significantly (Fig. 1). The relative liver weight of dams significantly increased in the PB-treated group (Fig. 1).

Findings of Western blot analysis

In the dam's liver, although CYP2B1 protein was not detected in the control group, it was easily detected in the PB-treated group (Figs. 2 and 3). CYP3A1 protein was significantly induced, while CYP2D1 protein was significantly decreased after PB-treatment (Figs. 2 and 3). CYP1A1, 2C6, 2C12, 2E1, and 4A1 proteins were not induced following the PB-treatment, and CYP2D4 protein was not detected in either the control or PB-treated groups.

In the placenta, only CYP3A1 protein was detected, and the value of band intensity was not significantly changed after the PB treatment (Figs. 2 and 3).

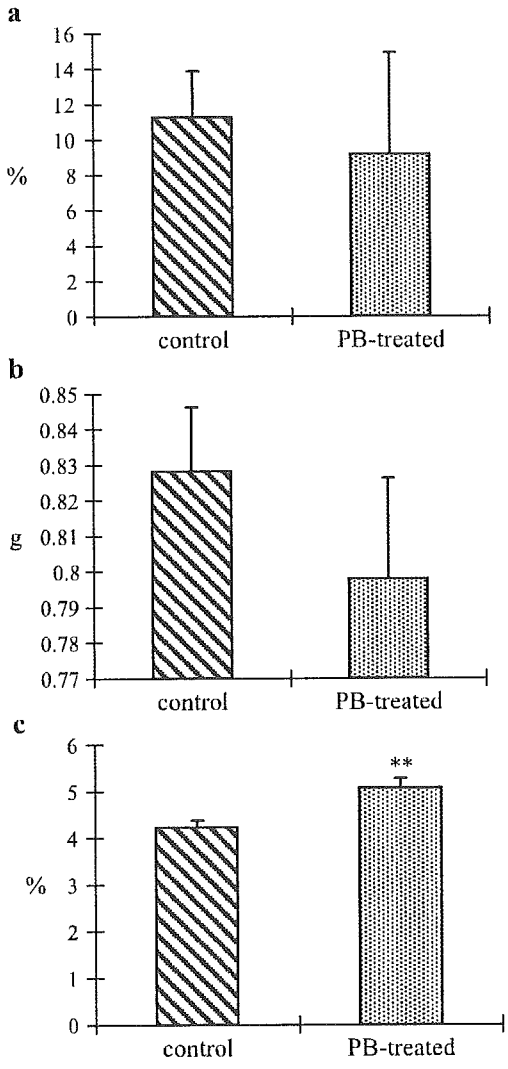

Fig. 1. Changes in body weight gain (a), fetal body weight (b) and relative liver weight in dams (c) after PB-treatment. (▨) control group, (▩): PB-treated group. Data are expressed as mean + SD of 5 dams. $*P < 0.05$ and $**P < 0.01$: significantly different from the control group.

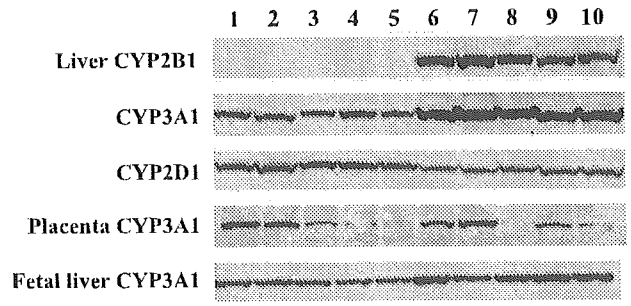

Fig. 2. The results of Western blot analysis. 1–5: rat number of the control group; 6–10: rat number of the PB-treated group.

In the fetal liver, CYP3A1 protein was significantly induced in the PB-treated group (Figs. 2 and 3). CYP2C6 protein was also significantly but slightly induced in the PB-treated group (Fig. 3). CYP2D1, 2E1, and 4A1 proteins showed no significant changes after the PB treatment, and CYP 1A1, 2B1, 2C12, and 2D4 proteins were not detected in both the control and PB-treated groups.

Histological findings

In the dam's liver, there were no histopathological changes in the control group (Fig. 4a). After the PB treatment, moderate vacuolization of hepatocytes was observed (Fig. 4b). There were no histopathological changes in the placenta and fetal liver in both the control and PB-treated groups.

Immunohistochemical findings

Among nine CYPs used in this study, CYP1A1, 2C6, 2D1, 2E1 and 3A1 proteins were clearly observed in the dam's liver in both the control and PB-treated groups. Positive signals for CYP3A1 were observed only in one layer of hepatocytes surrounding the central vein in the control group (Fig. 5a), while CYP3A1-positive hepatocytes were observed in the central and midzonal areas of the hepatic lobule in the PB-treated group (Fig. 5b). Positive signals for CYP1A1, 2C6, 2D1, and 2E1 were observed mainly in the central and midzonal areas of the hepatic lobule in the control group. The stainability for CYP2D1 protein was weakened in the PB-treated group compared with that in the control group (Figs. 5c and d), while the stainabilities for CYP1A1, 2C6, and 2E1 were not changed after the PB treatment.

Hepatocytes positive for CYP2B1 appeared after the PB treatment, although there were no CYP2B1-positive hepatocytes in the control group (Figs. 5e and f). A few hepatocytes weakly positive for CYP4A1 was observed in both the control and PB-treated groups, and there were no positive signals for CYP2C12 and 2D4 proteins in either the control or PB-treated groups.

In the placenta, CYP3A1 protein was detected immunohistochemically in the cytoplasm of trophoblastic giant cells

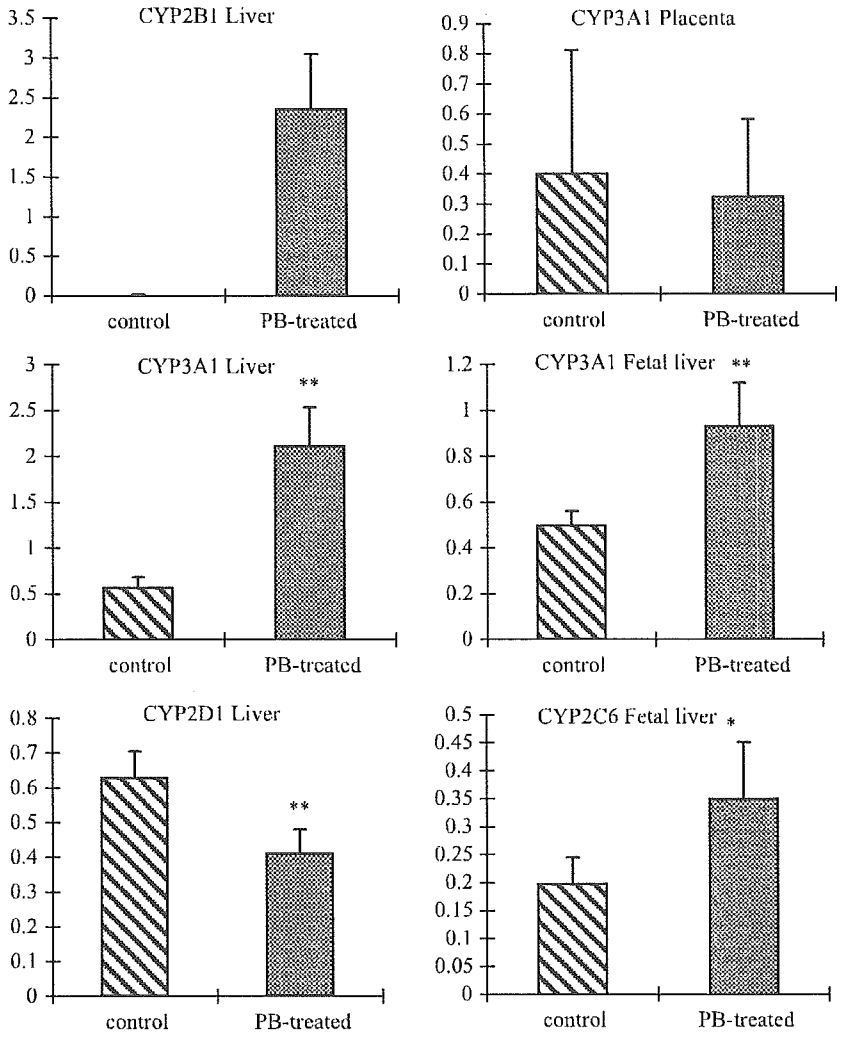

Fig. 3. Changes in CYP isozymes expression in dam's liver, placenta and fetal liver after PB treatment. (▨): control group; (▩): PB-treated group. *$P < 0.05$ and **$P < 0.01$: significantly different from the control group.

in both the control and PB-treated groups (Figs. 5g and h). Other CYPs used in this study were not detected immunohistochemically.

In the fetal liver, only a few signals positive for CYP3A1 and CYP2C6 proteins were detectable in both the control and PB-treated groups. The remaining CYPs were not detected, although Western blot analysis showed positive results on CYP2D1, 2E1, and 4A1.

Discussion

PB is commonly used as an antiepileptic drug. Because epileptic treatment is often long-term and pregnant women are sometimes treated with antiepileptics, there are many studies on adverse effects of antiepileptic drugs on mothers, fetuses, and newborn infants (Asoh et al., 1999; Bittigau et al., 2002, 2003;

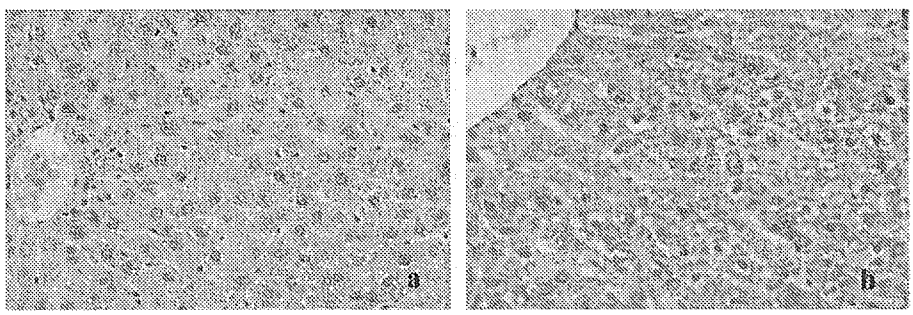

Fig. 4. Histology of dam's liver of the control (a) and PB-treated groups (b). Moderate vacuolization of hepatocytes is seen in b. HE, ×200.

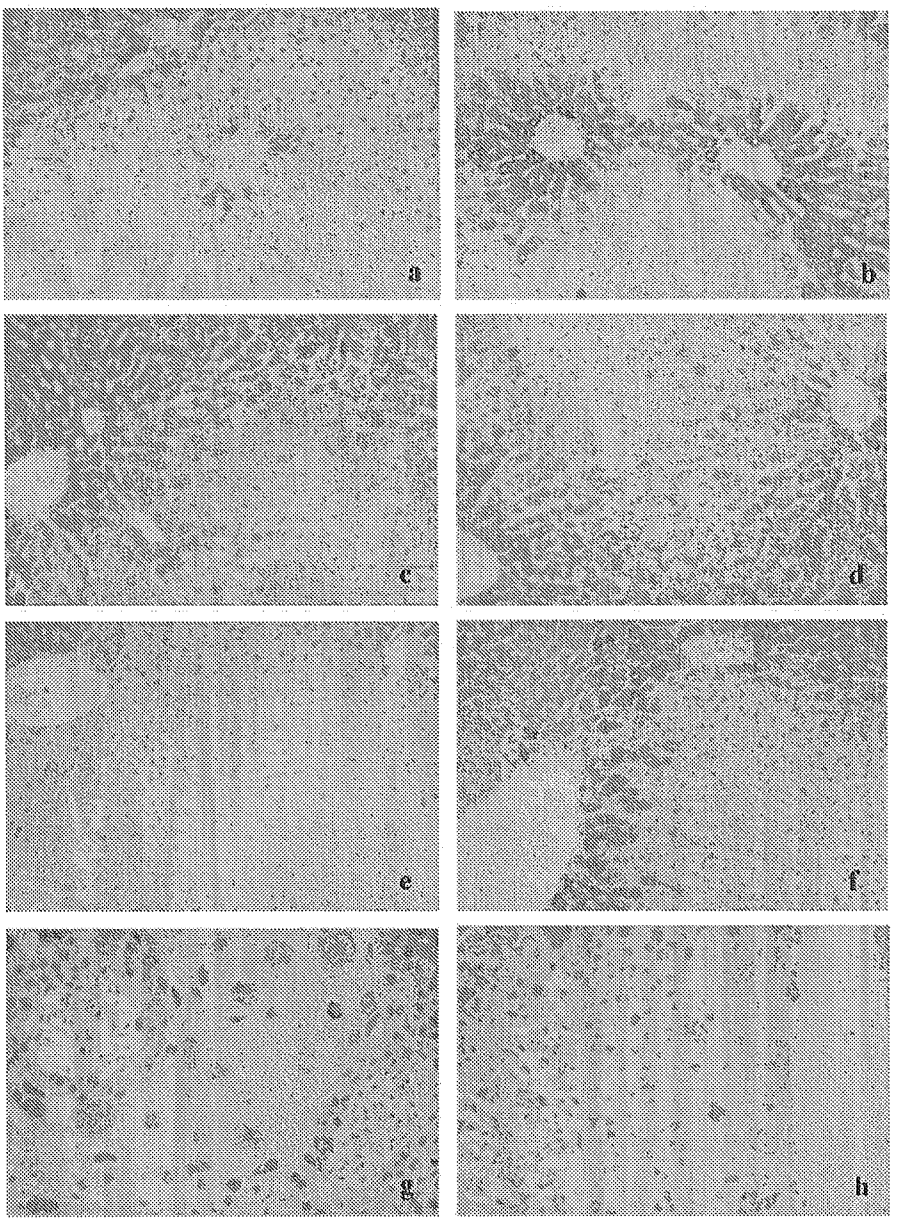

Fig. 5. Immunohistochemistry for CYP isozymes. CYP3A1 in dam's liver (a and b), CYP2D1 in dam's liver (c and d), CYP2B1 in dam's liver (e and f) and CYP3A1 in placenta (g and h). a, c, e, and g: control group; b, d, f, and h: PB-treated group. Immunostaining, ×100 (a, b, c, d, e, and f) or ×200 (g and h).

Bruno and Harden, 2002; Holmes et al., 2001; Nulman et al., 1999). As mentioned, antiepileptic drugs taken by pregnant women to prevent seizures are among the most common causes of malformations (neural tube defects, orofacial clefts, digital anomalies, and microcephaly), growth retardation, developmental delay, low birth weight, smaller head circumference, and so on (Asoh et al., 1999; Bittigau et al., 2003; Gupta and Yaffe, 1982; Holmes et al., 2001). In this study, although not significant, fetal body weight tended to be depressed. We did not examine the offspring for malformations. The relative liver weight was significantly increased after the PB-treatment, corresponding to the well-known fact that PB administration causes an increase in the liver weight.

In Western blot analysis, changes in the expression of CYP proteins were observed in the dam's liver, placenta, and fetal liver. In the dam's liver, seven (CYP1A1, 2C6, 2C12, 2D1, 2E1, 3A1 and 4A1) CYP proteins out of nine used in this study were detected spontaneously. Among these seven CYP proteins (CYP1A1, 2C6, 2C12, 2D1, 2E1, 3A1 and 4A1), only CYP3A1 was significantly increased after the PB treatment. On the other hand, CYP2B1 was not detected in the control group in the present study, although it is said that CYP2B1 protein is detected in the rat liver (Lupp et al., 2001). The reason for this difference is not clear, but it may be due to the difference in specificity and sensitivity of antibodies used, or the difference in the amount of CYP2B1 protein in the

liver. CYP2B1 protein was easily detected in the PB-treated group.

In the fetal liver of the control group, CYP proteins which can be detected (CYP2C6, CYP2D1, CYP2E1, CYP3A1 and CYP4A1) were more restricted than those in the dam's liver of the control group by Western blot analysis. In addition, they were barely detectable by immunohistochemical staining. Although CYP3A1 protein was prominently detected by Western blot analysis, it was not detected by immunohistochemical staining in either the control or PB-treated groups. It seems to depend on the quantity of protein in the fetal liver. In our previous study, CYP3A1 protein was easily detected in the fetal liver by Western blot analysis and immunohistochemical staining after exposure of pregnant rats to PCN and DEX (Ejiri et al., 2003). PCN and DEX may be more potent in the induction of CYP3A1 in the fetal liver than PB.

Moreover, in the fetal liver, CYP2B1 was not detected in either group, although it has been reported that the induction of CYP2B1 is observed in rat fetuses and neonates after the PB administration to dams (Asoh et al., 1999).

In the placenta, as reported in our previous paper (Ejiri et al., 2001, 2003), only CYP3A1 was detected in the control group, and there was no difference in the intensity of expression between the control and PB-treated groups; no other CYP proteins were induced after the PB administration by Western blot analysis and immunohistochemical staining.

The relative lack of enzyme induction in the placenta implies transplacental passage without chemical reaction therein. Accordingly, the placenta serves little or no role in fetal protection against phenobarbital as a prototype of maternal medication.

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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.

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

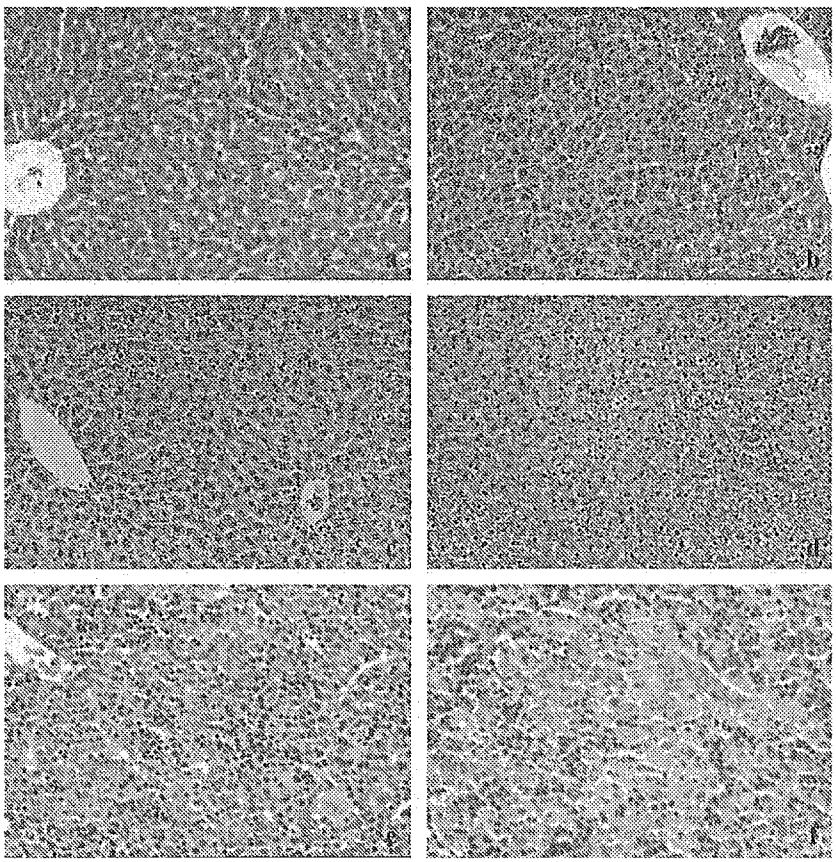

Fig. 1. Histology of dam's liver of CO group (a), PCN group (b), Sa group (c), and PB group (d), and histology of fetal liver of CO group (e) and PCN group (f). In the dam's liver, slight swelling of hepatocytes is observed in b, and moderate vacuolization of hepatocytes is observed in d. In the fetal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

Table 5
Changes in nuclear receptors genes expression in dam's liver, fetal liver, and placenta of pregnant rats treated with PCN

Gene	Accession no.		Fold change	*t* Test
Nuclear receptor subfamily 1, group 1, member 2 (Nr1i2)	NM_052980	Dam's liver	1.336*	0.000
		Fetal liver	0.963	0.622
		Placenta	1.093	0.753
Nuclear receptor (CAR) (Nr1i3)	NM_022941	Dam's liver	1.102	0.567
		Fetal liver	1.050	0.787
		Placenta	1.109	0.785

* $P < 0.05$.

normalization was performed in each experimental datum, fold changes (average of signals of treated groups/average of signals of control groups) were calculated. Students *t* test or Welchs *t* test was done. In this study, we picked up the probes focusing on drug metabolizing CYPs isozymes (Table 1) and two orphan nuclear receptors for CYP inducers, Nr1i2 and Nr1i3. Among CYP isozymes examined, probes of which significance level was $P < 0.05$ and Absolute Call was present, were picked up.

Reverse transcriptase polymerase chain reaction (RT–PCR) for CYP3A1 mRNA

Total RNA was prepared as described above. For RT–PCR analysis, we selected CYP3A1 gene because it was the only gene which was commonly up-regulated in dam's liver, placenta, and fetal liver. PCR was performed with pairs of oligonucleotide primers corresponding to the cDNA sequences of the rat mRNA. PCR was carried out with 1 μl of cDNA sample in a 100-μl reaction mixture containing 50 pM of sense and antisense primer, 1.25 U of rTaq, 10 × PCR buffer, and dNTP mixture (Takara, Ohtsu, Japan). This was immediately followed by preheating at 95°C for 7 min, denaturation at 95°C for 1 min, annealing for 1 min, and extension at 72°C for 1 min using Takara PCR Thermal Cycler SP (Takara). Annealing temperatures and cycle numbers are shown in Table 2. Optimal cycle numbers were determined in a preliminary experiment to ensure that the amplification was in the linear range and not during the plateau phase. PCR products were identified by electrophoresis on 2% agarose gel (Nippon Gene Co. Ltd.) followed by ethidium bromide (Invitrogen) staining. Fluorescent-gel imaging was carried out using an ultraviolet-CCD video system Fas-III (Toyobo, Tokyo, Japan). The relative band density against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was represented as the mean ± standard deviation (SD) for three dams, and statistical analysis was carried out using Students *t* test or Welchs *t* test.

Results

Histopathological findings

In the dam's liver, there were no histopathological changes in both CO and Sa groups (Figs. 1a, c). In PCN group, slight swelling of hepatocytes was observed (Fig. 1b). After the PB treatment, moderate vacuolization of hepatocytes was observed (Fig. 1d).

In the fetal liver, slight swelling of hepatocytes was observed in PCN group (Fig. 1f). There were no histopathological changes in CO (Fig. 1e), Sa, and PB groups. In the placenta, there were no histopathological changes in all groups.

Findings of microarray analysis

The selected results of microarray analysis on CYPs in the dam's liver, placenta and fetal liver are shown in Tables 3 and 4. Among 40 probes for drug metabolizing CYPs isozymes, probes showing significant changes were picked up. In Tables 5 and 6, the results of microarray analysis on two orphan nuclear receptors are shown.

In dam's liver, the gene expression of CYP3a18, Cyp3A1 (CYP3A subfamily), and cytochrome P-450e (CYP2B subfamily) prominently increased in PCN group (Table 3). The gene expression of Cyp2a1 (CYP2A subfamily) increased moderately, and that of Cyp2f1 (CYP2F subfamily), CYP4F4 (CYP4F subfamily), p450Md mRNA for cytochrome P-450 (CYP2C subfamily), CYP3A9 (CYP3A subfamily), and Cyp2b3 (CYP2B subfamily) also showed a

Table 6
Changes in nuclear receptors genes expression in dam's liver, fetal liver, and placenta of pregnant rats treated with PB

Gene	Accession no.		Fold change	*t* test
Nuclear receptor subfamily 1, group 1, member 2 (Nr1i2)	NM_052980	Dam's liver	0.901	0.265
		Fetal liver	1.000	0.998
		Placenta	0.982	0.957
Nuclear receptor (CAR) (Nr1i3)	NM_022941	Dam's liver	1.167	0.390
		Fetal liver	1.263	0.127
		Placenta	1.752	0.189

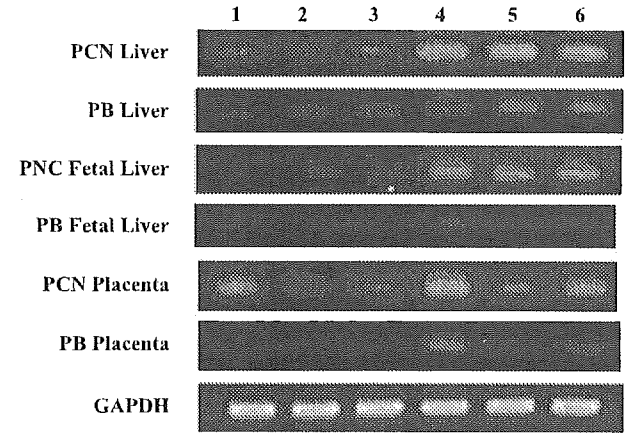

Fig. 2. The expression of CYP3A1 mRNA by RT–PCR in dam's liver, fetal liver, and placenta. Agarose gel electrophoresis. 1–3, rat number of control group; 4–6, rat number of treated group.

tendency of significant increase (Table 3). On the other hand, the gene expression of cytochrome P-450 ISF/BNF-G (CYP 1A subfamily) and Cyp4a1 (CYP4A subfamily) decreased in PCN group (Table 3). In PB group, the gene expression of Cyp3A1 and cytochrome P-450e prominently increased (Table 4), and that of Cyp2f1, Cyp2a1, Cyp2d2 (CYP2D subfamily), and cytochrome P450 PB1 (CYP2C subfamily) also showed a tendency of significant increase (Table 4). On the other hand, the gene expression of cytochrome P-450 ISF/BNF-G and Cyp2e1 (CYP2E subfamily) decreased in PB group (Table 4).

In fetal liver, the gene expression of Cyp3A1, pregnenolone 16-alpha-carbonitrile-inducible cytochrome P450 (CYP3A subfamily), and p450Md mRNA for cytochrome P-450 prominently increased in PCN group (Table 3), and that of Cyp2a1 also significantly increased (Table 3). On the other hand, significantly down-regulated genes coding CYPs were not detected in PCN group (Table 3). In PB group, the gene expression of Cyp3A1 and cytochrome P-450e prominently increased (Table 4), and that of Cyp2a1 showed a tendency of increase. The expression of CYP4A3 gene (CYP 4A subfamily) showed a tendency of decrease (Table 4).

In placenta, the expression of Cyp3A1 gene showed a non-significant increase in PCN group (Table 3), while it showed a significant increase in PB group (Table 4). There were no significantly down-regulated genes detected in either PCN or PB groups (Tables 3 and 4).

The expression of Nr1i2 gene was significantly elevated only in dam's liver of PCN group (Table 5), and the expression of Nr1i3 gene did not show significant changes in any groups (Table 6).

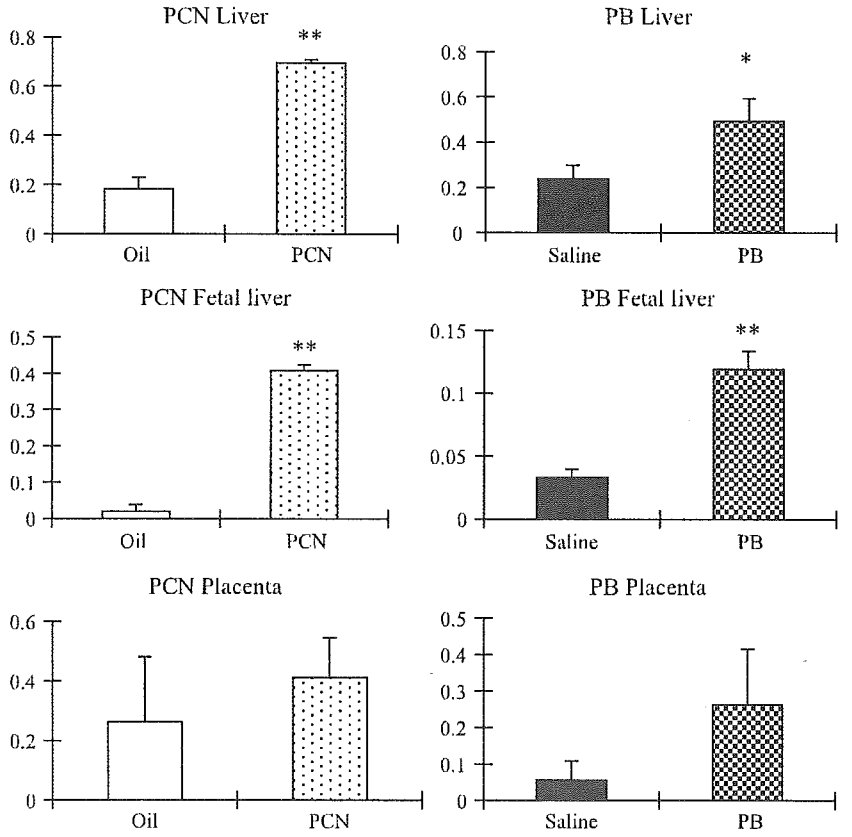

Fig. 3. The relative CYP3A1 band density to GAPDH by RT–PCR in dam's liver, fetal liver, and placenta. (□): CO group; (▨): PCN group; (■): Sa group; (▩): PB group. *$P < 0.05$ and **$P < 0.01$: significantly different from the control group.

Findings of RT–PCR for CYP3A1 mRNA

We performed RT–PCR on CYP3A1 mRNA, because it was the only gene which showed an up-regulation commonly in dam's and fetal livers and placenta. As shown in Figs. 2 and 3, a significant increase in CYP3A1 mRNA expression was observed in dam's and fetal livers. Although not significant, CYP3A1 mRNA expression also increased in placenta.

Discussion

Previously, we have reported the expression of nine CYP isozymes protein, against which antibodies were commercially available, in pregnant rat's liver, placenta, and fetal liver after treatment with dexamethasone, PCN or PB by Western blot analysis and immunohistochemistry (Ejiri et al., 2003, in press). In this study, the expression of CYPs genes was examined after PCN or PB treatment by microarray analysis, which can analyze 40 drug metabolizing CYP isozymes gene expression at one time.

In PCN group, the expression of Cyp3A1 gene was mainly elevated in both dam's and fetal livers, and its degree was more prominent in the latter. These findings of Cyp3A1 gene expression corresponded to the results of CYP3A1 protein expression (Ejiri et al., 2003). In placenta, although CYP3A1 protein was hardly induced by PCN (Ejiri et al., 2003), Cyp3A1 gene expression showed a mild but not significant increase. On the other hand, the expression of cytochrome P-450e and Cyp2b3 genes increased in dam's liver but not in fetal liver. This may be due to the difference of drug metabolizing enzyme profiles between dams and fetuses.

In PB group, the expressions of Cyp3a1 and cytochrome P-450e genes were elevated mainly in dam's and fetal livers. In our previous study on the inductions of CYPs proteins by PB, CYP3A1 protein was clearly induced in fetal liver by Western blot analysis, while CYP2B1 protein was not detected by either Western blot analysis or immunohistochemical staining (Ejiri et al., in press). Cyp3A1 gene expression in placenta significantly increased in PB group in the present study, but significant changes were not observed in Western blot analysis and immunohistochemical staining (Ejiri et al., in press).

In our previous report, the expression of CYP2B1 protein in dam's liver was observed in PB group but not in control group, and it was not detected in fetal liver even in PB group (Ejiri et al., in press). Among four probes for CYP2B subfamily, only cytochrome P-450e (phenobarbital-inducible) gene showed an apparently significant increase in this study. The relation between the increased gene expression of cytochrome P-450e and the protein expression of CYP2B1 in our previous report is still obscure.

As mentioned above, the existence of the orphan nuclear receptors, which induce CYPs by some inducers such as PCN or PB, is attracting researchers' attention (Masuyama et al., 2000; Mikamo et al., 2003; Xiong et al., 2002; Zhang et al., 1999). For example, it is said that Nr1i2 is concerned with an induction of CYP3A subfamily by PCN, and Nr1i3 is concerned with an induction of CYP2B subfamily by PB. In this study, a significant elevation in Nr1i2 gene expression was observed only in dam's liver of PCN group, while Nr1i3 gene expression showed no significant changes in all groups. In dam's liver, as mentioned above, Nr1i2 was induced in PCN group, followed by significant increase of Cyp3A1 gene expression. However, in fetal liver, Nr1i2 was not induced in PCN group, but a prominent induction of Cyp3A1 was observed. In addition, in dam's liver, although the expressions of cytochrome P-450e and Cyp3A1 genes were induced in PB group, the expression of Nr1i3 gene did not show significant change. Further study should be done to clarify the relationship between orphan receptors and CYPs gene expression.

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Ethylnitrosourea induces neural progenitor cell apoptosis after S-phase accumulation in a p53-dependent manner

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Neural progenitor cells populate the ventricular zone of the fetal central nervous system. In this study, immediately after the administration of ethylnitrosourea (ENU), an alkylating agent, an accumulation of neural progenitor cells in the S phase was observed. This event was caused by the inhibition or arrest of DNA replication rather than acceleration of the G1/S transition. Soon after this accumulation reached its peak, the number of cells in the G2/M phase decreased and the apoptotic cell count increased. In p53-deficient mice, both ENU-induced apoptosis and S-phase accumulation were almost completely abrogated. These findings indicate that ENU inhibits or arrests DNA replication in neural progenitor cells during the S phase and then evokes apoptosis before the cells enter the G2 phase. Furthermore, these data also demonstrate that both ENU-induced apoptosis and cell cycle perturbation in the S phase require p53.

Keywords: Apoptosis; Cell cycle arrest; Development; DNA damage; DNA replication; Ethylnitrosourea; Neural progenitor cell; p53

Introduction

In the fetal central nervous system (CNS), neural progenitor cells (NPCs) constitute a pseudostratified epithelium called the ventricular zone. NPCs are pluripotent cells that can self-renew and differentiate into cells of both the neuronal and glial lineage. In the ventricular zone, the nuclei of the NPCs undergo a series of characteristic movements called "elevator movements," and their locations are well correlated with the phase of the cell cycle. The nuclei of S-phase cells occupy the outer part of the ventricular zone. During the G2 phase, the nuclei move toward the ventricular surface, where the cells enter the M phase and complete mitosis. Nuclei of daughter cells that enter the G1 phase move outward, and then enter the S phase again in the outer part of the ventricular zone. In this way, NPCs proliferate and increase in number. During neurogenesis, after a division, one daughter cell differentiates into a neuroblast, escapes from the elevator movements, and migrates to the outside of the ventricular zone, where postmitotic neurons accumulate and form the cerebral neocortex (Yoshikawa, 2000).

Apoptosis is controlled by various signals during CNS development, and its precise regulation is indispensable for sound development (Oppenheim, 1991; Roth and D'Sa-Eipper, 2001). However, NPCs are quite susceptible to various types of stimuli, especially DNA-damaging agents, and easily enter the process of apoptotic cell death (D'Sa-Eipper and Roth, 2000; Kameyama and Inouye, 1994; Katayama et al., 2001). As a result of inappropriate apoptosis, congenital anomalies such as microencephaly, anencephaly, and cellular cytoarchitectural abnormalities may be induced in neonates (Katayama et al., 2000; Miki et al., 1995).

The tumor suppressor p53 has been implicated in cellular responses to DNA-damaging agents. In response to DNA damage, p53 is upregulated and transactivates a series of genes involved in the induction of apoptosis, cell cycle arrest, and DNA repair (Ko and Prives, 1996). NPC apoptosis induced by DNA-damaging agents is also mediated by the regulation of p53 and p53 target genes (Bolaris et al., 2001; Borovitskaya et al., 1996; Katayama et al., 2002; Ueno et al., 2002) and is efficiently inhibited in p53 knockout mice (D'Sa-Eipper et al., 2001; Kubota et al., 2000; Leonard et al., 2001).

Ethylnitrosourea (ENU) is a monofunctional alkylating agent with high mutagenicity (Shibuya and Morimoto, 1993). ENU selectively induces brain tumors in offspring when administered to pregnant animals, and this experimental model has been extensively used to investigate the pathogenesis of CNS tumors (Jang et al., 2004; Koestner, 1990). Most of the ENU-induced CNS tumors are regarded as gliomas, and the rat is the species most susceptible to neurogenic tumor development following ENU administration.

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In the present study, we investigated the relationship between apoptosis and the cell cycle in the fetal CNS after the administration of ENU to find out how fetal NPCs regulate the cell cycle and apoptosis and how they respond to DNA-damaging agents. Our findings indicate that ENU inhibits or arrests DNA replication in NPCs in the S phase and evokes apoptosis before the cells enter the G2 phase. Furthermore, our findings also demonstrate that ENU-induced apoptosis and cell cycle perturbation in the S phase both require p53.

Materials and methods

All procedures were performed in accordance with the protocol approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Chemicals

ENU, bromodeoxyuridine (BrdU), propidium iodide (PI), and RNaseA were obtained from Sigma (St. Louis, MO).

Animals

Pregnant F344 rats (plug day: day 0 of gestation) were obtained from Saitama Experimental Animal Company (Saitama, Japan), and pregnant ICR mice (plug day: day 0 of gestation) were purchased from Charles River Japan, Inc. (Kanagawa, Japan). $p53^{+/-}$ mice were purchased from Taconic (Germantown, NY). Heterozygous mice were crossed to generate wild-type, heterozygous, and homozygous gene-disrupted mice. Endogenous and disrupted genes were detected by polymerase chain reaction analysis of tail DNA extracts as described by Timme and Thompson (1994). Fas/CD95-mutant C57BL/6J-*lpr/lpr* mice and wild-type C57BL/6J mice were purchased from Japan SLC (Shizuoka, Japan). Fas/CD95-deficient fetuses were generated by crossing *lpr/lpr* mice, and wild-type fetuses were obtained by crossing wild-type mice.

Treatments for F344 rats and ICR mice

Pregnant animals were injected intraperitoneally (i.p.) with 60 mg/kg of ENU or an equivalent volume of buffer alone on day 11 (mice) or 13 (rats) of gestation, and fetuses were harvested at specific times after the treatment described in the text. Collected fetuses were subjected to histopathological examination, cell cycle analysis by flow cytometry, and Western blot analysis. For the histopathological analysis, fetuses were fixed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin sections (4 μm) were stained with hematoxylin and eosin. Some of the sections were subjected to immunohistochemical staining for cleaved caspase-3 as mentioned below.

Treatments for p53 knockout and lpr/lpr mice

Pregnant mice were injected i.p. with 60 mg/kg of ENU on day 11 of gestation; dams were euthanized and fetuses were collected at 6 and 12 h after the treatment, respectively. As a control, dams were injected i.p. with an equivalent volume of buffer alone on day 11 of gestation and euthanized at 6 h after the treatment. Collected fetuses were subjected to histopathological examination and cell cycle analysis.

Cell cycle analysis

The fetal telencephalon was dissected in Hanks' balanced salt solution (HBSS) and dissociated by brief mechanical trituration in HBSS. Isolated cells were washed with phosphate-buffered saline (PBS) and fixed in 70% ethanol. Cells were then washed with PBS, incubated in PBS containing RNaseA, and stained with PI. Cell cycle phase analysis was performed using a FACScan (Becton-Dickinson, San Jose, CA). Ten thousand cells were examined in each sample. Using the Cell Quest program (Becton-Dickinson), doublets and debris were discarded and then percentages of cells in the various phases of the cell cycle were calculated.

BrdU-incorporation assay

To analyze the DNA-replicating cells by flow cytometry, a BrdU incorporation assay was performed on the rat fetuses collected at 6 h after the ENU treatment. Pregnant dams were injected i.p. with 20 mg/kg of BrdU exactly 1 h before euthanasia. Cells were isolated from the fetal telencephalon, washed with PBS, and fixed in 70% ethanol. After being washed with PBS, the cells were resuspended in 2 M HCl containing 0.5% Triton X-100. The cells were neutralized in 0.1 M $Na_2B_4O_7$ and then incubated with fluorescein isothiocyanate (FITC)-labeled anti-BrdU monoclonal antibody (Pharmingen, San Diego, CA). Then, they were resuspended in PBS containing PI and analyzed using a FACScan (Becton-Dickinson). Ten thousand cells were examined in each sample. By using the Cell Quest program (Becton-Dickinson), doublets and debris were discarded, and then percentages of cells in the various phases of the cell cycle and FITC (BrdU)-positive cells were calculated.

Histological observation of the migration of BrdU-incorporated NPC nuclei

To analyze the migration of NPC nuclei histologically, ICR mice were injected i.p. with 60 mg/kg of ENU or an equivalent volume of buffer alone on day 11 of gestation. Dams were administered i.p. with 20 mg/kg of BrdU 3 h after the ENU treatment. Then, fetuses were collected at 1, 3, 6, and 9 h after the BrdU administration (i.e., 4, 6, 9, and 12 h after the ENU treatment). Collected fetuses were fixed in 10% neutral-buffered formalin and embedded in paraffin. The paraffin sections were subjected to immunohistochemical staining for BrdU as described below.

Immunohistochemistry

Immunohistochemical staining for cleaved caspase-3 and BrdU was carried out by the labeled streptavidin biotin (LSAB) method with streptavidin (Dako, Carpinteria, CA). Rabbit anti-cleaved caspase-3 polyclonal antibody (Cell Signaling Technology, Beverly, MA) and mouse anti-BrdU monoclonal antibody (Dako) were used as the primary antibodies. The positive signals were visualized using a peroxidase-diaminobenzidine reaction, and then the sections were counterstained with methyl green.

Western blot analysis

The fetal telencephalon was homogenized in a solution of 20 mM Tris–HCl (pH 7.4), containing 150 mM NaCl, 1 mM PMSF,

1% aprotinin, 2 mM EDTA, 2 mM Na_3VO_4, 1% NP-40, 0.1% SDS, and 1 mM DTT and centrifuged at 12,000 g for 20 min at 4°C. Approximately 30 μg of extract was loaded onto a 10% SDS-PAGE gel, electrophoresed, and transferred to a PVDF membrane (Bio-Rad, Hercules, CA). Blots were first probed with antibodies to p53 (Santa Cruz Biotechnology, Santa Cruz, CA), $p21^{waf1/cip1}$ (Pharmingen), cyclin D1 (Dako), cyclin-dependent kinase (CDK) 4 (Santa Cruz Biotechnology), and β-actin (Sigma). After incubation with the appropriate secondary antibody conjugated to horseradish peroxidase (Amersham, Buckinghamshire, UK), detection was performed with the ECL Plus kit (Amersham).

Results

ENU induces apoptosis in NPCs

In both rats and mice, an increase in the number of pyknotic cells, which fulfilled the morphological characteristics of apoptotic cells (Katayama et al., 2001), was observed from 3 to 24 h after the ENU treatment in the NPCs in the ventricular zone (Figs. 1a, b). Few pyknotic cells were observed in other zones. The nuclei of the pyknotic NPCs localized mainly in the outer part of the ventricular zone, which contains S-phase nuclei owing to the elevator movements. Immunohistochemically, pyknotic NPCs were also positively stained for cleaved caspase-3 (Figs. 1c, d), which is known to be involved in neural cell apoptosis during development as well as the apoptosis of neurons induced by DNA-damaging agents (Kuida et al., 1996; Keramaris et al., 2000).

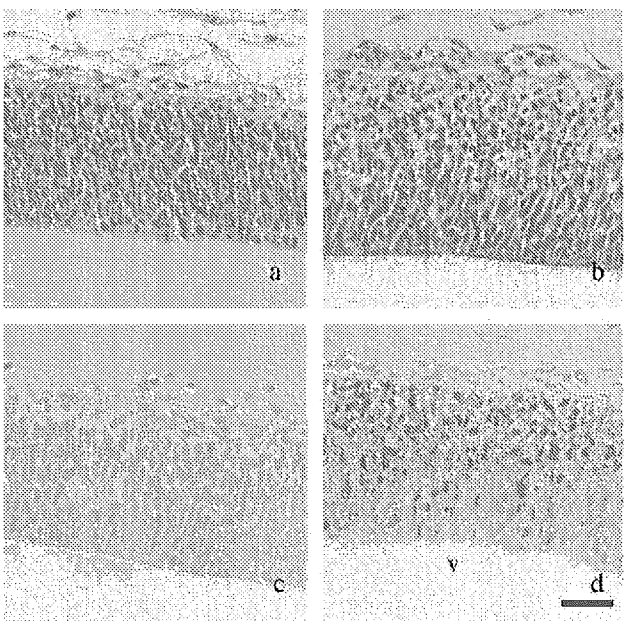

Fig. 1. ENU induces apoptosis and caspase-3 activation in NPCs in the fetal CNS. Transverse sections of the telencephalon at 12 h after the treatment. Hematoxylin and eosin-stained sections of the control (a) and ENU-treated (b) rat fetuses. Apoptotic NPCs bearing pyknotic nuclei are seen in the ENU-treated fetus. Apoptotic nuclei mainly localize in the outer part of the ventricular zone. Immunohistochemistry for cleaved caspase-3 of the control (c) and ENU-treated (d) rat fetuses. Extensive caspase-3 activation, as indicated by cleaved caspase-3 immunoreactivity, is observed in the ENU-treated fetus. V, ventricle; scale bar = 30 μm.

ENU induces S-phase accumulation in NPCs

Studies have shown that from gestational day 12 to 13, about 70% of mouse telencephalic cells are NPCs (D'Sa-Eipper and Roth, 2000); therefore, the results from our flow cytometric analyses are considered to accurately represent the cell cycle changes in NPCs. In both rats and mice, an accumulation of cells in the S phase was observed from 1 h after the ENU treatment, and this became most prominent at 6 h (Figs. 2, 3). Although the number of cells in S phase gradually decreased from 9 to 12 h, the number in G2/M phase also decreased at these time points. Furthermore, apoptotic cells (cells with sub-G1 DNA content) peaked at 12 h. The number of cells in G0/G1 phase decreased markedly between 6 and 12 h. The DNA content of the accumulated S-phase cells was close to that of the G1 cells, and it did not tend to increase with time (Fig. 2).

ENU inhibits or arrests DNA replication in NPCs

In our previous study, immunohistochemical staining for BrdU revealed a significant decrease in the number of BrdU-incorporated NPCs (DNA-replicating NPCs) in the fetal telencephalon after ENU administration, with a trough observed at 6 h (Katayama et al., 2001). Oyanagi et al. (1998) also reported that the number of BrdU-incorporated NPCs decreased immediately after the administration of ENU. However, in the present study, an accumulation of cells in the S phase was observed, and it became most prominent at 6 h after the ENU treatment. Therefore, we tried to analyze BrdU incorporation by flow cytometry.

At 6 h after the ENU treatment, BrdU incorporation in the fetal telencephalon was markedly reduced, as indicated by the decrease in FITC fluorescence intensity (Fig. 4). The number of BrdU-positive cells also decreased (mean ± standard deviation, control: 23.0 ± 1.6%, $n = 3$; ENU: 18.6 ± 1.0%, $n = 3$). In addition, there were many cells that contained DNA identical to that of the S-phase cells but did not incorporate BrdU (control: 0.9 ± 0.1%, $n = 3$; ENU: 15.2 ± 1.5%, $n = 3$). The DNA content of these cells was close to that of the G1 cells and identical to that of the accumulated S-phase cells observed in the cell cycle analysis. Almost the same results were obtained from mouse fetuses (data not shown). The results from the BrdU-incorporation assay suggest that S-phase accumulation is not brought about by acceleration of the G1/S transition but by the inhibition or arrest of DNA replication.

Migration of NPC nuclei is inhibited by ENU administration

To further analyze the cell cycle perturbation, we injected BrdU into pregnant mice at 3 h after the ENU treatment, when the accumulation of cells in the S phase began to be apparent, and analyzed the migration of BrdU-incorporated nuclei immunohistochemically. In both the control and ENU-treated fetuses, at 1 h after the BrdU administration, BrdU-incorporated NPC nuclei localized in the outer part of the ventricular zone, which contains S-phase nuclei owing to the elevator movements. In the control fetuses, BrdU-incorporated nuclei migrated toward the ventricular surface and showed mitotic figures from 3 to 6 h, and at 9 h, they began to move outward (Fig. 5). This result agrees well with the study of Takahashi et al. (1993), who reported that the S, G2/M, and G1 phases in mouse NPCs lasted 3.8, 2, and 9.3 h, respectively. In the ENU-treated fetuses, however, a large number of the BrdU-incorporated nuclei remained in the outer part of the

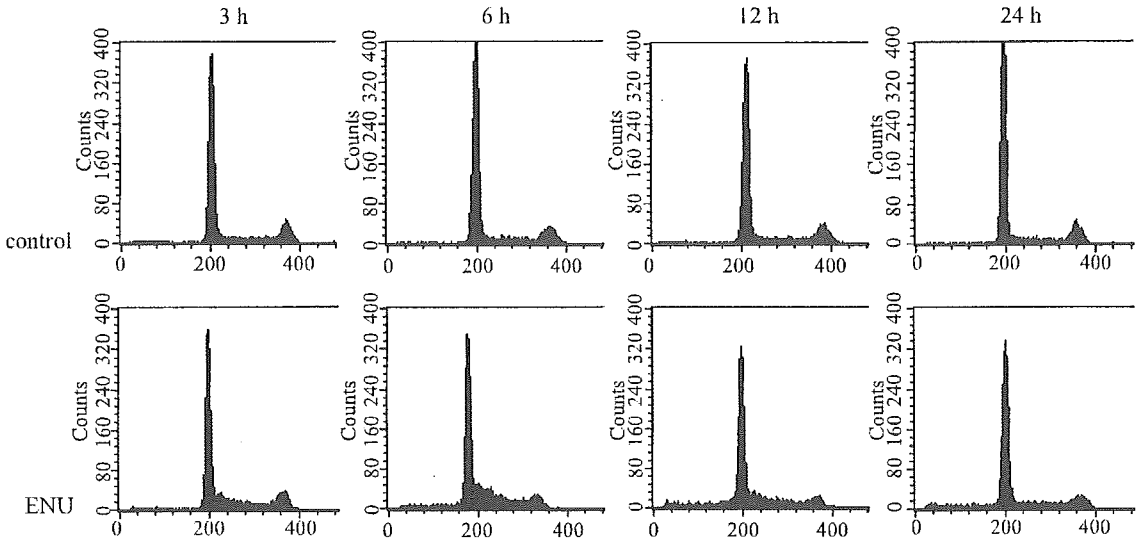

Fig. 2. ENU induces S-phase accumulation before the induction of apoptosis. Flow cytometric analysis of cells from rat fetal telencephalon. Horizontal and vertical axes represent PI fluorescence (DNA content) and cell number, respectively. S-phase accumulation was observed in the ENU-treated telencephalic cells and was most prominent at 6 h after the treatment. The number of cells in the G2/M phase decreased and that of apoptotic cells (cells with sub-G1 DNA content) increased at 12 h. The DNA content of the accumulated S-phase cells was close to that of the G1 cells and did not tend to increase with time.

ventricular zone from 3 to 6 h, finally reaching the ventricular surface at 9 h (Fig. 5). In addition, pyknotic nuclei positively stained by the anti-BrdU antibody were observed from 3 to 9 h after the BrdU administration, and these nuclei were mainly observed in the outer part of the ventricular zone; as a result, BrdU-positive nuclei markedly decreased at 9 h. These findings indicate that BrdU-incorporated NPC nuclei remain in the outer part of the ventricular zone for a long time and appear pyknotic before they move inward. According to the elevator movements, the results suggest that ENU induces cell cycle retardation in NPCs in the S phase and apoptosis occurs while they are still in the S phase. We performed the same experiment in rat fetuses, but BrdU-positive signals were very weak in the ENU-treated fetuses, and it was difficult to evaluate the migration of BrdU-incorporated nuclei.

S-phase accumulation is not accompanied by elevated expression of cell cycle promoting molecules

The expression of proteins involved in the G1/S transition was analyzed by Western blot using protein samples extracted from rat telencephalon (Fig. 6; Sherr and Roberts, 1999). The expression of cyclin D1, which promotes G1/S transition, decreased from 3 h after the ENU treatment. However, the expression of $p21^{waf1/cip1}$, a CDK inhibitor, increased at around 6 h, and the expression of p53, a transcriptional regulator of $p21^{waf1/cip1}$, also increased and peaked at 3 h. In our previous study, elevation in the levels of p53 and $p21^{waf1/cip1}$ proteins was also detected by immunohistochemistry (Katayama et al., 2002). The expression of CDK4 was nearly unchanged throughout the experimental period. Almost the same results were obtained from mouse fetuses (data not shown).

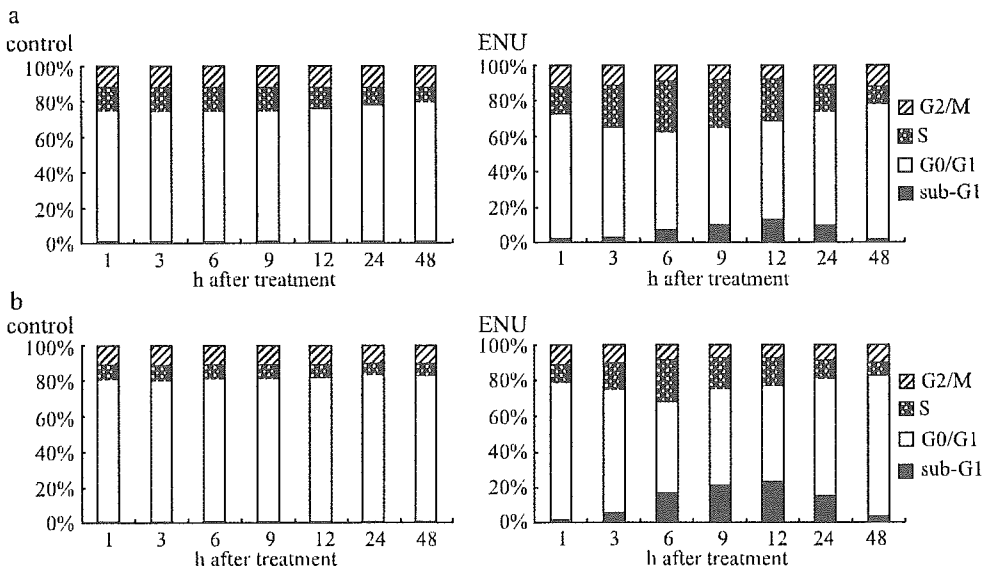

Fig. 3. Cell cycle distribution of control and ENU-treated rat (a) and mouse (b) fetal telencephalic cells. Percentages for each cell cycle phase are presented as the mean of three dams.

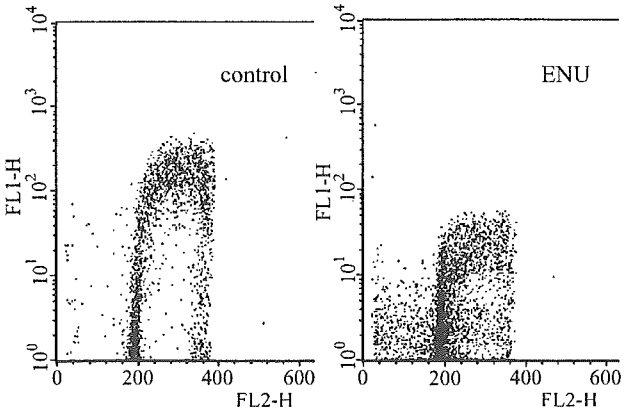

Fig. 4. ENU inhibits or arrests DNA replication in the telencephalic cells. Dot blots of cell cycle distribution and BrdU incorporation in rat fetal telencephalic cells. FITC-fluorescence (BrdU incorporation) on the vertical axis (log scale) and PI fluorescence (DNA content) on the horizontal axis. BrdU incorporation is severely inhibited in the ENU-treated fetus. In addition, there are many cells that contain DNA identical to that of the S-phase cells but do not incorporate BrdU.

These results also suggest that the accumulation of cells in the S phase is not brought about by the acceleration of the G1/S transition.

p53 is required for both ENU-induced apoptosis and S-phase accumulation

In the histopathology and cell cycle analysis of p53 knockout mice, we could not detect an increase in the number of apoptotic cells in the $p53^{-/-}$ mice after the administration of ENU (Figs. 7a, b, 8a). In addition, $p53^{-/-}$ mice showed almost no changes in the cell cycle distribution compared to the $p53^{+/+}$ and $p53^{+/-}$ mice (Fig. 8a). These findings suggest that p53 is required for both ENU-induced apoptosis and cell cycle perturbation in the S phase.

Fas/CD95 is not critical for ENU-induced apoptosis or S-phase accumulation

Fas/CD95 is a member of the tumor necrosis factor receptor superfamily and induces apoptosis when it binds to Fas/CD95 ligand (Itoh et al., 1991). Recent studies have indicated the involvement of the Fas/CD95 system in neuronal cell death in several neurological disorders (Hou et al., 2002; Padosch et al., 2003; Tan et al., 2001). Fas/CD95 is also known as a transcriptional target of p53 (Muller et al., 1998), and the expression of Fas/CD95 mRNA was upregulated after the activation of p53 in our previous study (Katayama et al., 2002). In *lpr/lpr* mice, which lack Fas/CD95 (Watanabe-Fukunaga et al., 1992), apoptosis and cell cycle perturbation were also observed upon administration of ENU, as in the wild-type C57BL/6J mice (Figs. 7c, d, 8b). Thus, Fas/CD95 is not critical for ENU-induced apoptosis or cell cycle alteration in the fetal CNS.

Discussion

In the present study, an accumulation of cells in the S phase was observed immediately after the administration of ENU. Our findings indicate that the accumulation is brought about by the inhibition or arrest of DNA replication rather than by the acceleration of the G1/S transition. Soon after the number of cells in the S phase reached its peak, the cells in the G2/M phase decreased and the number of apoptotic cells increased. In the p53-deficient mice, both ENU-induced NPC apoptosis and S-phase accumulation were almost completely abrogated. These findings indicate that ENU inhibits or arrests DNA replication in NPCs in the S phase and then signals for apoptosis before the cells enter the G2 phase. Furthermore, these data also demonstrate that both ENU-induced apoptosis and S-phase accumulation require p53.

After the administration of ENU to pregnant rats on day 15 of gestation, apoptotic cells were observed not only in the NPCs but also in the neuroblasts immediately after mitosis in the outside of

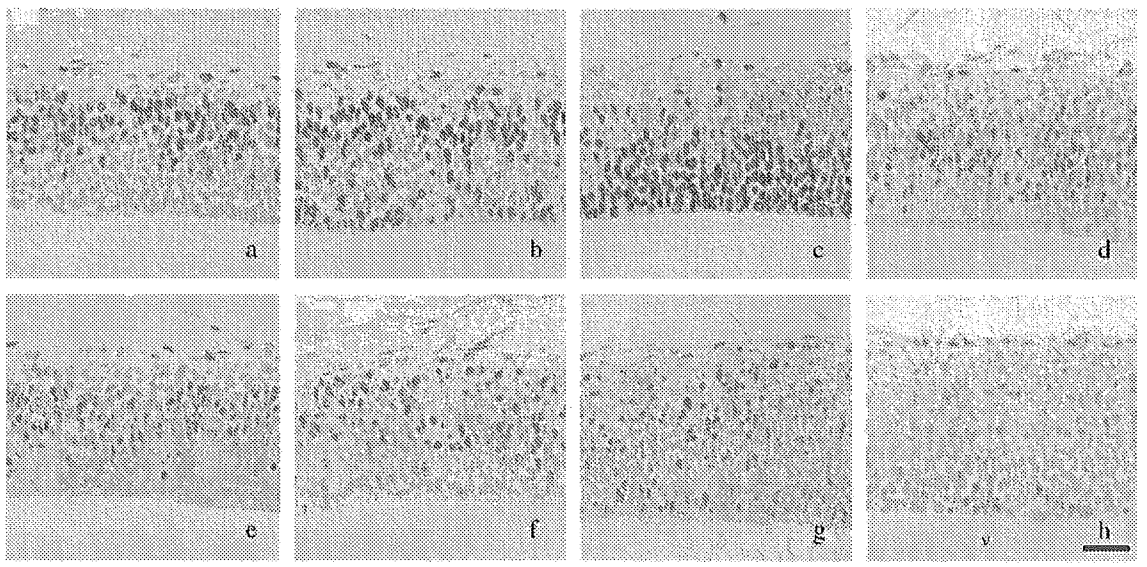

Fig. 5. Migration of BrdU-incorporated NPC nuclei is retarded by ENU administration. Immunohistochemistry for BrdU of the control (a–d) and ENU-treated (e–h) mouse fetal telencephalon at 1 (a, e), 3 (b, f), 6 (c, g), and 9 h (d, h) after the BrdU administration. The migration of BrdU-incorporated nuclei toward the ventricular surface is inhibited in the ENU-treated fetuses, indicating the cell cycle retardation in the S phase. In addition, some BrdU-positive nuclei remaining in the outer part of the ventricular zone appear pyknotic. V, ventricle; scale bar = 30 μm.

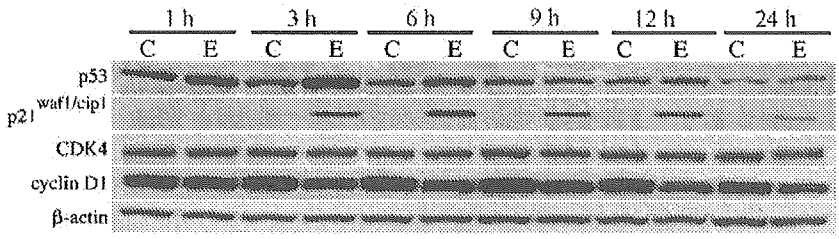

Fig. 6. Expression of cell cycle regulators during ENU-induced S-phase accumulation. Western blot analysis of p53, $p21^{waf1/cip1}$, CDK4, cyclin D1, and β-actin obtained from extracts from the control (C) and ENU-treated (E) rat telencephalon. The expression of p53 and $p21^{waf1/cip1}$ increased, cyclin D1 decreased, and CDK4 was unchanged in the ENU-treated fetuses.

the ventricular zone (Oyanagi et al., 1998). Day 15 of gestation in rats is the stage of neurogenesis. One NPC produces one neuroblast and one NPC by mitosis, and generated neuroblasts migrate to the outside of the ventricular zone. In the present experiment, at the time of the ENU administration (day 13 of gestation in rats and day 11 in mice), the fetal CNS was still undifferentiated, and consequently, the generation of neuroblasts was not very prominent. Thus, there was not marked apoptotic cell death in the neuroblasts in the present study. Although we cannot exclude the possibility that apoptosis is also induced in other ways, the results of this study strongly suggest that a large number of the NPCs die in the S phase after cell cycle retardation in the S phase.

ENU alkylates mainly the O^6 position of guanine (Shibuya and Morimoto, 1993), and the existence of alkyl lesions in the genomic DNA inhibits DNA replication in vitro (Ceccotti et al., 1993; Eckert et al., 1997). The retardation of the cells in S phase observed in the present study indicates that ENU also inhibits DNA replication in vivo. The O^6 alkylation of guanine induces GC-AT transitions. 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). Elimination of DNA-damaged cells by p53-dependent apoptosis is very important for the prevention of brain tumorigenesis (Leonard et al., 2001). However, it is still controversial that a high incidence of brain neoplasms is observed in rat neonates in spite of extensive apoptotic cell death in the fetal CNS after the administration of ENU.

Recent studies suggest the involvement of the cell cycle machinery of the G1/S transition such as activation of cyclin–CDK complexes, phosphorylation of retinoblastoma protein, and derepression of E2F-responsive genes in the postmitotic neuronal apoptosis induced by DNA damage (Liu and Greene, 2001;

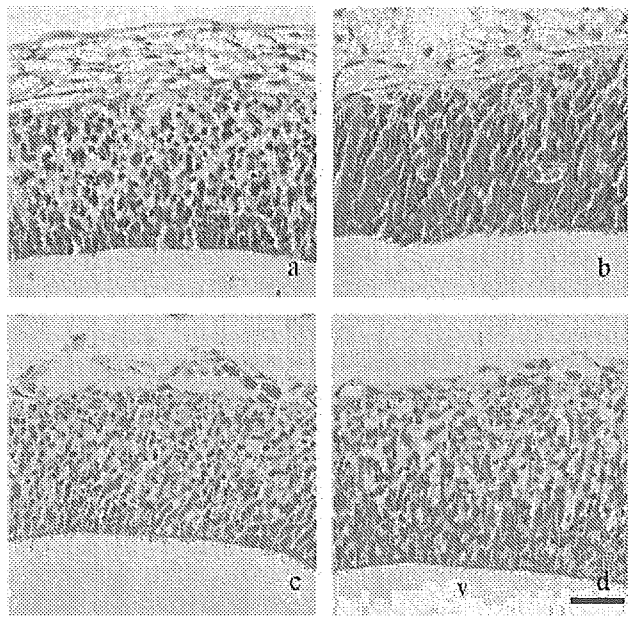

Fig. 7. p53, but not Fas/CD95, is required for ENU-induced NPC apoptosis. Hematoxylin and eosin-stained sections of the telencephalon from $p53^{+/+}$ (a), $p53^{-/-}$ (b), wild-type C57BL/6J (c), and *lpr/lpr* (d) mouse fetuses at 12 h after the ENU treatment. ENU-induced apoptotic cell death is almost completely abrogated in the p53-deficient mouse, and apoptosis is similarly induced in both the wild-type C57BL/6J and *lpr/lpr* mice. V, ventricle; scale bar = 30 μm.

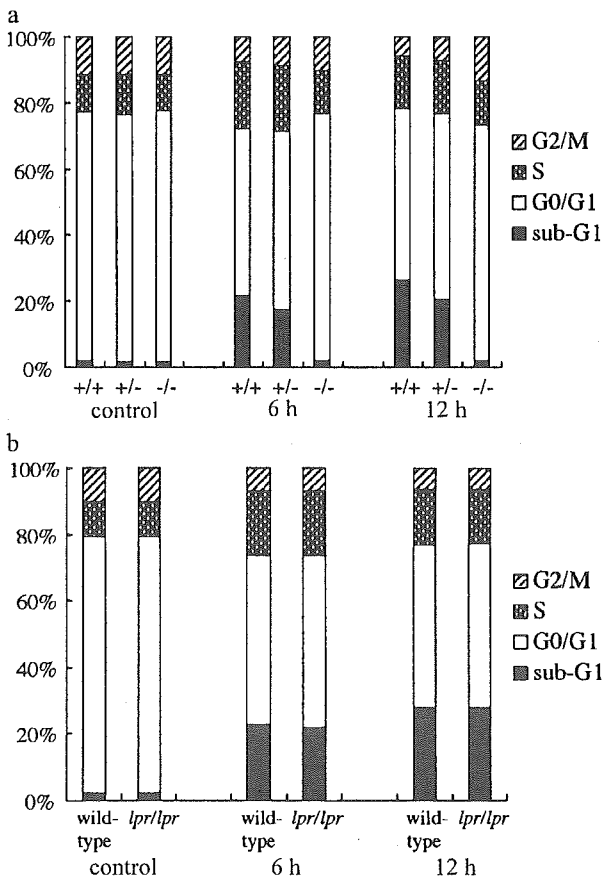

Fig. 8. Cell cycle distribution of p53 knockout (a) and *lpr/lpr* (b) mouse fetal telencephalic cells. Percentages for each cell cycle phase are presented as the mean of three dams. Both ENU-induced apoptosis and S-phase accumulation were almost completely abrogated in p53-deficient mice, and they were similarly induced in wild-type C57BL/6J and *lpr/lpr* mice.

Morris et al., 2001; Park et al., 1997, 1998, 2000). In the present study, the expression of cyclin D1 and CDK4 was not upregulated by ENU administration. Responses to DNA-damaging agents differ between postnatal and embryonic neurons (Johnson et al., 1999) and among embryonic neurons in various developmental stages (Lee et al., 2001). Thus, the findings of the present study would be a specific response of the fetal NPC, which are highly proliferative.

In the Western blot analysis, the expression of $p21^{waf1/cip1}$ was upregulated and peaked at around 6 h after the ENU treatment. Almost the same observation was made in an immunohistochemical analysis (Katayama et al., 2002). $p21^{waf1/cip1}$, a CDK inhibitor, is transactivated by p53 and induces cell cycle arrest in the G1 phase (Dulic et al., 1994; El-Deiry et al., 1993). We performed immunohistochemical staining for $p21^{waf1/cip1}$ in p53 knockout mice and found an increase in the number of $p21^{waf1/cip1}$-positive NPCs in the $p53^{+/+}$ and $p53^{+/-}$ mice, but not in the $p53^{-/-}$ mice after the administration of ENU (data not shown). This finding suggests that $p21^{waf1/cip1}$ is also upregulated in a p53-dependent manner. However, the number of cells in G0/G1 phase decreased and that in S phase increased on administration of ENU, indicating that the cell cycle is arrested in the S phase rather than in the G1 phase. Thus, the precise role of $p21^{waf1/cip1}$ is not clear. In our previous study, the number of $p21^{waf1/cip1}$-positive NPCs after ENU administration was much less than that of p53-positive cells (maximum index of $p21^{waf1/cip1}$ was about one fourth that of p53; Katayama et al., 2002); therefore, the fetal CNS would tend to eliminate DNA-damaged cells via apoptosis rather than arrest the cell cycle and repair the DNA lesions. Furthermore, the mechanism of the p53-dependent induction of the S-phase accumulation is not clear. Additional studies are needed to determine the key molecule required for the S-phase accumulation.

In the cell cycle analysis of p53 knockout mice, percentages of cells in the S phase at 6 and 12 h and G2/M phase at 12 h after the ENU treatment slightly increased in $p53^{-/-}$ mice. Though we cannot exclude the possibility that alkylation of the bases by ENU has some effect(s) on the cell cycle progression even in p53-deficient mice, the changes in the percentage of each cell cycle phase of $p53^{-/-}$ mice were not significantly different from the control ($P > 0.05$, Student's t test), whereas all the changes except G2/M phase of $p53^{+/-}$ mice at 6 h were significantly different in $p53^{+/+}$ and $p53^{+/-}$ mice ($P < 0.05$, Student's t test).

DNA damage-induced NPC apoptosis requires p53 and caspase-9 (D'Sa-Eipper et al., 2001; Leonard et al., 2001), and ataxia telangiectasia mutated (ATM) is required for the activation of p53 (Lee et al., 2001). However, little is known about how NPCs regulate apoptosis and the cell cycle. NPCs are highly proliferative and have the potential to differentiate into cells of both the neuronal and glial lineage. Thus, elucidating the mechanisms of how NPCs regulate the cell cycle and apoptosis and respond to exogenous stimuli is important for understanding both normal and abnormal development of the CNS.

Acknowledgment

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Birth Defects Research (Part B) 74:255–260 (2005)

Original Article

Microarray Analysis of Genes in Fetal Central Nervous System After Ethylnitrosourea Administration

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BACKGROUND: Ethylnitrosourea (ENU), a monofunctional alkylating agent, induces apoptosis and cell cycle arrest in neuroepithelial cells, neural stem cells in the fetal central nervous system (CNS). These effects occur immediately after the administration of ENU to pregnant animals resulting in fetal brain anomalies and long-term effects include brain tumors in the offspring. **METHODS:** Changes in gene expression were investigated in the fetal CNS after ENU administration to pregnant rats using microarray to identify the genes involved in the injury and recovery of the fetal CNS. **RESULTS:** 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. The genes up-regulated in the injury phase contained p53-target genes that mediate apoptosis and cell cycle arrest, and those in the recovery phase contained cell proliferation-promoting genes. The genes down-regulated in the injury phase contained cholesterol biosynthesis-related genes. In addition, there were some genes that have not been identified to be involved in the CNS injury and recovery. **CONCLUSIONS:** The present study will provide a better understanding of the mechanisms of development, regeneration and carcinogenesis of the CNS as well as the mechanisms of ENU-induced fetal CNS injury and recovery. *Birth Defects Res B* 74:255–260, 2005. © 2005 Wiley-Liss, Inc.

Key words: *apoptosis; cell cycle arrest; microarray; ethylnitrosourea; neuroepithelial cell; p53*

INTRODUCTION

Neuroepithelial cells are neural stem cells that exist in the fetal central nervous system (CNS). They are multipotent stem cells that can self-renew and differentiate into cells of both the neuronal and glial lineage (Yoshikawa, 2000). Ethylnitrosourea (ENU), a monofunctional alkylating agent, selectively induces brain tumors in offspring when administered to pregnant animals, and this experimental model has been extensively used to investigate the pathogenesis of CNS tumors (Koestner, 1990; Jang et al., 2004). ENU induces apoptosis and cell cycle arrest in neuroepithelial cells in the fetal CNS immediately after its administration to pregnant dams (Leonard et al., 2001; Katayama et al., 2001, 2005) and as a result, congenital brain anomalies can be induced in neonates (Katayama et al., 2000). It has been shown that neuroepithelial cell apoptosis and cell cycle arrest induced by ENU are mediated by the regulation of p53 and p53 target genes and efficiently inhibited in p53 knockout mice (Leonard et al., 2001; Katayama et al., 2002, 2005).

Precise mechanisms of ENU-induced CNS injury are not well understood and little is known about the restoration mechanisms after injury. Changes in gene expression at injury and recovery phases were investigated in the fetal CNS after ENU administration to pregnant rats on Day 13 of gestation using microarray to identify the genes involved in the injury and recovery of fetal CNS.

MATERIALS AND METHODS

All procedures were carried out in accordance with the protocol approved by the Animal Care and Use Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

Animals

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 lib.

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