

Mechanism of Ethanol-induced Purkinje Cell Death in Developing Rat Cerebellum: Its Implication in Apoptosis and Oxidative Damage

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ABSTRACT : Ethanol treatment during the brain growth spurt period has been known to induce the death of Purkinje cells. The underlying molecular mechanisms and the role of reactive oxygen species (ROS) in triggering ethanol-induced Purkinje cell death are, however, largely unresolved. We undertook TUNEL staining, western blotting assay and immunohistochemistry for the cleaved forms of caspase-3 and -9, with calbindin D28K double immunostaining to identify apoptotic Purkinje cells. The possibility of ROS-induced Purkinje cell death was immunohistochemically determined by using anti-8-hydroxy-2'-deoxyguanosine (8-OHdG), a specific cellular marker for oxidative damage. The results show that Purkinje cell death of PD 5 rat cerebellum following ethanol administration is mediated by the activation of caspase-3 and -9. However, unexpectedly, TUNEL staining did not reveal any positive Purkinje cells while there were some TUNEL-positive cells in the internal and external granular layer. 8-OHdG was detected in the Purkinje cell layers at 8 h, peaked at 12-24 h, but not at 30 h post-ethanol treatment. No 8-OHdG immunoreactive cells were detected in the internal and external granular layer. The lobule specific 8-OHdG staining patterns following ethanol exposure are consistent with that of ethanol-induced Purkinje cell loss. Thus, we suggest that ethanol-induced Purkinje cell death may not occur by the classical apoptotic pathway and oxidative damage is involved in ethanol-induced Purkinje cell death in the developing cerebellum.

Key words : Ethanol, Purkinje cell, Apoptosis, Oxidative damage

INTRODUCTION

Ethanol exposure during brain development produces a wide array of abnormalities, resulting from disruption of normal development. In humans such early ethanol exposure may cause a neurological syndrome called fetal alcohol syndrome (FAS), a condition characterized by a variety of neuropathologies accompanying behavioral and functional disturbances (West et al., 1994). The characteristic features

of FAS include facial dysmorphism, prenatal and postnatal growth retardation, and central nervous system dysfunction. Brain is particularly sensitive to the neurotoxic effect of ethanol during the period of synaptogenesis, also known as the brain growth spurt period, which occurs postnatally in rat but prenatally during the last trimester of gestation in humans (West et al., 1990; Ikonomidou et al., 2000). Microencephaly, or a reduced brain:body weight ratio is one of the most featured symptoms of FAS (Bellinger et al., 2002). Reduction of brain:body weight ratio may be attributed to loss of neurons, shrinkage of neuronal cell bodies or reduction in the number and extent of dendrites (Brooks, 2000).

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Of various regions of brain sensitive to early ethanol exposure, cerebellum is one of the most vulnerable regions (Goodlett et al., 1990; Thomas et al., 1998). Ethanol has been shown to produce a significant changes in neurogenesis, neuronal morphology, and enhanced cell death of differentiated neurons in cerebellum (Moulder et al., 2002). Therefore the rat cerebellum is an excellent model for evaluating anatomical deficits, as well as cellular and molecular events resulting from developmental alcohol exposure. In animal models, a single heavy exposure of ethanol on postnatal day 4-6, a period of brain growth spurt, results in severe loss of Purkinje cells and granule cells in cerebellum while similar exposure slightly later in the postnatal period produces little if any loss (Goodlett & Lundahl, 1996; Pierce et al., 1999). Purkinje cells within the different lobules of the cerebellum are differently vulnerable to the ethanol exposure. Lobules I, II, III, IX and X are the most vulnerable and lobules VI and VII are the least vulnerable (Hamre & West, 1993). In addition to timing and lobule specificity to alcohol-induced Purkinje cell loss, many cellular and molecular events have been identified that occur in close proximity to the loss of cells, and these cellular and molecular events have been assumed to drive the loss of Purkinje cells (Heaton et al., 2006). However, how the Purkinje cells exposed to ethanol are led to death is largely undetermined, although ethanol-induced Purkinje cell loss has been known to occur due to direct cell death not to defects of neuronal migration or neurogenesis during development.

The mechanisms of cell death induced by ethanol administration in the developing rodent brain has been investigated morphologically and biochemically, and revealed to occur via apoptosis (Dikranian et al., 2001; Light et al., 2002). Neurons begin showing apoptotic morphological changes 2 to 4 h after ethanol treatment, and by 10 to 12 h, almost all of the affected neurons are manifesting very advanced apoptotic changes, including condensation and disintegration of the cell body and dendritic tree into numerous small independent bodies. Biochemical studies also support the ethanol-induced apoptotic neurodegeneration in developing

rodent brain. Caspase-3, executioner of cell death in apoptosis, is activated and cytochrome c is released in various regions of brain in response to a single heavy exposure of ethanol (Olney et al., 2002). In homozygous Bax-deficient mice, neither caspase-3 activation nor cytochrome c release are induced and neurodegeneration does not occur (Young et al., 2003), the result implying that ethanol-induced neurodegeneration is Bax-dependent and thus involves the intrinsic pathway of apoptotic cell death.

One of various factors which can trigger apoptosis is oxidative stress, an excessive accumulation of free radicals in the cell. Free radicals are highly reactive molecules that can be formed during many biochemical reactions in the cell. Many of these free radicals contain oxygen and are called reactive oxygen species (ROS). To overcome oxidative stress, mammalian cells have a complex antioxidant defense system that includes non-enzymatic antioxidants (e.g. glutathione, thioredoxine) and enzymatic activities (e.g. superoxide dismutase, catalase). Thus the survival of a cell depends on the balance between ROS and antioxidants (Fleury et al., 2002). If ROS levels exceed the cell's ability to eliminate them, or if the normal antioxidant levels are reduced due to a toxic insult such as alcohol, then oxidative stress can occur. The cellular damage caused by oxidative stress is a consequence of lipid peroxidation (Sun et al., 1997), alteration of nucleic acid (Navasumrit et al., 2000), and proteins (Sun & Mayhan, 2001). These non-specific oxidative damages to cellular components have been known to provoke necrotic cell death. However, it was also suggested that ROS may induce cytochrome c release, and subsequent activation of caspase-9 and -3, which lead to apoptotic cell death (Yuan et al., 2003). Interactions between DNA and ROS produce DNA strand breaks and base modification (Won et al., 1999), which are frequently assessed by measurement of the nucleoside 8-hydroxy-2'-deoxyguanosine (8-OHdG) levels corresponding to the base lesion of 8-hydroxyguanosine. Thus 8-OHdG is employed as a good marker of oxidative DNA damage (Kasai, 1997).

The present study was designed to investigate the mecha-

nism of ethanol-induced Purkinje cell death using developing PD 5 rat. Immunohistochemistry and western blotting assay for the cleaved caspase-9 and -3 were carried out to show whether the degeneration of Purkinje cells in response to ethanol exposure occurs via apoptotic pathway. Furthermore, to confirm the possible role of oxidative stress in ethanol-mediated Purkinje cell death the production of 8-OHdG was assessed by immunohistochemical analysis.

MATERIALS AND METHODS

1. Animals and Ethanol Administration

Postnatal day (PD) 5 Sprague Dawley rats were used in this study. They were assigned to one of two groups: ethanol (n=8) or control (n=7). On PD5, ethanol (6 g/kg) in normal saline was administered subcutaneously in two separate treatments, 2 h apart, each treatment delivering 3 g/kg, and control rats were treated saline only. All experimental protocols in this study were approved by the Institutional Animal Care and Use Committee at the Jeju National University in accordance with the guidelines of Korea and Drug Administration.

2. Measurement of Blood Ethanol Concentration

Blood samples were collected from hearts of PD5 rats to determine the blood alcohol concentration (BAC). BAC was measured in all pups on 2, 4, 6, 8, 12, 18 and 24 h following ethanol treatment by using alcohol kit (Sigma, MO, USA). Briefly, the blood samples were centrifuged at 12,000 rpm for 5 min. Deionized water, ethanol standard solution (0.08%) and samples were added to alcohol reagent and then mixed. All mixtures were incubated for 10 min at room temperature. Absorbance of samples was measured at 340 nm wavelength.

3. Tissue Preparation

The tissue samples were prepared at 2, 4, 6, 8, 12, 18 and 24 h after ethanol or saline treatment by an overdose of pentobarbital and subsequently perfused with 0.9% saline

followed by 4% paraformaldehyde in 0.1M phosphate buffer. The cerebellum was removed from the brain and stored in the same fixative. Fixed cerebelli were immersed in 20% sucrose solution and sections of 40 μm thickness were obtained by cryostat sectioning. For the western blotting assay the cerebelli were rapidly removed after decapitation, and stored at -70°C until used.

4. Western Blot Analysis

The tissue samples were homogenized in lysis buffer (Upstate, USA), and the homogenates were centrifuged at 12,000 rpm for 15 min at 4°C . The supernatant was collected and protein concentration was determined with a BCA protein assay kit (Pierce, USA). The protein samples were separated by SDS-PAGE and transferred to PVDF membrane. Non-specific immunoreactivity was blocked overnight with 5% non-fat dry milk mixed in a solution of 0.1% Tween-20 in Tris buffered saline. Primary antibody for cleaved caspase-3, and -9 (1:3000, Cell Signalling, USA) were incubated overnight at 4°C . Membranes were washed and incubated with anti-rabbit IgG conjugated horseradish peroxidase (1:4000, Santa Cruz, USA) at room temperature for an hour. The signal was detected with enhanced chemiluminescence (ECL) detection system.

5. Immunohistochemistry

Immunohistochemistry for cleaved caspase-3 and -9 was carried out by free floating method. The sections were rinsed in 0.01M PBS and blocked in 1% H_2O_2 to inactivate endogenous peroxidase. The sections were incubated in PBS containing 3% normal horse serum at room temperature for 30 min, and then incubated with rabbit anti-cleaved caspase-3 and -9 (1:100, Cell Signalling, USA) overnight at 4°C . Sections were reacted for 90 min with biotinylated anti-rabbit IgG (1:200, Vector, USA) and then incubated with ABC reagents (Vector elite kit). For detection of 8-OHdG, sections were mounted on 2% gelatin coated slide, dried, rinsed in PBS, and blocked endogenous peroxidase in 0.3% H_2O_2 . Subsequently sections were treated with 150 $\mu\text{g}/\text{ml}$

RNase A for an hour at 37°C (to exclude interference effect of oxidative RNA products) and 50 nM sodium hydroxide in 40% ethanol for 10 min (for denaturation of DNA) and then incubated for 30 min in 3% normal goat serum. The sections were incubated with mouse anti-8-OHdG (1:100, Chemicon, USA) overnight, biotinylated anti-mouse IgG for 90 min and then ABC reagents. The sections were washed in PBS three times and incubated for 6 min in DAB solution and mounted by polymount.

For double fluorescent immunostaining with cleaved caspase-3, -9 and calbindin D28K, sections were incubated overnight with first primary antibody, cleaved caspase-3 or -9 at 4°C and then with FITC-conjugated goat anti-rabbit IgG at room temperature for 90 min. After washing, the sections were incubated with second primary antibody of mouse anti-calbindin D28K (1:1,000, Sigma, USA) for 90 min. The sections were incubated with Texas-Red conjugated anti-mouse IgG (Vector, USA) for 90 min, and mounted gelatin coated slide. Finally the sections were mounted by Vectashield Hard-set mounting medium and analysed by confocal laser scanning microscope (Olympus).

6. TUNEL Staining

For the analysis of DNA fragmentation, ApopTag kits (Intergen, NY, USA) were used according to the manufacturer's manual. Briefly, the paraffin-embedded tissue sections were deparaffinized with xylene and ethanol and then apply proteinase K (20 µg/ml) to the specimen for 15 min at room temperature. The sections were washed and incubated with the TdT enzyme solution containing the digoxigenin-dNTP at 37°C for 1 h. After several rinses, anti-digoxigenin peroxidase conjugate was applied to the slides for 30 minutes at room temperature and then develop color in DAB solution for 3 to 6 minute. Negative controls were obtained by exclusion of the TdT enzyme.

RESULTS

1. Blood Alcohol Concentration

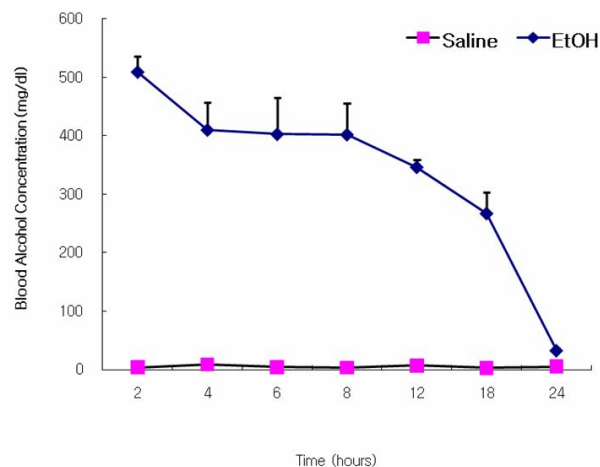


Fig. 1. Blood alcohol concentrations in PD 5 rats after ethanol and saline treatment. Ethanol was subcutaneously administered in two injections (3 g/kg×2) spaced 2 h apart. The peak blood ethanol concentrations were achieved 2 h after ethanol treatment and then decreased gradually to the control value at 24 h post-treatment. n=6-7 each treatment group.

Blood alcohol concentrations (BACs) were measured in PD5 rats after ethanol and saline administration. The mean BACs at various time points were shown in Figure 1. The peak BAC was 509 mg/dl and achieved 2 h after ethanol treatment in the pups, with near total clearance of ethanol at the end of 24 h post-treatment. Saline-treated pups showed basal levels of BACs.

2. Activation of Caspase-3/9 and TUNEL Staining

We previously showed that ethanol induces Purkinje cell degeneration in PD5 rat pups by Fluoro-Jade B staining (Lee et al., 2008). In the present study, we undertook immunohistochemistry for the cleaved forms of caspase-3 and -9, two important specific makers for apoptosis, to reveal that ethanol-induced Purkinje cell death occurs via apoptotic pathway. Cleaved caspase-3 and -9 immunoreactive cells began to appear in Purkinje cell layer of cerebellar vermis at 6-8 h, maximally detected between 12-18 h and almost absent 24 h after ethanol administration (Fig. 2A and 2B). To confirm these immunoreactive cells in the Purkinje cell layer are Purkinje cell per se, double

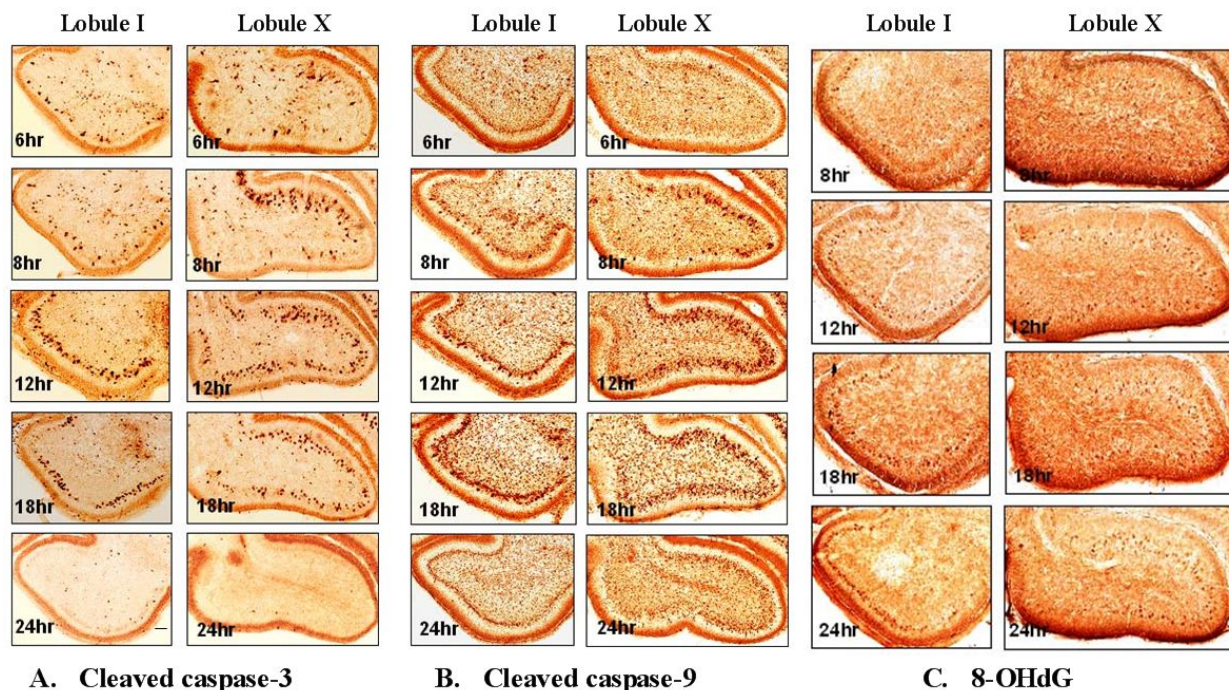


Fig. 2. Immunohistochemistry for the cleaved caspase-3 (A), caspase- 9 (B), and 8-OHdG (C) in the lobule I and X at various time points following ethanol exposure. All the positive cells were mostly confined to the Purkinje cell layer in the cerebellum of PD 5 rat. Cleaved caspase-3 and -9 immunoreactive cells began to appear at 6-8 h, were maximally detected at 12-18 h and were almost absent 24 h after ethanol treatment. The temporal and spatial pattern of 8-OHdG immunoreactivity were similar to those of the cleaved forms of caspases but 8-OHdG immunoreactive cells in the Purkinje cell layer were persisted at 24 h post-treatment. Scale bar = 50 μ m.

fluorescent immunostaining was conducted with anti-calbindin D28K, a marker for Purkinje cell. Confocal microscopic images with 0.5 μ m optical section showed that cleaved caspase-3 or -9 immunoreactive cells (green color) were

colocalized with calbindin D28K (red color) (Fig. 3). Thus most of active caspase-3 and -9 immunoreactive cells in the Purkinje cell layer proved to be Purkinje cells. As an attempt to evaluate any lobule-specific activation of caspases in the cerebellar vermis of ethanol-treated rat pups, we carried out immunohistochemistry with the whole vermis at 12 h after ethanol treatment. Lobules I, II, III, IX, and X showed the most numerous active caspase-3 and -9 immunoreactive cells in the Purkinje cell layer and lobules VI and VII did the least (Fig. 4).

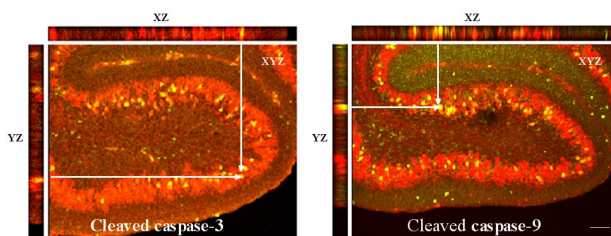


Fig. 3. Confocal microscopic images for the colocalization of caspase-3 or -9 (FITC), and calbindin D 28K (Texas red) in lobule X of cerebellum following ethanol exposure. Many calbindin D 28K-positive Purkinje cells (red color) contain the caspase-3 or -9 immunoreactivity (green color). XZ and YZ cross sections of arrows-indicated cells further prove the true colocalization events. Scale bar = 60 μ m.

To confirm the results of immunohistochemical analysis, we examined the cerebellar vermis extracts from control and ethanol-exposed pups at the same time points of immunohistochemical analysis using western blots with antibodies for cleaved caspase-3 and caspase-9. Caspase-3 was not activated in saline-treated control extracts, consistent with the low levels of naturally occurring cell death observed

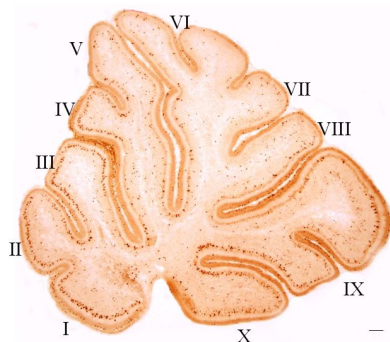


Fig. 4. Lobule-specific vulnerability following ethanol treatment in the midsagittal section of cerebellar vermis as revealed by caspase-3 immunohistochemistry. Caspase-3 immunoreactive cells are mainly confined to the Purkinje cell layer in all 10 lobules. However, lobule I, II, III, IX, and X of early maturing lobules show more numerous immunoreactive cells than the late maturing lobules V, VI, VII and VIII. Scale bar = 100 μ m.

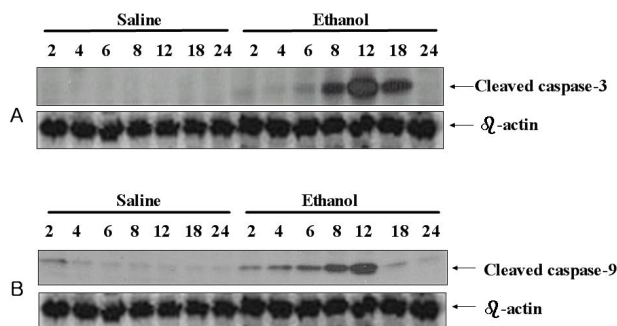


Fig. 5. Western blotting analysis for the temporal pattern of cleaved caspase-3 (A) and -9 (B) immunoreactivity. Caspase-3 and -9 are activated following ethanol exposure. Protein extracts of saline-treated control and ethanol-exposed vermis of cerebellum (2, 4, 6, 8, 12, 18, and 24 h posttreatment) were prepared and analyzed for cleaved caspases immunoreactivity. Cleaved caspase-3 and -9 immunoreactivity were readily detected by 6-8 h and peaked 12 h after ethanol exposure and then returned to the basal levels at 24 h. β -actin was used as a protein loading control.

immunohistochemically (Fig. 5). However the ethanol administration dramatically induced the activation of two caspases and the appearance of cleaved caspase-3 and -9 immunoreactivity was transient: It was increased between 6 and 8

h postethanol exposure, peaked at 12 h and then dropped abruptly to the saline control levels at 24 h after ethanol exposure (Fig. 5).

Next, we conducted TUNEL staining at the same time points of caspases immunostaining to obtain the further evidence of apoptotic Purkinje cell death induced by ethanol. Figure 6D shows that TUNEL-positive cells in the Purkinje cell layer were very rare although some positive cells were present in the internal and external granular layers.

3. Immunohistochemical Detection of 8-OHdG

To elucidate that ethanol-induced Purkinje cell death may be associated to ROS generation, immunohistochemistry for 8-OHdG was performed with the vermal section of PD5 rat cerebellum following ethanol administration. 8-OHdG immunoreactive cells began to be detected in the Purkinje cell layer of cerebellum at 8 h, peaked at 12-24 h, and disappeared almost completely at 30 h after ethanol treatment. Granular and molecular layer of cerebellum did not show

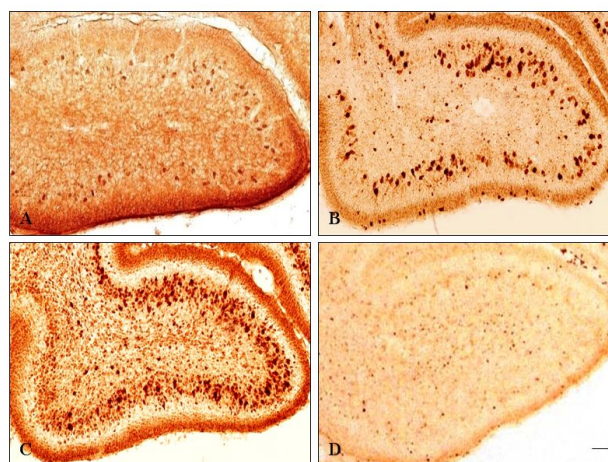


Fig. 6. Immunohistochemistry for 8-OHdG (A), the cleaved forms of caspase-3 (B), -9 (C), and TUNEL staining (D) to reveal the death mechanism of Purkinje cell following ethanol exposure. Immunoreactive cells for the 8-OHdG, cleaved caspase-3 and -9 were readily detected in the Purkinje cell layer, while TUNEL staining did not show any positive cells in the Purkinje layer. Note the numerous TUNEL positive cells in the internal granular layer. Scale bar = 50 μ m.

any 8-OHdG immunoreactivity (Fig. 2C, 6A). Cerebellum of saline treated animals did not show any 8-OHdG immunoreactivity.

DISCUSSION

This study was designed to investigate the possible mechanism of ethanol-induced Purkinje cell death in developing cerebellum. The cerebellar Purkinje cells are most vulnerable to ethanol during the early neonatal period in rat. Experimental studies suggested that ethanol activates the proapoptotic molecules (Heaton et al., 2003) and leads to neurodegeneration by activation of caspase-3 in developing brain (Ikonomidou et al., 2000; Onley et al., 2002). However, the mechanism leading to Purkinje cell degeneration following ethanol administration is not clearly understood.

Our previous study (Lee et al., 2008) showed that FJB-positive cells in the Purkinje cell layer after ethanol exposure on PD5 rat were more numerous in the early maturing lobules (lobules I, II, III, IX and X) than those found in late maturing lobules (lobules VI and VII). This pattern of neurodegeneration revealed by FJB staining was well consistent with that of lobule-specific Purkinje cell loss and confirmed that ethanol-induced Purkinje cell loss is due to direct Purkinje cell death not to the failure of their division or migration from the deep cerebellar nucleus. To further explore the underlying mechanism of ethanol-induced Purkinje cell death, we undertook the immunohistochemical staining for active caspase-3 and -9, the key regulator in the processes of apoptotic cell death. It revealed that ethanol-induced Purkinje cell death occurs by activation of caspase-3 and -9. The spatial and temporal patterns of caspase-3 and -9 immunoreactive Purkinje cell were very similar to those seen in FJB staining. These results well match the previous reports by other groups (Light et al., 2002). Next, we introduced TUNEL staining to determine whether Purkinje cell death is associated with DNA fragmentation. However, unexpectedly, TUNEL-positive Purkinje cell was very rare and there was no lobule-specific pattern of TUNEL staining

which was seen in the FJB staining, caspase-3 and -9 immunostaining. These results were contrary to the previous report by other group (Light et al., 2002). The TUNEL staining seems to be properly carried out since many TUNEL-positive cells could be seen in the granular layer and deep cerebellar nucleus of the same tissue section which showed negative staining for Purkinje cell. In the present, we have no idea why such different results were obtained between two studies. One possible explanation for our result is that ethanol may act as a NMDA receptor antagonist (Hoffman et al., 1989; Ikonomidou et al., 2000). In such circumstance, ethanol may block the entry of Ca^{2+} into the cell and the resulting low level of intracellular Ca^{2+} concentration could inhibit the $\text{Ca}^{2+}/\text{Mg}^{+}$ -dependent endonuclease activity, which lead to no DNA fragmentation (Cohen & Duke, 1984; Cohen et al., 1992). In support of this idea, some apoptotic cell death do not show DNA fragmentation although cytochrome c release and caspase activation are occurred (Schwartz et al., 1993; Sperandio et al., 2000). Thus, we tentatively suggest that Purkinje cell death in PD 5 rat cerebellum following ethanol administration may not occur via the classical apoptotic pathway.

8-OHdG immunoreactivity in response to ethanol treatment was observed in cells of Purkinje cell layer of PD5 rat cerebellum but was not detected at PD7 and PD14 (data not shown). Considering that vulnerability of Purkinje cells to ethanol treatment confines only to narrow developmental period (PD4-6) and is little if any at slightly later ethanol-resistant period (PD7-9 or later) (Pierce et al., 1999; Heaton et al., 2002), it implies that oxidative stress may play a role in the ethanol-mediated Purkinje cell death. In support of this suggestion, lobule-specific pattern of 8-OHdG immunoreactive cells in the Purkinje cell layer was very similar to those of active caspase-3/9 immunohistochemistry and FJB staining in the present and previous study, respectively.

The ability of oxidative stress to provoke necrotic cell death as a result of massive cellular damages associated to lipid peroxidation (Sun et al., 1997) and alteration of proteins (Sun & Mayhan, 2001) and nucleic acids (Navasumrit et

al., 2000) have been well documented for a long time. ROS has been also known to act as signalling molecules in apoptotic pathway. For example, some anti-oxidants can inhibit activation of caspase and subsequent steps leading to apoptotic cell death (Fleury et al., 2002). In the present study, 8-OHdG immunoreactivity in cells of Purkinje cell layer was observed for a longer time until 24 h after ethanol administration, at which time point the active caspase-3 and -9 immunoreactivities were gone by. This longer duration of 8-OHdG immunoreactivity may reflect the persistent production of ROS levels in response to ethanol exposure, since it has been demonstrated that ROS levels in the extract of whole cerebellum remains elevated by 24 h after ethanol administration (Heaton et al., 2002). Therefore, it is plausible that the later increase of ROS levels may convert the apoptotic pathway of Purkinje cell death into necrotic pathway, which may also reflect the absence of DNA fragmentation in TUNEL staining.

Taken together, this study has shown that ethanol-induced Purkinje cell death in developing cerebellum of rat is mediated by cleavages of caspase-3, -9 and oxidative DNA damage. However, the differential temporal pattern of cleaved caspases and 8-OHdG immunoreactivity in the present study suggest that the Purkinje cells committed to apoptosis may shift toward necrotic death due to a later burst in cellular ROS levels.

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