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

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발생중인 흰쥐 소뇌의 Purkinje 세포에서 에탄올에 의한 산화적 손상과 세포사멸 기작에 대한 연구

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ABSTRACT

It has been well established that ethanol treatment during the brain growth spurt period induces the loss of cerebellar Purkinje cells. Recently it has been reported that ethanol increases reactive oxygen species (ROS) in developing cerebellum and suggested that these ROS may contribute to the cytochrome c release, and subsequent caspase-9 and caspase-3 activation. Moreover, there is increasing evidence that the low levels of ROS can induce apoptosis while the accumulation of the high levels can lead apoptosis-committed cells toward necrotic cell death. However, the mechanism of ethanol-induced Purkinje cell death is not well understood. Thus, to elucidate the implication of apoptosis and oxidative damage in ethanol-induced Purkinje cell death in developing rat cerebellum, this study was carried out the Fluoro-jade B staining, TUNEL staining, immunohistochemistry and western blot analysis for caspase-9 and caspase-3, and immunohistochemistry for 8-OHdG.

Purkinje cell death of PD 5 rat cerebellum after ethanol administration was mediated by activation of caspase-9 and caspase-3, while TUNEL staining did not reveal any positive Purkinje cells in PD 5 rat cerebellum. These results suggested that ethanol-induced Purkinje cell death may not occur via the classical apoptotic pathway. Furthermore, the temporal patterns of 8-OHdG, which is generated under condition of severe oxidative stress was different from these of caspase-9 and caspase-3 activation.

Taken together, this study has shown that ethanol-induced Purkinje cell death

in developing rat cerebellum is mediated by the activation of caspase-9/-3 and oxidative DNA damage. But the differential temporal patterns of active caspases and 8-OHdG immunoreactivity suggest that apoptosis-committed Purkinje cells may shift toward necrotic death due to a later burst in cellular ROS levels.

Keywords : Fetal alcohol syndrome (FAS), Ethanol, Purkinje cell death, 8-hydroxy-2'-deoxyguanosine (8-OHdG), Reactive oxygen species (ROS)

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INTRODUCTION

Exposure to ethanol during nervous system development produces a wide array of abnormalities, resulting from disruption of many normal developmental event. In humans, such early ethanol exposure can lead to fetal alcohol syndrome (FAS), a condition characterized by a variety of neuropathologies, with accompanying behavioral and functional disturbances (West *et al*., 1994). The characteristic features of FAS include facial dysmorphology, prenatal and postnatal growth retardation, and central nervous system dysfunction (Chen and West, 1999). It is thought that the 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 human (West *et al*., 1990; Ikonomidou *et al*., 2000). One of the most serious symptoms of FAS is microencephaly, or a reduced brain : body weight ratio (Bellinger *et al.*, 2002). Reduction of brain:body weight ratio may be due to loss of neurons, shrinkage of neuronal cell bodies or reduction in the number and extent of dendrites (Brooks, 2000).

Ethanol toxicity has been demonstrated in many organs, but the most affected organ in FAS is the brain. One of the region in brain that is particularly sensitive to early ethanol exposure is the cerebellum (Goodlett *et al*., 1990; Thomas *et al*., 1998). Effects of ethanol on the cerebellum have been documented in great detail and previous works has demonstrated

changes in neurogenesis, neuronal morphology, and enhanced cell death of differentiated neurons (Moulder *et al*., 2002). The cerebellar Purkinje cell has been identified as a target of alcohol action in animal models of prenatal and postnatal alcohol exposure. The ethanol exposure during the early postnatal period in the rodent, on postnatal day (PD) 4 or 5, results in the loss of Purkinje and granule cells, while similar exposure slightly later in the postnatal period produces little if any neuronal death (Goodlett and Lundahl, 1996; Pierce *et al*., 1997). It also reported that ethanol reduced density of cerebellar Purkinje cell during the early postnatal brain growth spurt in rat (Hamre and West, 1993). Although it has been firmly established that the early postnatal treatment of ethanol induces the loss or death of neurons in various regions of central nervous system, how the neurons exposed to ethanol are led to death is largely undetermined. Thus, the main focus in this research area during the last few years has been laid on the mechanism of neuronal cell death following ethanol exposure to find the therapeutic intervention of FAS.

Apoptosis is a form of cell death which can be seen in both physiologic and pathologic conditions of central nervous system. It is an active form of cell death which is distinguished from classical necrosis by a number of criteria. In necrosis there is cell swelling, membrane lysis and release of cellular contents, resulting in inflammation (Kerr *et al*., 1972; Satry and Rao, 2000). DNA is degraded in a random manner, leading to the appearance of a continuous smear on agarose gel electrophoresis. Apoptosis, on the other hand, is characterized morphologically by chromatin

condensation and nuclear fragmentation. The dying cell shrinks and fragments, and is subsequently phagocytosed by neighboring cells. DNA degradation occurs into highly reproducible oligonucleosomal fragments of ~200 bp (Compton, 1992). Apoptosis is an active process and has been shown in many cases to be dependent on RNA and protein synthesis. The key components of apoptosis in neurons are Apaf-1, Bcl-2, and caspase families (Yuan and Yankner, 2000). The Bcl-2 family of protein includes both anti-apoptotic and pro-apoptotic proteins. The pro-apoptotic Bcl-2 family proteins, such as Bax and Bid, induce cytochrome C release from mitochodria (Robertson and Orrenius, 2000). These released cytochrome C forms a complex with Apaf-1 and pro-caspase-9, and then activates caspase-9. Subsequently, the activated caspase-9 activates caspase-3, which is known as the executioner of cell death. However, the boundaries between apoptotic and necrotic forms of cell death are not always all that sharp. There are some evidences that apoptosis-committed cell may convert to necrotic pathway under the unfavorable conditions for the completion of apoptosis, such as ATP depletion and severe oxidative stress (Hoek and Pastorino, 2002).

Ethanol exposure increases the expression of pro-apoptotic proteins, and decreases expression of anti-apoptotic proteins, while the overexpression of anti-apoptotic molecule Bcl-2 ameliorate ethanol-induced cell death (Heaton *et al*., 1999). Recently, it was shown that ethanol- induced neuronal death occurs via activation of caspase-3 in the developing forebrain of rat and mouse (Olney *et al.*, 2002). However, little is known on the death

mechanism of cerebellar Purkinje cell in response to ethanol administration, which is one of the most vulnerable neurons in the central nervous system.

Oxidative stress, an excessive accumulation of ROS, has been known to induce cell death in various organs. The production of ROS within a cell occurs mainly in mitochondria. The respiratory chain in mitochondria produces ROS at complex I and complex III, and the ubiquinone site in complex III is the major site of mitochondrial ROS production (Fleury *et al*., 2002). To overcome the 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. The cellular damage caused by free radicals and ROS is a consequence of the lipid peroxidation (Sun *et al*., 1997), and alteration of nucleic acid (Navasumrit *et al*., 2000), and proteins (Sun *et al*., 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 activations of caspase-9 and caspase-3 (Yuan *et al*., 2003), which lead to apoptotic cell death. Recently interactions between DNA and ROS have been known to produce DNA strand breaks and base modification (Moon *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 maker of oxidative DNA damage (Kasai, 1997).

Ethanol can induce oxidative stress directly and indirectly. The direct effect is achieved by the formation of free radical and ROS, and the indirect effect by reducing intracellular antioxidant capacity, such as the level of glutathione peroxidase (Kerem and Koren, 2003). Recently, it has been reported that ethanol generates ROS in developing PD 4 rat cerebellum, but slightly later exposure on PD 7 produces no immediate change in ROS (Heaton *et al*., 2002). This raises is the possibility that ethanol-induced Purkinje cell death during the brain growth spurt period may be mediated by oxidative stress.

The present study was designed to investigate the mechanism of ethanol-induced Purkinje cell death by using various experimental procedures. To determine whether ethanol treatment induces the degeneration of Purkinje cell Fluoro-Jade B (FJB) staining, which is known to detect both apoptotic and necrotic cell (Schmued and Hopkins, 2000), was carried out. Immunohistochemistry and western blotting assay for the cleaved caspase-9/-3 were also used 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=56) or control (n=28). On PD5, ethanol (6 g/kg) in normal saline was administered subcutaneously in two separate treatments, 2 hr apart, each treatment delivering 3 g/kg subcutaneously, control rats were treated saline only.

2. Measurement of blood ethanol concentration.

Blood samples were collected from the hearts of PD 5 rat to determine the blood ethanol concentration (BEC). BEC was measured in all pups on 2, 4, 6, 8, 12, 18 and 24 hr following the ethanol treatment using alcohol kit (Sigma, 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 (Sigma, USA), and then mixed by inversion. All mixtures were incubated for 10 min at room temperature. Absorbance of the samples were measured at 340 ㎚ wavelength.

3. Tissue preparation

The tissue samples were prepared at 2, 4, 6, 8, 12, 18, 24 and 30 hr after ethanol or control treatment by an overdose of anesthetic and subsequently perfused with 0.9% saline followed by 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The cerebellum was removed from the brain and stored in the same fixative. For the western blotting assay the cerebelli were rapidly removed after decapitation, and stored in the deep-freeger. Fixed cerebelli were immersed in 20% sucrose solution and sections of 40 ㎛ thickness were obtained by cryostat sectioning.

4. Western blot analysis

The tissue samples were homogenized in a lysis buffer (Upstate, USA), and the homogenates were centrifuged at 12,000 rpm for 15 min at 4℃. The supernatant was collected and assayed for BCA method. The proteins were separated by 4-20% SDS-polyacrylamide gel electrophoresis (125V, 90 min at 4℃) and transferred to PVDF membrane (25V, 90 min at 4°C). 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 (TBS-T). Primary antibody for cleaved caspase-3 (Cell Signaling, USA; 1:3000) and cleaved caspase-9 (Cell Signaling, USA; 1:3000) were

incubated overnight with membranes at 4℃, respectively. Membranes were washed and incubated with anti-rabbit IgG conjugated horseradish peroxidase (Santacruz, USA; 1:4000) at 22℃ for an hour. The signal was detected with enhanced chemiluminescence (ECL) detection system.

5. Immunohistochemistry

1) Cleaved form of caspase-3 and caspase-9

Immunohistochemistry for the cleaved forms of caspase-3 and caspase-9 was carried out by free-floating method. The sections were rinsed on 0.1 M PBS with triton-X 100 (PBST; pH 7.4), and blocked in 1% H_2O_2 to in activate endogenous peroxidase. The sections were incubated in 3% normal horse serum in PBST at room temperature for 30 min, and than incubated with rabbit anti-cleaved caspase-3 and cleaved caspase-9 (Cell Signaling, USA; 1:100) overnight at 4℃ cold lab chamber, respectively. The sections were incubated for 90 min with biotinylated anti-rabbit IgG (Vector Laboratory, USA; 1:200), and than incubated with avidin-biotin-horseradish peroxidase complex (Vector Laboratory, USA) for an hour. The sections were detected for 6 min with DAB solution and mounted by polymount (Polymount, USA).

2) 8-hydroxy-2'-deoxyguanosine (8-OHdG)

The sections were mounted on 2% gelatin coated slide glass and dried the slide at room temperature. The sections were rinsed in 0.1 M PBS (pH 7.4) and blocked in 0.3% H_2O_2 0.1 M PBS (pH 7.4) to inactivate endogenous peroxidase. Subsequently, sections were treated 150 ㎍/㎖ RNase A for an hour at 37℃ (to exclude interference effect of oxidative RNA products) and 50 mM sodum hydroxide in 40% ethanol for 10 min (for denaturation of DNA), and than incubated in 3% normal horse serum in PBST (pH 7.4) at room temperature for 30 min. The sections were incubated with diluted mouse anti-8-OHdG antibody (1:100) overnight at room temperature. The sections were incubated with biotinylated anti-mouse IgG (Vector Laboratory, USA) for 90 min, and were incubated with avidin-biotin-horseradish peroxidase complex (Vector Laboratory, USA) for an hour. The sections were detected for 6 min with DAB solution and coversliped by polymount (Polymount, USA).

6. Fluorescent double immunostaining

The sections were rinsed on 0.1 M PBST (pH 7.4) and blocked with 0.3% normal serum for 30 min. Its were incubated with first primary antibody; cleaved caspase-3 (Cell Signaling, USA; 1:50) and caspase-9 (Cell Signaling, USA; 1:50) overnight at 4℃ cold lab chamber. Its were incubated with FITC-conjugated goat anti-rabbit IgG (1:200) at room temperature for 90 min, from this step on, performed as possible away from light. The sections were incubated with second primary antibody

calbindin D28k (Sigma, USA; diluted in 1:1000) at room temperature for 90 min. The sections were incubated Taxas-Red conjugated anti-mouse IgG (Vector Laboratory, USA) for 90 min and than mounted on 0.5 % gelatin coated slide. Finally, the sections were mounted by mounting solution and analysed by confocal laser scanning microscopy (Olympus, USA).

7. Fluoro-Jade B Staining

The sections were mounted on 2% gelatin coated slide and than air dried on a slide warmer at 50℃ for at least half an hour. The slides were immersed in a solution containing 1% sodium hydroxide in 80% ethanol for 5 min. This was followed by 2 min in 70% ethanol and 2 min in distilled water. The slides transferred to a 0.06% potasium permanganate for 10 min, preferably on a shaker table to insure consistent block ground suppression between sections. The slides were than rinsed with distilled water for 2 min. The staining solution was prepared from a 0.01% stock solution (adding 10 ㎎ of the dye powder to 100 ㎖ of distilled water) of Fluoro-Jade B. The 0.0004% staining solution (4 ㎖ of stock solution was added to 96 ml of 0.1% acetic acid) was prepared within 10 min of use and was not reused. The sections were immersed in staining solution for 20 min. From this step on, perform as possible away from light. The slides were rinsed with distilled water, and than placed on a slide warmer, set at 50℃, until they were fully dry. The slides were coversliped with DPX (Fluka, USA).

RESULTS

1. Blood ethanol concentration

BECs were measured in PD 5 rat after ethanol and saline administration. The mean BECs at various time points are shown in Figure 1. The peak BEC (509 ㎎/㎗) achieved 2 hr after the ethanol treatment in the pups, with near total clearance of ethanol at the end of 24 hr after post treatment. None of the animals in either of control groups gave positive BEC reading.

2. Ethanol-induced Purkinje cell death

To confirm the ethanol-induced cell death in the vermal region of cerebellum, FJB staining which detects the apoptosic and necrotic cell death was conducted. Many positive cells were localized in the Purkinje cell layer while some cells in the granular layer and deep cerebellar nuclei were also positively stained. FJB-positive cells appeared at 8hr, peaked at 12-18 hr post-treatment and were almost disappeared by 24 hr after ethanol administration (Fig. 2). Lobule specific pattern of FJB staining in the Purkinje cell layer was that lobules I, II, III, IX, and X contained more numerous positive cells than lobules V, VI, and VII (data not shown).

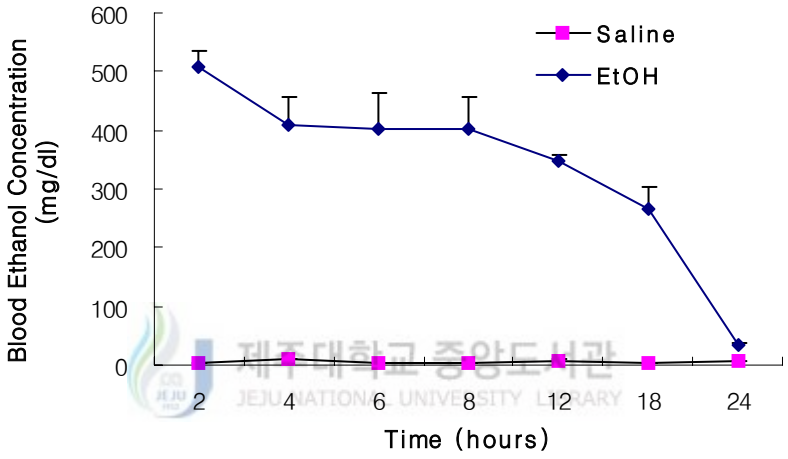

Figure 1. Time course of blood ethanol levels following ethanol administration on PD 5 rat. The profiles shown were obtained from a same group of pups used in the analyses of effects on the Purkinje cells at day 5.

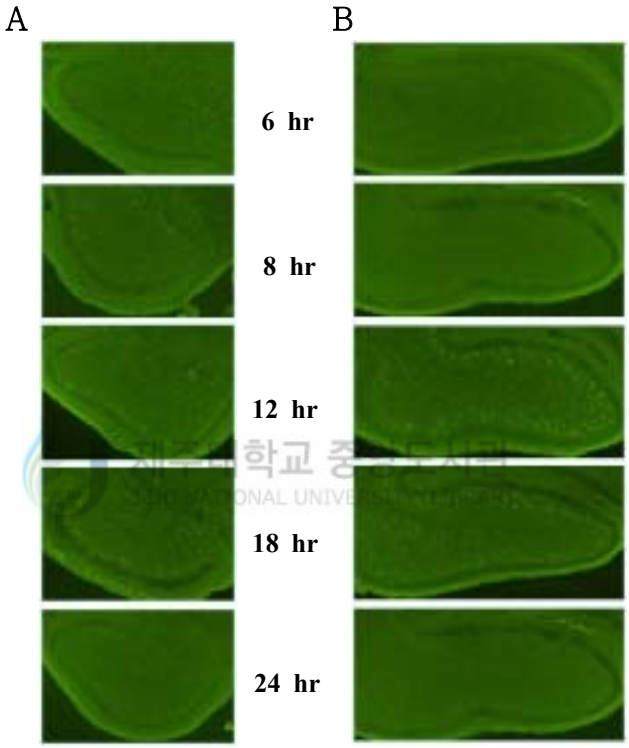

Figure 2. Temporal pattern of FJB staining from vermal section of PD 5 rat cerebellum after ethanol administration. Note that most of FJB-positive cells are located the Purkinje cell layer and these positive cells are most intensively stained at 12-18 hr after ethanol administration. A, lobule I; B, lobule X

3. Immunostaining and western blot analysis for cleaved aspase-9 and caspase-3

To determine whether the above FJB-positive cells in the Purkinje cell layer are degenerated via apoptotic pathway, immunohistochemistry and western blotting for the cleaved forms of caspase-9 and caspase-3 were undertaken by using vermal section and tissue homogenate of vermis respectively. Moreover double immunostaining for the cleaved caspase-9/-3 and calbindin D28k was carried out to confirm whether caspase-9/-3 immunoreactive cell in the Purkinje cell layer is Purkinje cell per se.

The expression of active forms of caspase-9 and caspase-3 was dramatically induced by ethanol administration. The temporal pattern of active caspase-9 was similar to that seen for the active caspase-3 and their expressions were transient: the peak expression of both active forms occurred 12 hr time points and then dropped abruptly to the saline control levels at 24 hr after ethanol administration (Fig. 3 and Fig. 4)

In immunohistochemical analysis, cleaved caspase-9 and caspase-3 immunoreactive cells began to appear in Purkinje cell layer and granular layer of cerebellum at 8 hr, maximally detected between 12-18 hr and were almost absent 24 hr after ethanol administration (Fig. 5 and Fig.6). To verify the identity of caspase-9 and caspase-9 immunoreactive cells in Purkinje cell layer as Purkinje cells, double immunolabeling was conducted. Figure 7 shows images from cerebellar sections for cleaved caspase-9/-3

and calbindin D28k. The red staining identifies the cell as Purkinje cell while the green staining represents the presence of cleaved caspase-9 or caspase-3. The active caspase-9 or caspase-3 positive Purkinje cells were those cells showing co-localization of active caspase-9/-3 (yellow) and calbindin D28k (red). Most of active caspase-9 or caspase-3 positive cells in the Purkinje cell layer proved to be Purkinje cells.

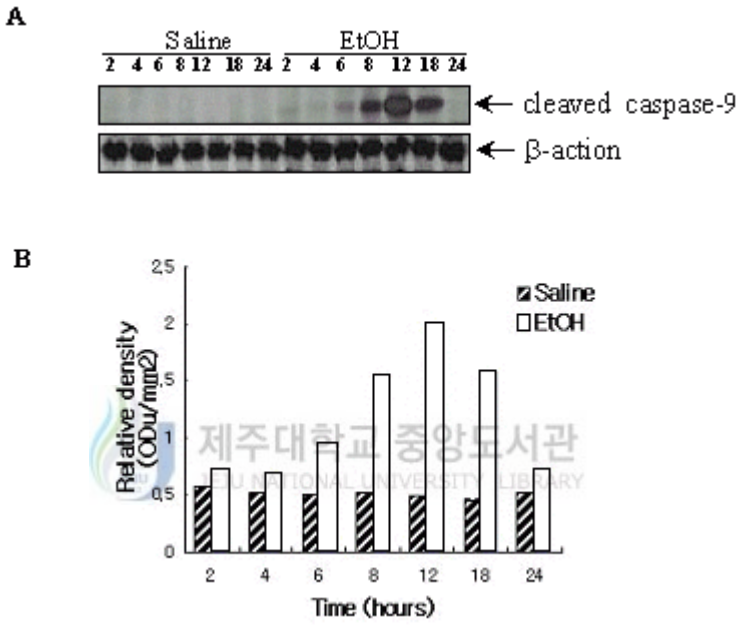

Figure 3. Western blot analysis for cleaved caspase-9 in PD 5 rat cerebellum after ethanol administration. cleaved caspase-9 Immunoreactivity was detected as a single band of 35 kDa (A), and quantified by densitometry (B).

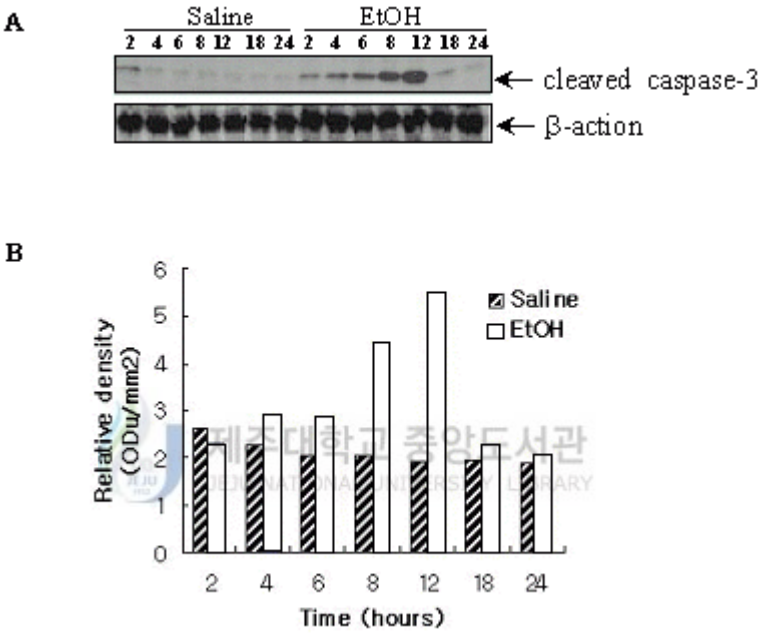

Figure 4. Western blot analysis for cleaved caspase-3 in PD 5 rat cerebellum after ethanol administration. Cleaved caspase-3 immunoreactivity was detected as a single band of 21 kDa (A), and quantified by densitometry (B).

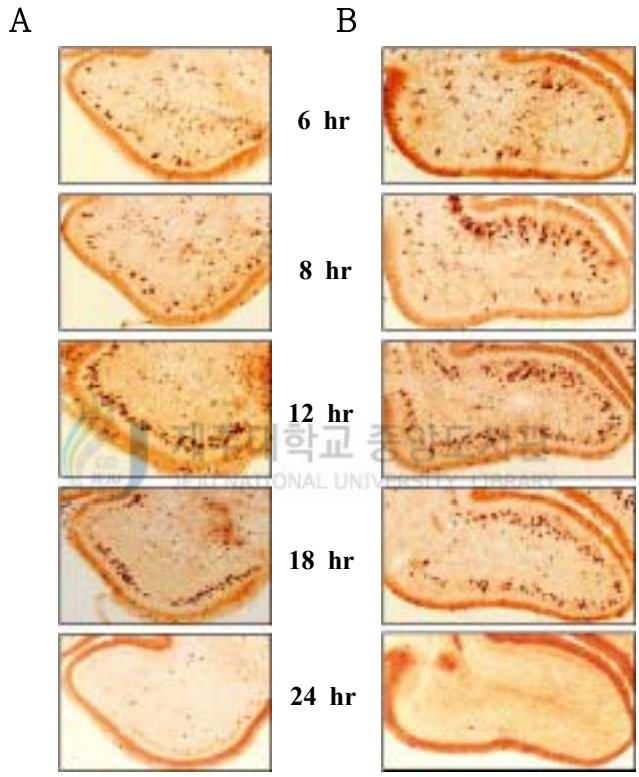

Figure 5. Immunohistochemical detection for cleaved caspase-9 in lobule I and X of PD 5 rat cerebelli after ethanol administration. A, lobule I; B, lobule X.

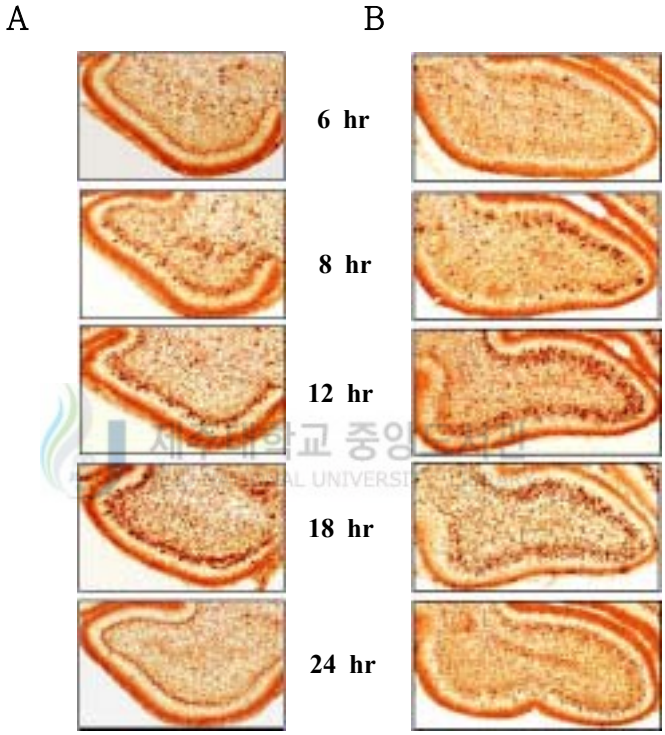

Figure 6. Immunohistochemical detection for cleaved caspase-3 in lobule I and X of PD 5 rat cerebelli after ethanol administration. A, lobule I; B, lobule X.

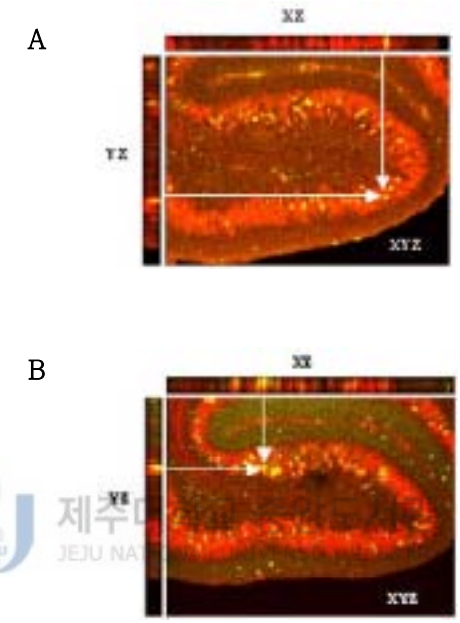

Figure 7. Confocal laser scanning microscopic images for the co-localization of caspase-3 or caspase-9 (FITC) and calbindin D28k (Texas red) in lobule X of cerebellum following ethanol administration. Left and upper figures in A and B show the co-localization of caspase-9 or caspase-3, and calbindin D28k in the plane of YZ and XZ axis of the arrow indicated cells. A, cleaved caspase-9; B, cleaved caspase-3.

4. 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 in PD 5 rat cerebellum following ethanol administration (Fig. 9). Cerebellum of saline treated animals did not show 8-OHdG immunoreactivities in any cells in lobule 1 and 10. On the other hand, 8-OHdG immunoreactive cells were detected in the Purkinje cell layer of cerebellum at 8 hr, and peaked 12-24 hr, but there was no positive cells at 30 hr following ethanol administration. In addition, 8-OHdG immunoreactive cells were not detected in granular and molecular cell layer.

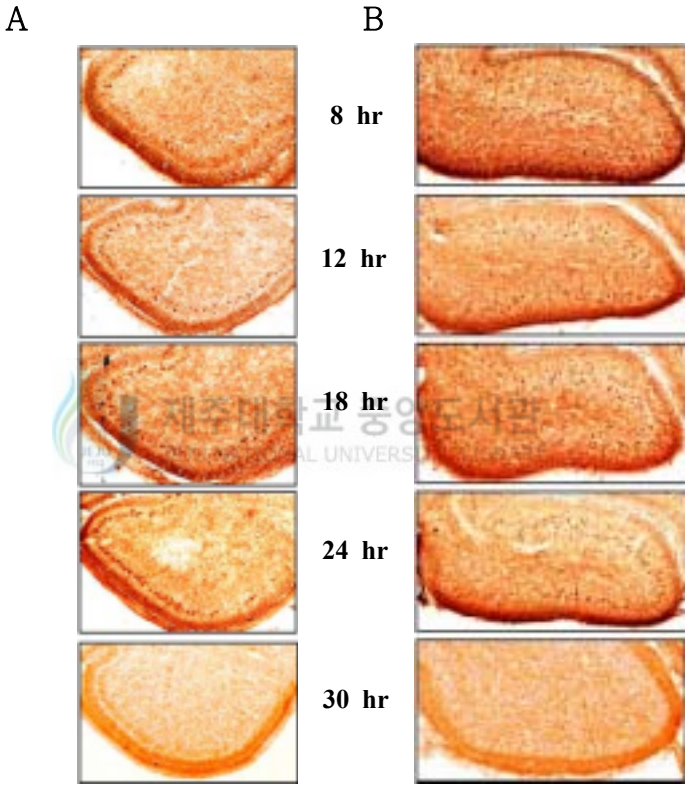

Figure 8. Immunohistochemical detection of 8-OHdG in lobule I and X of PD 5 rat cerebelli after ethanol administration. A, lobule I; B, lobule X.

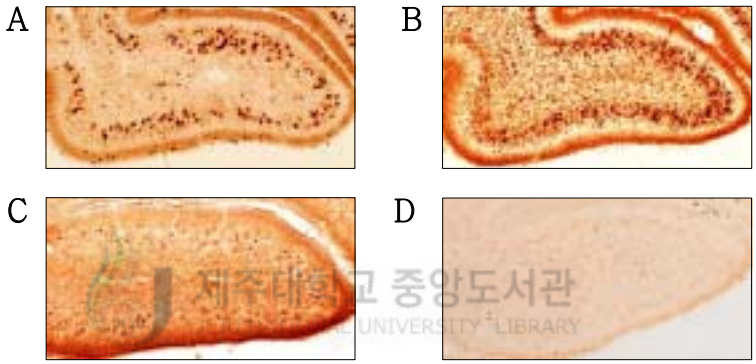

Figure 9. Immunohistochemistry for the cleaved forms of caspase-9 and caspase-3, 8-OHdG and TUNEL staining to reveal the death mechanism of Purkinje cell following ethanol administration. A, cleaved caspase-9; B, cleaved caspase-3; C, 8-OHdG; D, TUNEL.

Table 1. Comparative temporal patterns of caspase-3, caspase-9, and 8-OHdG immunoreactivity in cerebellar Purkinje cells.

	Cleaved caspase-9		Cleaved caspase-3		8-OHdG	
	lobule I	lobule X	lobule I	lobule X	lobule I	lobule X
2 hr	-	-	-	-	-	-
4 hr	-	-	-	-	-	-
6 hr	+	+	-	-	-	-
8 hr	++	++	++	++	+	+
12 hr	+++	+++	+++	+++	+++	+++
18 hr	++	++	++	++	+++	+++
24 hr	-	-	-	-	+++	+++
30 hr	-	-	-	-	-	-

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. Recently, experimental studies suggested that ethanol activate the pro-apoptotic molecules (Heaton *et al*., 2003) and lead to neurodegeneration by activation of caspase-3 in developing brain (Ikonomidou *et al*., 2000; Olney *et al*., 2000, 2002). However, the mechanism leading to Purkinje cell degeneration following ethanol administration is not clearly understood.

In present study, FJB positive cells in the Purkinje cell layer after ethanol exposure on PD 5 were more numerous in the early maturing lobules (lobules I, II, III, and IV) than those found in the late maturing lobules (lobules V, 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 understand the manner of Purkinje cell death, this study was imployed immunohistochemical technique for active caspase-3 and caspase-9, the key regulator in apoptotic cell death. The immunohistochemical results showed that ethanol-induced Purkinje cell death occurs by activation of caspase-9

and caspase-3. The spatial and temporal patterns of caspase-3 and caspase-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). Finally, this study introduced TUNEL technique in this studies to determine whether Purkinje cell death is involved in 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-9 and caspase-3 immunostaining. These results were contrary to the previous report by other group (Light *et al*., 2002). The TUNEL method 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. They delivered ethanol via intragastric intubation while we injected subcutaneously. but the peak BEC is very similar, being approximately 500 ㎎/dℓ. There are increasing evidences that ethanol may act as NMDA receptor antagonist (Hoffman *et al*., 1989; Ikonomidou *et al*., 2000). In this situation, ethanol may block Ca^{2+} can inhibit the activity of Ca^{2+}/Mg^{+}-dependent endonuclease, which lead to no DNA fragmentation (Cohen and Duke, 1984; Cohen *et al*., 1992). In some apoptotic pathway, it has been demonstrated that DNA fragmentation does not occur although cytochrome c release and caspase activation are observed. Thus, this study 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 administration was observed in cells of Purkinje cell layer PD 5 rat cerebellum but did not detect at PD 7 and PD 14 rat. Considering that vulnerability of Purkinje cells to ethanol treatment confines only to narrow developmental period (PD 4~6) and is little if any at the sightly later ethanol-resistant period (PD 7~9 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, the present study also shows that lobule-specific pattern of 8-OHdG immunoreactive cells in the Purkinje cell layer was very similar to those of active caspase-9/-3 immunohistochemistry and FJB staining.

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 and 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 signaling 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). ROS act upstream of mitochondrial membrane depolarization, Bax relocalization, cytochrome c release, executing caspase activation and nuclear fragmentation (Fleury *et al*., 2002; Yuan *et al*., 2003). In addition to these early regulation of apoptotic death pathway, some data raise the possiblity that ROS are also required for the execution of the death program (Kroemer *et al*., 1995; Schulz *et al*., 1996). For example, release of

cytochrome c trigger accumulation of ROS during the later stage of the death program, i.e. during the destruction phase when the cell is broken down and these increased ROS may be associated with a necrotic-type terminal degradation of the cell (Fleury *et al*., 2002).

In the present study, 8-OHdG immunoreactivity in cells of Purkinje cell layer as observed for a longer time until 24 hr after ethanol administration, at which time point the active caspase-9 or caspase-3 immunoreactivity is gone by. This longer duration of 8-OHdG immunoreactivity may reflect the persistent production of ROS in response to ethanol exposure, since it has been demonstrated that ROS levels in the extract of whole cerebellum remains to be elevated by 24 hr after ethanol administration. Therefore, the later increase of ROS level in this study may convert the apoptotic pathway of Purkinje cells into necrotic death. In such occur as shown in the present study.

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

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초 록

뇌 발생이 활발하게 일어나는 동안에 에탄올 섭취는 소뇌 Purkinje 세포 손실을 유도한다고 잘 알려져 있다. 최근에 에탄올이 발생중인 소뇌에서 활성 산소종(ROS)의 생성을 증가시킨다고 보고 되어졌으며, 이들 ROS는 cytochrome c 의 방출과 순차적인 caspase-9과 caspase-3의 활성화에 관여한다고 제안되었다. 더욱이 적은양의 ROS는 세포사멸을 유도하지만, 많은 양의 ROS 축적은 세포사멸을 수행하는 세포들이 세포괴사를 유도할 수 있다고 제안되었다. 그러나, 에탄올에 의해 유도되어지는 Purkinje 세포 죽음의 기전에 대하여 아직 잘 알려져 있지 않다. 따라서 본 연구는 발생중인 흰쥐 소뇌에서 에탄올에 의한 Purkinje 세포사멸과 산화적 손상과의 관계를 알아보기 위하여, FJB 염색, TUNEL 염색, caspase-9과 caspase-3의 면역조직화학적 염색과 western blotting, 그리고 8-OHdG 면역조직화학적 염색을 수행하였다.

본 연구에서 에탄올 처리 후 Purkinje 세포사멸이 caspase-9과 caspase-3의 활성화에 의해 중재되어진다는 것을 확인하였다. 그러나 TUNEL 염색을 수행한 결과, 출생 후 5일 되는 흰쥐 소뇌의 Purkinje cell에서는 전혀 염색이 되지 않았다. 이것은 에탄올에의해 유도되어지는 Purkinje 세포 죽음이 전형적인 세포사멸 경로를 통하여 일어나지 않을 것이라고 사료되어지며, 또한 강한 산화적 스트레스가 일어났을 때 생성되는 8-OHdG의 면역조직화학적 염색 결과는 caspase의 활성화 경향을 비교하였을 때 다른 양상으로 나타났다.

이상의 결과들로부터, 에탄올에 의해 의한 발생중인 흰쥐 소뇌의 Purkinje 세포 죽음은 caspase-9과 caspase-3의 활성화와 산화적 DNA 손상에 의해 일어난다. 그러나, caspase의 활성화와 8-OHdG 발현 양상의 차이는 세포사멸을 수행하는 Purkinje 세포가 세포내 ROS 양의 현저한 증가에 의해 세포괴사 경로로 변환되어질 것이라고 사료된다.

주요어: 태아 알콜 증후군, 에탄올, Purkinje 세포 사멸, 산화적 DNA 손상, 8-hydroxy-2'-deoxyguanosine (8-OHdG), 활성 산소종(ROS)

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