malignancy (Vandeputte et a., 2002; Iavarone and Lasorella, 2004). The results from the present study further support the importance of bHLH factors in neurogenesis and suggest that Id proteins work to maintain neuroepithelial cells in a proliferative state to recover the lost populations.

In addition, the expression of osteopontin was prominently elevated in the recovery phase. Osteopontin is expressed in macrophages of the developing brain and contribute to their migration and phagocytic function (Choi et al., 2004). At 24 h after the ENU administration, the number of apoptotic cells began to decrease and phagocytosis of apoptotic cells was frequently observed (Fig. 1. and Katayama et al., 2001). It is suggested that osteopontin plays an important role in the elimination of apoptotic cells by phagocytosis in the fetal CNS.

In both the injury and recovery phases, the up-regulation of genes involved in the drug metabolism was detected. Among them, the expression of epoxide hydrolase prominently increased in both the phases. Epoxide hydrolase is known to play an important role in the detoxification of some DNA-damaging agents (Herrero et al., 1997). Though, it is still controversial that fetal CNS really has the drug metabolizing activity, drug metabolizing enzymes are induced after the ENU administration at least in the mRNA level.

In the present study the up-regulation of 21 genes in injury and 15 genes in recovery phases and down-regulation of 5 genes in injury and 3 genes in recovery phases were identified. Some of these genes are already known to play an important role in the injury and recovery of CNS, but others are not. Despite the precise involvement of these genes and functions need to be elucidated, the data presented here will aid in the identification of genes that play a significant role in the cell death and proliferation of neuroepithelial cells. The results from the present study will provide a better understanding of the mechanisms of development, regeneration and carcinogenesis of CNS as well as the mechanisms of ENU-induced fetal CNS injury and recovery.

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Figure legends

Fig. 1. Cell cycle analysis of cells isolated from fetal telencephalon after the ENU administration. Cells were isolated from fetal telencephalon and stained with propidium iodide and cell cycle phase analysis was carried out by laser scanning cytometer. Percentages for sub-G1 (a), G0/G1 (b), S (c) and G2/M (d) phases. Δ control group, ♦ ENU-treated group. Percentages for each cell cycle phase are presented as the mean ± standard deviation (SD) of three dams.

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Table 1. Up-regulated genes at 6 h after the ENU treatment (injury phase).

	fold change	accession number
apoptosis, cell cycle control		
p21 (cip1)	10.05 ± 7.05	L41275
cyclin G1	6.41 ± 0.06	X70871
Mdm2	1.93 ± 0.39	AA875509
retinoblastoma protein	1.93 ± 0.37	D25233
Bax	1.66 ± 0.22	S76511
rBax alpha	1.65 ± 0.23	U49729
proliferating cell nuclear antigen (PCNA)	1.64 ± 0.26	M24604
DNA repair, replication, transcription		
DNA primase small subunit	1.55 ± 0.27	U67994
O^6-methylguanine-DNA methyltransferase	1.53 ± 0.26	M76704
metabolism		
epoxide hydrolase	3.44 ± 2.54	M26125
peroxisome forming factor	2.15 ± 0.52	E03344
UDP-glucose:ceramide glycosyltransferase	1.67 ± 0.33	AF0477007
pyruvate dehydrogenase phosphatase isoenzyme 1	1.59 ± 0.16	AF062740
phospholipase A-2-activating protein (plap)	1.59 ± 0.01	U17901
signal transduction		
extracellular signal-related kinase (ERK3)	1.71 ± 0.59	M64301
extracellular matrix-related		
lumican	2.01 ± 1.02	X84039
others		
5S rRNA	2.85 ± 0.83	X83747
beta-1,2-N-acetylglucosaminyltransferase II (Gnt II)	2.26 ± 0.82	U21662
chromogranin B (Chgb)	1.80 ± 0.12	AF019974
latexin	1.56 ± 0.24	X76985
cyclophilin	1.52 ± 0.41	M19533

Fold changes are expressed as the mean ± standard deviation (SD) of two experiments.

Table 2. Down-regulated genes at 6 h after the ENU treatment (injury phase).

	fold change	accession number
metabolism		
liver stearoyl-CoA desaturase	-2.02 ± 0.29	J02585
cytosolic 3-hydroxy 3-methylglutaryl coenzyme A synthase (EC 4.1.3.5)	-1.63 ± 0.25	X52625
squalene synthetase	-1.56 ± 0.18	M95591
others		
growth-respons protein (CL-6)	-1.91 ± 0.29	L13619
pituitary tumor transforming gene (PTTG)	-1.51 ± 0.08	U73030

Fold changes are expressed as the mean ± SD of two experiments.

Table 3. Up-regulated genes at 24 h after the ENU treatment (recovery phase).

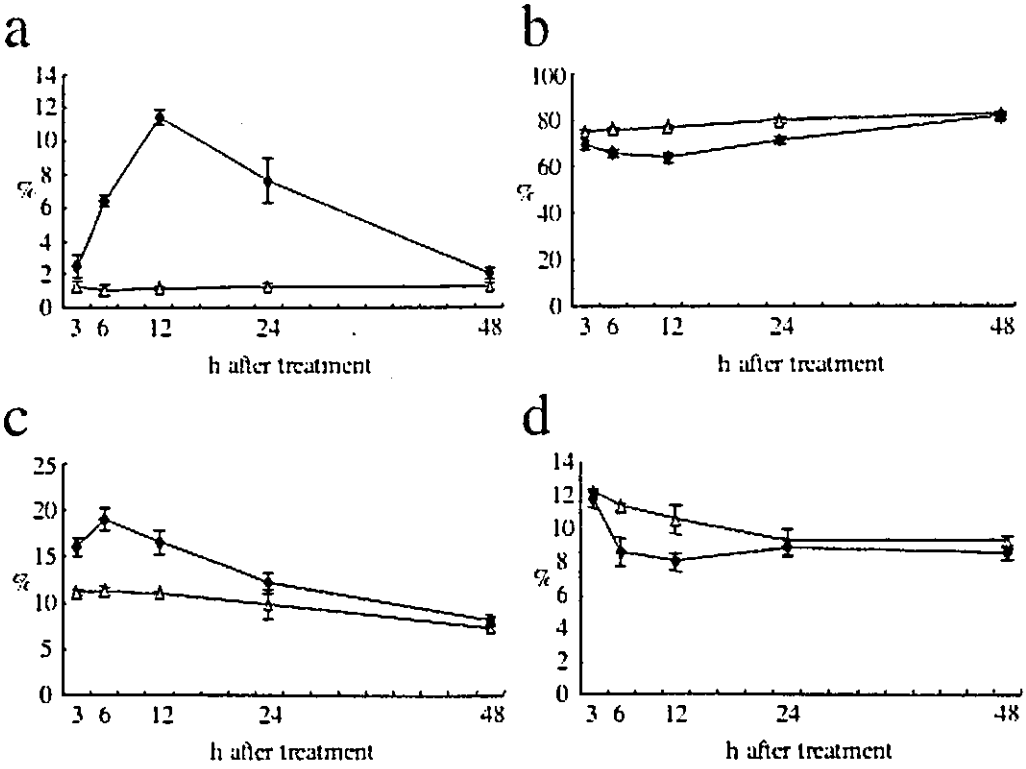
	fold change	accession number
apoptosis, cell cycle control		
cyclin G1	4.24 ± 0.02	X70871
cyclin D1	1.52 ± 0.27	D14014
DNA repair, replication, transcription		
inhibitor of DNA-binding, splice variant Id1.25	1.59 ± 0.24	L23148
Id3a	1.51 ± 0.44	AF000942
metabolism		
epoxide hydrolase	3.83 ± 2.81	M26125
long chain acyl-CoA dehydrogenase (LCAD)	1.62 ± 0.24	J05029
signal transduction		
type II cAMP-dependent protein kinase regulatory subunit	1.98 ± 0.73	M12492
serum and glucocorticoid-regulated kinase (sgk)	1.59 ± 0.33	L01624
cytoskeleton		
alpha-internexin	2.08 ± 0.51	M73049
alpha actinin	1.54 ± 0.33	U19893
extracellular matrix-related		
osteopontin	2.59 ± 0.08	M14656
SPARC	1.60 ± 0.62	U75929
others		
IgE binding protein	6.83 ± 6.01	J02962
ASM15	1.59 ± 0.20	X59864
stannin	1.54 ± 0.39	M81639

Fold changes are expressed as the mean ± SD of two experiments.

Table 4. Down-regulated genes at 24 h after the ENU treatment (recovery phase).

	fold change	accession number
DNA repair, replication, transcription		
NF1-X1	-1.81 ± 0.21	AB012234
LIM homeodomain protein (LH-2)	-1.51 ± 0.12	L06804
signal transduction		
RAB14	-1.63 ± 0.29	M83680

Fold changes are expressed as the mean ± SD of two experiments.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Microarray analysis on CYPs expression in pregnant rats after treatment with pregnenolone-16α-carbonitrile and phenobarbital

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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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(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

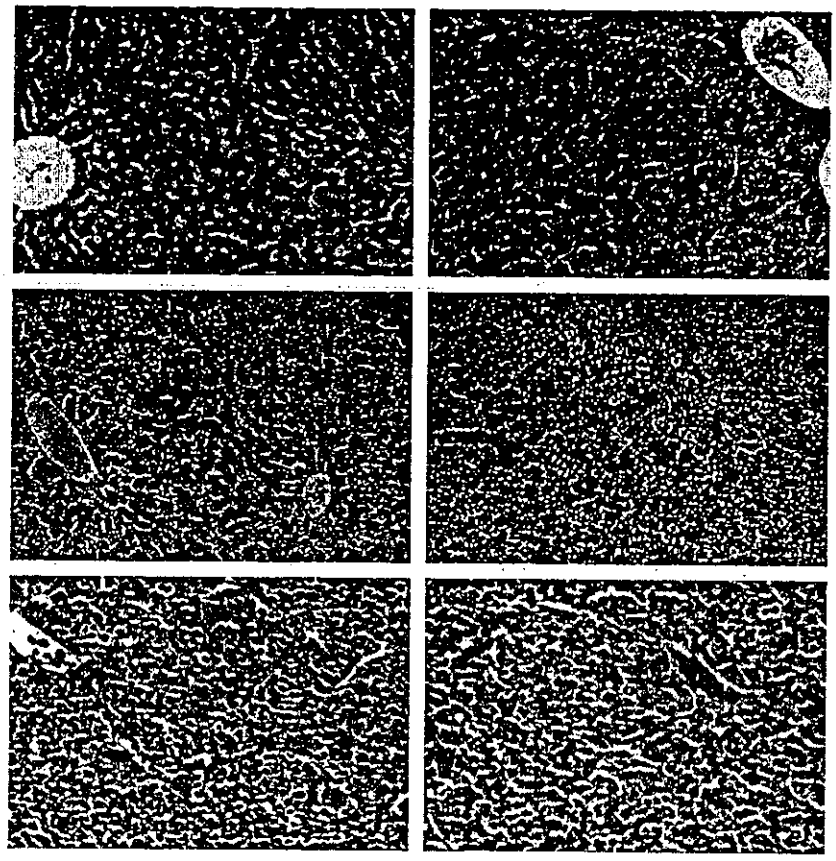

Fig. 1. Histology of dam's liver of CO group (a), PCN group (b), Sa group (c), and PB group (d), and histology of fetal liver of CO group (e) and PCN group (f). In the dam's liver, slight swelling of hepatocytes is observed in b, and moderate vacuolization of hepatocytes is observed in d. In the fetal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