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Ethanol induces apoptotic cell death of SK-N-SH neuroblastoma cells by stimulating p53-related cell cycle arrest

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Abstract

The role of mitogen-activated protein kinases in ethanol-induced damage was investigated in SK-N-SH neuroblastoma cells. Ethanol was shown to induce apoptotic cell death and cell cycle arrest, characterized by increased caspase-3 activity, DNA fragmentation, nuclear disruption, and G1 arrest of cell cycle. In addition, ethanol induced a lasting increase in c-Jun N-terminal protein kinase (JNK) activity and a transient increase in p38 kinase (p38K) activity. JNK or p38K 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 stimulating p53-related cell cycle arrest possibly through activation of the JNK-related cell death pathway.

Key Words

ethanol; apoptosis; p53; MAPK; JNK; p38K; neuroblastoma cell

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INTRODUCTION

R Alcohol is a toxic and dependence-producing substance that can damage most organs in the body, including 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. Alcohol-related brain damage describes 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. The reduced brain mass is probably due to a combination of actual loss of nerve cells and reduction in cell size[18, 19]. Data from tissue and quantitative morphometry studies demonstrate selective neuronal loss, reduced arborization, and reduction of synaptic complexity in specific brain regions of alcoholics. Alcohol also

damages the developing brain. The neuropathological features include microcephaly, abnormal cortical thickness, reduced cerebral white matter volume, and abnormalities of the corpus callosum and cerebellar vermis. Ethanol disrupts numerous developmental events in animal models, including neurogenesis, cell migration, cell adhesion, neuron survival, axon outgrowth, synapse formation, and neurotransmitter function[20-22].

Except structural alterations, chronic alcohol abuse clearly leads to changes in brain function, with the degree of dysfunction dependent upon the duration and amount of alcohol consumed. Prefrontal (the most anterior part of the cortex) damage typically is associated with changes in personality and cognitive abnormalities. Both clinical and experimental studies support a role for frontal cortical involvement in neuropsychological deficits in alcoholics, particularly those with Korsakoff's psychosis[22, 23]. These deficits include dysfunction in emotional control, problem-solving ability, and attention. Alcoholics who do not suffer from Wernicke-Korsakoff syndrome still show greater loss of neuropsychological performance than peer nonalcoholics do on tests of learning, memory, abstracting, problem solving, visuospatial and perceptual motor functioning, and information processing[24].

Executive cognitive function (ECF) is the ability to use higher mental processes such as attention, planning, organization, sequencing, abstract reasoning, and the use of external and internal feedback to adaptively shape future behavior[25]. ECF processes are dysfunctional in alcoholics[26]. Long-term follow-up of the fetal effects of ethanol demonstrates that mental retardation, abnormal behavior, and facial dysmorphism persist into adulthood[27]. It is also evident that cognitive and behavioral abnormalities can occur in the absence of dysmorphisms, so-called "fetal alcohol effects", "alcohol-related neurodevelopmental disorder", (ARND) or "fetal alcohol spectrum disorders" [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, yet 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, and increased levels of homocysteine, 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 elusive.

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), 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 initiate 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 lead to the activation of diverse signaling pathways and many outcomes in cells, including cell cycle arrest and apoptosis[46].

In the current study, we investigated the effect of ethanol on the JNK and p38K pathways and their roles in ethanol-induced cell death. In addition, we further studied expression of p53 protein and various proteins associated with cell cycle arrest and apoptosis after ethanol exposure in order to unveil signaling mechanisms during ethanol-induced cell death.

RESULTS

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

Phase contrast photomicrographs showed that most of the ethanol-treated SK-N-SH cells shrank into spherical shape and only a few exhibited normal spindle shape (Fig.1A).

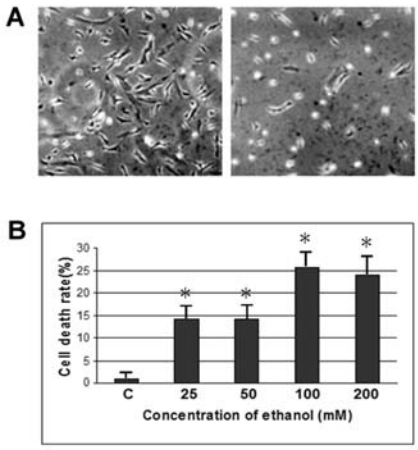

Figure 1 Ethanol-induced morphological alterations and cell death in SK-N-SH cells.

A: SK-N-SH cells grown in culture dishes without treatment with ethanol (CTL) and treated with ethanol for 24 h (EtOH). Cell morphology was examined by phase-contrast microscopy under 200 x 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 MTT assay after SK-N-SH cells were exposed for 24 h to different concentrations of ethanol. Significant increase in cell death rates was observed when ethanol concentration was increased to 25 mM. *P < 0.01, *vs.* control (C). mean ± SEM, *n* = 5.

MTT assay indicated that ethanol induced a concentration- and exposure time-dependent increase in cell death rates of the SK-N-SH neuroblastoma cells (Fig. 1B).

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

After treatment with ethanol, the levels of caspase-3 were increased (Fig. 2A). In addition, DNA fragmentation analysis showed that in the ethanol-treated cells there were fragmented DNA, which became apparent after the treatment time was prolonged (Fig. 2B). We stained the cells with 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 h treatment with 100 mM ethanol, the SK-N-SH cells exhibited typical alterations of apoptosis, such as nuclear condensation and disruption (Fig. 2C).

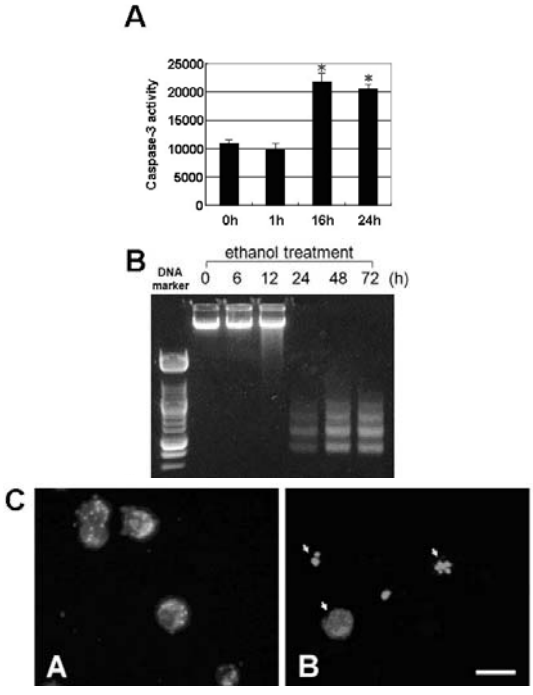

Figure 2 Ethanol-induced apoptotic alterations in SK-N-SH cells.

A: Caspase-3 activity in cells treated with 100 mM ethanol was determined. Significant increase in caspase-3 activity was observed at 16h after ethanol treatment. *P < 0.01, *vs.* control (C). *n* = 5.

B: DNA fragmentation was analyzed in SK-N-SH cells treated with 100 mM ethanol for indicated times. DNA fragmentation occurred 24 h after ethanol treatment.

C: Apoptotic nuclei from ethanol-treated SK-N-SH cells. SK-N-SH cells treated for 24 h with ethanol were fixed with 4% paraformaldehyde and stained with DAPI. The white arrow shows an intact nucleus, and the yellow arrows show apoptotic nuclei. Bar =15 μm.

Flow cytometrical analysis with the cellular DNA stained with propidium iodide revealed that the percentage of M1 cells, which indicated the cells in Sub-G1 stage of cell cycle, increased from 0.84% at 0 h to 15.82% at 36 h. In contrast, the cells at M3 and M4 phases, which represent S phase and G2/M phase, decreased from 8.33% and 27.52% at 0 h to 4.98% and 17.21% at 36 h, respectively (Fig. 3).

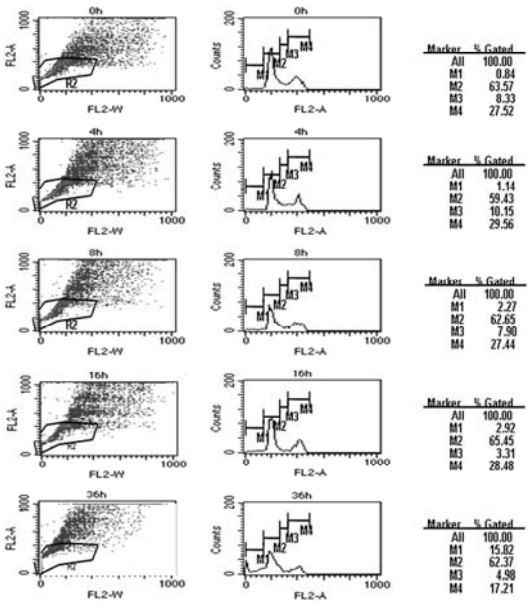

Figure 3 Flow cytometric analysis of cell cycle

SK-N-SH cells were treated with different ethanol concentrations, as indicated. Both attached and detached cells were collected at 0, 16, 36, 48, or 72 h after ethanol treatment, fixed, stained with propidium iodide, and subjected to the flow cytometric analysis of cell cycle. M1: Sub-G1 phase; M2: G0/G1 phase; M3: S phase; M4: G2/M phase. The percentage of M1 stage of cells increased dramatically after ethanol exposure.

Ethanol increased the levels of phosphorylated JNK and p38K

To determine the induction of JNK expression after ethanol exposure in SK-N-SH cells, JNK protein levels were determined by immunoblot analysis. As shown in Fig. 4, ethanol increased phosphorylated JNK (p-JNK) levels in a time- and concentration-dependent manner. Within 1 h after ethanol exposure, the p-JNK increased, and the elevated p-JNK persisted until 16 h after the exposure. Different from JNK, the phosphorylated p38 kinase (p-p38K) levels transiently increased between 1 h and 4 h 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 phosphorylated JNK and p38K. To examine the specific roles of JNK and p38K phosphorylation in ethanol-induced cell death, the cells were pretreated for 3 h with SP600125 (a JNK inhibitor) and SB203580 (a p38K inhibitor).

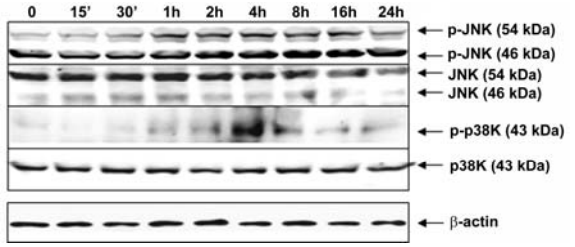

Figure 4 Ethanol-induced alterations of JNK and p38K

SK-N-SH cells were exposed to 100 mM ethanol for different times.

Whole cell extracts (60 μg protein/lane) were then subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblot analysis using the specific antibodies against phospho-JNK (p-JNK), JNK, phospho-p38K (p-p38K), and p38K proteins. The levels of p-JNK increased between 1 h to 16 h after ethanol exposure. The levels of p-p38K increased between 1 h to 4 h after ethanol exposure.

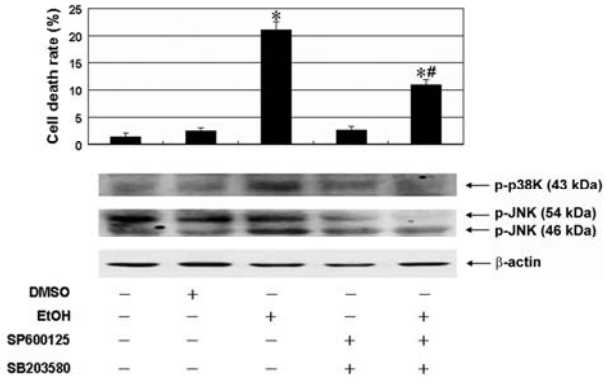

Figure 5 Effects of JNK and p38K inhibitors on ethanol-induced cell death.

A: SK-N-SH cells grown in microtiter plates were pretreated with DMSO (control) or 10 μM SB203580 (JNK inhibitor) or 500 nM SP600125 (p38K inhibitor) for 3 h before exposure to 100 mM ethanol for an additional 24 h. Cell viability was then determined using the MTT reduction assay (n = 5, data shown as mean ± SEM). *p < 0.01, compared with the ethanol-untreated control. #p < 0.05, compared with the ethanol-treated samples.

B: SK-N-SH cells were pretreated with 10 μM SB203580 or 500 nM SP600125 for 3 h and then treated with 100 mM ethanol for an additional 24 h before cells were collected for immunoblot analyses of the phospho-p38K (p-p38K) or phospho-JNK (p-JNK) proteins. Ethanol-induced increases in p-p38K and p-JNK were inhibited by SB203580 and SP600125.

As shown in Fig. 5, the inhibitors significantly reduced the ethanol-induced cell death as well as the levels of the phosphorylated JNK and p38K in SK-N-SH cells, suggesting that JNK and p38K phosphorylation are important during ethanol-mediated cell death.

Ethanol induced p53 phosphorylation in SK-N-SH cells

To determine the involvement of p53 in ethanol-mediated SK-N-SH cell death, the level of p53 was assayed by immunoblot in SK-N-SH cells treated with 100 mM ethanol. Ethanol induced the phosphorylation of p53, which led to accumulation of p53 protein at 1 h after ethanol exposure. Furthermore, this p53 activation was followed by an increase in the p21 tumor suppressor protein and a gradual decrease in phospho-Rb protein (Fig. 6).

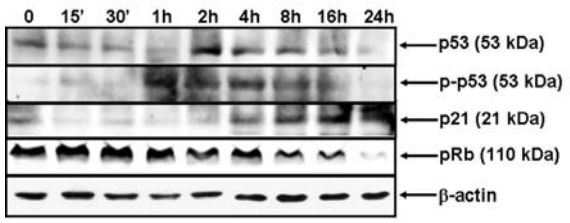

Figure 6 Immunoblot analysis for cell cycle regulatory proteins.

SK-N-SH cells were treated with 100 mM ethanol for the indicated time periods. The soluble fraction from each sample was separated by 12% SDS-PAGE followed by immunoblot analysis. Each antigenic protein was detected using antibodies against p53, phospho-p53 (p-p53), p21, or phospho-Rb (p-Rb).

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 Fig. 7A, the levels of Cdk2 and Cdk4 decreased in a time-dependent manner in SK-N-SH cells treated with 100 mM 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 cells treated with 100 mM ethanol (Fig. 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].

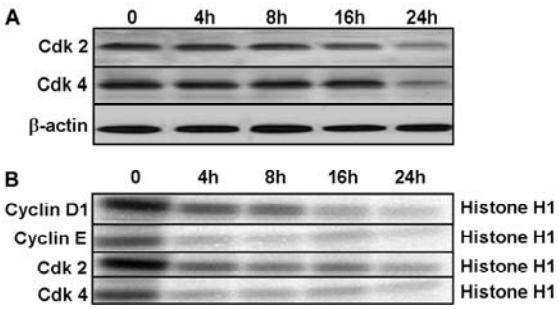

Figure 7 Changes in the levels and activity of cyclin-dependent protein kinases.

A: The soluble fractions from SK-N-SH cells treated with 100 mM ethanol for different times were subjected to 12% SDS-PAGE followed by immunoblot analysis using the specific antibodies against each target protein.

B: Protein kinase activity associated with the immunoprecipitated CDK and cyclin proteins was determined using Histone H1 as the substrate.

However, whether the toxic effect of ethanol on nerve cells is due to indirect or direct mechanism remains elusive. 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 ethanol-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 cytometrical analysis identified that the percentage of G1 cells in cell cycle increased dramatically, indicating that ethanol could also induce cell cycle arrest of the SK-N-SH neuroblastoma cells.

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 stress stimuli, were shown to be activated by ethanol exposure[42-44].

However, how their activation initiate 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 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 h 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 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 important mediator in p53-dependent cell cycle arrest and apoptosis[49, 50]. Another CDK inhibitor is retinoblastoma protein (pRb) that works in the late G1, phosphorylation of pRb is found to be essential for G1/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 cells treated with ethanol. Since The cyclin D1/Cdk4 complex can activate cell cycle progression early in the G1 phase by phosphorylation of pRb, while the Cdk2/cyclin E complex plays a role in the transition from the G1 to S phase[57-59], the above results can well explain our flow cytometrical analysis showing that the cells treated with ethanol arrested in G1 stage. The p53 protein-mediated cell cycle arrest can further lead to apoptosis if the DNA can not 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 was 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).

Cell culture

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

Cell viability assay

Cell viability was measured after ethanol exposure using the 3-[4, 5-dimethylthiazol 2-y] 2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, the medium was removed and replaced with 20 µl of tetrazolium (MTT, 5 mg/ml, Sigma) in phosphate-buffered saline (PBS). The plates were incubated at 37°C for 4 h, followed by addition of 100 µl of dimethyl sulfoxide (DMSO). The multi-well plates were then shaken for 15 s, and the signals were detected with a microplate reader at a wavelength of 595 nm. Cell viability was expressed as a percentage of the control cells treated with vehicle and was designated as 100%.

DAPI Staining

The cells were fixed at room temperature with 4% paraformaldehyde, and stained for diamidino-2-phenylindole (DAPI) using Cell Apoptosis DAPI Detection Kit (Genscript,USA) according to the instruction provided. The stained cell nuclei were examined under a fluorescence microscope.

Analysis of DNA Fragmentation

DNA fragmentation in the SK-N-SH 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 ligasive buffer (Boehringer Mannheim, Indianapolis). Oligonucleotides were annealed. 3U 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 photographed on a UV transilluminator.

Flow Cytometry

After trypsin digestion, approximately 106 cells were collected by centrifugation at 1000 × g for 5 min. The cells were then washed in PBS followed by re-suspension and fixation in 70% ethanol for approximately 2 h. The cells were washed once with PBS, re-suspended in 0.5 ml PBS containing 0.1 mg RNAase, and incubated for 30 min 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).

Immunoblot Analyses

Cells were washed twice with ice-cold PBS and lysed with a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2.0% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS. The lysate was centrifuged at 15,000 × g for 5 min at 4°C, and the supernatant was used as whole cell extracts. 50–100 µg of protein were separated by 10% SDS-PAGE, transferred onto a polyvinylidene difluoride (Millipore, MA, USA) membrane, and incubated with each primary antibody (1/1000–1/2000, Abcam, USA) against each target protein (phospho-JNK, JNK, phospho-p38 kinase, and p38 kinase protein, phospho-p53, p53, p21, pRb, Cdk2, and Cdk4, β-actin). The immunoreactivity was detected using an ECF detection system (Millipore, USA)[62].

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% sodium dodecyl sulfate (SDS), 0.15 M NaCl and 0.01 M Tris, pH 7.4). Lysates were clarified by centrifugation at 15,000 × g for 30 min at 4°C. Samples 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 min with Protein A-Sepharose CL-4B (Pharmacia, USA). Immune complexes were centrifuged, pellets were washed in RIPA-buffer, resuspended, boiled in SDS sample buffer and analysed 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 ANOVA analysis of variance was performed to determine the significance among mutual groups by SPSS11.5 software. $P < 0.05$ was considered statistically significant.

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Author contributors: Yong Moon and Yongil Kwon were in charge of experimental design and revised the content of the manuscript. Maria Lee, Byoung-Joon Song, and Jiyoung Park were responsible for conducting the experiments. Shun Yu participated in the revision of the manuscript.

Conflicts of interest: no conflicts of interest or financial ties to disclose.

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