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# Gene Expression Profiles in the Fetal Mouse Brain after Etoposide (VP-16) Administration

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**Abstract:** The aim of this study was to analyze the response of gene expression caused by etoposide (VP-16) in the fetal mouse brain. Four miligrams/kilogram of VP-16 was intraperitoneally injected into pregnant mice on day 12 of gestation (GD 12). Gene expression profiling of the VP-16-treated fetal mouse brain by DNA microarray was performed. The expression changes of the target genes of p53 were also examined by real-time RT-PCR. VP-16 induced S-phase accumulation, G2/M arrest, and eventually apoptosis of neuroepithelial cells in the fetal brain. DNA microarray analysis revealed that 8 of cell cycle control- and apoptosis-related genes were upregulated and that 5 of DNA damage, repair, replication, and transcription genes were also upregulated in the fetal telencephalons at 4 h after VP-16 treatment (HAT). The results of real-time RT-PCR demonstrated that the expression of *topoisomerase IIα* was increased at 4 and 8 HAT. The expression of pro-apoptotic factors such as *puma*, *noxa*, *bax*, and *cyclin G* was also increased from 4 to 12 HAT. These results suggest that VP-16 induces DNA damage, DNA repair, cell cycle alternation, and apoptosis in the fetal mouse brain. In addition, VP-16-induced apoptosis is mediated through the mitochondrial pathway in a p53-related manner. The present study will provide a better understanding of the mechanisms of VP-16-induced fetal brain injury.

**Key words:** apoptosis, cell cycle arrest, etoposide (VP-16), neuroepithelial cell, p53

## Introduction

Neuroepithelial cells (NPCs) constitute the pseudostratified epithelium in the ventricular zone of the fetal central nervous system (CNS) [3]. The cells are multipotnent neural stem cells and differentiate into neurons, astrocytes, and oligodendrocytes [19]. During the CNS development, apoptosis of neuroepithelial cells is controlled by various signals, and its precise regulation is indispensable for brain development [13, 14]. On the other hand, neuroepithelial stem cells are quite susceptible to various types of chemical stimuli, such as DNA-damaging agents and mycotoxins, and easily undergo apoptosis [6, 7, 16, 18].

Etoposide (VP-16), a topoisomerase II inhibitor, that stabilizes both topoisomerase II- and topoisomerase II-DNA covalent complexes, is widely used as a DNA damaging agent with a mechanism for inducing DNA double-strand breaks [17, 20]. It has been shown that embryotoxicity and teratogenicity arise when VP-16 is injected into rodents on gestation days 6, 7, and 8, and that the drug induces dose-dependent fetal anomalies

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such as major skeletal abnormalities, exencephaly, and anophthalmia [15]. In our previous studies, VP-16 induces cell cycle arrest and apoptosis in neuroepithelial cells of the fetal brain immediately after its administration to a pregnant dam, and such cell cycle arrest and apoptosis occur in a p53-related manner [9–11].

There were no effects on litter size and mortalities of fetus (embryo days 12 to 14) neonates and pups after 4 mg/kg VP-16 administration to dams on GD 12 in our previous studies (data were not reported). And, these 4-week-old pupils showed only a decrease of cerebral cortex thickness without other anomalies (data were not reported). Therefore, this study was focused on cell damage and its mechanisms in NPCs of the fetal mouse telencephalons. Precise mechanisms of VP-16-induced neuroepithelial cell damage in the fetal CNS are not well understood and little known. In the present study, we investigated changes in gene expression using DNA microarray analysis in the fetal CNS after administration of VP-16 to pregnant dams on GD 12. We also examined serial changes in the expression of p53 and its target genes using the real-time reverse transcription-polymerase chain reaction (real-time RT-PCR) method.

## Materials and Methods

*Animals*

Eight-week-old pregnant ICR (Crlj:CD-1) mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). Mice were kept in an isolator cage (Niki Shoji, Tokyo, Japan) under a controlled conditions (23 ± 2°C, 55 ± 5% humidity, and a 14-h light/10-h dark cycle) and provided with commercial pellets (MF, Oriental Yeast, Tokyo, Japan) and tap water *ad libitum*. All experimental procedures were approved by the Animal Use and Care Committee of the Graduated School of Agricultural and Life Sciences, The University of Tokyo.

*Chemical*

VP-16 was purchased from Sigma (St. Louis, MO, USA), and dissolved in a 1% dimethyl sulphoxide (DMSO) physiological saline before injection.

*Treatment for ICR mice*

Pregnant mice were injected intraperitoneally (i.p.) with 4 mg/kg of VP-16 on GD 12. As controls, pregnant mice were injected i.p. with the vehicle on the same gestation day. Dam mice were sacrificed by exsanguination under a deep ether anesthesia at 1, 2, 4, 8, 12, 24, and 48 h after treatment (HAT). Collected fetal telencephalons were subjected to the flow cytometric analysis, DNA microarray, and real-time RT-PCR.

*Cell cycle analysis*

Forty-two pregnant dams were subjected to cell cycle analysis. Telencephalons of three fetuses from each dam (n=3) were analyzed following the method described previously [8]. Cell cycle analysis was performed with a FACSCalibur Flow Cytometer (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Using Cell Quest program (Becton, Dickinson and Co.), the percentages of cells at various phases of the cell cycle were calculated after doublets and debris were discarded.

*RNA extraction for DNA microarray and real-time RT-PCR analysis*

Based on cell cycle analysis and our previous study [10], VP-16 exposure in NPCs of the fetal mouse brain markedly induced apoptosis from 4 to 24 HAT and upregulated p53-related genes from 4 to 12 HAT [10, 11]. Based on the above data, DNA microarray and real-time RT-PCR analyses were pweformed on 4, 8, and 12 HAT.

Twelve and eighteen pregnant dams were subjected to DNA microarray and real-time RT-PCR analyses, respectively. Eight fetal telencephalons were acquired from each dam (test groups and controls; n=2 dams per time point for microarray analysis; n=3 dams for real-time RT-PCR) and total RNA was extracted with an RNeasy Mini Kit (Qiagen, Germantown, MD, USA). The quality and quantity of the extracted RNA samples were examined by agarose gel electrophoresis.

*DNA microarray analysis*

Microarray analysis was performed with a GeneChip system (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions. Double-stranded cDNA was synthesized from total extract RNA. The first cDNA strand was prepared from total RNA with SuperScript II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and the T7-(dT)24 primer (primer sequence, 5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-[dT]24-3', Amersham Bioscience, Tokyo, Japan). The second strand was synthesized with a SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen). Biotin-labeled cRNA was then synthesized from the double-stranded cDNA with a BioArray High

**Table 1.** Oligonucleotide primer sequences and annealing temperatures for real time RT-PCR

| Gene | Sense primer | Antisense primer | Annealing temperature (°C) |
|---|---|---|---|
| *topoisomerase IIα* | CCAGCGGAAACTGAACAGTCAA | TTCCGAGACACGGCACTCAA | 64 |
| *puma* | CCTCCTTTCTCCGGAGTGTTCA | ATACAGCGGAGGGCATCAGG | 68 |
| *noxa* | GGCACTCGCGATTCATCTTG | GAGCTGCGAACTCAGGTGGTA | 64 |
| *bid* | AGACAGCTAGCCGCACAGTTCA | TTGGCCAACAGCATGGTCA | 64 |
| *bax* | CAGGATGCGTCCACCAAGAA | GTTGAAGTTGCCATCAGCAAACA | 64 |
| *cyclin G1* | GTAGCAGGATTACAGCGCAGCA | CCCTAACCGTGAGGCCATTC | 64 |
| *gapdh* | TGTGTCCGTCGTGGATCTGA | TTGCTGTTGAAGTCGCAGGAG | 64 |

Yield RNA transcription labeling kit (Enzo Diagnostics, Farmingdale, NY, USA) and purified with an RNeasy Mini Kit (Qiagen). Biotin-labeled cRNA (20 $\mu$g) was then fragmented in a fragmentation buffer and mixed in a hybridization solution prepared with a GeneChip eukaryotic hybridization control kit (Affymetrix). cRNA was then hybridized to the Affymetrix rat expression array 230A chip at 45°C for 16 h while being rotated at 60 rpm in a GeneChip Hybridization Oven 640 (Affymetrix). The chips were then washed and stained automatically with a fluidics station (Affymetrix) and scanned with a GeneArray scanner (Hewlett Packard, Palo Alto, CA, USA).

*Microarray data analysis*

Microarray imaging data were analyzed and calculated automatically with Microarray Suite v. 5.0 (Affymetrix). In the pairwise comparison of results, the patterns of changes of the whole probe set were used to perform a statistical analysis as described in the manufacturer's guide (Microarray Suite v. 5.0 User's Guide). A qualitative call (a "difference call") of increase, decrease, marginal increase, marginal decrease, or no change was made. Then, the data were extracted from the groups of genes corresponding to significant changes in both samples. The change in gene expression was calculated as the ratio of the average value compared with that of control arrays. Fold changes in expression of greater than a 1.4-fold increase or less than a −1.4-fold decrease were used as criteria for meaningful changes between VP-16-treated and control groups.

*Real-time RT-PCR*

For real-time RT-PCR assay, first-strand cDNA was synthesized from the total extract RNA by reverse transcription, using a PrimeScript™ RT Reagent Kit (Takara, Otsu, Japan). For real-time RT-PCR, oligonucleotide primer sets of *topoisomerase IIα*, *bax*, *puma*, *noxa*, *bid*, *cyclin G*, and *gapdh* were purchased from Takara (Table 1). The reaction mixture containing SYBR® Premix Ex Taq™ (Takara), ROX reference dye, sense primer, antisense primer, and a cDNA sample underwent 40 cycles of amplification (denaturation at 95°C for 5 s and annealing at 64 or 68°C for 30 s) using an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Expression levels of the genes were calculated relative to that of *gapdh*, and the fold changes relative to control values were represented as the mean ± SE of three dams. Statistical analysis was performed by Student's *t*-test.

## Results

*Cell cycle analysis*

Results of flow cytometric analysis are shown in Fig. 1. The S and G2/M fractions were increased significantly at 4 and 8 HAT by VP-16 treatment. Furthermore, the sub-G1 fraction (apoptotic cells) of VP-16-exposed fetuses began to increase significantly at 4 HAT, peaked at 12 HAT, decreased gradually at 24 HAT, and then returned to the control level at 48 HAT. It was, therefore, concluded that VP-16 induced S-phase accumulation, G2/M arrest, and apoptosis of neural progenitor cells in the fetal telencephalon.

*DNA microarray analysis*

Results of the DNA microarray analysis are shown in Tables 2 and 3. To investigate detailed mechanisms of cell damage, two time points were selected for microarray analysis. It was expected that the expression of genes involved in cell cycle control and apoptosis would be changed at 4 and 8 HAT (cell damage phase). At 4 HAT (Table 2), the expressions of 5 cell cycle control and apoptosis-related genes including p53-target factors (p21, noxa, and cyclin G) were upregulated, and 5 genes involved in DNA damage, repair, replication, and transcription were also upregulated in the fetal telencephalon

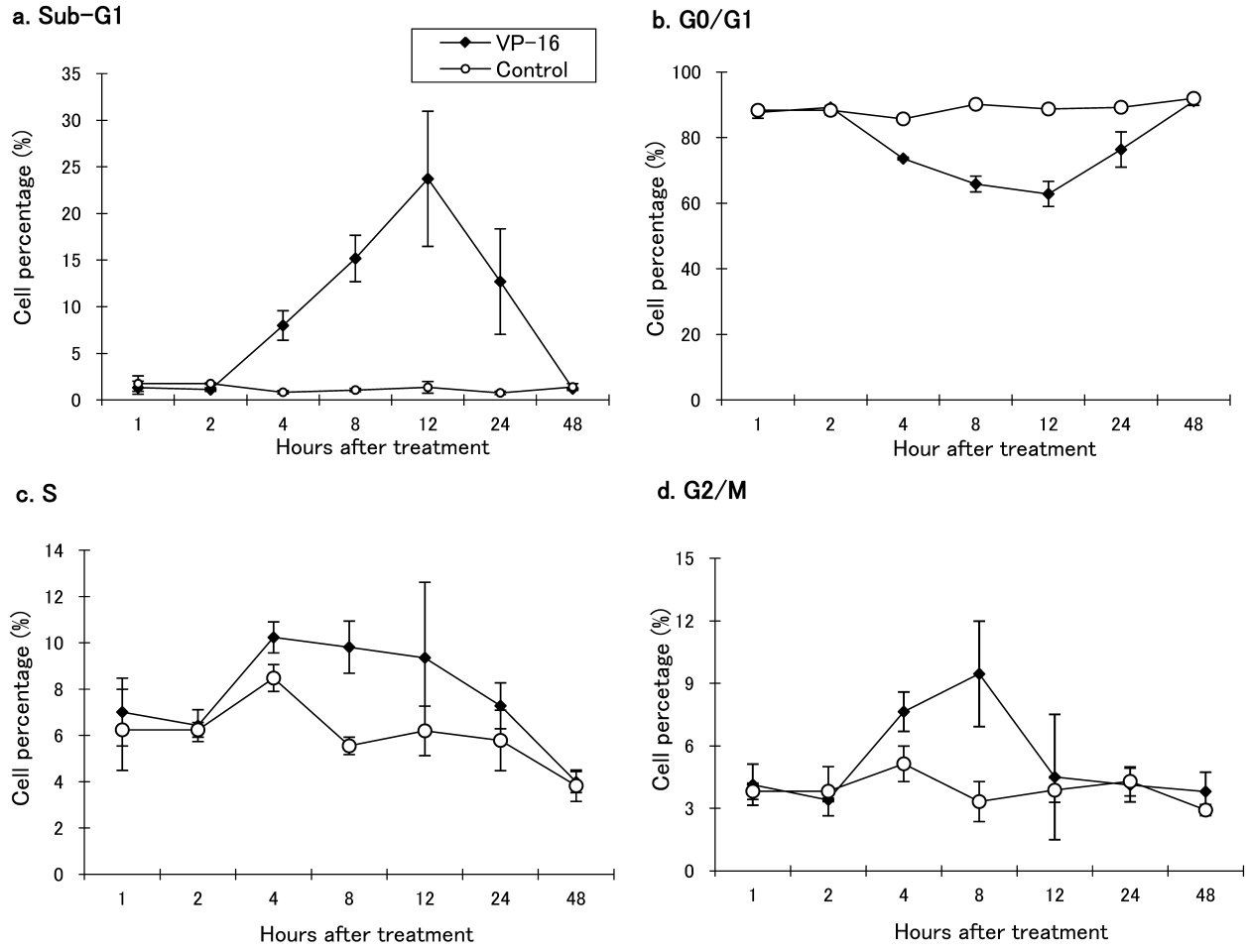


**Fig. 1.** Cell cycle analysis of fetal telencephalic cells after VP-16 administration. VP-16 induces S-phase accumulation, G2/M arrest, and apoptosis. Percentages for each cell cycle phase [sub-G1 (a), G0/G1 (b), S (c), and G2/M (d)] are presented as the mean ± SD of two fetuses from each dam (n=3).

by VP-16 treatment. However, only two genes were downregulated at 4 HAT by VP-16 administration (Table 2). At 8 HAT, there were only several genes with alterd changed in their expression as a result of VP-16 treatment (Table 3). There were almost no changes of gene expression at 12 HAT (data were not shown).

*Findings of real-time RT-PCR*

Results of the real-time RT-PCR analysis are shown in Fig. 2. The expression of *topoisomerase IIα* was slightly increased at 4 and 8 HAT, which might have occur in response to the inhibition of topoisomerase IIα by VP-16. The expressions of *puma* and *noxa* were predominantly increased from 4 to 12 HAT. The expression of *bax* was also slightly increased from 4 to 12 HAT. The expression of *cyclin G1* was also prominently increased from 4 to 12 HAT.

## Discussion

Our previous studies verified that VP-16 administration induced DNA damage, induced cell cycle alteration such as S-phase accumulation and G2/M arrest, and then underwent apoptotic cell death in NPCs of the fetal mouse telencephalon [9–11]. NPCs following VP-16 exposure showed prominent histopathological changes such as a decrease of mitotic cells, nuclear piknosis, and apoptotic appearances. These cells also reacted positively in TUNEL staining and immunohistochemistry for cleaved-caspase 3 by VP-16 [10]. VP-16-induced cell damage increased p53 and p21 protein and upregulated p53 transcriptional factors such as *p21*, *fas*, and *puma* [11]. It was also clarified that VP-16 caused DNA damage and induced cell cycle arrest and apoptosis of the neural progenitor cells via the ATM/p53 pathway [9].

**Table 2.** Changes in gene expression at 4 h after VP-16 treatment

| Accession No. | Gene | Fold change |
|---|---|---|
| Upregulataed genes | | |
| *Apoptosis, cell cycle control* | | |
| AK007630.1 | cyclin-dependent kinase inhibitor 1A (p21) | 1.4 ± 0.141 |
| BG065754 | cyclin G | 2.15 ± 0.071 |
| AV308638 | sestrin 2 | 2.6 ± 0.141 |
| NM_021451.1 | noxa | 1.75 ± 0.354 |
| U67321.1 | caspase 7 | 2.8 ± 0.566 |
| *DNA damage, repair, replication, transcription* | | |
| AY028963.1 | zinc finger and BTB domain containing 20 | 1.45 ± 0.071 |
| L04961.1 | inactive X specific transcripts | 2.3 ± 0.071 |
| BG806300 | X-specific transcript antisense | 1.4 ± 0.141 |
| BI152524 | far upstream element binding protein 1 | 1.75 ± 0.212 |
| BI664122 | nuclear factor I/B | 1.95 ± 0.071 |
| *Signal transduction* | | |
| AA560093 | receptor activity modifying protein 2 | 1.6 ± 0.566 |
| NM_010181.1 | fibrillin 2 | 1.4 ± 0.141 |
| *Cytoskeletion or extramertix related* | | |
| NM_007993.1 | fibrillin 1 | 1.4 ± 0.424 |
| BM251152 | versican | 1.4 ± 0.424 |
| BB700837 | rotatin | 2.75 ± 0.212 |
| *Others* | | |
| NM_013750.1 | pleckstrin homology-like domain, family A, member 3 | 1.4 ± 0.424 |
| BM119226 | Gtl2 | 1.4 ± 0.424 |
| BB475271 | LUC7-like 2 | 2.3 ± 0.141 |
| BG09162 | formin binding protein 4 | 2.65 ± 0.071 |
| BM239446 | RNA binding motif protein 39 | 1.55 ± 0.212 |
| Downregulated genes | | |
| *Metabolism* | | |
| AA561726 | 3-phosphoglycerate dehydrogenase | –2.9 ± 0.071 |
| *Cytoskeleton* | | |
| NM_021050.1 | cystic fibrosis transmembrane conductance regulator homolog | –3.3 ± 0.849 |

**Table 3.** Changes in gene expression at 8 h after VP-16 treatment

| Accession No. | Gene | Fold change |
|---|---|---|
| Upregulated genes | | |
| DNA damage, repair, replication, transcription | | |
| NM_053141.1 | protocadherin beta 16 | 1.7 ± 0.424 |
| Signal transduction | | |
| M61000.1 | gastrin-releasing peptide receptor | 2.6 ± 1.555 |
| Mm.8585.2 | Casitas B-lineage lymphoma | 1.4 ± 0.099 |

The present study clarified that administration of VP-16 to pregnant mice induced 1) cell cycle alternation and apoptosis, promoted 2) the upregulation of DNA damage- and repair-, cell cycle alternation-, and apoptosis-related genes, and 3) increased in mRNA expressions of p53 target genes, such as *p21, puma*, *noxa*, *bax*, and *cyclin G1*, in the fetal telencephalons.

It is well known that p53 is a transcriptional activator regulating the genes involved in growth arrest, DNA repair, and apoptosis [2, 4, 5]. Using DNA microarray and real-time RT-PCR analyses, it was observed that VP-16 exposure in the fetal telencephalons upregulated p53 transcription factors related to cell cycle control and apoptosis. *Cyclin G* and *p21* were also up-regulated by p53 in response to the DNA damage after VP-16 treatment. Cyclin G1 is a negative regulation factor in response to the degradation of p53 protein like mdm2 resulting in p53 increase [6]. p53 pathway activation following DNA damage induces p21 up-regulation which may inhibit G1-S phase transition by inhibiting Cdk4/

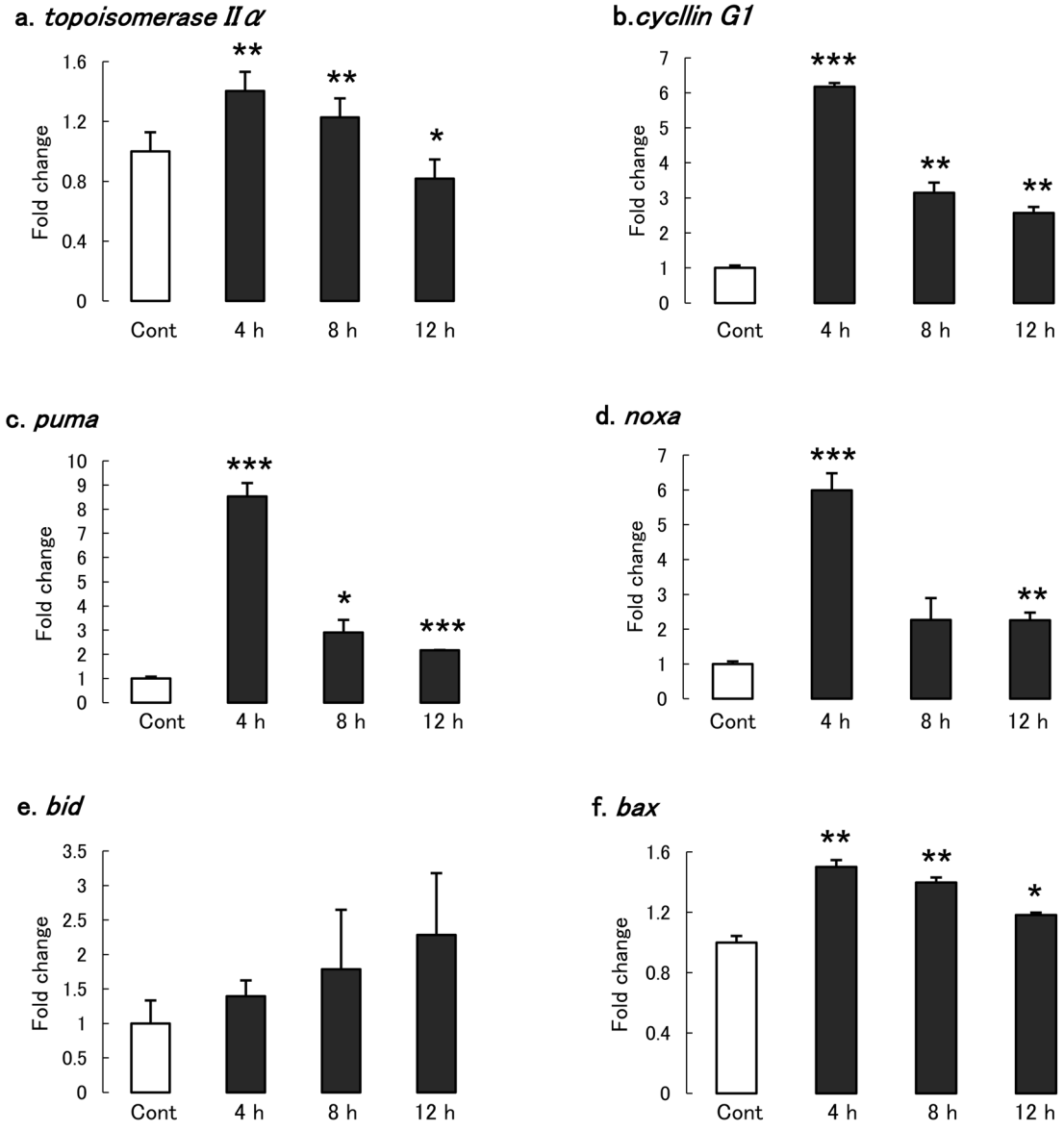


**Fig. 2.** mRNA expression of *topoisomerase IIα*, *cyclin G1*, *puma*, *noxa*, *bid*, and *bax*, as determined by real-time RT-PCR. Open and closed bars indicate control (8 h) and VP-16-treated groups, respectively. Data represent the means of 8 fetuses obtained from each dam (n=3) ± SE. **P*<0.05; ***P*<0.01; ****P*<0.001: significantly different from controls by the Student's *t*-test.

cyclin and/or Cdk2/cyclin E activities [5]. Otherwise, p21 induction by p53 following DNA damage may lead to G2/M arrest by inhibition of Cdc2/cyclin B complex function. [4]. Therefore, it was presumed that DNA damage by VP-16 might induce G2/M arrest by p21 upregulation following p53 activation. It has been reported that p21 protein by exposure of VP-16 was prominently increased in immunohistochemistry and western blot [9, 10].

The upregulation of pro-apoptotic factors like *puma*, *noxa*, and *bax* was directly induced by p53 in response to the DNA damage by VP-16. In response to genotoxic stress, puma, noxa, and bax induced apoptosis in neural progenitor cells of the fetal brain via the p53-dependent pathway [1, 2]. Puma is an essential factor for DNA damage-induced neuroepithelial cell death [1]. When overexpressed, noxa underwent BH3 motif-dependent localization to mitochondria and interacted with anti-apoptotic Bcl-2 family members, resulting in the activation of caspase-9 [12]. Bax, a pro-apoptotic factor, is

directly activated by p53 and it mediates mitochondrial membrane permeabilization and apoptosis [1, 2]. From these results, it is clear that VP-16-induced apoptosis of fetal neuroepithelial cells might involve the activation of mitochondrial pro-apoptotic factors.

In addition, the expression of *topoisomerase IIα* was upregulated at 4 and 8 HAT according the real-time RT-PCR analysis. It was assumed that upregulation of *topoisomerase IIα* might be a response to quick degradation of topoisomerase IIα-DNA-VP-16 complex.

In conclusion, the present gene expression data might explain the mechanism of DNA damage, DNA repair, cell cycle alternation, and apoptosis, caused by exposure of the fetal mouse brain to VP-16.

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