How does ethanol induce apoptotic cell death of SK-N-SH neuroblastoma cells?

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Research Highlights

(1) Previous studies have demonstrated that ethanol influences the secretion of neurotrophins, promotes oxidative stress, reduces the absorption of nutritive substances, and thereby induces neuronal damage.

(2) Recent evidence has demonstrated that ethanol can directly induce apoptotic cell death of the neurons and initiate c-Jun N-terminal protein kinase and p38 kinase pathway.

(3) By using SK-N-SH neuroblastoma cells, this study investigated the mechanism behind ethanol-induced neural cell apoptosis.

(4) Results from this study indicate that ethanol mediates apoptosis of SK-N-SH neuroblastoma cells by activating p53-related cell cycle arrest possibly through activation of the c-Jun N-terminal protein kinase-related cell death pathway.

Abstract

A body of evidence suggests that ethanol can lead to damage of neuronal cells. However, the mechanism underlying the ethanol-induced damage of neuronal cells remains unclear. The role of mitogen-activated protein kinases in ethanol-induced damage was investigated in SK-N-SH neuroblastoma cells. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide cell viability assay, DNA fragmentation detection, and flow cytometric analysis showed that ethanol induced apoptotic cell death and cell cycle arrest, characterized by increased caspase-3 activity, DNA fragmentation, nuclear disruption, and G_1 arrest of cell cycle of the SK-N-SH neuroblastoma cells. In addition, western blot analysis indicated that ethanol induced a lasting increase in c-Jun N-terminal protein kinase activity and a transient increase in p38 kinase activity of the neuroblastoma cells. c-Jun N-terminal protein kinase or p38 kinase inhibitors significantly reduced the ethanol-induced cell death. Ethanol also increased p53 phosphorylation, followed by an increase in p21 tumor suppressor protein and a decrease in phospho-Rb (retinoblastoma) protein, leading to alterations in the expressions and activity of cyclin dependent protein kinases. Our results suggest that ethanol mediates apoptosis of SK-N-SH neuroblastoma cells by activating p53-related cell cycle arrest possibly through activation of the c-Jun N-terminal protein kinase-related cell death pathway.

Key Words

neural regeneration; ethanol; apoptosis; nerve cell; p53; mitogen-activated protein kinases; c-Jun amino-terminal kinases; p38K; neuroblastoma cell; grants-supported paper; neuroregeneration

INTRODUCTION

Alcohol is a toxic and dependence-producing substance that can damage most organs in the body, including the liver[1-3], pancreas[4-6], skeletal and cardiac muscle[7], and brain[8-9]. The brain is particularly sensitive to the toxic effects of alcohol. The model of alcohol-related brain damage can be used to investigate the effects of chronic alcohol consumption on human brain structure and function in the absence of well-characterized neurological concomitants of alcoholism[10-15]. For example, structural imaging techniques have revealed that chronic alcohol use is accompanied by volume reductions of gray and white matter, microstructural disruption of various white matter tracts, and enlargement of cerebral ventricles and sulci[16-17]. Postmortem studies of brain tissue in both humans and animals confirmed the observation by structural imaging techniques, showing significant reductions in the weight of the cerebral hemispheres and the cerebellum in severe alcoholics[18-19]. Except the structure of normal adult brain, alcohol also damages neurogenesis of the developing brain as well as adult neuroregeneration of the adult brain. Ethanol has been shown to disrupt numerous events in the developing brain, including neurogenesis, cell migration, cell adhesion, neuron survival, axon outgrowth, synapse formation, and neurotransmitter function[20-22]. Since similar events also happen during neuroregeneration of the adult brain, it is possible that ethanol also affects adult neuroregeneration. Inhibition of neuroregeneration by ethanol has been demonstrated in animal models that receive low dose of ethanol.

The structural alterations due to alcohol abuse clearly lead to changes in brain function, with the degree of dysfunction dependent upon the duration and amount of alcohol consumed, which include neuropsychological deficits in alcoholics, particularly in those with Korsakoff's psychosis[22-24]. Alcoholics also show the abnormality of executive cognitive function, the ability to use higher mental processes to adaptively shape future behavior[25-26]. Long-term follow-up of the fetal effects of ethanol demonstrates that mental retardation, abnormal behavior, and facial dysmorphism persist into adulthood[27]. In rodents exposed *in utero* to ethanol, the hippocampi display reduced number of neurons and dendritic spine density, correlating with the animals' impaired learning and memory[27].

A large number of works have been done to unveil the mechanisms for the toxicity of ethanol to the brain. Although the exact mechanism behind alcoholic neuropathy is not well understood, several explanations have been proposed. It is believed that chronic alcohol use can damage the brain by inducing malnutrition and thiamine deficiency leading to Wernicke-Korsakoff syndrome. This indirect toxic effect of ethanol results from the compromised absorption and abnormal metabolism of thiamine and other vitamins induced by ethanol[28]. In addition, reduced availability of neurotrophins, increased levels of homocysteine, and activated microglia are also proposed to be responsible for the neurodegeneration induced by ethanol[28]. Except the indirect toxic effect, studies support a direct toxic effect of ethanol to neurons, since a dose-dependent relationship has been observed between severity of neuropathy and total lifetime dose of ethanol[29-30]. For example, axonal degeneration has been documented in rats receiving ethanol while maintaining normal thiamine status[31]. The direct toxic effect of ethanol on nerve cells has been directly observed in cultured cells. For example, the moderate or high concentration of ethanol could lead to morphological changes and cytoskeleton organization of the cultured neurons[32-33]. Ethanol can affect the differentiation of neural stem cells[34]. Numerous recent *in vitro* and *in vivo* studies provide evidence showing that ethanol can directly induce apoptotic cell death of the neurons[35-38]. However, the signaling mechanism of neuronal apoptosis induced by ethanol remains unclear. It is known that the initiation and execution of apoptosis depend on activation of the extrinsic and/or intrinsic death pathways. Mitogen-activated protein

kinases (MAPKs) are protein Ser/Thr kinases that convert extracellular stimuli into a wide range of cellular responses[39-40]. MAPKs are among the most ancient signal transduction pathways and are widely used throughout evolution in many physiological processes[39-41]. In mammals, there are more than a dozen MAPK enzymes that coordinately regulate cell proliferation, differentiation, motility, survival, and apoptosis. The best known are the conventional MAPKs, which include the extracellular signal-regulated kinases (ERK), c-Jun amino-terminal kinases (JNK), and p38 MAP kinases (p38K). While ERKs are key transducers of proliferation signals and are often activated by mitogens, the JNKs and p38K are poorly activated by mitogens but strongly activated by cellular stress inducers[39-41]. It has been shown that both the JNK and p38K can be activated by ethanol exposure[42-44]. However, how their activation initiates neuronal apoptosis has yet to be identified. The p53 tumor suppressor protein exerts its growth inhibitory activity by activating and interacting with diverse signaling pathways. As a downstream target, p53 protein is phosphorylated and activated by a number of protein kinases including JNK and p38K in response to stressful stimuli[45]. As an upstream activator, activated p53 acts as a transcription factor to induce and/or suppress a number of genes whose expression leads to the activation of diverse signaling pathways and many outcomes in cells, including cell cycle arrest and apoptosis[46].

SK-N-SH neuroblastoma cells are hybrid cells of neurons and blastomas that are phenotypically similar to neurons but able to proliferate. Therefore, this cell line has been extensively used to study the effect of ethanol on neuronal cells. By using SK-N-SH neuroblastoma cells, the current study was designed to investigate the effect of ethanol on the JNK and p38K pathways and their roles in ethanol-induced cell death of neuronal cells. In addition, the expression levels of p53 protein and various proteins associated with cell cycle arrest and apoptosis were measured after ethanol exposure in order to unveil the signaling mechanisms in the ethanol-induced cell death.

RESULTS

Ethanol reduced cell viability of SK-N-SH neuroblastoma cells

SK-N-SH neuroblastoma cells were divided into a control (C) group and four ethanol treatment groups and received PBS and various concentrations (25, 50, 100, 200 mmol/L) of ethanol treatment for 24 hours. Phase contrast photomicrographs showed that most of the

ethanol-treated SK-N-SH neuroblastoma cells shrank into spherical shape and only a few exhibited normal spindle shape (Figure 1A). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay indicated that ethanol induced a concentration- and exposure time-dependent increase in cell death rates of the SK-N-SH neuroblastoma cells ($P < 0.01$; Figure 1B).
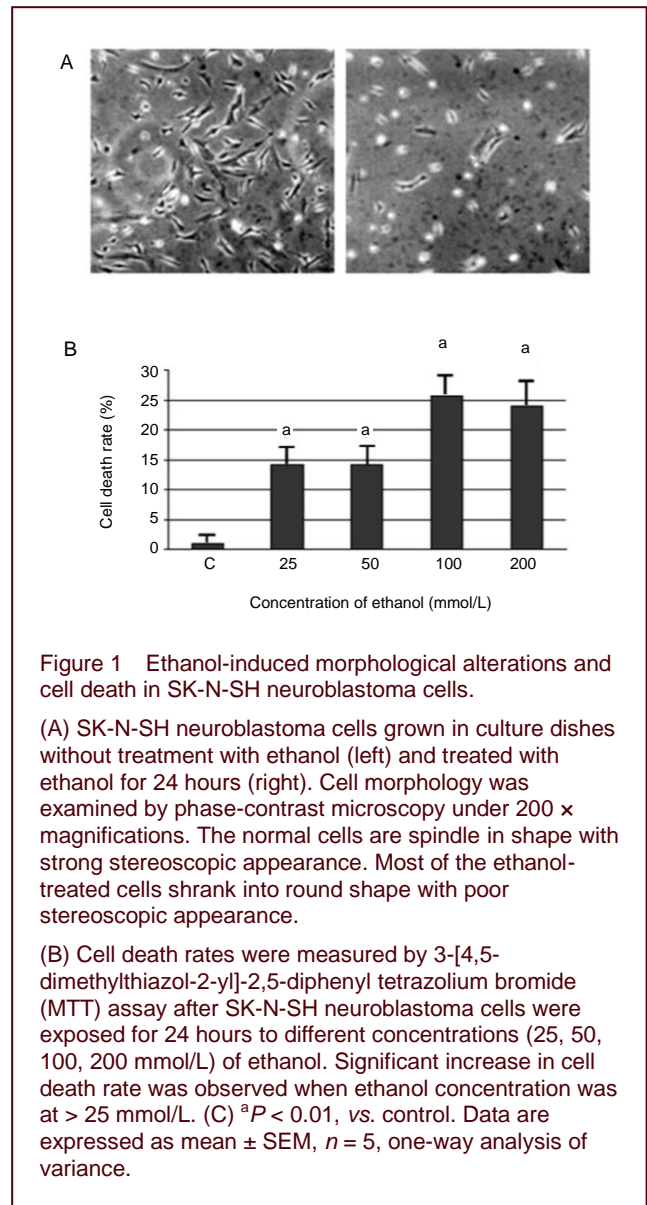

Figure 1   Ethanol-induced morphological alterations and cell death in SK-N-SH neuroblastoma cells.

(A) SK-N-SH neuroblastoma cells grown in culture dishes without treatment with ethanol (left) and treated with ethanol for 24 hours (right). Cell morphology was examined by phase-contrast microscopy under 200 × magnifications. The normal cells are spindle in shape with strong stereoscopic appearance. Most of the ethanol-treated cells shrank into round shape with poor stereoscopic appearance.

(B) Cell death rates were measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay after SK-N-SH neuroblastoma cells were exposed for 24 hours to different concentrations (25, 50, 100, 200 mmol/L) of ethanol. Significant increase in cell death rate was observed when ethanol concentration was at > 25 mmol/L. (C) $^aP < 0.01$, vs. control. Data are expressed as mean ± SEM, $n = 5$, one-way analysis of variance.

Ethanol induced apoptotic alterations and cell cycle arrest in SK-N-SH neuroblastoma cells

After treatment with 100 mmol/L ethanol, the levels of caspase-3 were increased after 16 hours, which still kept at high levels at 24 hours ($P < 0.01$; Figure 2A). In addition, DNA fragmentation analysis showed that in the ethanol-treated cells, there were fragmented DNA at 24 hours, which became apparent after the treatment time was prolonged from 24 to 72 hours (Figure 2B). We stained the cells with 4',6-diamidino-2-phenylindole

(DAPI), a sensitive assay for apoptosis. Without ethanol treatment, the nuclei of control cells showed uniform staining, indicating that these cells were healthy and nuclei intact. In contrast, after 24 hour treatment with 100 mmol/L ethanol, the SK-N-SH neuroblastoma cells exhibited typical alterations of apoptosis, such as nuclear condensation and disruption (Figure 2C). Flow cytometric analysis with the cellular DNA stained with propidium iodide revealed that the percentage of M1 cells, which indicated the cells in sub-G_1 stage of cell cycle, increased from 0.84% at 0 hour to 15.82% at 36 hours. In contrast, the cells at M3 and M4 phases, which represented S phase and G_2/M phase, decreased from 8.33% and 27.52% at 0 hour to 4.98% and 17.21% at 36 hours, respectively (Figure 3).
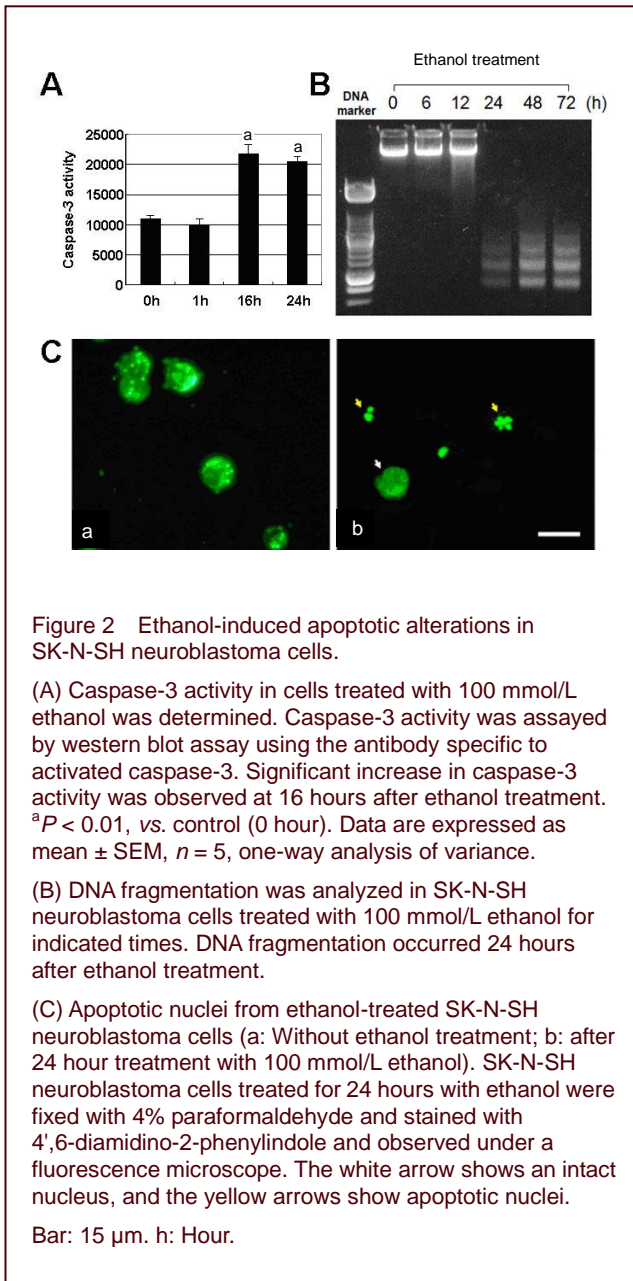

Figure 2　Ethanol-induced apoptotic alterations in SK-N-SH neuroblastoma cells.

(A) Caspase-3 activity in cells treated with 100 mmol/L ethanol was determined. Caspase-3 activity was assayed by western blot assay using the antibody specific to activated caspase-3. Significant increase in caspase-3 activity was observed at 16 hours after ethanol treatment. $^a P < 0.01$, *vs.* control (0 hour). Data are expressed as mean ± SEM, $n = 5$, one-way analysis of variance.

(B) DNA fragmentation was analyzed in SK-N-SH neuroblastoma cells treated with 100 mmol/L ethanol for indicated times. DNA fragmentation occurred 24 hours after ethanol treatment.

(C) Apoptotic nuclei from ethanol-treated SK-N-SH neuroblastoma cells (a: Without ethanol treatment; b: after 24 hour treatment with 100 mmol/L ethanol). SK-N-SH neuroblastoma cells treated for 24 hours with ethanol were fixed with 4% paraformaldehyde and stained with 4',6-diamidino-2-phenylindole and observed under a fluorescence microscope. The white arrow shows an intact nucleus, and the yellow arrows show apoptotic nuclei.

Bar: 15 μm. h: Hour.
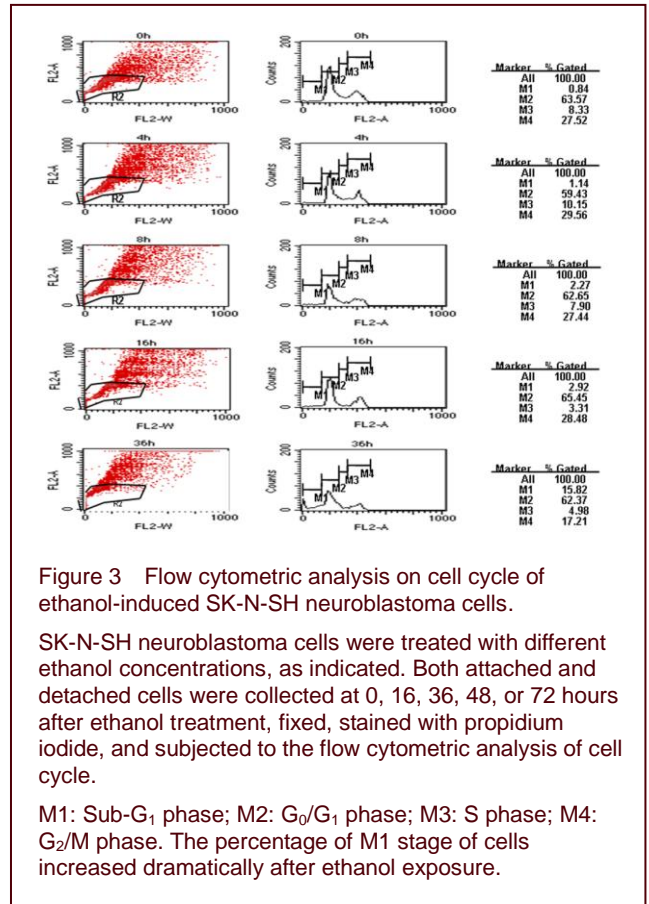
Figure 3　Flow cytometric analysis on cell cycle of ethanol-induced SK-N-SH neuroblastoma cells.

SK-N-SH neuroblastoma cells were treated with different ethanol concentrations, as indicated. Both attached and detached cells were collected at 0, 16, 36, 48, or 72 hours after ethanol treatment, fixed, stained with propidium iodide, and subjected to the flow cytometric analysis of cell cycle.

M1: Sub-G_1 phase; M2: G_0/G_1 phase; M3: S phase; M4: G_2/M phase. The percentage of M1 stage of cells increased dramatically after ethanol exposure.

Ethanol increased the levels of phosphorylated JNK (p-JNK) and p38K (p-p38K)

To determine the induction of JNK expression after ethanol exposure in SK-N-SH neuroblastoma cells, JNK protein levels were determined by immunoblot analysis after exposure to 100 mmol/L ethanol at different time points. As shown in Figure 4, ethanol increased p-JNK levels in a time- and concentration-dependent manner.
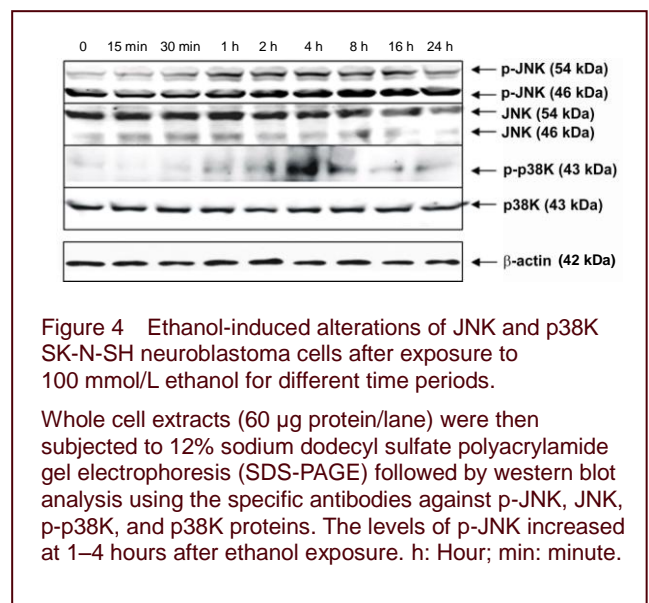
Figure 4　Ethanol-induced alterations of JNK and p38K SK-N-SH neuroblastoma cells after exposure to 100 mmol/L ethanol for different time periods.

Whole cell extracts (60 μg protein/lane) were then subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis using the specific antibodies against p-JNK, JNK, p-p38K, and p38K proteins. The levels of p-JNK increased at 1–4 hours after ethanol exposure. h: Hour; min: minute.

Within 1 hour after ethanol exposure, p-JNK levels increased, and the elevated p-JNK persisted until 16 hours after ethanol exposure. Different from JNK, p-p38K levels transiently increased at 1–4 hours after ethanol treatment before returning to control levels.

Inhibition of JNK and p38K phosphorylation reduced ethanol-induced cell death

As described above, ethanol treatment led to remarkable increases in the levels of p-JNK and p-p38K. To examine the specific roles of JNK and p38K phosphorylation in ethanol-induced cell death, the cells were pretreated for 3 hours with SP600125 (a JNK inhibitor) and SB203580 (a p38K inhibitor). As shown in Figure 5, the inhibitors significantly reduced ethanol-induced cell death as well as p-JNK and p-p38K levels in SK-N-SH cells, suggesting that JNK and p38K phosphorylation are important during ethanol-mediated cell death.

Figure 5 Effects of JNK and p38K inhibitors on ethanol (EtOH)-induced SK-N-SH neuroblastoma cell death.

(A) SK-N-SH neuroblastoma cells grown in microtiter plates were pretreated with dimethyl sulfoxide (DMSO; control) or 10 μmol/L SB203580 (JNK inhibitor) or 500 nmol/L SP600125 (p38K inhibitor) for 3 hours before exposure to 100 mmol/L ethanol for an additional 24 hours. Cell viability was then determined using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. Data are expressed as mean ± SEM, $n = 5$, one-way analysis of variance. [a]$P < 0.01$, *vs.* ethanol-untreated control. [b]$P < 0.05$, *vs.* ethanol-treated samples.
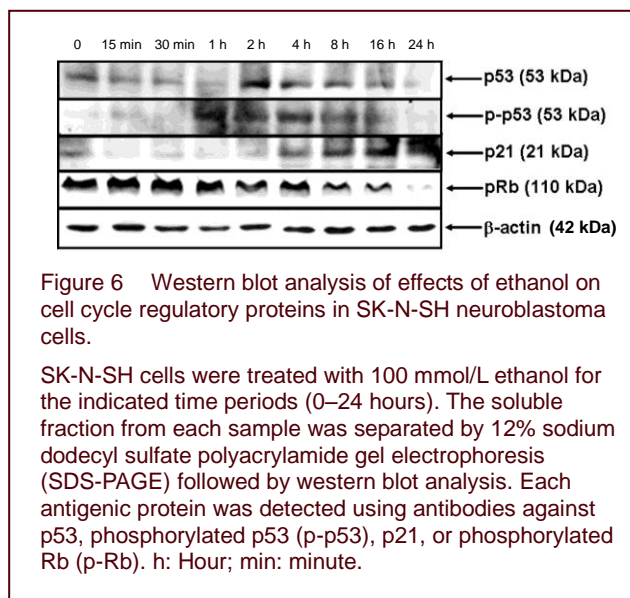
(B) SK-N-SH neuroblastoma cells were pretreated with 10 μmol/L SB203580 or 500 nmol/L SP600125 for 3 hours and then treated with 100 mmol/L ethanol for an additional 24 hours before cells were collected for immunoblot analyses of the phosphorylated-p38K (p-p38K) or phosphorylated JNK (p-JNK) proteins. Ethanol-induced increases in p-p38K and p-JNK were inhibited by SB203580 and SP600125.

Ethanol induced p53 phosphorylation in SK-N-SH neuroblastoma cells

To determine the involvement of p53 in ethanol-mediated SK-N-SH neuroblastoma cell death, the level of p53 was assayed by western blot analysis in SK-N-SH neuroblastoma cells treated with 100 mmol/L ethanol. Ethanol induced the phosphorylation of p53, which led to accumulation of p53 protein at 1 hour after ethanol exposure. Furthermore, this p53 activation was followed by an increase in the p21 tumor suppressor protein and a gradual decrease in phosphorylated Rb protein (Figure 6).

Figure 6 Western blot analysis of effects of ethanol on cell cycle regulatory proteins in SK-N-SH neuroblastoma cells.

SK-N-SH cells were treated with 100 mmol/L ethanol for the indicated time periods (0–24 hours). The soluble fraction from each sample was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis. Each antigenic protein was detected using antibodies against p53, phosphorylated p53 (p-p53), p21, or phosphorylated Rb (p-Rb). h: Hour; min: minute.

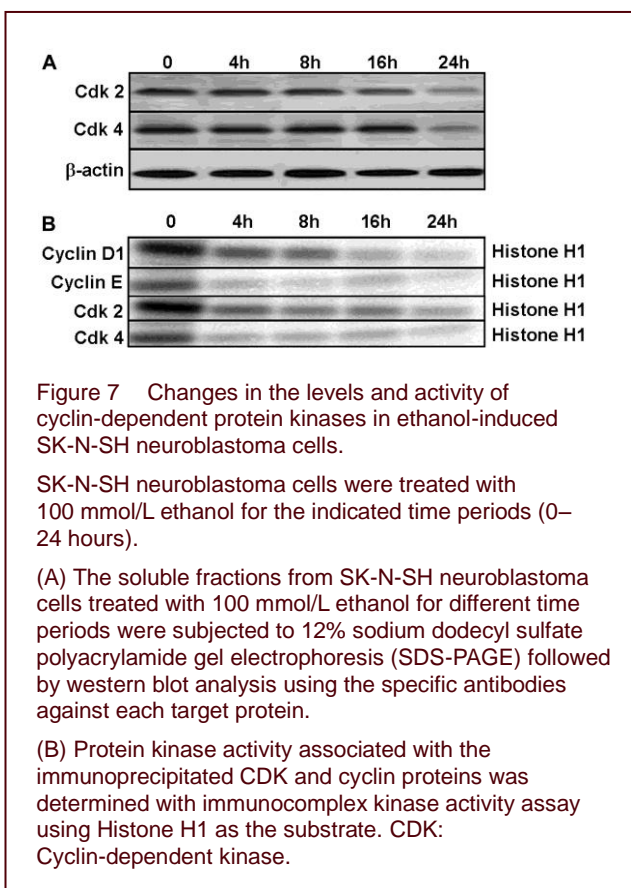## Ethanol reduced expression and activity of cyclin dependent protein kinases

To investigate the effect of ethanol on cell cycle, the expressions and activity of the cyclin-dependent protein kinases were examined. As shown in Figure 7A, the levels of Cdk2 and Cdk4 decreased in a time-dependent manner in SK-N-SH neuroblastoma cells treated with 100 mmol/L ethanol. In addition, the protein kinase activity associated with the immunoprecipitated CDK (Cdk2 and Cdk4) and cyclin proteins (cyclin D1 and cyclin E) also reduced in a time-dependent manner in SK-N-SH neuroblastoma cells treated with 100 mmol/L ethanol (Figure 7B).

DISCUSSION

Long term alcohol exposure has been shown to be toxic to the nerve cells in either the developing or the adult brain[21]. However, whether the toxic effect of ethanol on nerve cells is due to indirect or direct mechanism remains unclear. While some studies show that ethanol may induce apoptotic neurodegeneration by some indirect mechanisms such as increase in oxidative stress, induction of proinflammatory cytokines, deficiency in thiamine, and accumulation in GM2 ganglioside and sphingosine 1-phosphate[28, 47-48], some others suggest that a direct mechanism may play a role in the etha-

nol-induced neuronal cell death[35-38]. By using cultured SK-N-SH neuroblastoma cells treated for various periods of time by different concentrations of ethanol, we showed that ethanol could significantly reduce the cell viability of the SK-N-SH neuroblastoma cells.

The reduction of cell viability induced by ethanol may result from increased apoptotic cell death and decreased cell proliferation. That the ethanol-treated SK-N-SH neuroblastoma cells underwent apoptotic cell death was evidenced by several typical apoptotic changes the cells presented after ethanol exposure. These include caspase-3 activation, DNA fragmentation, and nuclear condensation and disruption. In addition, flow cytometric analysis identified that the percentage of G_1 cells in cell cycle increased dramatically, indicating that ethanol could also induce cell cycle arrest of the SK-N-SH neuroblastoma cells.

Figure 7   Changes in the levels and activity of cyclin-dependent protein kinases in ethanol-induced SK-N-SH neuroblastoma cells.

SK-N-SH neuroblastoma cells were treated with 100 mmol/L ethanol for the indicated time periods (0–24 hours).

(A) The soluble fractions from SK-N-SH neuroblastoma cells treated with 100 mmol/L ethanol for different time periods were subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis using the specific antibodies against each target protein.

(B) Protein kinase activity associated with the immunoprecipitated CDK and cyclin proteins was determined with immunocomplex kinase activity assay using Histone H1 as the substrate. CDK: Cyclin-dependent kinase.

To further understand the potential mechanism for the ethanol-induced apoptotic cell death and cell cycle arrest in SK-N-SH neuroblastoma cells, we tried to identify the possible signal transduction pathways related to the ethanol-induced apoptosis and cell cycle arrest. As have demonstrated previously, MAPKs appear to participate in the ethanol-induced cell death. Both the JNK and p38K, the two subfamilies of MAPKs that are usually activated

by stressful stimuli, were shown to be activated by ethanol exposure[42-44]. However, how their activation initiates neuronal apoptosis and cell cycle arrest has yet to be elucidated. Our results showed that both the levels of phosphorylated JNK and p38K were increased by ethanol treatment, indicating these two pathways were activated during ethanol exposure. To demonstrate that the ethanol-induced activation of JNK and p38K is associated with the ethanol-induced cell death, we used JNK inhibitor SP600125 and p38K inhibitor SB203580 to treat the cells before ethanol exposure. The results showed that the inhibitors significantly reduced the ethanol-induced cell death as well as the levels of the phosphorylated JNK and p38K in SK-N-SH neuroblastoma cells, suggesting that the ethanol-mediated cell death is mediated by JNK and p38K activation.

p53 is the most commonly mutated gene in human cancer. The p53 tumor suppressor protein is a nuclear phosphoprotein with a short half life that is regulated mainly through post-translational modifications. Upon stressful stimuli, p53 protein is modified through multiple post-translational events, including phosphorylation and acetylation. These modifications stabilize and activate p53 protein. Once p53 protein is activated, it acts as a transcription factor for many genes that contain the consensus p53-binding sites in their promoters or intronic sequences. It is accepted that activation of p53 protein triggers a number of signaling pathways that lead to cell cycle arrest, apoptosis, senescence, DNA repair and antiangiogenesis[45]. It has been shown that the MAP kinases including p38 and JNKs can phosphorylate p53 in response to different stressful stimuli, and such phosphorylation can initiate p53 response, leading to cell cycle arrest and apoptosis[45-46]. To determine the involvement of p53 in ethanol-mediated SK-N-SH cell death and cell cycle arrest, the level of p53 was assayed by immunoblot in the SK-N-SH cells treated with ethanol. We found that ethanol induced the phosphorylation of p53, which led to accumulation of p53 protein at 1 hour after ethanol exposure. This result indicates that p53 protein is involved in the apoptotic cell death and cell cycle arrest after modification by activated p38K and JNK in the ethanol-treated SK-N-SH neuroblastoma cells.

It is known that cell cycle progression is controlled by a set of cyclin-dependent kinases (CDKs), which are activated by their associated cyclins, but inhibited by two classes of CDK inhibitors. One of the CDK inhibitors is p21, which is a small 165 amino acid protein also known as p21WAF1/Cip1 and has been shown to be an impor-

tant mediator in p53-dependent cell cycle arrest and apoptosis[49-50]. Another CDK inhibitor is retinoblastoma protein (pRb) that works in the late G_1, and phosphorylation of pRb is found to be essential for G_1/S transition[51-52]. It is established that the p53 protein can enhance the transcription of p21[53]. Binding of p21 to the cyclin-Cdk complex therefore results in an inhibition of a kinase activity, thereby interfering with phosphorylation of pRb and inducing arrest of cell growth[54-56].

In accordance with the above theory, we showed that p53 activation by ethanol was followed by an increase in the p21 tumor suppressor protein and a gradual decrease in phospho-Rb protein. In addition, we showed that both the levels of Cdk2 and Cdk4, the protein kinase activity associated with CDK (Cdk2 and Cdk4) and cyclin proteins (cyclin D1 and cyclin E) decreased in a time-dependent manner in SK-N-SH neuroblastoma cells treated with ethanol. Since the cyclin D1/Cdk4 complex can activate cell cycle progression early in the G_1 phase by phosphorylation of pRb, while the Cdk2/cyclin E complex plays a role in the transition from the G_1 to S phase[57-59], the above results can well explain our flow cytometric analysis showing that the cells treated with ethanol arrested in G_1 stage. The p53 protein-mediated cell cycle arrest can further lead to apoptosis if the DNA cannot be repaired effectively. This may be one of the mechanisms for the ethanol-induced apoptotic cell death in the SK-N-SH neuroblastoma cells. However, since p53 can also induce apoptosis through cascade of caspases[60], and caspase-3 is activated in the ethanol-treated cells, it is also possible that ethanol-induced apoptotic cell death may be partially mediated through activation of caspase-3 by p53.

In conclusion, the present study strongly indicates that ethanol can directly induce cell cycle arrest and apoptosis in SK-N-SH neuroblastoma cells. Ethanol may first activate p53 protein through phosphorylation of JNK and p38K, and further initiate the cell death pathways leading to cell cycle arrest and apoptosis.

MATERIALS AND METHODS

Design
A randomized, controlled, *in vitro* experimental study.

Time and setting
The experiment was performed at Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, Kangdong Sacred Heart Hospital, Hallym University, Seoul, Korea from March 2011 to April 2012.

Materials
SK-N-SH cells were obtained from the American Type Culture Collection (Rockville, MD, USA).

Methods
SK-N-SH neuroblastoma cell culture
SK-N-SH neuroblastoma cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Fisher Bioblock Scientific, Rhine Province, France) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator under 5% CO_2/95% air at 37°C.

Detection of ethanol-treated SK-N-SH neuroblastoma cell viability by MTT assay
SK-N-SH neuroblastoma cell viability was measured after ethanol exposure using MTT assay (Sigma-Aldrich, St. Louis, MO, USA). Briefly, the medium was removed and replaced with 20 μL of MTT (5 mg/mL; Sigma-Aldrich) in PBS. The plates were incubated at 37°C for 4 hours, followed by addition of 100 μL of dimethyl sulfoxide (DMSO). The multi-well plates were then shaken for 15 seconds, and the signals were detected with a microplate reader at a wavelength of 595 nm. Cell death rate was expressed as the percentage of the ethanol-treated cells in the vehicle-treated control cells.

Detection of ethanol-treated SK-N-SH neuroblastoma cell apoptosis by DAPI staining
The SK-N-SH neuroblastoma cells were fixed at room temperature with 4% paraformaldehyde, and stained for DAPI using Cell Apoptosis DAPI Detection Kit (GenScript USA Inc., Piscataway, NJ, USA) according to the instruction provided. The stained cell nuclei were examined under a fluorescence microscope (Nikon TE2000, Tokyo, Japan).

Analysis of DNA fragmentation in ethanol-treated SK-N-SH neuroblastoma cells
DNA fragmentation in the SK-N-SH neuroblastoma cells was measured using a previously published method[61]. Briefly, genomic DNA isolated from ethanol-treated and untreated cells was mixed with unphosphorylated oligonucleotides in T4 DNA ligase buffer (Boehringer Mannheim, Stuttgart, Germany). Oligonucleotides were annealed. 3 U of T4 DNA ligase (Boehringer Mannheim) were added for ligations. The reactions were then diluted with TE buffer to a final concentration of 5 ng/mL. Samples were stored at 20°C until PCR. The ligated DNA was amplified by PCR using a specific linker primer. The PCR products were analyzed by electrophoresis through 1.2% agarose gels. After electrophoresis, the gels were stained by ethidium bromide and photo-

graphed on a UV transilluminator (JUNYI, Beijing, China).

Detection of ethanol-treated SK-N-SH neuroblastoma cells by flow cytometry

After trypsin digestion, approximately 10^6 cells were collected by centrifugation at 1 000 × g for 5 minutes. The cells were then washed in PBS followed by re-suspension and fixation in 70% ethanol for approximately 2 hours. The cells were washed once with PBS, re-suspended in 0.5 mL PBS containing 0.1 mg RNAase, and incubated for 30 minutes at 37°C. Cellular DNA was then stained with 10 μg of propidium iodide. The stained cells were subsequently analyzed on a FACScan with the Cellquest software (Becton Dickinson, Franklin Lakes, NJ, USA).

Western blot assay

Cells were washed twice with ice-cold PBS and lysed with a lysis buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 2.0% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate, and 0.1% (w/v) sodium dodecyl sulfate (SDS). The lysate was centrifuged at 15 000 × g for 5 minutes at 4°C, and the supernatant was used as whole cell extracts. 50–100 μg of protein was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene difluoride (Millipore, Billerica, MA, USA) membrane, and incubated with each primary antibody (1/1 000–1/2 000, Abcam, USA) against each target protein (activated caspase-3, p-JNK, JNK, phospho-p38 kinase, and p38 kinase protein, phospho-p53, p53, p21, pRb, Cdk2, Cdk4, and β-actin). The immunoreactivity was detected using an executive cognitive function detection system (Millipore)[62]. Caspase-3 activity in cells treated with 100 mmol/L ethanol was determined. Caspase-3 activity was assayed by western blot assay using the antibody specific to activated caspase-3.

Immunocomplex kinase activity assay

SK-N-SH neuroblastoma cells treated with ethanol for the indicated times were harvested, homogenized in ice cold lysis buffer, and used to determine the activities of cdk2, cdk4, cyclin D1, and cyclin E in the soluble fraction (300 μg per reaction) according to the published method[63]. Briefly, cells were then washed twice in cold PBS and lysed by the addition of RIPA-buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 mol/L NaCl and 0.01 mol/L Tris, pH 7.4). Lysates were clarified by centrifugation at 15 000 × g for 30 minutes at 4°C. Sam-

ples were incubated with each rabbit polyclonal antibody against Cdk2, Cdk4, cyclin D1, and cyclin E overnight at 4°C, followed by incubation for another 60 minutes with Protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology, Piscataway, NJ, USA). Immune complexes were centrifuged, and pellets were washed in RIPA-buffer, resuspended, boiled in SDS sample buffer and analyzed on discontinuous 12.5% SDS-polyacrylamide slab gels followed by fluorography. The protein kinase activity associated with the immunoprecipitated CDK (Cdk2 and Cdk4) and cyclin proteins (cyclin D1 and cyclin E) was measured using purified histone H1 as the substrate.

Statistical analysis

All experimental results shown were repeated five times unless otherwise indicated. The results were expressed as mean ± SEM. One-way analysis of variance was performed to determine the significance among groups using SPSS 11.5 software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

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