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Histopathological changes in the brain of mouse fetuses by etoposide-administration

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Summary. Etoposide (VP-16), a topoisomerase II inhibitor, is an anti-tumor agent which is also known to show embryotoxicity, and teratogenicity when administered to pregnant rodents. We examined VP-16-induced histopathological changes in the brain of mouse fetuses. Pregnant mice were intraperitoneally injected with VP-16 (4 mg/kg) on day 12 of gestation (GD 12), and fetuses were collected from 1 to 48 hours after treatment (HAT). Mitotic neuroepithelial cells in the telencephalic wall prominently decreased at 2 HAT, and were hardly observed at 4 HAT. The number of pyknotic neuroepithelial cells in the fetal brain began to increase at 4 HAT, and became prominent from 8 to 24 HAT. These pyknotic cells were also positively stained by TUNEL method, which can detect fragmented DNA, and showed ultrastructural characteristics of apoptosis. Additionally, these cells were also positive for cleaved caspase-3, an essential executioner of apoptosis. This indicated that excessive neuroepithelial cell apoptosis was induced in the brain of mouse fetuses following VP-16 treatment on GD 12.

Key words: Apoptosis, Brain, Etoposide, Fetus, Mouse

Introduction

Epotoside (VP-16) is a semisynthetic derivative of podophyllotoxin, which is extracted from a plant *Podophyllum peltatum* and has an antitumor activity (Sieber et al., 1978). VP-16 is widely used as an anticancer chemotherapeutic agent for small cell lung cancer, testicular cancers and lymphomas (Hande, 1998). VP-16, a topoisomerase II inhibitor, impairs DNA synthesis by forming complexes with the cleaved DNA and prevents relegation of the double-strand breaks in the replicating cells (Chen and Liu, 1994). On the other hand, VP-16 is embryocidal and teratogenic in rats and mice. For example, a single intraperitoneal injection of VP-16 to pregnant mice on day 6, 7, or 8 of gestation (GD 6, 7 or 8) caused embryotoxicity, cranial abnormality, and major skeletal malformation (Sieber et al., 1978).

However, details of histopathological changes were not investigated in the brain of fetuses following VP-16 administration to their dams. Therefore, we carried out detailed histopathological examination on the fetal brain obtained from pregnant mice which were treated with VP-16 on GD 12.

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Materials and methods

Animals and treatments

Eight-week-old pregnant ICR (Crj:CD-1) mice were obtained from Charles River Japan Co., Yokohama, Japan. Mice were kept using an isolator caging system (Niki Shoji Co., Tokyo) under controlled conditions (23±2°C with 55±5% humidity and a 14-hr light/10-hr dark cycle) and fed commercial pellets (MF, Oriental Yeast Co., Ltd., Tokyo) and tap water ad libitum. VP-16 (Sigma, St. Louis, MO) was first dissolved in 1% dimethyl sulphoxide (DMSO) solution in physiologic saline.

Thirty-five 8-week-old pregnant mice were injected with 4 mg/kg of VP-16 intraperitonially (i.p.) on GD 12, and each group including five dams were sacrificed by exsanguinations under ether anesthesia at 1, 2, 4, 8, 12, 24, and 48 hours after treatment (HAT), respectively. Twenty-one age-matched pregnant mice were injected i.p. with 1% DMSO solution on GD 12, and three dams were sacrificed in the same way at 1, 2, 4, 8, 12, 24, and 48 HAT, respectively. Fetuses were collected by Caesarian section from dams.

The protocol of the present study was approved by

the Animal Use and Care Committee of the Graduated School of Agricultural and Life Sciences, the University of Tokyo.

Histopathology

The fetuses obtained from the dams as scheduled were fixed in 10% neutral-buffered formalin and embedded in paraffin. Four-μm paraffin sections were then stained with hematoxylin and eosin (HE) for histopathological examination.

Immunohistochemistry

Immunohistochemical staining was carried out by LSAB method. Anti-cleaved caspase-3 antibody was obtained from Cell Signaling Technology., USA, and anti-proliferating cell nuclear antigen (PCNA) antibody was purchased from Novacostra Laboratories, UK. Paraffin sections were deparaffinized and immersed in 10mM citrate buffer, pH 6.0, and autoclaved for 10 min at 121°C. After washing in Tris-buffered saline (TBS), the sections were placed in 0.3% H_2O_2-containing methanol for 30 min to inactivate endogenous peroxidase. After incubation in skimmed milk at 37°C for 40 min to reduce non-specific staining, the sections were reacted with primary antibodies at 4°C overnight, with secondary antibody at room temperature for 40 min, and then with peroxidase-labeled streptoavidine (Dako, CA) at room temperature for 40 min, respectively. The sections were visualized by peroxidase-diaminobenzidine (DAB) reaction and then counterstained with methyl green.

In situ detection of fragmented DNA (apoptosis)

DNA fragmentation was examined on the paraffin sections by a modified TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) method, which was first proposed by Gavrieli et al. (1992) and has been widely used for the detection of apoptotic cells. A commercial apoptosis detection kit (ApopTag® Peroxidase *In situ* Apoptosis Detection Kit; Chemicon, CA) was used in the present study. In brief, multiple fragmented DNA 3'-OH ends on a deparaffinized section were labeled with digoxigenin-dUTP in the presence of terminal deoxynucleotidyl transferase (TdT). Peroxidase-conjugated anti-digoxigenin antibody was then reacted with the section. Apoptotic nuclei were visualized by diaminobenzidine (DAB) reaction. The sections were then counterstained with methyl green.

Electron microscopy

Some fetal brain tissues were subjected to electron microscopic examination. Small pieces of the tissues were fixed in 2% glutaraldehyde in 0.1M phosphate buffer (pH 7.4), postfixed in 1% osmium tetroxide in the same buffer, and embedded in an epoxy resin (Oken, Shoji Co., Tokyo). Semithin sections were stained with toluidine blue for light microscopic survey. Ultrathin sections of selected areas were then double-stained with uranyl acetate and lead citrate, and observed using a JOEL 1200 EX electron microscope (Nippon Denshi Co., Ltd., Tokyo).

Cell counting

The number of mitotic cells in the ventricular zone (VZ) of telencephalon, was counted on one HE-stained section each for randomly chosen two fetuses per dam. TUNEL-positive and cleaved caspase-3-positive neuroepithelial cells in the telencephalon were counted on one immunostained section each for six fetuses per dam. One thousand cells were counted on each section under a light microscope (X400). Values were expressed as mean ± standard deviation (SD) at each point of examination, and statistical analysis was done by Student's t-test.

Results

There were no significant differences in body weight changes of fetuses between the VP-treated and control groups.

VP-16 administration induced histopathological changes in the fetal brain. Mitotic-index of neuroepithelial cells of the telencephalon began to decrease significantly at 2 and 4 HAT, and then recovered to the control level at 8 HAT (Fig. 1). At 4 HAT, there were only few mitotic neuroepithelial cells in the VZ of telencephalon.

Pyknotic cells in the telencephalon of VP-16 treated mice began to increase at 4 HAT, peaked at 12 HAT, gradually decreased at 24 HAT, and then returned to the control level at 48 HAT (Fig. 2). Pyknotic cells in the

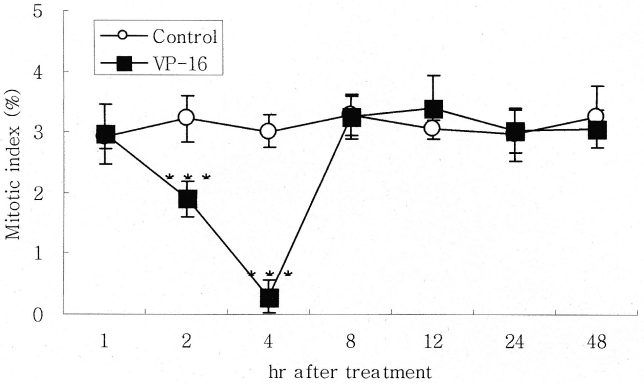

Fig. 1. Changes in the mitotic index (%) in the telencephalic wall of the fetal brain. Each value represents the mean ± SD of 2 fetuses obtained from each dam. ***p<0.001: Significantly different from control.

telencephalon were observed mainly in the ventral layer at 4 HAT and mainly in the middle and dorsal layers of the VZ at 8, 12, and 24 HAT, respectively (Fig. 2). Futhermore, in some fetuses, neuroepithelial cell arrangement was affected by the loss of pyknotic cells. These cells fell into the ventricular apace at 12 and 24 HAT.

Almost all of the pyknotic cells in the VP-16 treated fetal tissues were positive for TUNEL and cleaved caspase-3 (Figs. 3, 5). The time course of the number of TUNEL-positive cells in the telencephalic wall corresponded well to that of pyknotic cells. The number of TUNEL-positive cells increased significantly from 8 HAT, reached the peak at 12 HAT, decreased at 24 HAT, and then returned to the control level at 48 HAT (Fig. 4). Only a few TUNEL-positive cells were observed in controls. Cleaved caspase-3-positive cells in the VZ increased moderately at 4 HAT, peaked at 8 and 12 HAT, and decreased at 24 HAT (Fig. 6). On the other hand, PCNA-positive cells in the VZ slightly decreased only at 12 HAT compared to controls.

Electron-microscopically, the pyknotic cells were characterized by shrinkage of the cell body, condensation of nuclear chromatin and margination of condensed chromatin along the nuclear membrane (Fig 7a). Some apoptotic cells were fragmented into small pieces, which were frequently ingested by adjacent cells and macrophages (Fig 7b).

A similar but less prominent pyknotic changes were also observed in the diencephalon, mesencephalon, metencepahlon, spinal cord (only near the spinal canal area), and mesenchyme of limbs in VP-16-treated fetuses. Almost of these cells were also positive for TUNEL and cleaved caspase-3.

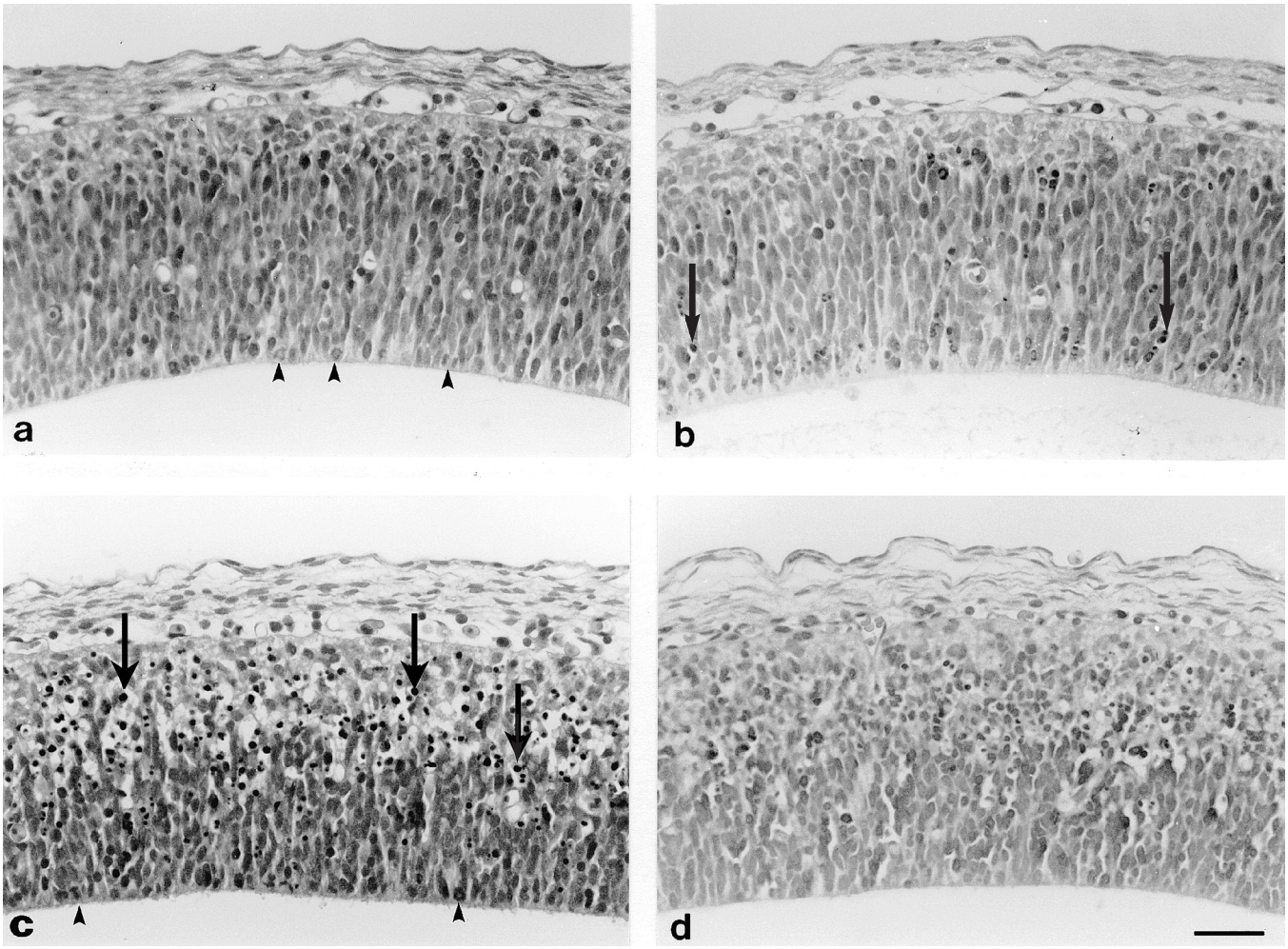

Fig. 2. Histological appearances of the telencephalic wall of a control fetus **(a)** and those of VP-16-treated fetuses at 4 HAT **(b)**, 12 HAT **(c)**, and 24 HAT **(d)**. HE stain, bar: 31 μm. Pyknotic cells were observed from 4 HAT. The number of pyknotic cells peaked at 12 HAT, and then gradually decreased at 24 HAT. Arrowheads: mitotic cell; Arrows: pyknotic cell. x 400

Discussion

Etoposide (VP-16), a topoisomerase II inhibitor, has been widely used as an antitumor drug since 1970. VP-16 is also known as a genotoxic and teratogenic chemical. This study was focused on morphological characteristics of the fetal mouse brain lesions treated with VP-16 on GD 12.

Almost all of the nuclei of VP-16-induced pyknotic cells were positive for TUNEL stain and cleaved caspase-3. Activation of caspase-3 is an essential event for apoptosis, and it is either partially or totally responsible for the proteolytic cleavage of many key proteins during the apoptotic cascade such as the nuclear enzyme poly (ADP-ribose) polymerase (PARP) (Fernandes-Alnemri et al., 1994). Moreover, electron microscopic features of these cells accorded well with the ultrastructural characteristics of apoptotic cells (Ihara et al., 1998). TUNEL method, which can detect fragmented DNA *in situ*, is widely used for the evaluation of apoptotic cells. Even though some researchers have recently demonstrated that TUNEL technique may not be specific for apoptotic cells, and it also detects a small population of necrotic cells (de Torres et al., 1997; Levin et al., 1999), the detection of cleaved caspase-3 and ultrastructural morphology in the VP-16-treated fetal mouse brain in this study verified apoptotic changes. Therefore, it is reasonable to consider that the pyknotic cells observed in the fetal brain of the VP-16-treated fetuses are apoptotic ones.

Following VP-16 treatment to pregnant mice on GD 12, a marked decrease of mitosis of neuroepithial cells was observed in the VZ from 2 HAT to 4 HAT. The cleaved caspase-3-positive cells began to increase at 4 HAT, peaked at 8 and 12 HAT, and then decreased weakly at 24 HAT. This indicates that VP-16-induced apoptotic changes of neuroepithelial cells began to occur at 4 HAT. Decreased mitotic index before apoptotic

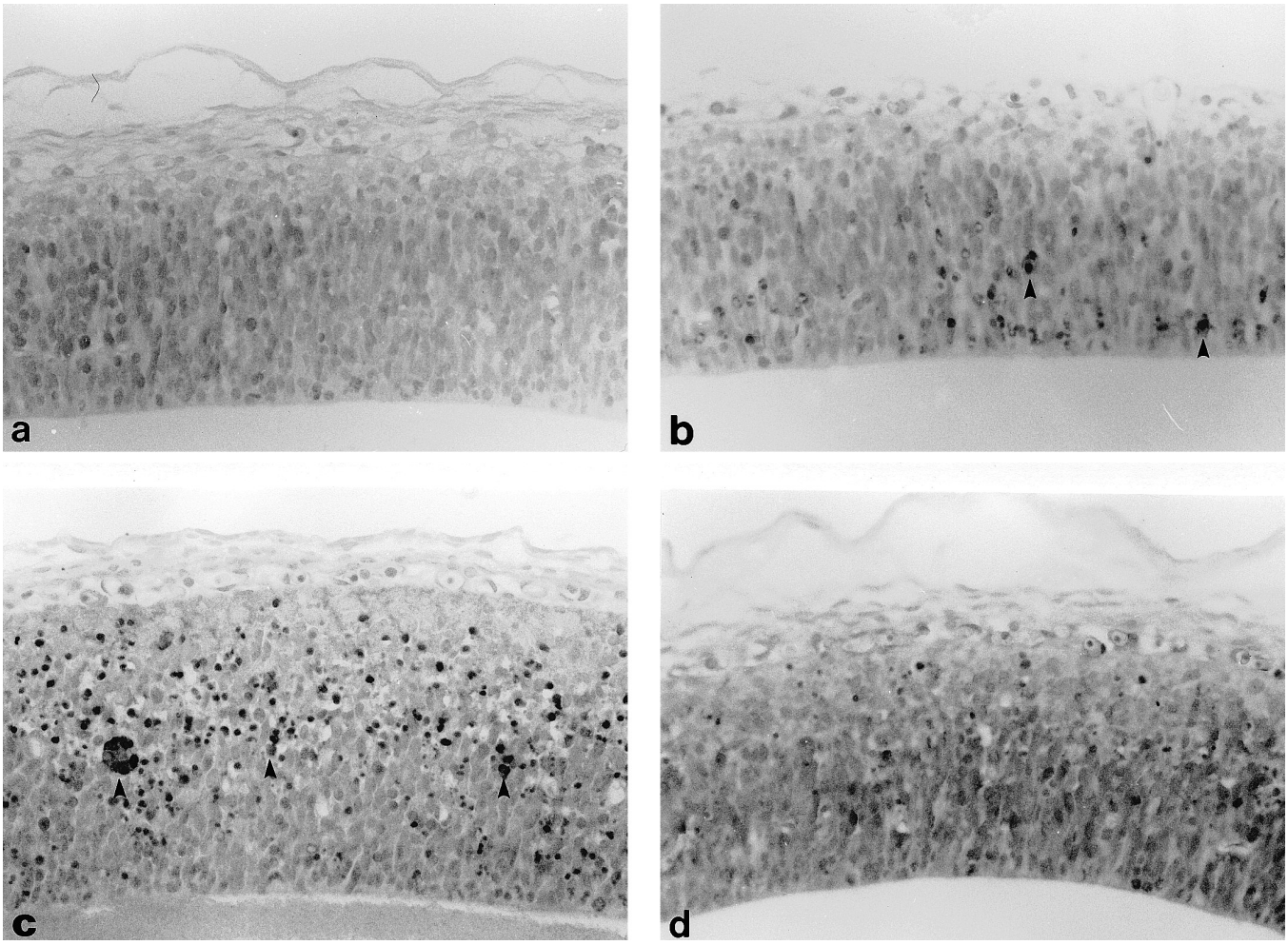

Fig. 3. TUNEL-positive cells in the telencephalic wall of the fetal brain of a control **(a)** and those of VP-16-treated fetuses at 4 HAT **(b)**, 12 HAT **(c)**, and 24 HAT **(d)**. The number of TUNEL-positive cells began to increase from 4 HAT, peaked at 12 HAT, and decreased at 24 HAT. Arrowheads: TUNEL-positive cells. x 400

changes demonstrated in the present study suggested that the initiation of neuroepithelial damage might occur in the pre-mitotic phase of the cell cycle. Apoptotic neuroepithelial cells were mainly observed in the middle and dorsal layers of the VZ at 8, 12, and 24 HAT. It is reported that the neuroepithelial cells in the dorsal layer actively synthesize DNA (S-phase), and those of the middle layer are in the G1 or G2 phase of the cell cycle (Langman et al., 1966). VP-16 has also been reported to induce apoptosis throughout late G1/S or G1 arrest in mouse embryo fibroblasts (Attardi et al., 2004).

Apoptotic changes in neuroepithelial cells of the fetal brain were reported in rats and mice following prenatal treatment with other teratogenic drugs such as 5-azacytidine (Lu et al., 1998), ethylnitrosourea (Katayama et al., 2000), hydroxyurea (Woo et al., 2003), and 1-ß-arabinofuranosylcytosine (Yamauchi et al, 2003). The present study showed that VP-16 can also

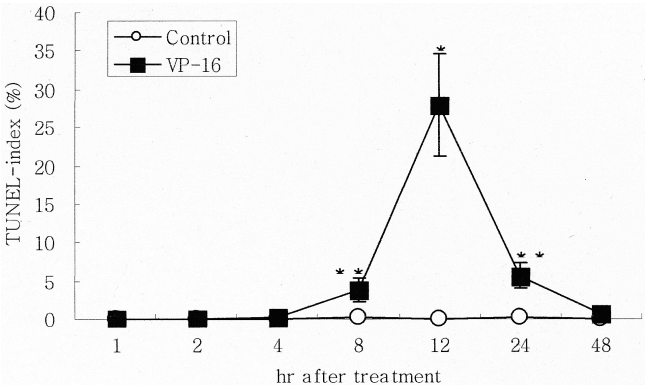

Fig. 4. Changes in the TUNEL-index (%) in the telencephalic wall of the fetal brain. Each value represents the mean ± SD of 6 fetuses obtained from each dam. *p<0.05: Significantly different from controls, **p<0.01: significantly different from controls.

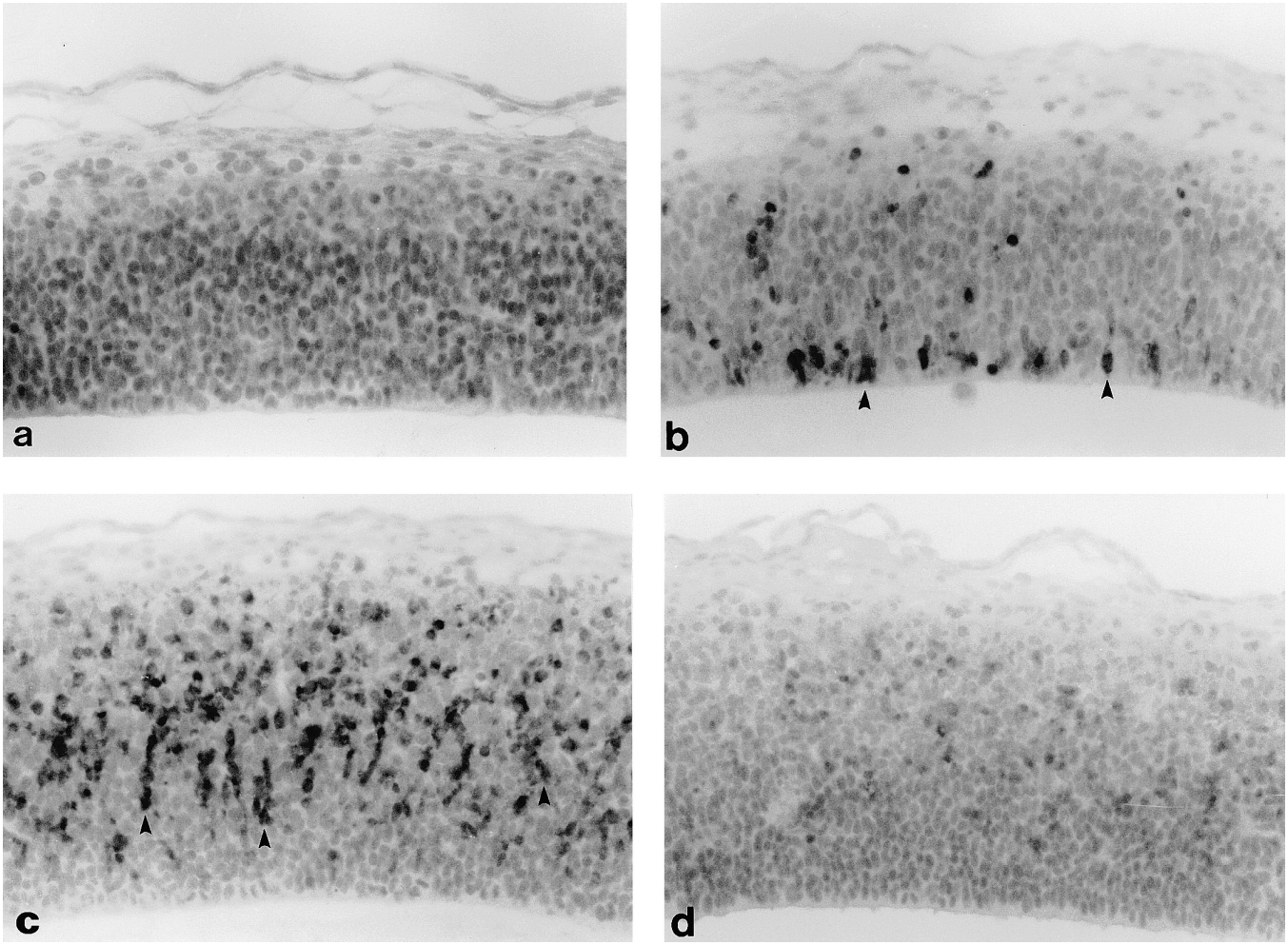

Fig. 5. Immunostaining for cleaved caspase-3 in the telencephalic wall of a control fetus **(a)** and of VP-16-treated fetuses at 4 HAT **(b)**, 12 HAT **(c)**, and 24 HAT **(d)**. The cleaved caspase-3 positive cells began to increase at 4 HAT, peaked at 8 and 12 HAT, and decreased at 24 HAT. Arrowheads: cleaved caspase-3-positive cell. x 400

induce similar morphological changes in the fetal brain. These findings suggest that the fetal brain in the organogensis phase might be very sensitive to such teratogenic chemical exposures.

VP-16 alters microtubule assembly, and mainly poisons topoisomerase II by increasing the steady-state concentrate of their covalent DNA cleavage complexes. Therefore this action converts topoisomerases into physiological toxins that cause high levels of transcript of transient-associated DNA breaks in the genome (Chen and Liu, 1994; Jesen and Sehested, 1997; Hande, 1998). When VP-16 was removed, DNA breakage was quickly repaired (Wozniak and Ross, 1983; Hande, 1998). Plasma half –life of VP-16 is less than 2 hr in mice when injected i.p. and 6.4 hr in human via intravenous route (Dorr et al., 1989; Hande, 1998). According to our present results, rapid recovery from VP-16-induced cellular damage caused by VP-16 corresponded well to the pharmacokinetics of VP-16.

Single injection of VP-16 (1.5, 2, and 3 mg/kg) at the early gestation period caused embryocidal or more severe teratogenic effects such as major skeletal malformation and/or various cranial abnormalities (Sieber et al., 1978). In the present study, administration of VP-16 of 4 mg/kg to pregnant mice on GD 12 (fetal organogenesis phase) induced apoptotic cell death only in the fetal CNS and mesenchyme of limbs. Accordingly, VP-16 administration into dams might induce various embryotoxic and teratotoxic effects, depending on the

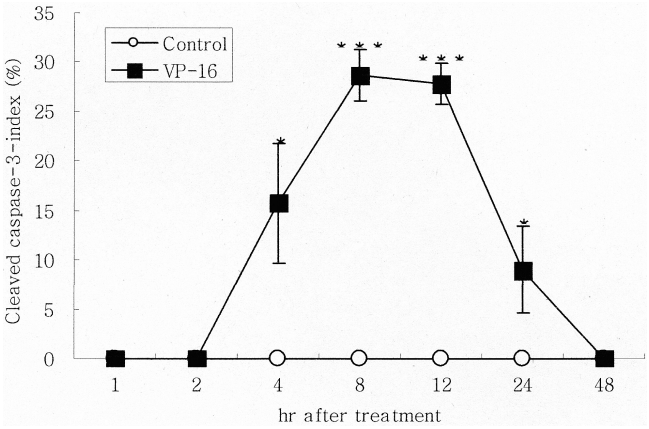

Fig. 6. Changes in the cleaved caspase-3-index (%) in the telencephalic wall of the fetal brain. Each value represents the mean ± SD of 6 fetuses obtained from each dam. *p<0.05: significantly different from control, ***p<0.001: significantly different from control.

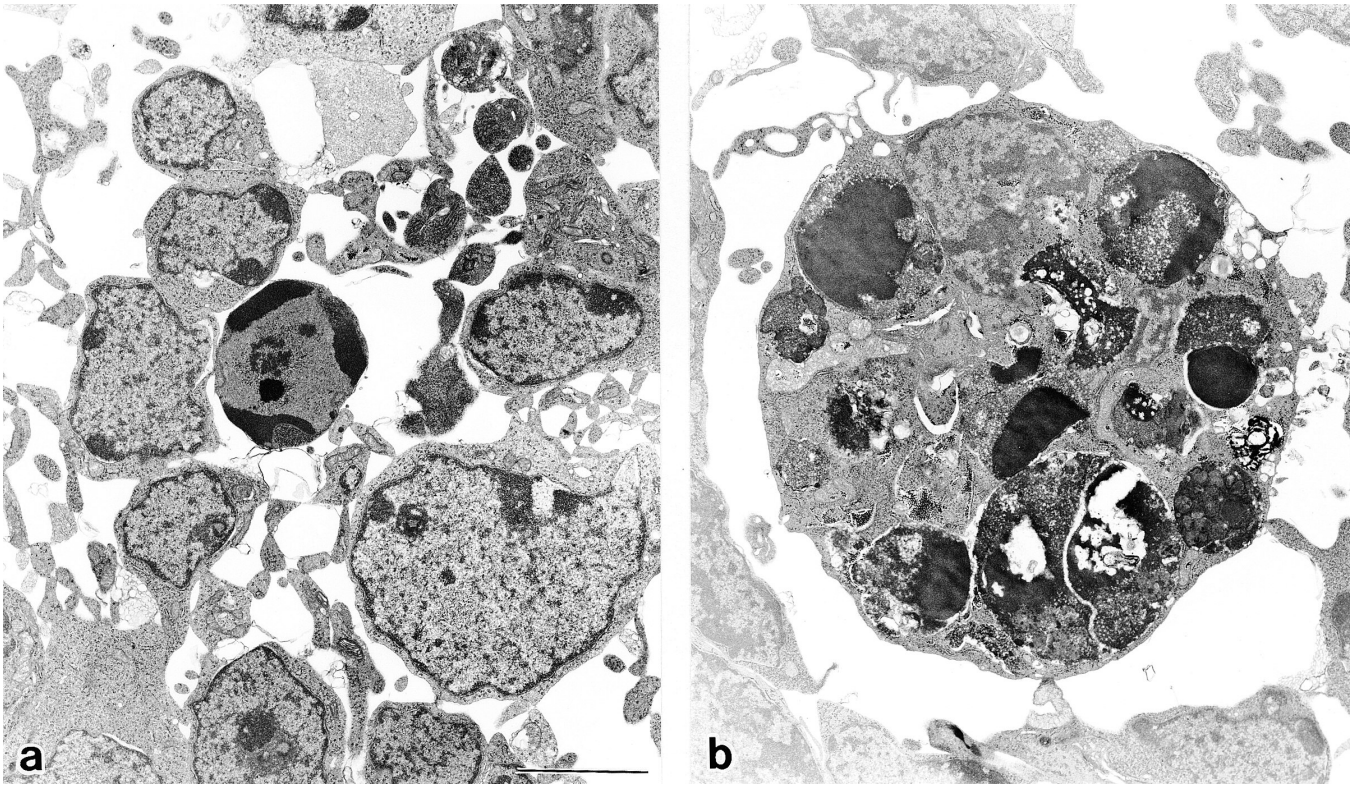

Fig 7. Electron microscopy of the telencenphalic wall of the VP-16-treated mouse fetal brain. **a.** An apoptotic cell at the center shows margination of condensed nuclear chromatin along the nuclear membrane. **b.** Apoptotic bodies are ingested by a macrophage. Bar: 2.0 μm.

gestational day of VP-16 injection.

In conclusion, cellular damage in the fetal brain obtained from pregnant mice treated with VP-16 on GD 12 was apoptotic cell death. The present results offer a clue for studies on the mechanisms of fetotoxicity and teratogenicity induced by VP-16. Further studies on gene expression profiles are now in progress in the fetal brain after VP-16 administration to dams.

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