

# Expression of p53 and its transcriptional target genes mRNAs in the ethylnitrosourea-induced apoptosis and cell cycle arrest in the fetal central nervous system

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**Summary.** Ethylnitrosourea (ENU) is an alkylating agent and we previously clarified that it induced apoptosis and cell cycle arrest in the fetal central nervous system (CNS). In the present study, we studied the expression of p53 and its transcriptional target genes to investigate the role of p53 in the ENU-induced apoptosis and cell cycle arrest in the fetal CNS following the administration to dams on day 13 of gestation (GD13). Although the enhancement of *p53* mRNA expression was not detected by reverse transcription and polymerase chain reaction (RT-PCR), p53-positive signals were detected immunohistochemically in the nuclei of neuroepithelial cells of the ENU-administered fetuses from 1 hour after treatment (HAT) to 12HAT, and they were most intensive at 3HAT. On the other hand, p53-positive signals were scarcely detected in the control fetuses. Among the p53 target genes, the expression of *p21*, *bax*, *cyclinG1* and *fas* mRNAs increased and peaked at 6HAT. In addition, strong immunoreactivity for p21 was detected in the nuclei of neuroepithelial cells of the fetuses at 6HAT. The expression of p53 protein increased prior to the induction of apoptosis and cell cycle arrest, and transcription of its target genes was also activated. The present results suggest that ENU may induce apoptosis and cell cycle arrest in the fetal neuroepithelial cells in a p53-dependent manner.

**Key words:** Apoptosis, Cell cycle arrest, Ethylnitrosourea, p21, p53

## Introduction

Ethylnitrosourea (ENU), a well known DNA alkylating agent, induces brain tumors in offsprings when administered to pregnant rats (Druckrey et al.,

1967). ENU is also known as a teratogen, and it induces anomalies especially in the central nervous system (CNS) (Pfaffenroth et al., 1974; Hallas and Das, 1978, 1979; Oyanagi et al., 1988; Katayama et al. 2000a). In our previous study, microencephaly was observed in all of the neonates from dams administered with ENU on day 13 of gestation (GD13) (Katayama et al. 2000a). ENU induces apoptosis and cell cycle arrest in the CNS of the rat fetuses immediately after its administration to pregnant rats (Oyanagi et al., 1998; Katayama et al., 2000b, 2001), and this is supposed to be a cause of the above-mentioned brain anomalies.

p53 is activated by DNA-damaging agents or mitotic inhibitors. It transactivates a series of genes including *p21* (el-Deiry et al., 1993), *bax* (Selvakumaran et al., 1994), *cyclinG1* (Okamoto and Beach, 1994), *fas* (Muller et al., 1998) and *gadd45* (Kastan et al., 1992). p21 is an inhibitor of cyclin-dependent kinases and it induces cell cycle arrest at G1 phase (Dulic et al., 1994). Bax is a pro-apoptotic member of the bcl-2 family. Increased bax/bcl-2 ratio enforces dimerization of bax, and finally induces apoptosis (Gross et al., 1998). Although its function is not well understood, *cyclinG1* is thought to be involved in DNA replication because it localizes at replication foci at S phase (Reimer et al., 1999). Fas is a type I membrane protein which belongs to Tumor Necrosis Factor Receptor/Nerve Growth Factor Receptor family (Itoh et al., 1991), and it induces apoptosis when it binds to fas ligand. Gadd45 is reported to play an important role in DNA repair (Smith et al., 1994).

In this study, we examined the changes in the amount of p53 mRNA and protein, and expression of its target genes (*p21*, *bax*, *cyclinG1*, *fas* and *gadd45*) in the rat fetal CNS after ENU-administration to pregnant rats at GD13, which is reported to be the most sensitive period of the rat fetal CNS to ENU-administration (Pfaffenroth et al., 1974; Hallas and Das, 1978).

The present study was approved by The Laboratory Animal Use and Care Committee of the Graduate School of Agricultural and Life Sciences, The University of Tokyo.

## Materials and methods

### Animals

Seventy-two F344/Jcl rats (plug day: day 0 of gestation) were obtained from Saitama Experimental Animal Co., 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 water *ad libitum*.

### Chemicals

ENU (Sigma, St. Louis, MO) was dissolved in 2 mM sodium citrate buffer (pH 4.5) immediately before the treatment, and the concentration was adjusted to 10 mg/ml.

### Treatments

Pregnant rats were injected with 60 mg/kg of ENU intraperitoneally at GD13, and eight dams were sacrificed by heart puncture under ether anesthesia at 1, 3, 6, 12, 24 and 48 hours after treatment (HAT), respectively. Eight untreated rats were sacrificed in the same way at GD13, 14 and 15, respectively. Five dams obtained at each time point were used for reverse transcription and polymerase chain reaction (RT-PCR) analysis and three dams for immunohistochemical analysis, respectively.

### Immunohistochemistry

Collected fetuses were fixed in 10% neutral-buffered formalin and embedded in paraffin. Paraffin sections (4 μm) were deparaffinized and placed in 0.3% H<sub>2</sub>O<sub>2</sub>-containing methanol for 30 minutes to inactivate endogenous peroxidase. After washing in tris-buffered saline (TBS) the sections were immersed in 10 mM citrate buffer, pH 6.0, and heated for 10 minutes at 121 °C by autoclave. Then the sections were incubated in skimmed milk for 40 minutes at 37 °C to reduce non-specific staining, and successively incubated in the rabbit anti-p53 polyclonal antibody (Santa Cruz, CA) or mouse anti-p21 monoclonal antibody (PharMingen, San Diego, CA) overnight at 4 °C, and then EnVision+

polymer reagent (Dako, Carpinteria, CA) for 30 minutes at room temperature. These sections were washed in TBS for 15 minutes after each step. The positive signals were visualized by peroxidase-diaminobenzidine reaction and the sections were then counterstained with methylgreen.

### RNA extraction and semi-quantitative RT-PCR

Five to seven fetal heads from a dam were pooled and total RNA was extracted using Isogen (Nippon Gene Co. Ltd., Toyama, Japan). Reverse transcriptase reaction for the first strand cDNA synthesis was carried out using oligo(dT)<sub>12-18</sub> primer and SUPERScript Preamplification System (GibcoBRL, Grand Island, NY). Polymerase chain reaction was performed with pairs of oligonucleotide primers corresponding to the cDNA sequences of rat mRNA (Table 1). PCR was carried out in a 50 μl reaction mixture containing 50 pM of sense and antisense primer, 1.25 U rTaq, 10 x PCR buffer and dNTP mixture (Takara, Ohtsu, Japan). This was immediately followed by pre-heating at 94 °C for 7 minutes, denaturation at 94 °C for 1 minute, annealing at 58.5 °C for 1 minute and extension at 72 °C for 1 minute using Takara PCR Thermal Cycler MP (Takara, Ohtsu, Japan). Cycle numbers for different PCR reactions are shown in Table 1. Optimal cycle numbers were determined by the preliminary experiment. PCR products were identified by electrophoresis on 2% agarose gels (Nippon Gene Co. Ltd., Toyama, Japan) followed by ethidium bromide (GibcoBRL, Grand Island, NY) staining. Fluorescence gel imaging was carried out using an UV-CCD video system Fas-III (Toyobo, Tokyo, Japan). The results were shown as a relative ratio to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The relative band density is presented as mean ± standard deviation (SD) of 5 dams and statistical analysis was carried out by using the Student's *t*-test.

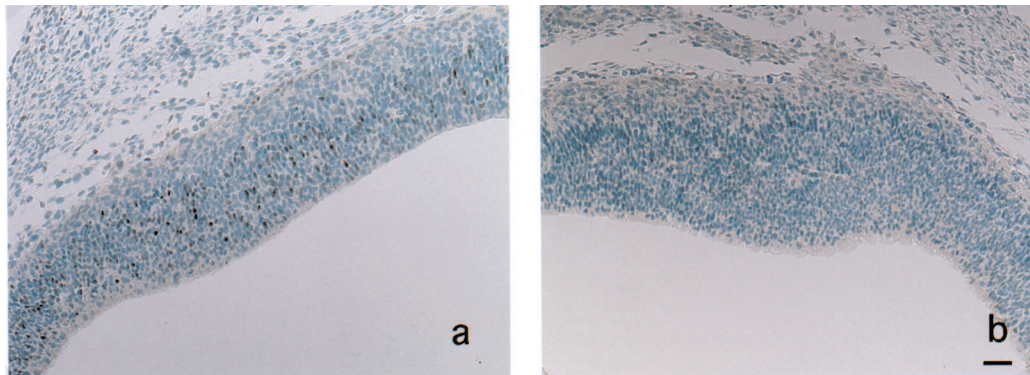
### Morphometry

p53 or p21-positive surviving neuroepithelial cells in the telencephalic wall were counted in two randomly chosen fetuses from a dam on the immunohistochemically stained sections under light microscope. Five hundred cells were counted in each fetus. The p53

**Table 1.** Primer sequences and cycle numbers.

GENE	SENSE PRIMER	ANTISENSE PRIMER	CYCLE NUMBER
p53	ATATGAGCATCGAGCTCCCTCT	CACAACCTGCACAGGGCATGT	24
p21	AAGTATGCCGTCGTCTGTTCG	GGCACTTCAGGGCTTTCTCTT	30
cyclinG1	GTGTCGGAAGTCTGAGCTGCTTTT	TTGGGAGGTGGGTTATCCTGT	23
bax	TTCATCCAGGATCGAGCAGAG	TGAGGACTCCAGCCACAAAGAT	25
fas	AAGAGGAGCGTTCGTGAAACC	GATCAGCAGCCAAAGGAGCTTA	31
gadd45	GATCGAAAGGATGGACACGG	CCCCTGATCCATGTAGCGA	26
GAPDH	GCTTACCACCTTCTTGATGTC	GAGTATGTCTGGAGTCTACTG	20

## p53 expression in ENU-induced apoptosis



**Fig. 1.** Immunostaining for p53 in the telencephalic wall of rat fetuses. **a.** ENU-treated group at 3HAT shows positive signal for p53 in numerous nuclei of the neuroepithelial cells of the ventricular zone. **b.)** In control group at GD13, positive nuclei are less abundant. Bar: 30  $\mu$ m.

and p21-labeling indices (%) are expressed as the mean  $\pm$  SD of 3 dams and statistical analysis was carried out by using the Student's *t*-test.

## Results

### Immunohistochemistry

In the CNS of the control group, a few neuroblasts in the outside of the ventricular zone (marginal zone or cortical plate) and almost no neuroepithelial cells were positive for p53. In the ENU-administered fetuses, p53-positive signals were also detected in the nuclei of the surviving neuroepithelial cells of the ventricular zone from 1HAT to 12HAT, and they were most numerous at 3HAT (Fig. 1). Immunoreactivity for p53 returned to the control level at 24HAT (Fig. 2).

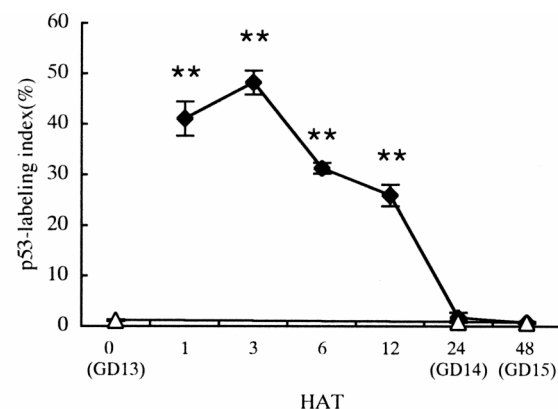
In the CNS of the control fetuses, positive signals for p21 were observed in some neuroblasts in the outside of the ventricular zone (marginal zone or cortical plate), endothelial cells and only a few neuroepithelial cells in the ventricular zone. In the ENU-administered fetuses, p21-positive signals were also detected in the nuclei of the neuroepithelial cells of the ventricular zone from 3HAT. They were most numerous at 6HAT, and returned to the control level at 48HAT (Figs. 3, 4).

### Semi-quantitative RT-PCR

The expression of *p21*, *bax*, *cyclinG1* and *fas* mRNAs significantly increased in the ENU-administered fetuses. The expression of these mRNAs peaked at 6HAT and then returned to the control level at 48HAT. The expression of *p53* and *gadd45* mRNAs did not increase throughout the experimental period (Figs. 5, 6).

## Discussion

In the present study, we used untreated dams as controls. Histologically, there was no difference between untreated controls and vehicle controls in our previous studies (Katayama et al., 2000b, 2001). Thus, we thought it was appropriate to use untreated dams as controls in the present study.



**Fig. 2.** p53-positive neuroepithelial cells were increased from 1HAT, peaked at 3HAT and returned to the control level at 24HAT.  $\blacklozenge$ : ENU-treated group ( $n = 3$ );  $\Delta$ , control group ( $n = 3$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$ : Significantly different from the control of the same day of gestation (Student's *t*-test).

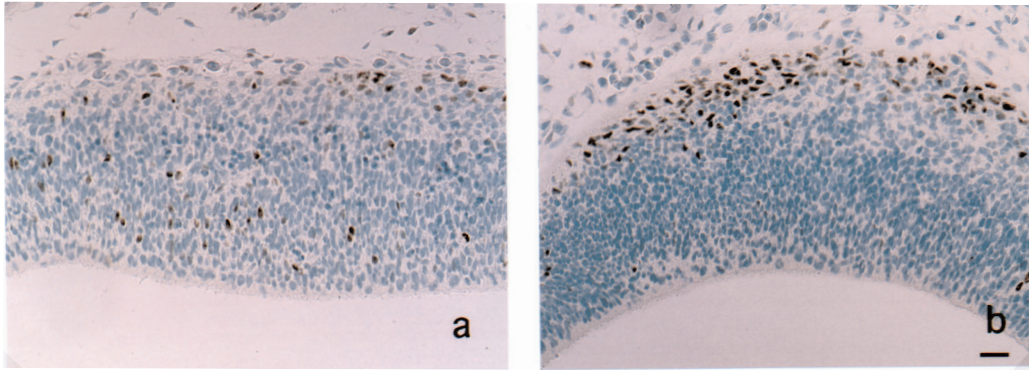
In our previous study (Katayama et al., 2001), we investigated the changes in the number of apoptotic cells and cell proliferative activity after ENU-administration to pregnant rats at GD13, and obtained the following results. Namely, the enhancement of apoptotic cell death was detected in neuroepithelial cells of the fetal CNS from 3HAT. The number of apoptotic cells peaked at 12HAT, gradually decreased towards 24HAT, and returned to the control level at 48HAT. DNA-replicating cells significantly decreased in accordance with the increase in the number of apoptotic cells at 6HAT, indicating the induction of cell cycle arrest. In the present study, the increase of p53 protein was detected prior to the induction of apoptosis and cell cycle arrest, and the activation of its transcriptional target genes was also detected. In addition, in the immunohistochemistry for p21, of which mRNA expression was most prominently elevated among six genes examined, we could detect the enhancement of p21 expression at protein level. Thus, ENU is thought to induce apoptosis and cell cycle arrest in the fetal neuroepithelial cells in a p53-dependent way. The expression of p21 mRNA and

p53 expression in ENU-induced apoptosis

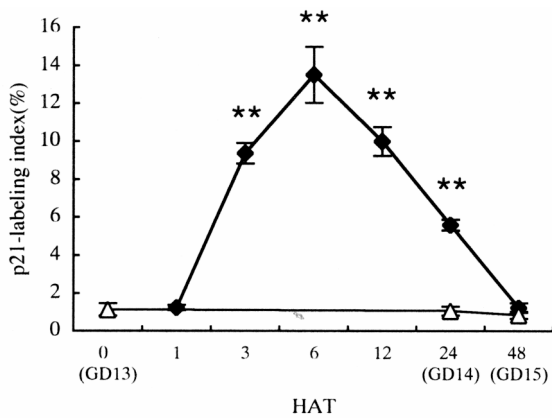
protein was most intensively detected at 6HAT when the number of DNA-replicating cells most strikingly decreased in our previous study (Katayama et al., 2001). Therefore, p21 may play an important role in the cell

cycle arrest induced by ENU.

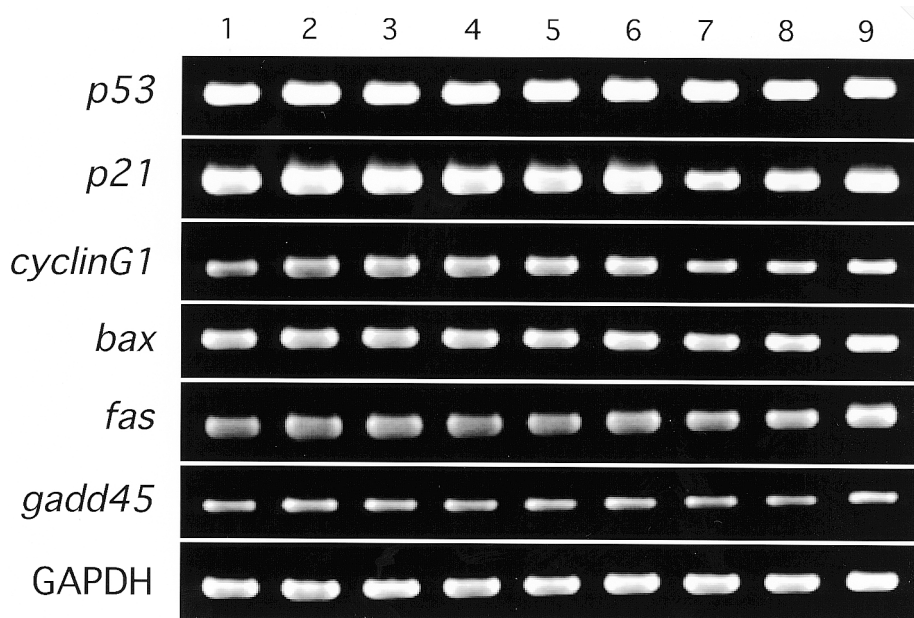
In response to DNA damage, the p53 protein is phosphorylated and becomes stabilized upon disruption of an interaction with its negative regulator, mdm2. In a



**Fig. 3.** Immunostaining for p21 in the telencephalic wall of rat fetuses. **a.** ENU-treated group at 6HAT shows positive signal for p21 in numerous nuclei of the neuroepithelial cells of the ventricular zone and some neuroblasts in the outside of the ventricular zone (marginal zone or cortical plate). **b.** In control group at GD13, positive signals are less abundant in the neuroepithelial cells of the ventricular zone. Bar: 24  $\mu$ m.



**Fig. 4.** p21-positive neuroepithelial cells were increased from 3HAT, peaked at 6HAT, gradually decreased towards 12HAT and returned to the control level at 48HAT.  $\blacklozenge$ , ENU-treated group (n = 3);  $\Delta$ , control group (n = 3). , p < 0.05, \*\* p < 0.01: Significantly different from the control of the same day of gestation (Student's t-test).



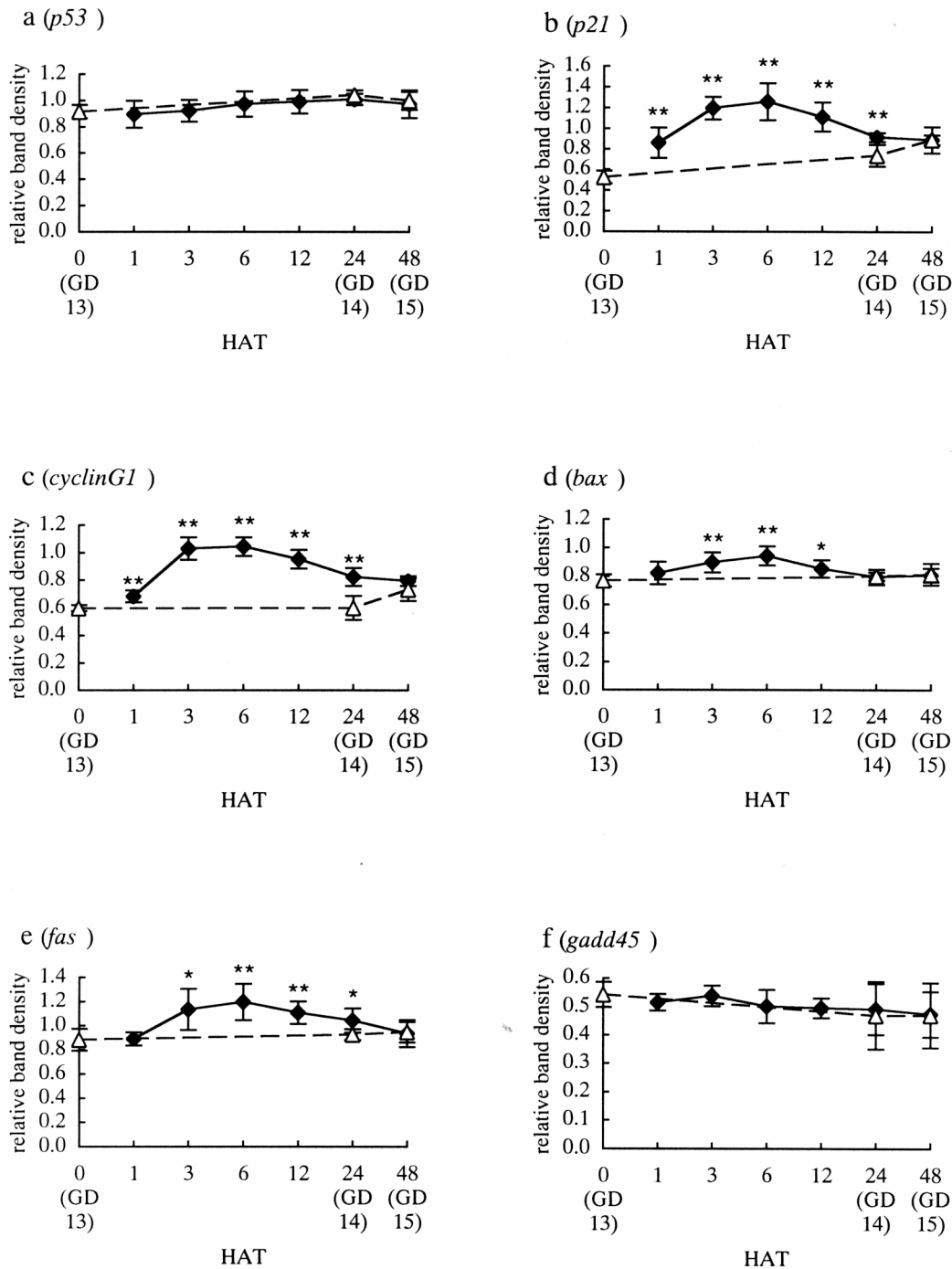
**Fig. 5.** Sequential changes of mRNA expression of p53 and its target genes. Agarose gel electrophoresis. Lane1: 1HAT, 2: 3HAT, 3: 6HAT, 4: 12HAT, 5: 24HAT, 6: 48HAT, 7: Control(GD13), 8: Control(GD14), 9: Control(GD15). Expression of p53 target genes except gadd45 are elevated in the ENU-treated group around 6HAT.

## p53 expression in ENU-induced apoptosis

lot of cases, the activity of p53 is known to be mediated by such a post-transcriptional protein stabilization mechanism. However, in the nervous system, p53 induction was detected not only at protein level but also at mRNA level in accordance with the induction of apoptosis caused by radiation (Bolaris et al., 2001) and excitotoxicity (Sakhi et al., 1994). The induction of p53 mRNA was not detected by RT-PCR in the present study. Therefore, p53 may be regulated by the protein stabilization mechanism in our case.

Besides ENU, radiation induces apoptosis in the rat fetal CNS in a p53-dependent manner (Bolaris et al., 2001), and brain anomalies including microencephaly are detected after birth (Miki et al., 1995). These findings suggest that fetal CNS is susceptible to genotoxic stress, easily develops apoptotic cell death, and decrease of neuronal cells induced by apoptosis may result in formation of brain anomalies including microencephaly.

Among the p53 target genes, *cyclinG1* is known to



**Fig. 6.** Sequential changes of mRNA expression of p53 (a), p21 (b), cyclinG1 (c), bax (d), fas (e) and gadd45 (f). (♦) ENU-treated group (n = 5); (Δ) control group (n = 5). \* p < 0.05, \*\* p < 0.01: Significantly different from the control of the same day of gestation (Student's t-test).

be induced after nerve injury in a p53-independent way, and it is suggested that the alternative regulatory pathway of *cyclinG1* may exist in the nervous system (Morita et al., 1996). Further investigation is needed for clarifying that the induction of *cyclinG1* which was observed in the present study is whether p53-dependent or not. However, the elevation pattern of *cyclinG1* mRNA expression was almost the same as those of other p53 target genes. Therefore, *cyclinG1* may be regulated in a p53-dependent way in our experiment.

At GD15, the expression of *p21* and *cyclinG1* mRNAs was elevated even in the control fetuses compared to that at GD13. It is reported that in the normal rat fetal CNS, both *p21* and *cyclinG1* are expressed only in the postmitotic neurons (Van Lookeren Campagne and Gill, 1998). It is natural to consider that this elevation is brought about by the increase in the number of postmitotic neurons with the progress of differentiation.

It is well known that *gadd45* plays a central role in DNA repair (Smith et al., 1994). In the present study, the expression of *gadd45* mRNA, however, did not increase following the ENU administration. This little induction activity of *gadd45* brings about the remaining of the DNA damage, and finally results in the high incidence of brain neoplasms after birth.

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