Ethanol Mediates Cell Cycle Arrest and Apoptosis in SK-N-SH Neuroblastoma Cells

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Background: The mechanisms of cell or organ damage by chronic alcohol consumption are still poorly understood. The present study aimed to investigate the role of the mitogen-activated protein kinases during ethanol-induced damage to SK-N-SH neuroblastoma cells.

Methods: Cells were treated with ethanol and subsequently analyzed for cell morphology, viability, and DNA fragmentation. Immunoblot analysis was performed to assess various proteins levels associated with cell cycle arrest and apoptosis after ethanol exposure.

Results: Ethanol induced time- and dose-dependent cell death in SK-N-SH cells and increased c-Jun N-terminal protein kinase (JNK) activity in a time- and concentration dependent manner. In contrast, p38 kinase activity increased transiently. After treatment with JNK or p38 kinase inhibitors, ethanol-induced cell death significantly reduced. Ethanol-induced cell death was accompanied by increased cytochrome c release and caspase 3 activity observed at 12 h. In contrast, the level of anti-apoptotic Bcl-2 protein did not change. Ethanol also increased the phosphorylation of p53 and p53 activation was followed by an increase in the p21 tumor suppressor protein accompanied by a gradual decrease in phospho-Rb protein.

Conclusion: Our results suggest that ethanol mediates apoptosis of neuroblastoma cells by stimulating p53-related cell cycle arrest mediated through activation of the JNK-related pathway. (J Cancer Prev 2014;19:39-46)

Key Words: Ethanol, Apoptosis, p53, MAPK, Neuroblastoma cell line

INTRODUCTION

Chronic alcohol consumption can damage many organs, including the liver, pancreas, and brain.⁹⁻¹⁸ In addition, numerous studies show that ethanol can damage various cells in culture and is a strong risk factor for cancer in the upper aerodigestive tract, liver, colorectum, and breast.⁹⁻¹¹ Alcohol-related brain damage describes the effects of chronic alcohol consumption on human brain structure and function in the absence of more discrete and well-characterized neurological concomitants of alcoholism.¹²⁻¹⁵ However, the signaling mechanism of cell or organ damage is still poorly understood with respect to early signaling cascades, including the mitogen activated protein kinases (MAPKs).

MAPKs comprise a family of protein kinases whose function and regulation were conserved during evolution from unicellular organisms to complex organisms, including humans.¹⁶ Because MAPKs modulate cellular activities, such as proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis,¹⁷ we hypothesized that changes in the early signaling cascades are
critically important in ethanol-mediated cell death. McAlhany et al. reported that ethanol caused the apoptosis of SK-N-SH neuroblastoma cells, possibly by activating c-Jun N-terminal protein kinase (JNK) in a concentration- and time-dependent manner. In addition, ethanol-induced apoptosis was prevented by treatment with glial-derived neurotropic factor. However, the effect of ethanol on the activities of other MAPKs and their potential roles in ethanol-induced apoptosis were not reported.

JNK, which is a subfamily of the MAPK superfamily, and p38 kinase have a well-characterized role in apoptosis. Therefore, we hypothesized that ethanol may also activate p38 kinase and JNK during ethanol-induced cell death. In the current study, we investigated the effect of ethanol on all three MAPKs and their roles in ethanol-induced cell death. We also studied the levels of various proteins associated with cell cycle arrest and apoptosis after ethanol exposure to understand signaling mechanisms during ethanol-induced cell death.

MATERIALS AND METHODS

1. Cell culture

SK-N-SH cells were obtained from the American Type Culture Collection (Rockville, MD). 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% CO2/95% air at 37°C.

2. Cell viability

Cell viability was measured after ethanol exposure using the 3-[4, 5-dimethylthiazol-2-yl] 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 dimethyl sulfoxide (DMSO). The multi-well plates were then shaken for 15 s, and the signals were detected with a micro-plate 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%.

The cells were fixed at room temperature with 4% paraformaldehyde, and apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) followed by incubation with a FITC-labeled anti-avidine antibody. The stained cell nuclei were examined under a fluorescence microscope at 400 nm.

3. Analysis of DNA Fragmentation

DNA fragmentation in the SK-N-SH cells was measured using a previously published method. SK-N-SH cells were treated with 100 mM EtOH for indicated times and DNA was extracted 6 h later with the DNA Extraction Kit (Stratagene, La Jolla, California), and the DNA was used for ligation-mediated polymerase chain reaction (LM-PCR). LM-PCR for detecting DNA fragmentation was performed using the ApoAlert LM-PCR Ladder Assay kit (Clontech Laboratories, Incorporated, Palo Alto, California). Briefly, adaptor-ligated DNA (100 ng) was prepared and added to 10X LM-PCR Mix (10 μl) and 50X Advantage cDNA Polymerase Mix (2 μl) in a total volume of 100 μl. The PCR was performed on a GenAmp 9700 Thermocycler (Applied Biophysics, Foster City, California): initial denaturation step at 72°C (8 min), followed by 94°C (1 min), 72°C (3 min) × 20 cycles, final extension step 72°C (15 min). Each 10-μl amplified DNA sample was electrophoresed on 1.2% agarose/ETBr gel at 6 V/cm for 2.5 h. Samples of 100 bp were used as a standard to determine DNA fragment size.

4. Flow Cytometry

After trypsin digestion, approximately 10^6 cells were collected by centrifugation at 1000 × g for 5 min. The cells were then washed in PBS followed by resuspension and fixation in 70% ethanol for approximately 2 h. The cells were washed once with PBS, resuspended in 0.5 ml PBS containing 0.1 mg RNAse, and incubated for 30 min at 37°C. Cellular DNA was then stained with 10 μg propidium iodide. The stained cells were subsequently analyzed on a FACScan with the Cellquest software (Becton Dickinson).

5. Immunoblot Analyses

Whole cell extracts or cytosolic fractions (50-100 μg protein) were used for immunoblot analyses with anti-
bodies against each target protein (phospho-JNK, JNK, phospho-p38 kinase, and p38 kinase protein). Each antigen was then detected with an ECF detection system (Amersham).

6. 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 MAPK activity using the method of Park et al. The activities of cdk2, cdk4, cyclin D1, and cyclin E in the soluble fraction (300 μg per reaction) were measured according to the published method.

7. Measurement of Caspase-3 Activity

Caspase 3 activity was measured in whole cell homogenates (50 μg/protein) using a method described previously. Specifically, the fluorescence generated from the proteolytic cleavage of 20 μM Ac-DEVD-AMC was measured with a fluorescence plate reader (Cytoflor 2300; Millipore Corporation) with excitation at 360 nm and emission at 450 nm.

8. Statistical Analysis

All experimental results shown were repeated two or three times unless otherwise indicated. The results are reported as mean±standard error of the mean. The mean values were compared using a Student’s t-test. A P value of <0.05 was considered statistically significant.

RESULTS

1. Ethanol induced cell death in SK-N-SH cells

SK-N-SH cell morphology was changed after treatment with ethanol for 24 h and examined by phase-contrast microscopy (Fig. 1A). We used an MTT assay to study the effects of ethanol on cell death in SK-N-SH cells. The results indicated that SK-N-SH cells were significantly
inhibited with increasing ethanol dose and exposure time (Fig. 1B). DNA fragmentation was also examined by labeling the fragmented DNA ends with fluorescent nucleotides (TUNEL assay) (Fig. 1C). As expected, the ethanol-treated SK-N-SH cells stained positive compared to the control cells. Thus, ethanol treatment mediated time- and dose-dependent cell death in SK-N-SH cells, most likely via apoptosis.

2. Cytochrome c release and caspase 3 activity after ethanol treatment

After ethanol treatment, both cytochrome c release and caspase 3 activities were elevated at 12 h in a successive manner (Fig. 2).

3. Ethanol dependent apoptosis

To verify the type of cell death, we stained the cells with DAPI, a sensitive assay for apoptosis (Fig. 3). Without ethanol treatment, the nuclei of control cells showed uniform staining, indicating that cells were healthy and nuclei intact. In contrast, after 24 h treatment with 100 mM

![Fig. 2. Changes in caspase activity and flow cytometric analysis in SK-N-SH cells after exposure to 100 mM ethanol. (A) Caspase-3 activity in whole cell extracts from SK-N-SH cells treated with 100 mM ethanol for the indicated times was determined as described in the Methods section. (B) 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 flow cytometric cell cycle analysis.](image-url)

![Fig. 3. 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, as described in the Methods section. The white arrow shows an intact nucleus, and the red and yellow arrows show apoptotic nuclei. Bar indicates 15 μm.](image-url)
ethanol, SK-N-SH cells exhibited typical characteristics of apoptosis, such as nuclear condensation as determined by DAPI staining. These data confirm that ethanol-induced cell death is mediated via apoptosis.

4. Ethanol increased the phosphorylation of JNK

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 JNK activity in a time- and concentration-dependent manner. Within 15 min after ethanol exposure, JNK activity increased, and the elevated JNK activity persisted until 16 h after the exposure.

5. Transient activation of p38 MAP kinase activity after ethanol treatment

In contrast to JNK levels, p38 kinase activity transiently increased between 15 min and 4 h after ethanol treatment before returning to control levels (Fig. 4).

6. Activation of JNK and p38 kinase during cell death

As described above, ethanol treatment led to remarkable increases in the levels of JNK and p38 kinase. To examine the specific roles of JNK and p38 kinase in ethanol-induced cell death, the cells were pretreated for 3 h with SP600125 (a JNK inhibitor) and SB203580 (p38 inhibitor). As shown in Fig. 5, the inhibitors significantly reduced cell death in SK-N-SH cells after ethanol treatment, suggesting that JNK and p38 kinase activation are important during ethanol-mediated cell death.

7. 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. In addition to JNK activation, 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). CDK2, CDK4, cyclin D1 and E activity were decreased by ethanol exposure (Fig. 7).
Fig. 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, p21, or phospho-Rb (pRb).

DISCUSSION

It has been accepted that excessive alcohol consumption can cause structural and functional abnormalities of the brain and other organ.1-11 In the brain, structural abnormalities such as macroscopic shrinkage of the brain, reduced viability of neuronal cells and axonal degradation are presented as cognitive dysfunction in alcoholics.12,13 Alcohol-related brain damages appear to be affected by lifetime alcohol consumption although underlying mechanisms are still largely unknown. When alcoholics are medically complicated, damage appears to be more widespread precipitating more neurocognitive dysfunction. Wernicke-Korsakoff syndrome is one of the most important complications affecting the brains of alcoholics, which is caused by thiamine shortage/deficiency.14,15 In a few years, quantitative studies and improvements in neuroimaging have contributed significantly to the documentation of alcohol-related brain changes.25,26 However, there have been few studies on the subject of the mechanism of ethanol-mediated damage to cells.

A previous study revealed that ethanol specifically induced the phosphorylation of JNK and MAPKs specifically associated with apoptosis.14 Another report showed that ethanol increased the phosphorylation of c-Jun, and p38 is activated by the receptor-mediated cell death pathway.27,28 The MAPK signaling pathways (e.g., extracellular signal regulated kinase, JNK, and p38 MAPK) are known to play important roles in regulating cell proliferation, apoptosis, and tumorigenesis. Furthermore, the transducers of stress signals are largely dependent on JNK and p38 MAPK. It was reported by Chen et al.29 that treatment of rat hepatocytes with ethanol in vitro selectively increased JNK activity, but did not affect p38 MAPK activity. No significant changes in the levels of phosphorylated p38 were found in the different treatment groups, indicating that chronic ethanol consumption by rats may specifically induce JNK activity. In ethanol-fed rats, the levels of phosphorylated JNK protein were significantly increased by more than four-fold compared to control rats.30 In our study, JNK and p38 kinase activity were both activated after ethanol exposure. Moreover, we found that cell death was inhibited after treatment with both JNK and p38 kinase inhibitors. These findings demonstrate that ethanol affects apoptosis in SK-N-SH cells through both JNK and p38 kinase-associated pathways. Further experiments by inhibition JNK would also be carried out to establish the role JNK signaling in ethanol-induced cell death.

p21 is a potent cyclin-dependent kinase (CDK) inhibitor. The p21 protein binds to and inhibits the activity of cyclin-CDK2 or -CDK1 complexes, and thus functions as a regulator of cell cycle progression in the G1 phase.31 The expression of this gene is tightly controlled by the tumor suppressor protein p53, through which this protein mediates G1 phase cell cycle arrest in response to a variety of stress stimuli. In the hypophosphorylated state, pRb is
active and carries out its tumor suppressive role by inhibiting cell cycle progression.\textsuperscript{32}

In this study, phosphorylated p53 protein levels were increased after ethanol exposure. The activation of p53 induced an increase in p21 protein in addition to a gradual decrease in phospho-Rb protein. These results indicate that ethanol treatment increases cell cycle arrest by activating the p53 pathway. Moreover, CDK2, CDK4, cyclin D1 and E activity were decreased by ethanol exposure, suggesting that the p21 protein is involved in the apoptosis of SK-N-SH cells.

In conclusion, the present study strongly indicates that ethanol mediates apoptosis in SK-N-SH neuroblastoma cells by stimulating p53-related cell cycle arrest. Furthermore, this may be mediated through the activation of the JNK-related cell death pathway.

**DISCLOSURES**

Maria Lee, Byoung-Joon Song and Yongil Kwon have no conflicts of interest or financial ties to disclose.

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